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The Differential Tissue Distribution of the Citrus Flavanone Naringenin Following Gastric Instillation

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Citrus flavonoids have been investigated for their biological activity, with both anti-inflammatory and -carcinogenic effects being reported. However, little information is known on the bioavailability of these compounds *in vivo*. The objectives of this study were to determine the tissue distribution of naringenin after gastric gavage of [^{3}H]-naringenin to rats. Unlabelled naringenin was also used to quantify the levels of naringenin and its major metabolites in tissues and eliminated in the urine and faeces. Significant radioactivity was detected in the plasma as well as all tissues examined 2 h post-gavage. After 18 h, higher levels of radioactivity were retained in plasma and tissues (55% of the administered radioactivity). Investigation of the nature of metabolites, using unlabelled naringenin, revealed that the glucuronides were the major components in plasma, tissues and urine, in addition to the colonic metabolite 3-(4-hydroxyphenyl) propionic acid, detected in the urine. The aglycone was the form extensively retained in tissues after 18 h post-gavage. Total identified metabolites detected after 18 h in most tissues were only 1–5% of the levels detected after 2 h. However, the brain, lungs and heart retained 27, 20 and 11%, respectively, relative to the total metabolites detected at 2 h. While radioactive detection suggests increased levels of breakdown products of naringenin after 18 h versus 2 h, the products identified using unlabelled naringenin are not consistent with this, suggesting that a predominant proportion of the naringenin breakdown products at 18 h are retained as smaller decomposition molecules which cannot yet be identified.

Keywords: Naringenin; Flavonoids; Absorption; Distribution; Metabolism; Radioactive

INTRODUCTION

There is considerable interest in the role of dietary flavonoids as health beneficial agents in terms of cardioprotection,[1,2] cerebrovascular and neuroprotection [3–5] as well as chemoprevention.[6] Their modes of action remain to be elucidated and, in particular, the identification of the specific chemical forms which elicit bioactivity.

Flavonoids are known to be metabolised and conjugated during their absorption across the small intestine [7] and on circulating through the liver,[8] yielding glucuronides, methylated forms and sulphates. Our previous studies with naringenin have detected the glucuronide as the major metabolite in urine following either oral or intravenous administration.[9] Flavonoids were also shown to undergo colonic biotransformation to cleavage products such as hydroxylated phenyl acetates, phenyl propionates and benzoates.[10]

Flavonoids *in vitro* have been shown to elicit powerful H-donating properties,[11–13] as indicated by their reduction potentials[14] and by the ability to scavenge reactive oxygen and nitrogen species.[15,16] Their roles in inhibiting oxidative stress-induced activation of signalling pathways to apoptosis, especially the flavanol epicatechin and its methylated forms[17,18] and UV-induced necrosis, in

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particular hesperetin glucuronide, the citrus flavanone,[19] are currently proposed to be their most likely modes of action in cytoprotection. The contrasting abilities of structures such as the flavonol quercetin and its *in vivo* methylated forms to facilitate cell death processes via oxidative metabolism in fibroblasts[20] or inhibiting AKT-dependent cell survival pathways in neurons,[21] demonstrate that bioactivities and cytoprotective/cytotoxic effects in cells *ex vivo* are structure-dependent.

Thus, it is important to understand the distribution of flavonoids *in vivo*, to delineate the forms in which they access tissues and the influence of time on their retention and metabolism at the target sites. Our previous studies have demonstrated the ability of epicatechin, and its physiological form epicatechin glucuronide and methylated glucuronide to reach the brain.[22] In the present study, we have investigated the distribution of naringenin to heart, brain, lungs, spleen, liver and kidneys, in relation to the levels of the circulating forms of naringenin and those in the gastrointestinal tract, following gastric gavage of [^{3}H]-naringenin. The nature of the conjugates/metabolites of naringenin accessing these tissues has also been assessed following gastric gavage with unlabelled naringenin.

MATERIALS AND METHODS

Chemicals

Ring-[^{3}H]-naringenin (4′,5,7-trihydroxyflavanone), was custom-synthesised by Amersham Biosciences (Buckinghamshire, UK). Radiochemical purity was established to be 98.3% using HPLC analysis. Specific activity determined by mass spectrometry was 37.0 GBq/mmol (1 Ci/mmol). Naringenin, 3-(4-hydroxyphenyl) propionic acid (*p*-HPPA), 3,4-dimethoxycinnamic acid, trichloroacetic acid, β-glucuronidase enzyme (type L-II from Limpets) and sulfatase enzyme (type H-5 from Helix pomatia) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium acetate and *o*-phosphoric acid (85%) were obtained from BDH laboratory Supplies (Poole, Dorset, UK). Acetonitrile (HPLC grade), acetone, and methanol were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK).

Animals and Sample Preparation

The study used 250 g male Sprague-Dawley rats obtained from the Comparative Biology Unit at the Royal Free amp; University College Medical School, and maintained on standard rat chow (Diet RM1, SDS Ltd, Witham, Essex, UK). Rats ($n = 9$/group) were administered via gastric gavage, 10 or 50 mg/kg body weight naringenin containing [^{3}H]-naringenin (dissolved in 1 ml 50% ethanol), the final specific activities being 0.496 and 0.1 MBq/μmol, respectively. Animals were killed either 2 or 18 h following gavage. Similar experiments were performed whereby two additional groups of rats received unlabelled naringenin (50 mg/kg) for the same time intervals. Three animals from each group served as controls and received only 50% aqueous ethanol. In all groups, animals were fasted for 4 h prior to gavage.

At 2 or 18 h post-gavage, animals were anaesthetised with intraperitoneal pentobarbitone sodium (90 mg/kg; Sagital; Rhone-Merieux, Harlow, Essex, UK). Heparin (0.3 ml, 5000 I.U./ml; CP Pharmaceuticals Ltd, Wrexham, UK) was injected into a tail vein and 2 ml of blood was removed by cardiac puncture and stored in heparinized tubes. Animals were then exsanguinated with 200 ml ice-cold heparinized 0.9% (w/v) saline and the organs under study (liver, kidneys, spleen, heart, small intestine, large intestine, lung and brain) were rapidly removed. Plasma was obtained by centrifugation of blood at 600*g* for 30 min. The haemoglobin concentration of the cardiac blood sample and exsanguinated fluid was measured using a haemoglobin assay kit (Sigma, Poole, Dorset, UK). These data allowed blood volume to be calculated, the latter being equal to total amount of haemoglobin recovered divided by its concentration in the cardiac blood sample. Total haemoglobin was determined from the volume of perfusate collected after exsanguination together with the concentration of haemoglobin in this mixture. The removed tissues were weighed and then homogenized in ice-cold 0.9% (w/v) saline (10 vol/g tissue) using a Ultra Turrax homogeniser (Janke & Kunkel, FRG) for 40 s at half speed. For the 18-h experiments, urine and faeces from rats housed in metabolic cages were collected. Intestinal contents were obtained by finger pressure applied to the intestinal wall, followed by flushing with 0.9% (w/v) saline alone and then saline containing a 10-fold excess of unlabelled naringenin. The contents were retained for measurement of radioactivity.

Measurement of Radioactivity

Plasma (100 μl) or tissue homogenate (400 μl) was mixed with 4 ml of Ultima Gold scintillation cocktail (Packard Biosciences, UK) designed to withstand quenching and to solubilise protein, before a determination of radioactivity using a Packard Liquid Scintillation Counter (Tri-Carb 2900TR). Data for organ weight and DPM of digested sample, allowed calculation of organ content of radioactivity after 2 or 18 h. Percentage detected in each organ was then calculated. Percentage excretion was similarly determined in urine and faeces collected for 18 h following naringenin administration.

Autoradiography

Autoradiography was performed on tissue taken from rats gavaged with 50 mg/kg naringenin, containing [^{3}H]-naringenin (specific activity 1 MBq/μmol). Tissues were fixed *in vivo* using 400 ml of 4% glutaraldehyde (in PBS with 2% sucrose), removed from the animal and post-fixed overnight at room temperature in 4% formal saline. Tissues were then dehydrated, mounted in wax and sectioned at 7 μm using a sledge microtome (Anglia Scientific, Cambridge, UK). Sections were adhered to the slides by over-night heating at 60°C, followed by dewaxing, re-hydration and drying. Slides were dipped in molten nuclear emulsion (K2; Ilford, Moberly, Cheshire, UK), laid flat on an ice-cold platform to set and stored in the dark at room temperature for 7 days. Slides, developed in Kodak D19, fixed in Hypam (Ilford, Moberly, Cheshire, UK) and counterstained using haemotoxylin and eosin, were viewed using a Leica DMR and photographed using a Nikon Coolpix 990.

Extraction of Plasma and Tissues for HPLC Analysis

Plasma (300 μl) was deproteinised with 60 μl 20% (w/v) trichloroacetic acid and then extracted with 300 μl ice-cold acetone. The extract obtained following centrifugation was evaporated to dryness under nitrogen and the residue dissolved in 300 μl 20% (v/v) methanol in 1% (w/v) acetic acid for quantitative HPLC analysis. For β-glucuronidase enzyme treatment, plasma was mixed 100 mM sodium acetate buffer (pH 3.8) containing β-glucuronidase enzyme (final enzyme concentration of 2000 unit/ml). Samples were incubated for 1 h in a shaking water bath at 37°C and extraction was undertaken as described above. Another aliquot of plasma was alternatively deproteinised and extracted using an equal volume of ice-cold methanol and the obtained extract treated as previously described.

Extraction of naringenin and its metabolites from rat tissues was performed by mixing the homogenates with equal volume of ice-cold methanol followed by vigorous vortexing for 1 min. The methanolic extract obtained after centrifugation was dried under vacuum at 30°C by rotary evaporation and then the extracts were treated as described above. Additional aliquots of small intestine tissue homogenates were subjected to sulfatase enzyme treatment (100 U/ml) in sodium acetate buffer (pH 5.0) for 2 h.

Quantitative determination of naringenin and its metabolites was based on external standards. An internal standard (IST) 3,4-dimethoxycinnamic acid was added to the samples before extraction and to the external standards to give a final concentration of 5 μM. Calibration curves were conduced over the range of 0.3–10 μM for plasma and tissue samples and up to 500 μM for urine samples. Peak area ratios (compound/IST) were plotted against concentration of the compound/metabolite. Calibration curves thus obtained were linear over the entire range with correlation coefficient values ≥ 0.995. Recovery experiments using control plasma and tissue homogenates spiked with unlabelled standards, showed 85 and 100% recovery for naringenin, and 3 (4-hydroxyphenyl) propionic acid, respectively, using the described method. Applying the same extraction and evaporation procedure to plasma and tissue samples obtained from [^{3}H]-naringenin-fed animals resulted in a remarkable loss of pre-evaporation counts, which was observed to differ between 2 and 18 h samples. Evaporated extracts of 2 h plasma retained approximately 12.4% of counts detected in original plasma, while 18 h samples retained only 0.7%. Similarly, 2 h tissue extracts retained 37.7% while 18 h samples retained only 10.4% of original activity.

Urine Samples for HPLC Analysis

Urine samples were either filtered through a micropore filter (MILLEX-GP, 0.22 μm filter unit) or extracted with an equal volume of methanol prior to quantitative HPLC analysis. For β-glucuronidase treatment, urine was mixed with an equal volume of sodium acetate buffer (pH 3.8) containing 12,000 U/ml of enzyme and incubated for 2 h at 37°C before analysis.

Sample Purification for GC-MS

Urine samples or extracted faeces were diluted with 1 ml of water/methanol/acetic acid (94/5/1 v/v/v) and then applied to a C-18 cartridge (Waters Sep-Pak) pre-washed with 3 ml of methanol and pre-conditioned by washing with 6 ml water/methanol/acetic acid (94/5/1 v/v/v). Following the sample application, the cartridge was washed with 6 ml water/methanol/acetic acid (94/5/1 v/v/v) and the polyphenols eluted from the cartridge material with 3 ml methanol. The SPE extract was dried under nitrogen, and the residue used for GC-MS analysis.

HPLC Analysis

HPLC analysis used a Waters system consisting of controller 600, auto sampler 717 plus, photodiode array detector 996, and on-line degasser. Samples were analysed on a Phenomenex Luna Phenyl-Hexyl column, 4.6 × 150 mm, with 5 μm particle size and a guard column of the same material, 4.6 × 15 mm.

Column temperature was set at 30°C. Mobile phase A consisted of water/85% *o*-phosphoric acid (99.5/0.5 v/v) and mobile phase B of acetonitrile/water/85% *o*-phosphoric acid (50/49.5/0.5). The gradient applied was as follows: from 0 to 5 min 90% A and 10% B, from 5 to 40 min to 100% B, from 40 to 45 min 100% B, and from 45.1 min 90% A and 10% B. Run time was 50 min followed by a 10 min delay prior to the next injection. Injection volume was 75 μl for plasma and tissue samples and 50 μl for urine samples. Components were identified according to retention times (RT), UV/visible spectra and spiking with commercially available relevant standards. Quantification of naringenin and naringenin glucuronides was undertaken at 300 nm, and for 3-(4-hydroxyphenyl) propionic acid at 220 nm.

LC-MS Analysis

The samples were analysed using a Finnigan LCQ Deca XP quadrupole ion trap mass spectrometer. Separation was performed using a C18 column (50 mm × 2.5 mm, Phenomenex) with the following gradient (Phase A: 0.1% (w/v) formic acid in water, phase B: 50% (w/v) acetonitrile in water, 0.1% formic acid). The LC-MS gradient was 0–5 min 100% A, 5–40 min from 100% A to 50% A, 40–60 min to 0% A, 60–65 min 0% A. Compounds were detected using a full ion scan and identified by performing product ion scans on selected ions.

GC-MS Analysis

The samples, purified and dried as described earlier, as well as standard compounds, were dissolved in 50 μl dry acetonitrile and 20 μl N-(*t*-butylydimethylsilyl)-N-methyltrifluoroacetamide (TBDMS) containing 1% (w/v) N-(*t*-butylydimethylsilyl)-N-methyltrifluoroacetamide chlorosilane (TBDMSCL). After 30 min, 10 μl of the derivatized samples were dried under nitrogen and dissolved in 20 μl undecane before analysis by a Fisons GC8000 gas chromatography using a DB-1701 column and a Fisons Trio 1000 with EI positive and full scan mode.

Calculations and Statistics

Values are given as means ± standard error of the mean (SEM). Statistical analysis was performed using an unpaired *t*-test (Instat) and considered significant at $P < 0.05$.

RESULTS

Distribution of Radioactivity in Rat Tissues After Administration of [^{3}H]-naringenin

Figure 1a demonstrates the overall recovery of [^{3}H]-naringenin radioactivity, 2 and 18 h following gastric gavage. These values were representative of [^{3}H]-naringenin detected in the brain, lungs, heart, liver, spleen, kidney, plasma and intestinal contents, including the faeces for the 18-h experiments. The failure to recover 100% of the gavaged counts is likely to be a consequence of accumulation of the flavonoid and its metabolites in the skin, muscle, testis, fat and adipose tissue, none of which was included in the present study. Approximately 87% of the administered dose was shown to disappear from the gastrointestinal tract 18 h after gavage (Fig. 1b), which was significantly higher than that seen 2 h post-gavage (47%). This amount of radioactivity is assumed to enter the animal from the intestinal tract. However, as shown in Fig. 1c, lower amounts were found in the tissues examined, particularly 2 h post gavage, again implying that radioactivity was present in tissues not studied during the present work.

The percentage of the gavaged [^{3}H]-naringenin that was detected in each of the excised tissues plus the plasma, urine and faeces is shown in Table I. At 18 h post-gavage, the highest level of [^{3}H]-naringenin and metabolites was detected in the urine, demonstrating that the compound is efficiently excreted by the kidney. Relative to the plasma, lower levels of [^{3}H]-naringenin and metabolites were detected in the tissues. The percentage detected in all tissues and body fluids calculated as the percentage of that gavaged was not different when comparing the 50 mg/kg with 10 mg/kg dose. However, significantly higher levels of radioactivity were seen at 18 h compared to 2 h in most tissues, but specifically in the brain and heart, reaching approximately 10-fold of those found at 2 h post-gavage.

Autoradiographs clearly demonstrated the presence of radioactivity within blood vessels of all tissues studied but not within cells of these tissues (Fig. 2). In small intestine, silver grains were also noted in the lacteals of villi whilst in the kidney, strong radioactivity was detected in the tubular lumen and peritubular fluid. In the liver, silver grains were noted in the sinusoids. For all tissues, the adequacy of the vascular perfusion technique is evidenced by the noticeable absence of red blood cells within blood vessels.

Identification of Naringenin Metabolites in Rat Tissue Extracts

Analysis of plasma and tissue extracts after gavage of unlabelled naringenin was carried out using HPLC with photodiode array detection, followed by LC-MS. In all experiments, purified standard naringenin glucuronides (a mixture of 5- and 7-*o*-β-glucuronides), previously-synthesized,[23] were used to aid identification of comparable peaks in samples. A standard mixture of the glucuronides, the naringenin aglycone, and 3-(4-hydroxyphenyl)

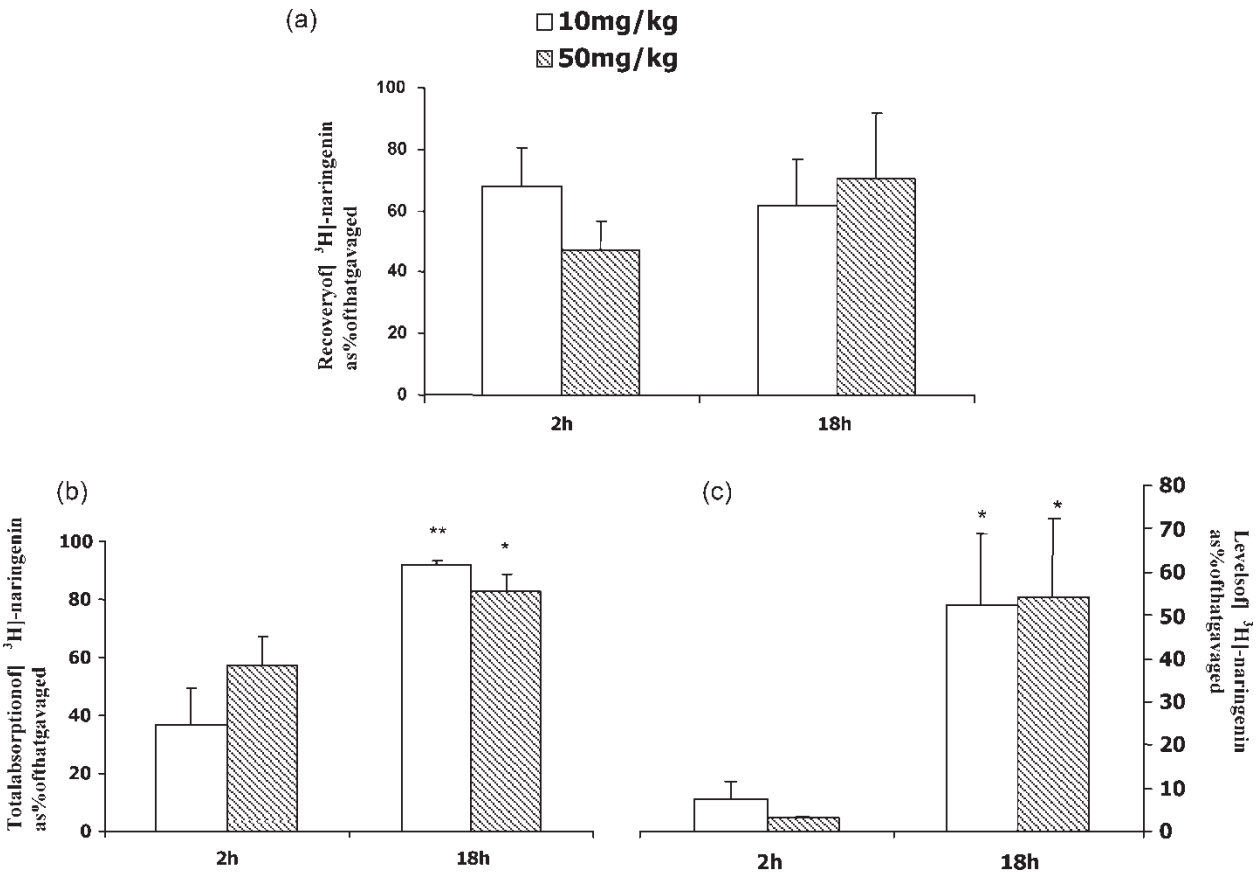

FIGURE 1 (a) Recovery of [^{3}H]-naringenin activity at 2 h and 18 h post-gavage. The summated total counts detected in excised tissues, plasma, intestinal contents, faeces and urine were expressed as a percentage of the gavaged DPM. (b) Calculated total absorption of [^{3}H]-naringenin at 2 h and 18 h post-gavage, expressed as the amount of radioactivity of the gavage solution, which disappeared from the gastrointestinal lumen. This was calculated by subtracting the DPM of the intestinal contents (and faeces for 18 h experiments) from that in the gavage solution and the difference expressed as a percentage of the gavaged counts. (c) Accumulation of [^{3}H]-naringenin in all excised tissues, urine and plasma (but not intestinal flushes and faeces) at 2 h and 18 h post-gavage. This data was calculated as total DPM detected as a percentage of the [^{3}H]-naringenin DPM gavaged. In all cases, values represent the mean ± SEM of 9 animals per group. Significant from the same dose at 2 h at $^*P < 0.05$, and $^{**}P < 0.001$.

propionic acid together with the internal standard, were included in all HPLC runs.

Representative chromatographic profiles of small intestinal and brain extracts are shown in Fig. 3a and b, respectively. HPLC analysis of most tissues as well as plasma taken from animals 2 h after dosing with naringenin, revealed 2 peaks (1,2) which had the same UV spectra and retention times as naringenin-5- and

TABLE I Levels of [^{3}H]-naringenin in tissues, plasma, urine and faeces

	2 h		18 h	
	10 mg/Kg	50 mg/Kg	10 mg/Kg	50 mg/Kg
Brain	0.026 ± 0.007	0.017 ± 0.003	0.24 ± 0.10*	0.15 ± 0.04**
Lung	0.025 ± 0.004	0.025 ± 0.005	0.095 ± 0.026*	0.086 ± 0.030
Heart	0.013 ± 0.002	0.013 ± 0.002	0.12 ± 0.05	0.12 ± 0.04*
Liver	0.79 ± 0.16	0.68 ± 0.08	1.30 ± 0.45	1.35 ± 0.44
Spleen	0.008 ± 0.001	0.01 ± 0.004	0.068 ± 0.023*	0.071 ± 0.015**
Kidney	0.16 ± 0.04	0.12 ± 0.02	0.42 ± 0.14	0.32 ± 0.07*
Small intestine	1.62 ± 0.31	0.86 ± 0.26	0.39 ± 0.14**	0.95 ± 0.55
Large intestine	0.12 ± 0.03	0.08 ± 0.02	0.25 ± 0.13	0.16 ± 0.03
Plasma	4.94 ± 3.63	1.44 ± 0.21	7.75 ± 2.55	9.18 ± 2.60*
Faeces	–	–	2.58 ± 0.44	7.31 ± 2.63
Urine	–	–	42.11 ± 12.9	41.69 ± 14.9

Levels were calculated as a percentage of that gavaged and expressed as the mean ± SEM of 9 animals per group. Significant difference between same dose at 2 h and 18 h at $^*P < 0.05$ and $^{**}P < 0.001$.

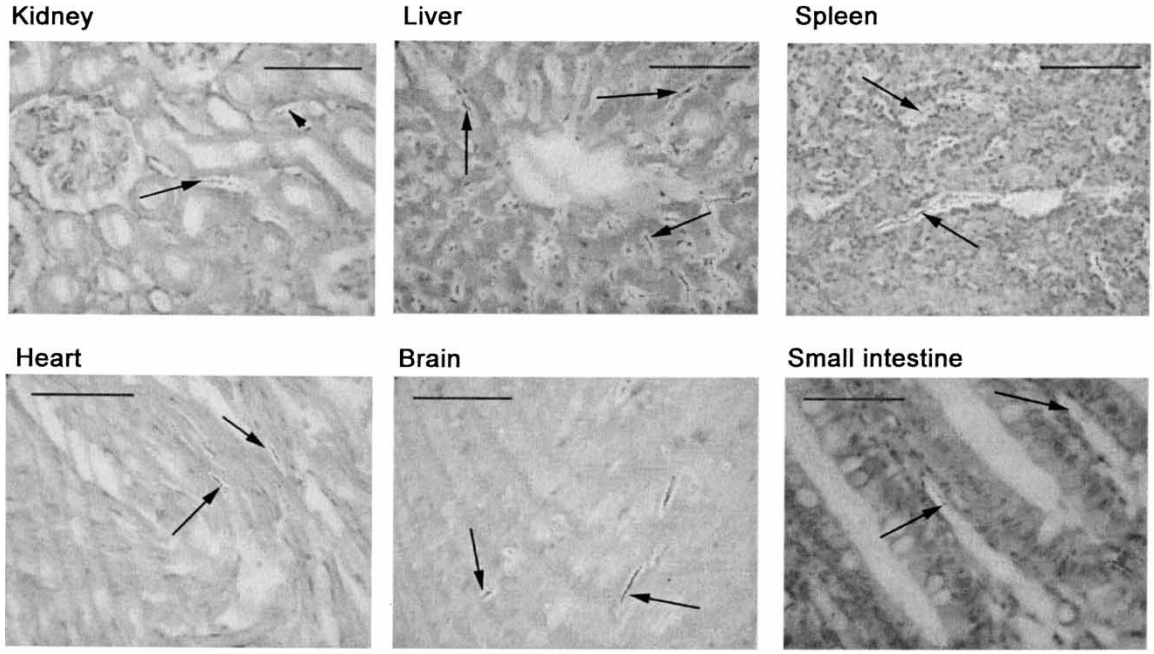

FIGURE 2 Autoradiographs of tissue sections obtained from rats at 2 h after gastric gavage of [^{3}H]-naringenin after staining with haemotoxylin and eosin. Arrows show grains representing the presence of [^{3}H]-naringenin in the blood vessels and also lacteals in the intestine. Arrowhead shows grains in tubular fluid in kidney. Scalebar = 50 μm.

7-*o*-β-glucuronides, at RT 29.6 and 30.3 min, respectively and peak (6) corresponding to naringenin, at RT 38.2 min. Further confirmation of identification was provided by spiking with purified standards as well as by treatment with β-glucuronidase. Following enzyme treatment, peaks attributed to the glucuronides, disappeared and a corresponding increase was observed in the naringenin aglycone peak. In some tissue samples, including the brain (Fig. 3b), it was possible to detect only the naringenin-5-*o*-β-glucuronide (peak 1) and naringenin (peak 6). The HPLC profiles for control samples revealed no trace of these peaks.

LC-MS analysis of samples, relative to standards, confirmed the presence of the aforementioned metabolites detected by HPLC. Two abundant signals at $m/z = 449$, and $m/z = 273$, corresponding to naringenin glucuronides and aglycone, respectively, were detected. The product ion spectrum of $m/z = 449$ led to an intense signal of $m/z = 273$, on fragmentation, consistent with an assignment to naringenin, after a neutral loss of glucuronic acid (Fig. 4) and consistent with signals from the relevant standard compounds.

In the small intestine, however, 4 additional peaks (peaks 3, 4, 5 and 7), having similar UV spectra to naringenin, were detected at RT 32, 32.8, 35.7, and 41.2 min, respectively (Fig. 3a). Treatment with β-glucuronidase resulted in the disappearance of peaks 5 and 7, confirming that these peaks are also glucuronide conjugates of naringenin-related metabolites. Peak 4 disappeared only after sulfatase enzyme treatment but because the enzyme also possesses β-glucuronidase activity, it is only possible to assume that this peak corresponded to either a sulfo- or glucurono-sulfoconjugated derivative. However, peak 3 was resistant to hydrolysis with both enzymes. LC-MS investigations of the identity of peaks 3, 4, 5, and 7 showed a strong signal of $m/z = 479$, eluting earlier than naringenin, and thus more polar. This mass could not account for a sulpho- or glucurono-sulfoconjugate of naringenin. In addition, the product ion spectrum of this peak did not show any signal corresponding to loss of glucuronic acid or sulphate moiety. Based upon these data, combined with enzyme experiments, it thus seems likely that this peak $m/z = 479$ corresponded to peak 3 detected by HPLC. Another signal of $m/z = 426$ was detected and assigned to peak 7 as shown in the HPLC trace, since it is the only peak that eluted after naringenin. However, the exact identity of these metabolites was not elucidated.

In addition to the identification of the glucuronides and aglycone, HPLC analysis of urine and faecal samples, collected for 18h following naringenin administration, revealed a peak corresponding to 3-(4-hyroxyphenyl) propionic acid which was clearly detected using PDA detection at 220 nm (Fig. 3c). GC-MS analysis was used to confirm the precise identity of this phenolic acid in urine by comparing the mass spectra and retention times with standard data after derivatization with TBDMS (Fig. 5).

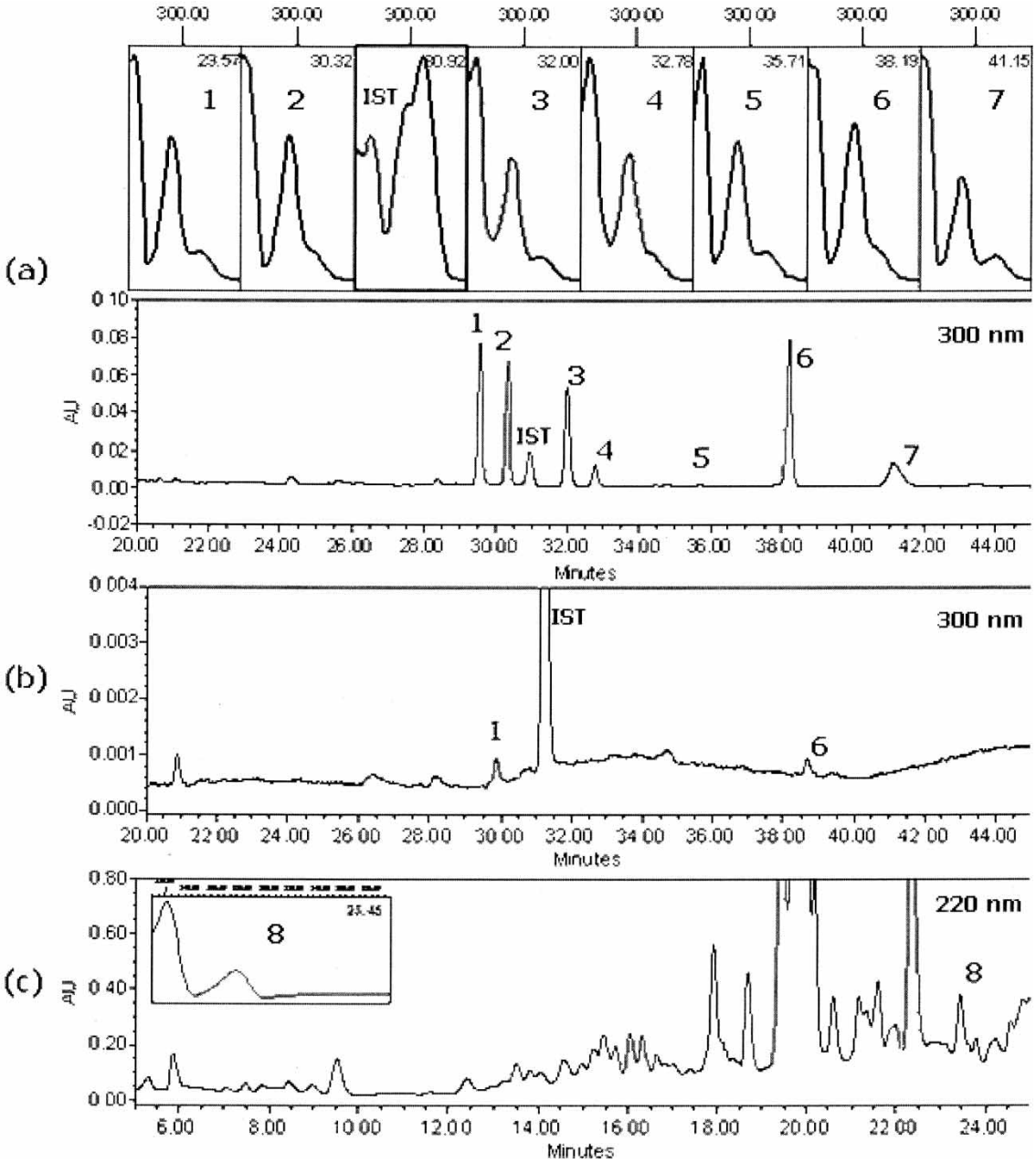

FIGURE 3 Representative chromatograms of tissues obtained from naringenin-gavaged rats. (a) Small intestine extract at 300 nm. Peaks: (1, 2) naringenin-5-and 7-*O*-β-D-glucuroindes, (3, 4, 5, 7) unidentified, (6) naringenin. (b) Brain extract at 300 nm. (c)] Enzyme-treated urine at 220 nm. Peaks: (8) p-hydroxyphenyl propionic acid. [IST] internal standard.

Concentration of Naringenin and its Metabolites in Plasma and Tissue Extracts

The concentrations of naringenin and its identified metabolites were measured in plasma and tissue extracts of rats 2 and 18 h following gavage of naringenin at a dose of 50 mg/kg body weight. In the plasma, the main metabolites detected after 2 h were naringenin-*o*-β-D-glucuronides, reaching concentrations of 5 μM, which constituted 98% of total detected metabolites in plasma (Fig. 6a). These glucuronides were quantified from the increase in naringenin peak after enzyme treatment. In most of the tissues, the detected metabolites 2 h post-gavage were mainly glucuronides. However, the naringenin aglycone was detected at higher levels in tissues compared to the plasma. Highest levels of metabolites were found in the small intestine followed by the kidney, liver and lungs. To eliminate the possibility that the detected aglycone was derived from the breakdown of glucuronides during extraction, recovery experiments were performed, which showed the stability of the purified glucuronide standard spiked onto control tissue and treated with the same extraction procedure.

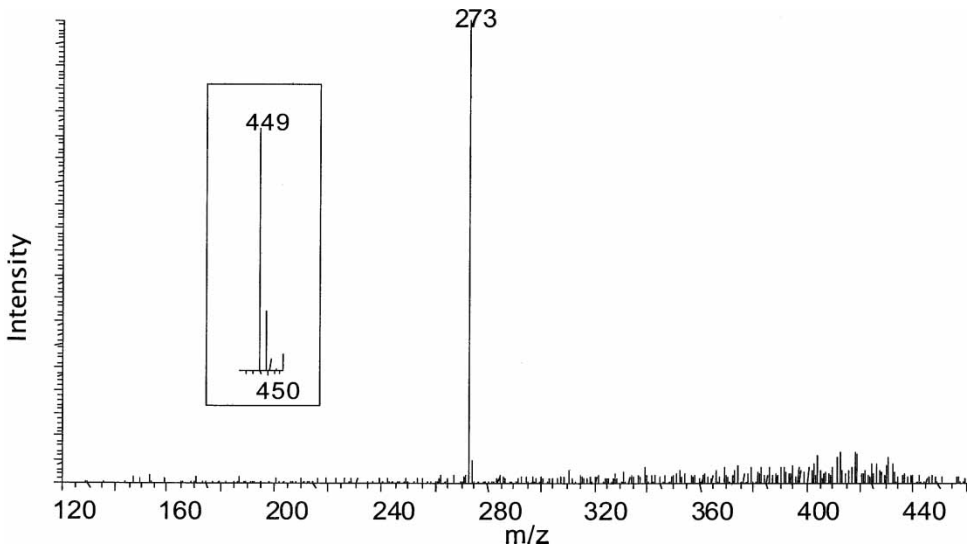

FIGURE 4 MS/MS spectrum of naringenin-O-glucuronide detected in the small intestine of rats 2 h following gastric gavage of 50 mg/kg body weight of naringenin. The spectrum shows a single fragment at $m/z = 273$, the $[M^+H^+]^+$ ion of the aglycone, indicating the neutral loss of glucuronic acid. The insert shows the spectrum of the base peak ($[M^+H^+]^+$).

Contrary to the metabolites identified 2 h post-gavage, the detected metabolites in plasma and tissues after 18 h were mainly the aglycone (Fig. 6b). Total metabolites detected after 18 h in most tissues were only 1–5% of the levels detected after 2 h. However, the brain, lungs and heart still retained to 27, 20 and 11% relative to the total identified metabolites detected at 2 h, respectively. It has to be noted that this time-dependant changes in the concentration of naringenin aglycone and glucuronide might not apply to other unidentified metabolites, since the radioactive data obtained from plasma and tissue homogenates before extraction and evaporation revealed an increase in total counts at 18 h compared to 2 h. As revealed from the recovery experiments performed on radioactive plasma and tissue samples, these metabolites are likely to be small volatile breakdown molecules, lost from the extracts and thus could not be detected and identified.

The amounts of naringenin and its metabolites excreted in urine and faeces collected during

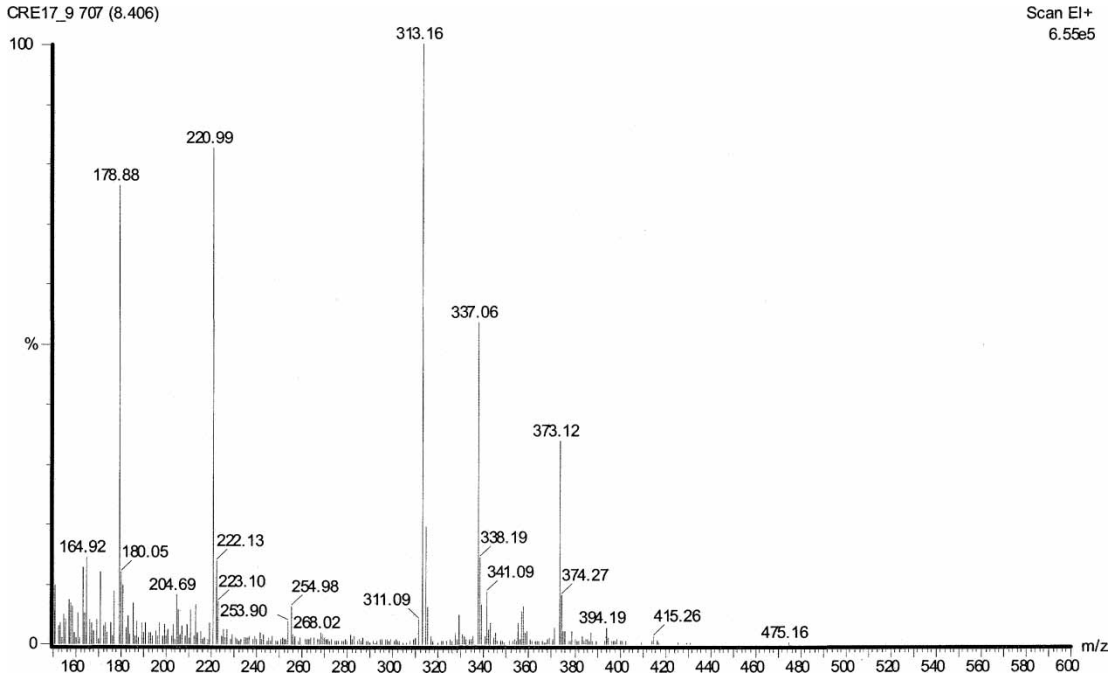

FIGURE 5 Mass spectrum of the derivatized derivatives of p-hydroxyphenyl propionic acid as detected in urine. The spectrum shows a signal at $m/z = 394$ (M^+) and a major fragment at $m/z = 337$, resulting from the loss of a *t*-butyl group of the derived compound.

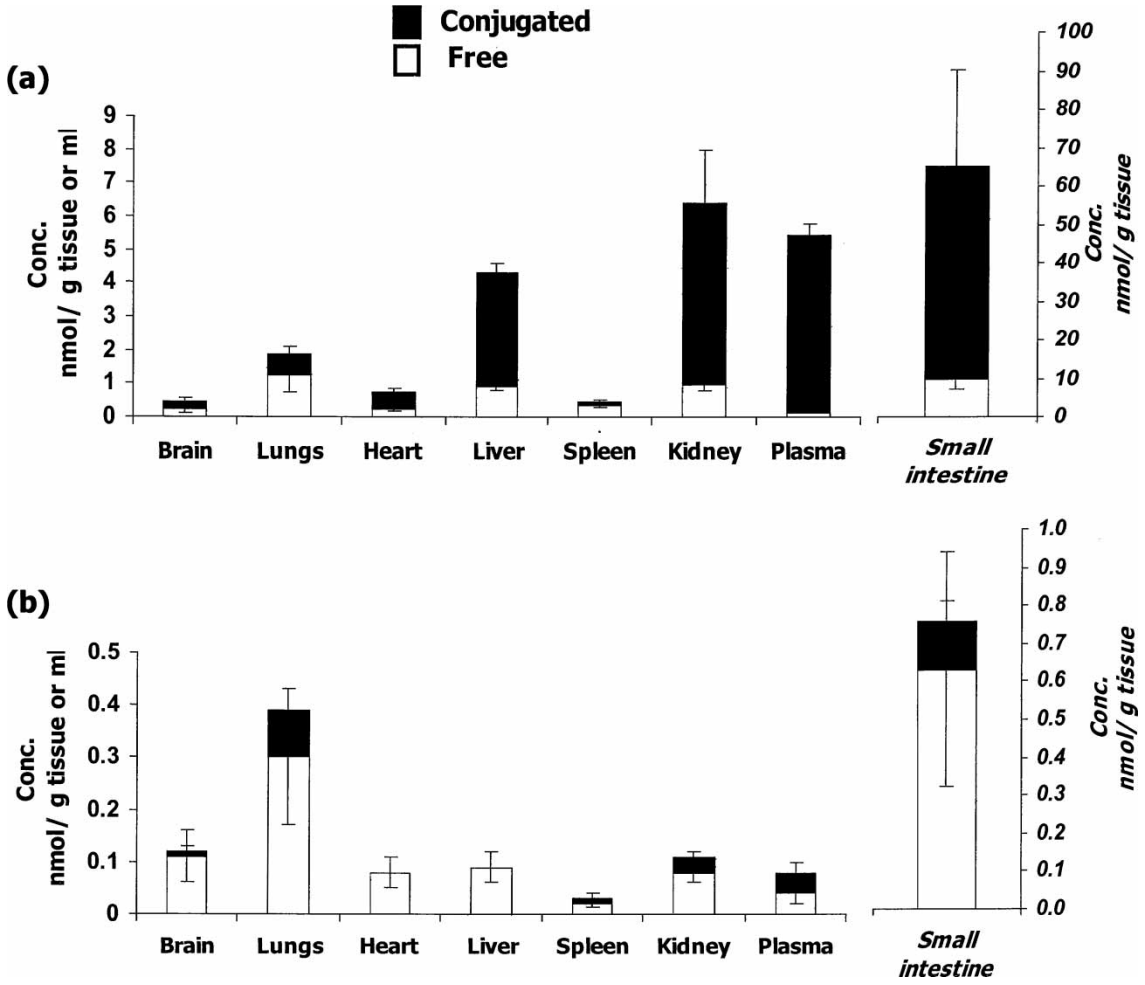

FIGURE 6 Concentrations of naringenin and its metabolites in plasma and tissues obtained at 2 h (A) and 18 h (B) after gastric gavage of 50 mg/kg naringenin. Panels show concentration of naringenin in its free and conjugated forms extracted and quantified by HPLC with PDA detection. Left scale indicates levels in all tissues while right scale (italics) shows levels in the small intestine. Values are expressed as mean ± SEM, $n = 6$.

the 18 h following gavage of naringenin are shown in Table II. High levels of naringenin metabolites, greater than 3 μmol, were detected in a total urine volume of 6.3 ml, compared to lower total amounts (0.5 μmol) in 7.3 g faeces. The results show, however, that urinary glucuronide excretion was approximately double that of free naringenin, but in faeces the naringenin excretion was more than double that of glucuronides. High levels of the phenolic acid *p*-HPPA were detected in both urine and faeces (0.48 and 0.31 μmol/18 h, respectively).

DISCUSSION

Much recent attention has been given to the biological activity of dietary flavonoids. Epidemiological studies have indicated a protective relationship between the consumption of citrus fruits or

TABLE II Excretion of naringenin and its metabolites 18 h after the oral administration of 50 mg/kg body weight of naringenin. Values are mean ± SEM, $n = 8$ rats

	Urine (ml) (6.3 ± 1.6)		Faeces (g) (7.3 ± 0.49)	
Excretion (μmol)	Pre-gavage	Post-gavage	Pre-gavage	Post-gavage
Total metabolites*	ND	3.13 ± 0.22	ND	0.33 ± 0.11
Naringenin	ND	0.88 ± 0.35	ND	0.13 ± 0.04
Naringenin glucuronides	ND	1.83 ± 0.26	ND	0.05 ± 0.02
p-Hydroxyphenyl propionic acid	0.06 ± 0.012	0.48 ± 0.09	0.14 ± 0.066	0.31 ± 0.08

ND, Non-detectable. *The total metabolites are calculated as the sum of (free naringenin + glucuronides + increase in p-hydroxybenzoic acid above control levels). Calculations were made on individual animal data not on mean values.

juices and the risk of ischemic stroke[24] and lung cancer.[25] Feeding of naringenin, a citrus flavanone, to rats was previously reported to result in high detectable levels in plasma, reaching concentrations of 40 μM under certain conditions.[25] However, little information on the distribution of naringenin and associated metabolites to tissues is available. Few studies have provided evidence for the localization of the citrus flavonoids, naringenin and hesperetin within the brain,[26,27] but only after intravenous injection and thus, are not representative of normal dietary consumption and the effects and consequences of gastrointestinal metabolism. The present study reports, for the first time, the uptake and tissue distribution of naringenin and its metabolites after oral administration of this flavanone.

The finding that the glucuronide conjugates constitute 98% of plasma metabolites is consistent with previous studies involving naringenin[28] or other flavonoids,[29] showing that glucuronidation is the first major metabolic step post-absorption. It is interesting that the plasma levels of metabolites, shown here reaching 5 μM at 2 h post-gavage of 50 mg/kg naringenin, are similar to plasma concentrations of naringenin in healthy human volunteers after ingestion of grape fruit juice.[30] The results also show that naringenin glucuronides are the main metabolites detected in urine collected for 18 h post-gavage, although *p*-HPPA, a colonic metabolite of naringenin, is also detected at high levels in urine. A previous study, using an *in vitro* model of the human colon, has demonstrated the formation of this phenolic acid by the activities of the intestinal microflora on naringenin.[31] Our results showing the appearance of this phenolic product in urine 18 h after gastric gavage supports the conclusion that ingested naringenin was hydrolysed by the colonic microflora, leading to the formation of easily absorbed flavonoid ring fission products.

The nature of metabolites in plasma suggests that the glucuronidated conjugates are the only metabolites likely to reach the tissues. Indeed, we found moderately high levels of naringenin glucuronides in liver, kidney, heart and brain. However, compared to the plasma, even though the glucuronides are the major metabolite, a higher ratio of naringenin aglycone to total metabolites is detected in the tissues. The likelihood that glucuronides enter tissue cells is low, owing to the increased polarity that reduces their ability to cross the lipid cell membrane. It is possible that naringenin glucuronides could be hydrolysed by tissue β-glucuronidase, resulting in the penetration of free naringenin into the tissues, followed by subsequent conjugation with glucuronic acid. This assumption is also plausible because of the presence of both UDP-glucuronosyltransferase and β-glucuronidase in close subcellular proximity within hepatocytes[32] and cells of other organs.[33,34] Our results have also shown a longer retention of the aglycone form compared to glucuronides over 18 h by most of the tissues, but mainly by the brain and lungs. It is probable that the higher rate of excretion of glucuronides is responsible for these differences. The idea that the aglycone form could be released from its glucuronide is supported by studies showing that beta-glucuronidase could be useful in tumour specific bio-activation of glucuronidated pro-drugs of anticancer agents.[35]

Our findings of the detection of radioactivity in all tissues under study, showed that the radioactivity associated with naringenin and its cleavage products was retained by the tissues extensively. However, autoradiography demonstrated the presence of radioactivity in blood vessels but not cells of these tissues. The marked absence of blood cells within blood vessels implies that radioactivity within vessels is due to its binding to the endothelial layer. Although radioactivity overlying cells was similar to background level, it cannot be conclusively stated that cell uptake of naringenin or its metabolites did not occur, since the amounts involved may be below the detection level of autoradiography. It may also be possible that cells possess mechanisms for exporting these substances back to the extracellular fluid or they may be retained extracellularly by binding to cell membrane components. In this context, it has been shown that naringenin, kaempferol and quercetin-3-*o*-β-D-glucoside bind to benzodiazepine binding sites of different receptors, including the GABA-A-receptor.[36] It has also been demonstrated that, despite no detectable cell uptake of hesperetin glucuronides by FEK4 fibroblasts, these conjugates were able to elicit cytoprotective effects against UVA-induced necrotic cell death.[19]

Similar amounts of radioactivity by the tissues and plasma, calculated as percentage of the given dose, was observed at the two very different gavaged doses of naringenin suggests a dose-dependent relationship between consumption levels of this flavanone and its tissue or body fluid level. A similar effect has also been demonstrated between plasma concentration of epicatechin and the amount of oral dose of pure compound given to rats[37] or the degree of consumption of procyanidin-rich chocolate by humans.[38]

While high levels of radioactivity were detected in the urine, implying its efficient excretion, plasma and tissue radioactivity levels at 18 h post-gavage did not decline compared to 2 h. The extent of this absorption, expressed by the exceptionally high levels of radioactivity incorporated into the tissues, is far greater than that may have been envisaged from studies applying unlabeled flavonoids. In accordance with our findings with the unlabelled naringenin, plasma kinetic studies of polyphenols have previously shown that peak levels of administered

compounds and their metabolites are often reached between 2 and 4 h following consumption, both in animals[39] and humans.[40] More specifically, the plasma concentration of naringenin metabolites was found to decline to its initial pre-consumption level 2 h following feeding of naringenin-enriched diet to rats[28] and 14 h following intake of orange juice by human subjects.[30] The contradiction between these reports and the results of the present study provide strong evidence for the existence of further unidentified metabolites of naringenin, which are either absorbed slowly or strongly retained by body organs. The use of [^{3}H]-naringenin allowed us to assess to what extent flavanones and their possible metabolites accumulate in tissues where they might be expected to exert their biological effects.

In conclusion, our findings show that 2 h after gastric gavage of naringenin to rats, glucuronide metabolites were the major components in plasma and tissues. After 18 h, total levels of the aglycone form and glucuronides in plasma and tissues were significantly lower, and the aglycone was predominant form detected. The colonic metabolite 3-(4-hydroxyphenyl) propionic acid was also eliminated after 18 h, particularly in the urine. Radioactive detection suggests increased levels of breakdown products of naringenin after 18 h compared to 2 h, in contrast to LC detection of unlabelled naringenin and its metabolites, suggesting that a substantial proportion of the breakdown products of naringenin at 18 h, are retained as smaller cleavage products which have not yet been identified. This indicates the importance of considering absorbed metabolic products formed at a later stage, possibly in the colon, when investigating the potential biological activity of flavonoids.

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