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RESEARCH ARTICLE

Anthocyanin-derived phenolic acids form glucuronides following simulated gastrointestinal digestion and microsomal glucuronidation

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Scope: Current research indicates that anthocyanins are primarily degraded to form phenolic acid products. However, no studies have yet demonstrated the metabolic conjugation of these anthocyanin-derived phenolic acids in humans.
Methods and results: Within the present study, a simulated gastrointestinal digestion model was used to evaluate the potential degradation of anthocyanins post-consumption. Subsequently, cyanidin (Cy) and pelargonidin and their degradation products, protocatechuic acid and 4-hydroxybenzoic acid, were incubated in the presence of human liver microsomes to assess their potential to form hepatic glucuronide conjugates. For structural conformation, phenolic glucuronides were chemically synthesised and compared to the microsomal metabolites. During the simulated gastric digestion, anthocyanin glycosides (200 μM) remained stable however their aglycone derivatives were significantly degraded (20% loss), while during subsequent pancreatic/intestinal digestion only pelargonidin-3-glucoside remained stable while cyanidin-3-glucoside (30% loss) and Cy and pelagonidin aglycones were significantly degraded (100% loss, respectively). Following microsomal metabolism, pelargonidin formed 4-hydroxybenzoic acid, which was further metabolised (65%) to form two additional glucuronide conjugates, while Cy formed protocatechuic acid, which was further metabolised (43%) to form three glucuronide conjugates.
Conclusions: We propose that following ingestion, anthocyanins may be found in the systemic circulation as free or conjugated phenolic acids, which should be a focus of future dietary interventions.

Keywords:
Anthocyanins / Glucuronide / Metabolism / Phenolic acids

1 Introduction

In recent years, it has been accepted that inflammation plays a central role in the development of chronic diseases. As such, novel pharmaceutical and dietary strategies are being developed to prevent the progression of chronic inflammatory conditions, such as atherosclerosis. Among these strategies, dietary polyphenols, including anthocyanins, have been shown to be associated with reduced chronic disease risk and mortality [1–3]. However, current evidence in the literature poorly describes the human metabolism of anthocyanins, with no information on the biological effects of their metabolites. Thus, a full appreciation of the health-

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Abbreviations: 4GBA, 4-(1-carboxyphenyl) β-D-glucuronic acid; **3OH4GBA,** 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid; **4OH3GBA,** 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid; **Cy,** cyanidin; **Cy-3-Glc,** cyanidin-3-glucoside; **equiv.,** equivalents; **GIT,** gastrointestinal tract; **HBA,** 4-hydroxybenzoic acid; **MeOH,** methanol; **PCA,** protocatechuic acid; **Pg,** pelargonidin; **Pg-3-Glc,** pelargonidin-3-glucoside; **UDP-GA,** 4-uridine 5′-diphospho-glucuronic acid; **UV,** ultraviolet

related benefits of ingested anthocyanins has yet to be achieved and requires a greater understanding of their metabolism and associated mechanisms of action.

It has been demonstrated that less than 1% of ingested anthocyanins are detectable in urine or plasma [4] and it has been suggested that anthocyanins form intermediate or end stage degradation products during digestion, absorption and metabolism that have yet to be sufficiently established *in vitro* or *in vivo* [5]. To this end, the anthocyanin degradation products, protocatechuic acid (PCA) and 4-hydroxybenzoic acid (HBA) [6, 7] (Fig. 1), have been identified in the plasma of rats following high dose consumption of cyanidin (Cy) and pelargonidin (Pg) (Fig. 1). Furthermore, data from a recent study by Vitaglione *et al.* explain the low systemic presence of anthocyanins in humans, as almost 73% of ingested Cy was accounted for by the formation of PCA.

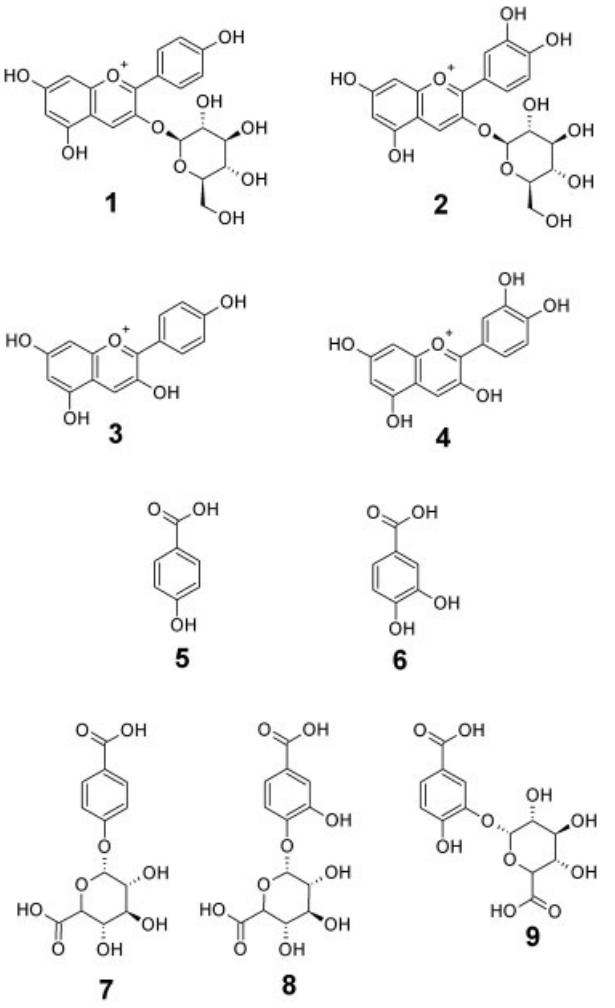

Figure 1. Chemical structures of anthocyanins and their potential metabolites. 1, pelargonidin-3-glucoside; 2, cyanidin-3-glucoside; 3, pelargonidin; 4, cyanidin; 5, 4-hydroxybenzoic acid; 6, protocatechuic acid; 7, 4-(1-carboxyphenyl) β-D-glucuronic acid; 8, 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid; 9, 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid.

This suggests that ingested anthocyanins are primarily available to the systemic circulation as phenolic acid derivatives. However, the fate of these derivatives remains unknown. Indeed, current opinion suggests that anthocyanins are likely metabolised to their phenolic acid constituents [5, 7–9]. We propose that the low recovery of anthocyanins post-consumption may not only be accounted for by the formation of free phenolic acids but also their conjugated metabolites following absorption and/or first pass metabolism. Human studies have yet to demonstrate the phase II conjugation of the anthocyanin-derived phenolic acids, PCA and HBA, but a recent study has reported the *in vivo* metabolic conjugation of PCA to monoglucuronide, methyl-monoglucuronide and methyl-monoglycine conjugates in rats [10].

In the current study, the potential degradation of anthocyanins (Pg, Cy, pelargonidin-3-glucoside (Pg-3-Glc) and cyanidin-3-glucoside (Cy-3-Glc)) during the early stages of gastrointestinal digestion was explored using a simulated *in vitro* gastrointestinal tract (GIT) digestion model. The potential for anthocyanin-derived degradation products, HBA and PCA, to undergo hepatic glucuronidation, both as free phenolic acids and as constituents of their precursor anthocyanidin structures was also evaluated. Furthermore, to assess the conjugation position of the phenolic acid metabolites, the glucuronide derivatives of PCA and HBA, namely 4-(1-carboxyphenyl) β-D-glucuronic acid (4GBA), 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid (3OH4GBA) and 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid (4OH3GBA) (Fig. 1), were chemically synthesised and compared to the biologically derived metabolites.

The focus of the present study was to establish the potential sites of metabolic conjugation of anthocyanins and their products of degradation. Therefore, the utilised experimental system was designed and optimised for maximal conjugation efficiency of each of the phenolic substrates. The system was not designed to mimic *in vivo* situations, but to establish potential targets for searching for biological metabolites of anthocyanins in future human trials and/or cell studies aimed at unravelling the mechanisms behind anthocyanins' proposed bioactivity.

2 Materials and methods

For metabolism and digestion studies, pooled human liver microsomes (male), pepsin, panceatin, bile extract, alamethicin, saccharolactone and 4-uridine 5′-diphosphoglucuronic acid trisodium salt (UDP-GA) were purchased from Sigma-Aldrich (UK). The phenolic compounds HBA, PCA, kaempferol, Cy chloride, Pg chloride, Cy-3-Glc chloride and Pg-3-Glc chloride were purchased from Extrasynthese (France) or Sigma-Aldrich. For chemical synthesis, methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosylurate bromide was purchased from Apollo Scientific (UK) and methyl 4-hydroxybenzoate and

methyl 3,4-dihydroxybenzoic acid was purchased from Alfa Aesar (UK). All water used was 18 MΩ/cm milliQ water and solvents were of HPLC grade.

2.1 Anthocyanin degradation during simulated gastrointestinal digestion

To assess the degradation of anthocyanins during gastrointestinal digestion, Cy-3-Glc, Pg-3-Glc, Cy and Pg were, individually, subjected to a simulated gastrointestinal digestion. Simulated digestions were based on previously established methods with modifications [11]. Briefly, to purify pepsin for gastric phase digestion, 0.1 g of pepsin was made up to 2.5 mL in 0.1 M HCl, added to 1.25 g Chelex-100 and shaken gently on a platform tabletop shaker (EMLI, Sky Line) for 30 min to remove any residual iron that may chelate the catechol anthocyanins. The solution was then poured into a 1.6-cm-diameter glass column (acid washed) and the eluent collected. A further 2.5 mL of 0.1 M HCl was added to the column and the filtrate collected. The final pepsin solution (filtrate) was 20 mg/mL pepsin. To purify pancreatin for duodenal (intestinal) digestion, 0.0125 g of pancreatin and 0.075 g of bile extract was made up to 6.25 mL with 0.1 M $NaHCO_3$ and shaken gently with 3.125 g Chelex-100 for 30 min. The pancreatin/bile solution was then poured into a 1.6-cm-diameter glass column to filter out the chelex. An additional 2.5 mL of 0.1 M $NaHCO_3$ was added to the column and the eluent collected. The final pancreatin/bile solution contained 1.4 mg/mL pancreatin and 8.6 mg/mL bile extract. Both enzyme solutions were stored at 4°C and used within 24 h.

To simulate gastric phase digestion, the individual anthocyanins (final concentrations of 200 μM) were added to a 140 mM NaCl, 5 mM KCl buffer solution (pH 2) containing 0.15 mL pepsin solution and 25 μg *trans*-cinnamic acid as an internal standard (3.15 mL final volume). The individual solutions were incubated at 37°C for 30 min on a platform oscillating tabletop shaker (EMLI, Sky Line) and at 0, 15 and 30 min, 500-μL aliquots were taken, acidified with 0.2 M HCl in ethanol (1:1 v/v final sample dilution) and stored at −80°C until HPLC analysis. To simulate duodenal (intestinal) digestion, the pH of the gastric phase digest was titrated to pH 6.7 by the addition of 1 M $NaHCO_3$, followed by the addition of 0.417 mL pancreatin/bile solution (0.6 mg pancreatin and 3.6 mg bile extract). The digest was made up to a final volume of 2.5 mL with 140 mM NaCl, 5 mM KCl buffer solution (pH 6.7, degassed under vacuum for 30 min prior to use) and incubated for 120 min at 37°C on a platform oscillating tabletop shaker. At 0, 60 and 120 min, 500-μL aliquots were removed from the incubation mixture and acidified with 0.2 M HCl in ethanol (1:1 v/v final sample dilution) and stored at −80°C until HPLC analysis. Control samples consisted of initial samples (0 min) spiked with 0.1 M HCl and stored at −80°C until HPLC analysis. Sample concentrations were normalised to the final incubation volumes for the gastric and intestinal phases respectively. For quantification, samples were thawed at room temperature, vortexed and centrifuged at 10 000 rpm for 20 min at room temperature and the supernatants analysed by HPLC.

2.2 Microsomal conjugation of anthocyanidins and phenolic acids

Microsomal conjugation was performed as previously described [12, 13] with some adjustments. Substrate concentration and incubation times were optimised for each respective compound (Cy, Pg, HBAs and PCA) to achieve optimal concentrations and chromatographic clarity of phenolic acid glucuronides. Briefly, within a 0.1 M potassium phosphate buffer (pH 7.4), 0.25 mg human liver microsomes and 12.5 μg alamethicin were vortexed and placed on ice for 15 min. $MgCl_2$ (1 mM), saccharolactone (5 mM) and the respective test compounds (1–2.5 mM) were added (as individual treatments) and each mixture was pre-incubated for 3 min at 37°C. To initiate the reaction, UDP-GA (5 mM) was added to give a final incubation volume of 0.1 mL. The reaction mixtures were then incubated at 37°C for 4–12 h. Control incubations were performed without the addition of UDP-GA. Incubations were also performed with 0.3 mM kaempferol under the above stated conditions as a reaction control, since its microsomal conjugation has previously been characterised. The reactions were stopped with the addition of 100 μL ice-cold 1% HCl in ACN. After equilibration on ice for 15 min, stopped reaction mixtures were centrifuged (13 000 rpm for 15 min at room temperature) to pellet precipitate proteins and the supernatants analysed by HPLC.

2.3 Chemical synthesis of phenolic acid glucuronides

In general, all solvents were dried over freshly activated 3 Å molecular sieves. Evaporations were performed *in vacuo* at 50°C and solids were dried overnight *in vacuo* over P_2O_5 before use. TLC was performed on Macherey-Nagel Silica Gel 60/UV254 plates using ultraviolet (UV) light, or 50% sulphuric acid and charring for visualisation. Flash chromatography used pre-packed silica cartridges (Isolute Flash Si, BiotageTechnologies) and UV detection. The final reaction products were characterised by ^{1}H NMR, run on a JEOL GX-400 spectrometer at room temperature.

2.3.1 3OH4GBA synthesis

Briefly, methyl 3,4-dihydroxybenzoate (3 g, 17.8 mmol) was stirred in acetic anhydride (50 mL) at room temperature and H_2SO_4 (5 mL) was added dropwise. After 40 min, the

mixture was poured into ice-water (250 mL) and extracted with ethylacetate (2 × 200 mL). The combined organic extracts were washed with water (2 × 100 mL) and dried ($MgSO_4$). Evaporation, followed by co-evaporation with toluene produced methyl 3,4-diacetoxybenzoate [^{1}H NMR: ($CDCl_3$) 2.30 (6H, s, 2 × $COCH_3$), 3.90 (3H, s, OCH_3), 7.29 (1H, d, J = 10.4 Hz, H-5), 7.87 (1H, d, J = 1.8 Hz, H-2), 7.94 (1H, dd, H-6)]. Subsequently, methyl 3,4-diacetoxybenzoate (1 g, 3.96 mmol) was dissolved in a mixture of THF (50 mL) and imidazolium hydrochloride (pH 7; 50 mL) at room temperature. After 30 min, the mixture was extracted with CH_2Cl_2 (2 × 200 mL) and the combined extracts were washed with water (100 mL), $NaHCO_3$ (100 mL) and water (100 mL), dried ($MgSO_4$) and evaporated. Purification by flash chromatography (100% CH_2Cl_2 to 5% MeOH in CH_2Cl_2) gave pure methyl 3-acetoxy-4-hydroxybenzoate [^{1}H NMR: ($CDCl_3$ 2.37 (3H, s, $COCH_3$), 3.87 (3H, s, OCH_3), 7.02 (1H, d, J = 8.6 Hz, H-5), 7.82 (1H, d, J = 1.8 Hz, H-2), 7.84 (1H, dd, H-6)]. Methyl 3-acetoxy-4-hydroxybenzoate (0.50 g, 2.38 mmol) was dissolved in dry CH_2Cl_2 (25 mL) under Ar gas at room temperature. Subsequently, powdered 3 Å molecular sieves (0.5 g), methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate bromide (1.04 g, 1.1 equivalents (equiv.)), silver (I) carbonate (0.72 g, 1.1 equiv.) and collidine (0.34 mL, 1.1 equiv.) were added to the methyl 3-acetoxy-4-hydroxybenzoate solution. The reaction was stirred for 7 days in the dark and then filtered through filter aid powder. The latter was washed with acetone (30 mL) and CH_2Cl_2 (30 mL) and the combined organics evaporated. The resulting red oil was dissolved in CH_2Cl_2 (50 mL) and washed with 1 M HCl aqueous (2 × 50 mL) and water (1 × 50 mL), and dried ($MgSO_4$). Flash chromatography (100% CH_2Cl_2 to 4% MeOH in CH_2Cl_2) produced 4-(1-methoxycarboxy-3-acetoxyhydroxyphenyl)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate methyl ester, which was de-protected as previously described [14, 15] and purified by preparative HPLC to yield 3OH4GBA [^{1}H NMR (dmso-d_6)– 3.34–3.40 (m, partially obscured by HOD peak, H-2′, H-3′, H-4′), 3.92(1H,d, J = 9.8 Hz, H-5′), 5.06 (1H, J = 7.3 Hz, 1H′), 7.09 (1H, d, J = 9.2 Hz, H-5), 7.36–7.38 (2H, m, H-2, H-6)].

2.3.2 4GBA synthesis

4GBA was synthesised as described previously [16] for the glucuronidation of 4-hydroxybenzaldehyde. Briefly, methyl 4-hydroxybenzoate (100 mg), methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosylurate bromide (234 mg, 1 equiv.), Ag_2O (200 mg, 2 equiv.) and ACN (0.66 mL) were stirred in the dark under Ar gas. After 16 h, the reaction product was purified by medium pressure LC (20 g silica, 60% ethylactetate/40% hexane isocratic elution) and de-protected as previously described [15]. The final product was precipitated in 50% MeOH aqueous at 4°C to yield 4GBA as a yellow powder [^{1}H NMR (dmso d_6)– [δH 7.89 (2H, d, J = 8.4 Hz, H-2, H-6), 7.09 (2H, d, J = 8.4 Hz, H-3, H-5), 4.97 (1H, d, J = 7.2 Hz, H-1′), 3.48 (1H, d, J = 10.0 Hz, H-5′), 3.15–3.40 (3H, m, H-2, H-3, H-4)].

2.3.3 4OH3GBA synthesis

The reaction precursor, methyl 3-benzyloxy-4-hydroxybenzoate was synthesised as previously described [14]. Subsequently, methyl 4-benzyloxy-3-hydroxybenzoate (100 mg), methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosylurate bromide (153 mg, 1 equiv.), ACN (1 mL) and Ag_2O (179 mg, 2 equiv.) were stirred continually for 24 h on ice under Ar gas. The mixture was then purified by medium pressure LC (20 g silica, 20% ethylactetate/80% hexane isocratic elution) and the main reaction product de-protected as previously described [14, 15]. Due to poor reaction yields, synthesised metabolite, 4OH3GBA was tentatively identified based on ESI-MS and UV–visible spectral data following comparison with biologically derived glucuronide conjugates.

2.4 HPLC conditions

HPLC analysis was performed on an Agilent 1100 series HPLC equipped with a DAD. Simulated GIT digestion samples were analysed on a 4 μm, 250 × 4.6 mm Synergi Max-RP reverse phase column (Phenomenex, Macclesfield, UK) with a SecurityGuard guard cartridge (Phenomenex AJO-6074) and microsmal samples on a 5 μm, 250 × 4.6 mm Lunar C18 column (Phenomenex) with a SecurityGuard guard cartridge (Phenomenex AJO-6074) at a column temperature of 37°C. The mobile phase consisted of 1% formic acid v/v in water (A) and 1% formic acid v/v in MeOH (B). The solvent gradient consisted of 2.5% B at 0 min, 25% B at 15 min, 45% B at 20 min and 100% B at 25–30 min for simulated GI digestion samples and 0% B at 0 min, 3% B at 4 min, 3% B at 10 min, 15% B at 20 min, 18% B at 25 min, 35% B at 30 min, 40% B at 35 min, 50% B at 40 min and 100% B at 45–50 min for microsomal samples. The flow rate was 1 mL/min and absorbance was recorded at 520, 360, 280 and 265 nm. Quantification and identification of known compounds was performed using authentic analytical standards.

2.5 ESI-MS and MS/MS conditions

Full scan LC/MS analysis of all microsomal samples and synthesised metabolites was conducted on an Agilent 1100 series LC/MSD SL single quadrupole mass spectrometer coupled to an Agilent 1100 series HPLC with DAD detector. Electrospray ionisation was performed in full scan mode (mass range: 100–1000 Da) with the following spray chamber conditions: drying gas flow 13 L/min; nebulizer pressure 50 psi; drying gas temperature 350°C. Negative mode ionisation was performed at a capillary voltage of −3000 V and a

fragmentor setting of 100. For further structural elucidation of a selected number of samples, MS/MS analysis was conducted on an Applied Biosystems 3200 Q-Trap LC/MS/MS system coupled to an Agilent 1200 series HPLC with DAD detector. Source parameters were as follows: electrospray flow rate 1 mL/min, source temperature 500°C, ion spray voltage −3500 V, curtain gas 10 psi, collision gas medium, ion source gas 1, 65 psi and ion source gas 2, 65 psi. For HBA glucuronide metabolites negative mode product ion scanning parameters were as follows: Q1 mass 313.3, declustering potential −61 V, entrance potential −4 V, collision energy −19 V and collision cell exit potential −2 V. For PCA glucuronide metabolites: Q1 mass 329.2, declustering potential −55 V, entrance potential −3 V, collision energy −18 V and collision cell exit potential −2 V. HPLC conditions were as previously described.

2.6 Statistical analysis

Simulated GIT digestion samples were compared by one-way analysis of variance with Tukey post-hoc tests (Windows SPSS, version 15) on concentration data derived from standard curves of authentic analytical standards. Significance was determined at $p<0.05$ across triplicate experiments.

3 Results

3.1 *In vitro* gastrointestinal digestion of anthocyanins

To assess the degradation of Pg-3-Glc, Cy-3-Glc, Pg and Cy during GIT digestion, the anthocyanins were exposed to simulated gastric and duodenal digestion (Figs. 2 and 3). During the simulated gastric digestion (0–30 min; Fig. 2A), no significant anthocyanin degradation was observed. However, significant loss of anthocyanin aglycones was observed following 15 min of gastric digestion ($p<0.05$; Fig. 3A). During post-gastric duodenal digestion (0–120 min), a significant loss of Cy-3-Glc was observed (Fig. 2B), while significant losses of both anthocyanin aglycones were observed across 0–120 min ($p<0.05$; Fig. 3B) with complete loss of the aglycones within 60 min of digestion. No phenolic acid degradation products were identified within the 120 min digestion.

3.2 Microsomal glucuronidation of anthocyanin-derived phenoic acids

To assess the potential for anthocyanins to form glucuronidated phenolic acid metabolites, Pg and Cy, and their reported degradation products, HBA and PCA, were incubated with human liver microsomes in the presence of UDP-GA. As a reaction control, kaempferol was subjected to the incubation conditions described for anthocyanins and phenolic acids. Following 30 min, kaempferol demonstrated complete conjugation to form two glucuronide metabolites in a manner consistent with previously reported data [13]. Following microsomal metabolism, Pg formed two phenolic acid metabolite peaks (Fig. 4A), which were identified as HBA (138 m/z) and its monoglucuronide metabolite **M1** (314 m/z; demonstrating a loss of 176 m/z under ESI-MS and MS/MS conditions). In addition, Cy formed one phenolic acid metabolite peak (Fig. 5B), which was identified as PCA (154 m/z). No PCA glucuronide metabolites were observed using UV–visible, however, LC/MS/MS analysis using product ion scanning parameters optimised

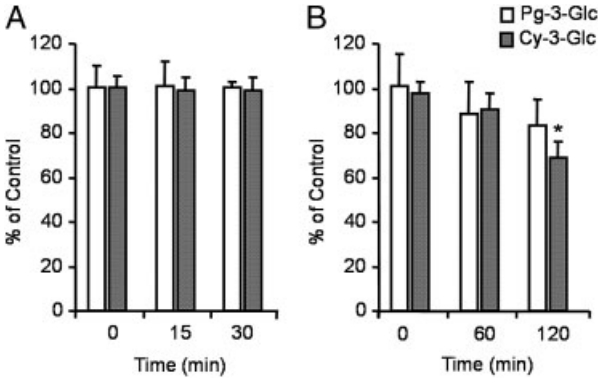

Figure 2. Anthocyanin (glycoside) loss during *in vitro* gastrointestinal digestion. (A) Gastric phase digestion in the presence of pepsin (pH 2). (B) Duodenal phase digestion in the presence of pancreatin and bile salts (pH 6.7). *Significance from control (initial anthocyanin spiked samples maintained at pH 2 (0.1 M HCl) throughout digestion; $t=0$; $p<0.05$). Pg-3-Glc, pelargonidin-3-glucoside; Cy-3-Glc, cyanidin-3-glucoside. Data represent means ± SD ($n=3$).

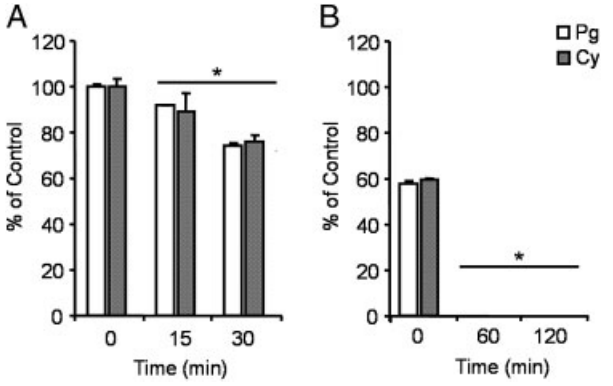

Figure 3. Anthocyanidin (aglycone) loss during *in vitro* gastrointestinal digestion. (A) Gastric phase digestion in the presence of pepsin (pH 2). (B) Duodenal phase digestion in the presence of pancreatin and bile salts (pH 6.7). *Significance from control (initial anthocyanin spiked samples maintained at pH 2 (0.1 M HCl) throughout digestion; $t=0$; $p<0.05$). Pg, pelargonidin; Cy, cyanidin. Data represent means ± SD ($n=3$).

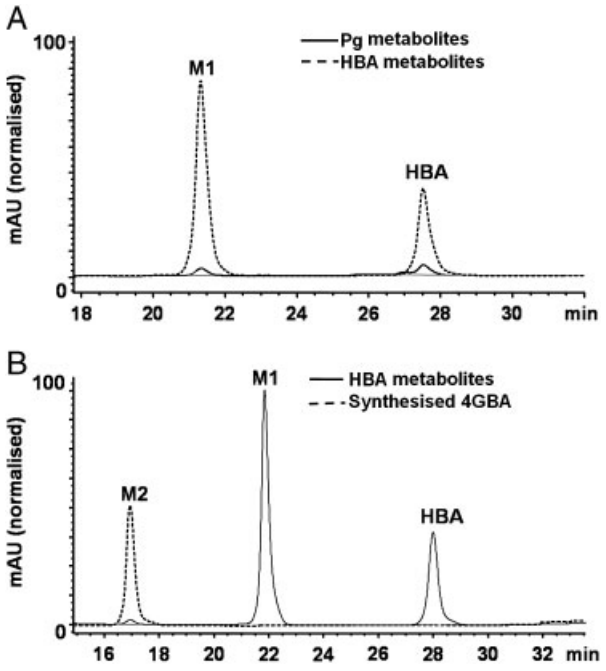

Figure 4. Representative chromatograms of pelargonidin and 4-hydroxybenzoic acid post incubation with human liver microsomes in the presence of UDP-GA. (A) Individual incubations with pelargonidin (Pg) or its degradation product 4-hydroxybenzoic acid (HBA) (with chromatograms overlaid). (B) Incubations with 4-hydroxybenzoic acid overlaid with the chromatogram of the chemically synthesised metabolite, 4-(1-carboxyphenyl) β-D-glucuronic acid (4GBA). M1 and M2 refers to microsomal metabolites 1 (4-hydroxybenzoyl glucuronide) and 2 (4GBA). For metabolite and synthetic standard spectral data, refer to Table 1.

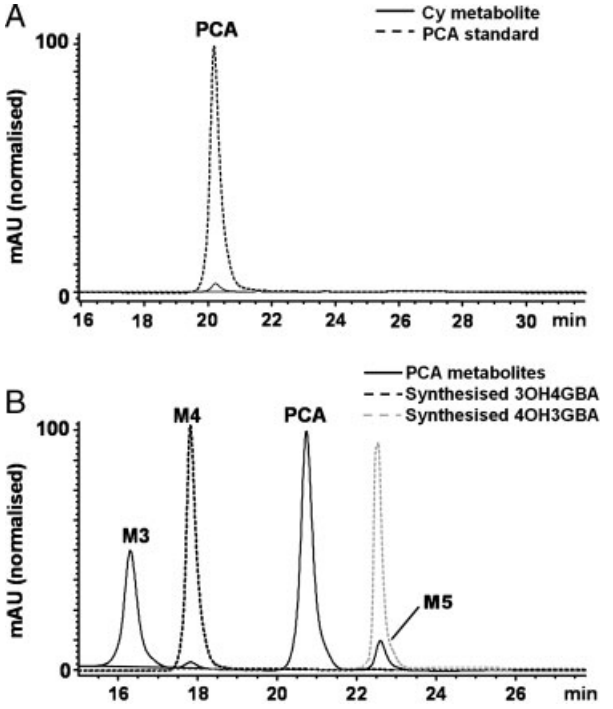

Figure 5. Representative chromatographs of cyanidin and protocatechuic acid post incubation with human liver microsomes in the presence of UDP-GA. (A) Incubation with cyanidin (Cy) overlaid with the chromatogram of the commercial standard protocatechuic acid (PCA). (B) Incubation with PCA overlaid with the chromatograms of the chemically synthesised metabolites, 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid (4OH3GBA) and 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid (3OH4GBA). M3-5 refers to microsomal metabolites 3 (protocatecuic acid-acyl-glucuronide), 4 (protocatechuic acid-3′-glucuronide) and 5 (protocatechuic acid-4′-glucuronide). For metabolite and synthetic or commercial standard spectral data, refer to Table 1.

for the synthesised standards revealed (based on MS^2 fragmentation patterns) the presence of multiple PCA glucuronides (Table 1; M4-7).

Following microsomal incubations, the Pg degradation product HBA (138 *m/z*) was metabolised to form two glucuronide conjugates (**M1** and **M2**; 314 *m/z*; Fig. 4B) which both demonstrated a loss of 176 *m/z* under ESI-MS and MS/MS conditions. It should be noted that the microsomal metabolite **M1** was observed during the incubation of both Pg and HBA (individually). PCA (154 *m/z*), when incubated in the presence of liver microsomes, was metabolised to form three glucuronide conjugates (**M3**, **M4** and **M5**; 330 *m/z*; Fig. 5B), which all demonstrated a loss of 176 *m/z* under ESI-MS and MS/MS conditions. The spectral and chromatographic data for metabolites **M1-5** are described in Table 1 and were confirmed using synthetic standards.

3.3 Structural elucidation of phenolic acid metabolites

To aid structural elucidation, the synthesised glucuronide conjugates of HBA and PCA were chemically synthesised and compared with the biologically derived anthocyanin and phenolic acid metabolites. It was confirmed that the synthesised metabolite 4GBA had the same retention time, UV maxima and mass fragmentation pattern as HBA's microsomal derived metabolite **M2** (Fig. 4B). Synthesised metabolites 3OH4GBA and 4OH3GBA were also confirmed to have the same retention time, UV maxima and mass fragmentation pattern as PCA's microsomal derived metabolites **M4** and **M5**, respectively (Fig. 5B).

4 Discussion

Although anthocyanins possess a number of beneficial bioactivities [17], their apparent systemic bioavailability has been shown to be very low [18]. Previous studies have suggested that this is likely due to spontaneous degradation under physiological conditions [19] or following microbial metabolism [8, 20]. In this study, the aim was to identify the

Table 1. Summery of HPLC-MS/MS characteristics of phenolic acid metabolites[a)]

Compound	Rt (min)	Mass ion spectrum $[M\text{-}H]^-$, (m/z)[b)]		UV maximum (nm)	Relative abundance[c)] (% peak area)	Identification[d)]
		Precursor	Fragment (product)			
Metabolites						
M1[e)]	21.8	313	193, 175, 137, 113	262	99±18	HBA-acyl-GlcA[f)]
M2[e)]	16.9	313	175, 137, 113	248	1±1	HBA-4′-GlcA
M3[e)]	16.3	329	193, 175, 153, 113	266, 298	81±3	PCA-acyl-GlcA[f)]
M4[e)]	17.8	329	193, 175, 153, 113	252, 292	3±0	PCA-3′-GlcA
M5[e)]	22.5	329	175, 153, 113	258, 288	15±0	PCA-4′-GlcA
M6[g)]	27.5	329	193, 175, 153, 113	–	–	Unknown
M7[g)]	28.8	329	193, 175, 153, 113	–	–	Unknown
Commercial Standards						
HBA	27.9	137	93, 75, 65	254	–	–
4GBA	16.9	313	175, 137, 113	248	–	–
PCA	20.7	153	109, 91, 65	260, 294	–	–
Synthesised Standards						
4OH3GBA	17.8	329	175, 153, 113	252, 292	–	–
3OH4GBA	22.5	329	175, 153, 113	258, 288	–	–

a) Spectral and chromatographic data for phenolic metabolites derived following microsomal incubations in the presence of UDP-GA.
b) Mass fragmentation patterns as determined by full scan MS analysis and confirmed by product ion MS/MS scanning (refer to Section 2).
c) Tentative abundance based on peak areas relative to controls.
d) Tentative identification based on comparisons with synthesised and commercial standards.
e) M1-5, microsomal metabolites 1–5 as illustrated in Figs. 4 and 5.
f) Metabolite identification based on plausible remaining conjugation site and indirect comparisons with aryl-synthetic standards and HPLC-MS/MS data.
g) Microsomal metabolites 6 and 7 were identified in cyanidin microsomal incubations based on product ion HPLC-MS/MS scans only. Rt, retention time; HBA, 4-hydroxybenzoic acid; 4GBA, synthesised 4-(1-carboxyphenyl) β-D-glucuronic acid; HBA-acyl-GlcA, acyl glucuronide of 4-hydrocybenzoic acid; HBA-4′-GlcA, 4′ glucuronide of 4-hydroxybenzoic acid; PCA, protocatechuic acid; 4OH3GBA, synthesised 3-(1-carboxy-4-hydroxyphenyl) β-D-glucuronic acid; 3OH4GBA, synthesised 4-(1-carboxy-3-hydroxyphenyl) β-D-glucuronic acid; PCA-acyl-GlcA, acyl glucuronide of protocatechuic acid; PCA-3′-GlcA, 3′ glucuronide of protocatechuic acid; PCA-4′-GlcA, 4′ glucuronide of protocatechuic acid; GlcA, glucuronic acid; UDP-GA, uridine 5′-diphosphoglucuronic acid.

plausible alternative occurrences of anthocyanins following metabolism and it was hypothesised that anthocyanins are degraded to form phenolic acids and subsequently conjugated *via* phase II metabolism.

Here it was observed that anthocyanins were stable during gastric digestion, but were degraded during intestinal digestion. Although this is consistent with previous studies [21–23], little account for phenolic acid formation or spontaneous degradation has been previously given. Importantly, the loss of anthocyanins and anthocyanidins during GIT digestion in this study did not result in the formation of their representative phenolic acid degradation products. This was likely due to the short incubation time (2 h) at neutral pH [19, 20], where we have previously described an initial lag in phenolic acid formation following anthocyanin degradation at neutral pH and 37°C; potentially as a result of the formation of relatively stable 'ring-open' transition products [19].

Degradation of anthocyanins was similar both in the presence and absence of digestive enzymes (experimental control data not shown), which was consistent with previous observations regarding anthocyanin degradation under simulated physiological conditions [19] and suggests that anthocyanin degradation during digestion occurred spontaneously. The extent to which anthocyanins are hydrolysed *in vivo* by the human intestinal brush border enzymes [24, 25]remains to be established; however, the efficient deglycosylation of Pg has been previously reported [26], suggesting significant degradation following deglycosylation should occur *in vivo*, which is consistent with our previous findings and those of others that indicate anthocyanin glycosides are more stable than their aglycones at neutral pH [19]. Although this study does not account for such *in vivo* intricacies as the hydrolytic potential of brush border enzymes or microbial metabolism, which would likely increase the rate of anthocyanin degradation due to increasing pH and the loss of the stabilising glycoside, it may be speculated that anthocyanins are likely to be degraded in the GIT prior to or during absorption.

This study demonstrated that both the degradation products of anthocyanins (Pg and Cy) HBA and PCA were efficiently metabolised by human liver microsomes to form monoglucuronide conjugates. Indeed, HBA was metabolised to form two monoglucuronide species and PCA was

metabolised to form three monoglucuronide species. This pattern of mono-glucuronidation is similar to that demonstrated by Cao *et al.* [10] in rats and is consistent with the number of free hydroxyl groups present on these phenolic structures. Comparison of the microsomal metabolites for HBA with the chemically synthesised glucuronides confirmed that metabolite **M2** was the 4′-glucuronide conjugate of HBA. This evidence suggests that Pg and its HBA metabolite **M1** is an acyl glucuronide. Similarly, comparison of the PCA microsomal metabolites with the chemically synthesised glucuronides confirmed that metabolites **M4** and **M5** were the 4′ and 3′ glucuronide conjugate of PCA. This indicates that the PCA metabolite **M3** is an acyl glucuronide. Interestingly, the fragmentation of these phenolic acyl glucuronides gives a characteristic mass fragmentation ion at 193 *m/z*, which may prove useful in distinguishing these conjugation sits (acyl *versus* aryl) in future human studies. Herein we provide evidence that suggests acyl and aryl mono-glucuronides of HBA and PCA may be common *in vivo* biological conjugates following anthocyanin consumption. Further research should be conducted to evaluate the extent to which acyl glucuronide products of anthocyanin-derived carboxylates are formed *in vivo*. Indeed, the occurrence of acyl glucuronides may be of particular biological interest, since they may cause covalent modifications to endogenous proteins and other macromolecules [27] and may impact pharmaceutical drug metabolism/bioactivity [27]. In addition, future work is needed to establish the sites of methyl and glycine conjugation as indicated in recent rodent studies [10]. Even though this study may establish metabolic targets for future human trials exploring anthocyanin absorption and bioavailability, and increase the number of targets for use in future cell studies, the bioavailability and pharmacokinetics of these compounds requires further research (*i.e.* human intervention).

It should be noted that the focus of the utilised experimental system was to establish potential sites of metabolic conjugation and therefore the methods were developed to produce a system optimised for conjugation efficiency of each of the phenolic substrates. Consequently, the results cannot be implied or translated relative to physiological events such as systemic absorption, bioavailability or excretion. Therefore, future research should aim to identify these metabolic targets *in vivo*, in order to determine their systemic absorption, bioavailability and excretion.

In conclusion, evidence is presented to suggest that anthocyanins can be degraded during gastrointestinal digestion and hepatic metabolism to form several monoglucuronide metabolites. Hence, it is proposed that anthocyanins may additionally be found in the systemic circulation as free or conjugated phenolic acids.

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5 References

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