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The effects of phytoestrogenic isoflavones on the formation and disposition of paracetamol sulfate in the isolated perfused rat liver

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Abstract

This study examines the potential for the phytoestrogenic isoflavones, a type of complementary medicine, to be involved in pharmacokinetic interactions in the liver. Rat livers were isolated and perfused to steady state, in single-pass mode, with either 5 μM paracetamol (n = 6), or 5 μM paracetamol with a 50:50 molar mixture of genistein and biochanin A or daidzein and formononetin, at a total isoflavone concentration of 1 and 10 μM (n = 6 for each mixture at each concentration). At 1 μM, neither isoflavone mixture had any effect, while at 10 μM both mixtures decreased the clearance of paracetamol and the formation clearance to paracetamol sulfate. Genistein and biochanin A (10 μM) also increased the biliary extraction of hepatically-generated paracetamol sulfate. Additional livers were perfused with an infusion of 5 μM ^{14}C-paracetamol in the absence (n = 4), or presence, of a 10 μM genistein and biochanin A mixture (n = 4). Analysis of washout perfusate and bile samples (up to 30 min after stopping the infusion) revealed that the isoflavones reduced the first-order rate constant for paracetamol sulfate transport into perfusate, but not for transport into bile. The results indicate that isoflavones can reduce the formation of paracetamol sulfate and that its enhanced excretion into bile arises from the inhibition of sinusoidal efflux transport.

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Introduction

Studies in the USA, UK and Australia report that 49–64% of people use complementary medicines (MacLennan et al 1996; Tsen et al 2000; Paltiel et al 2001; Standish et al 2001; Thomas et al 2001) and that this use is increasing (MacLennan et al 2002). It is probable that many of these people were also taking conventional drugs, and whenever complementary medicines and conventional drugs are concurrently administered, the potential for a pharmacokinetic interaction exists. A recent example of this is the effect of the extract of St John's wort to decrease the plasma concentration of drugs such as digoxin, HIV protease inhibitors and cyclosporin (Johne et al 1999; Piscitelli et al 2000; Barnes et al 2001), by decreasing the intestinal absorption or increasing the hepatic clearance of the drugs (Durr et al 2000; Roby et al 2000). However, despite the potential for complementary medicine–drug interactions to occur, therapeutic registering bodies of many countries do not require the completion of systematic drug interaction studies before complementary medicines become available over the counter for self-medication.

The phytoestrogenic isoflavones are structurally related to flavonoids in St John's wort and are administered as complementary medicines, with reported benefits including a reduction in the severity of menopausal symptoms and the prevention of cardiovascular disease, osteoporosis and reproductive system neoplasia (Knight & Eden 1996; Murkies et al 1998). The phytoestrogenic isoflavones include genistein and daidzein, which can be consumed in the diet, in foods such as soy and legumes (Hutchins et al 1995; Reinli & Block 1996), or in over-the-counter preparations for self-medication (Setchell et al 2001). In food, genistein and daidzein are predominantly

present as the corresponding glycosides, genistin and daidzin, with less as the methylated products biochanin A and formononetin (Barnes et al 1994; Reinli & Block 1996). The type, proportion and amount of the isoflavones in over-the-counter medications varies with the brand (Setchell et al 2001). When ingested, the isoflavone glycosides are hydrolysed to the corresponding aglycones in the intestine (Day et al 2000), and the aglycones and methylated derivatives are readily absorbed (Setchell et al 2001). The isoflavones are then subject to extensive first-pass metabolism by the liver (Sfakianos et al 1997), where isozymes of phenolsulfotransferase and uridinediphosphate-glucuronosyltransferase catalyse the formation of various sulfate and glucuronide conjugates, which can be recovered from bile and urine (Yasuda et al 1994, 1996; Jager et al 1997; Sfakianos et al 1997).

This study investigated the pharmacokinetic interaction potential of the phytoestrogenic isoflavones by examining the effects of mixtures of the phytoestrogenic isoflavones on the hepatic formation and transport of a sulfate metabolite. Paracetamol was chosen as the marker compound because it has a well-characterised hepatic disposition that is similar to the hepatic disposition of the isoflavones (Watari et al 1983) and at the chosen inflow paracetamol concentration of 5 μM the major metabolite of paracetamol formed in the rat isolated perfused liver (IPL) is paracetamol sulfate (Pang et al 1995).

Materials and Methods

Chemicals

Paracetamol, ^{14}C-paracetamol, paracetamol glucuronide, biochanin A, sodium taurocholate, sulfatase (type VI: from *Aerobacter aerogenes*), glucuronidase (EC 3.2.1.31) and tetrabutyl ammonium bromide were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Daidzein, formononetin and genistein were purchased from Indofine Chemical Company (Somerville, NJ). Paracetamol sulfate was synthesised following a previously published procedure (Fendler & Fendler 1968) with modifications (Williams et al 1983). The purity of paracetamol sulfate and paracetamol glucuronide was determined by quantifying the molar amount of paracetamol produced following enzyme hydrolysis. Water was purified with a Milli-R/Q purifier system (Millipore Australia Pty. Ltd, Victoria, Australia). All other chemicals were manufactured by Merck (Kilsyth, Victoria, Australia).

IPL preparation

The Animal Ethics Committee of the Institute of Medical and Veterinary Science, Adelaide, Australia, approved the study. Male Sprague-Dawley rats (200–350 g) were purchased from the Gilles Plains Animal Resource Centre, Adelaide.

Livers were perfused in-situ, at 37 °C, using surgical techniques and apparatus described previously (Evans & Shanahan 1995), allowing sampling from the cannulated bile duct and portal inflow and venous outflow cannulas. The liver perfusion medium consisted of freshly prepared, albumin- and erythrocyte-free Krebs-Henseleit buffer (containing 1.2 mM inorganic sulfate), supplemented with 16.5 mM glucose and 8.33 μM sodium taurocholate. This solution was filtered (0.45 μm), warmed to 37 °C, bubbled with humidified 95% O_2/5% CO_2 and adjusted to a pH of 7.4.

Liver viability was confirmed throughout each perfusion by measuring hepatic oxygen consumption (>10 μmol min^{-1}; 820 Dissolved Oxygen Meter; Orion, MA), bile flow rate (>5 μL min^{-1}, determined gravimetrically assuming a specific gravity of 1.0), the decrease in pH between inflow and outflow perfusate (>0.05 pH units), and by observing for changes in the gross appearance of the liver (Ross 1972).

Steady-state experiments

Thirty livers were each perfused for a 15-min equilibration period with drug-free perfusate and then for a further 50 min with perfusate containing 5 μM paracetamol (control, n = 6) or with 5 μM paracetamol plus a 50:50 molar mixture of genistein and biochanin A or daidzein and formononetin. The isoflavones were perfused at concentrations of 1 and 10 μM (n = 6 for each mixture at each concentration).

After the perfusion with paracetamol was commenced, inflow perfusate samples were collected from the reservoir at 20, 40 and 50 min and venous outflow perfusate samples at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 min. Bile samples were collected in 5-min periods throughout the perfusion. All samples were stored at −20 °C until analysed.

Loading-washout experiments

In additional experiments in eight livers, paracetamol (5 μCi ^{14}C-paracetamol/liver) was infused into the portal vein catheter using a syringe pump, to generate an inflow paracetamol concentration of 5 μM. At the same time, the inflowing perfusate reservoir was changed to one containing either drug-free perfusate (n = 4), or perfusate containing genistein and biochanin A in a 50:50 molar ratio with a total isoflavone concentration of 10 μM (n = 4).

Samples of outflow perfusate were collected at 0, 5, 10, 15, 20, 25, 30, 35 and 40 min after starting the paracetamol infusion. The paracetamol infusion was stopped after 40 min and outflow perfusate samples were collected at 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 15, 20 and 30 min thereafter. Bile was collected in 5-min periods throughout the experimental period. At the end of the washout phase, the catheter was removed from the portal vein and the syringe pump was restarted. After 3 min, three samples (1.5 mL) of inflow perfusate were collected directly from the catheter. All samples were stored at −20 °C until analysed.

Sample analysis

Paracetamol, paracetamol sulfate and paracetamol glucuronide were simultaneously quantified in perfusate and

bile by reversed-phase ion-pair HPLC, based on a previously reported method (Kamali & Herd 1990). Perfusate (30 μL) was injected directly, while bile was diluted (1:50) in water and 150 μL injected onto an Alltima C_{18} 5-μm pre-column (Alltech, Deerfield, IL) followed by an Econosil C_8 10-μm 250 mm × 4.6 mm column (Alltech, Deerfield, IL). Paracetamol, paracetamol sulfate and paracetamol glucuronide were eluted with a mobile phase of 5% methanol, 4% glacial acetic acid and 6 μM tetrabutyl ammonium bromide for perfusate, or 2.5% methanol, 2.5% glacial acetic acid and 6 μM tetrabutyl ammonium bromide for bile, both at a flow rate of 0.7 mL min^{-1}. The HPLC system consisted of a SIL-10A auto injector, LC-10ADVP pump, SCL-10A system controller, SPD-10AV spectrophotometric detector and C-R5A chromatopac integrator (Shimadzu, Kyoto, Japan). UV absorbance was measured at 254 nm and typical retention times for paracetamol sulfate, paracetamol glucuronide and paracetamol in perfusate were 8, 10 and 15 min, respectively; corresponding times for the bile assay were 11, 13 and 21 min.

The limit of quantification in perfusate was 0.4 μM for paracetamol sulfate and 0.5 μM for paracetamol glucuronide and paracetamol, while in undiluted bile the limit was 7.9 μM for paracetamol sulfate, 10 μM for paracetamol glucuronide and 5 μM for paracetamol. From repeated analysis of the high and low quality control samples, the accuracy for each compound was within 10% of the nominal values and the precision was 8%.

For the analysis of radiolabelled paracetamol and metabolites (loading–washout experiments), the outflow from the HPLC was diverted to a fraction collector (FRAC-100; Pharmacia Biotech, Sweden) and collected into scintillation vials, with the sample collection times corresponding to the retention times of paracetamol, paracetamol sulfate and paracetamol glucuronide. The eluent in scintillation vials was mixed with 3 mL aqueous counting scintillant (Amersham Corporation, Arlington Heights, IL) and counted on a liquid scintillation counter (Tri-Carb 2200CA; Packard Instrument Company, Downers Grove, IL).

Data analysis

Steady-state experiments

Steady state corresponds to the time at which the rates of recovery of the analytes in both perfusate and bile were stable. Concentrations at steady state were calculated as the arithmetic mean concentration from samples collected during this period.

The hepatic extraction ratio of paracetamol (E) was calculated as:

$$E = 1 - (C_{out}/C_{in}) \quad (1)$$

where C_{in} and C_{out} are the concentrations of paracetamol in inflow and outflow perfusate at steady state, respectively.

The clearance of paracetamol (CL) was the product of the hepatic extraction ratio and the perfusate flow rate. The rate of formation of a paracetamol metabolite ($R_f^{Metabolite}$) was assumed to be the sum of the rate of recovery of that metabolite in perfusate ($R_{perf}^{Metabolite}$) and bile ($R_{bile}^{Metabolite}$) at steady state. The formation clearance to a paracetamol metabolite ($CL_f^{Metabolite}$) was calculated by dividing its rate of formation by the inflow concentration of paracetamol. The biliary extraction ratio of a hepatically-generated metabolite ($E_B^{Metabolite}$) was calculated as the rate of recovery of the metabolite in bile divided by its rate of formation. The mass balance was taken to be the total rate of recovery of paracetamol, paracetamol sulfate and paracetamol glucuronide in outflow perfusate and bile at steady state, relative to the inflow rate of paracetamol.

Loading–washout experiments

The following assumptions were made during data analysis and modelling.

The hepatic uptake of paracetamol was flow limited. This assumption is supported by multiple-indicator dilution experiments in the IPL (Pang et al 1995).

Paracetamol and its metabolites were unbound in perfusate (fraction unbound, $f_u = 1$). This assumption was supported by the use of albumin-free perfusion medium in a single-pass mode.

Paracetamol metabolites formed in the liver were either excreted into bile or appeared in outflow perfusate, and did not undergo further metabolism, hydrolysis to paracetamol or irreversible sequestration within the hepatocytes. This assumption is supported by previous observations (Studenberg & Brouwer 1993).

The vascular space of the perfused liver acts as a well-stirred compartment for paracetamol and paracetamol sulfate.

Once transported into the vascular space, paracetamol sulfate did not re-enter downstream hepatocytes and therefore $k_{12} = 0$. This assumption is supported by multiple-indicator dilution experiments with paracetamol sulfate in the IPL (Goresky et al 1992).

Production of paracetamol sulfate ceased immediately upon stopping the paracetamol infusion. This assumption is supported by the immediate monoexponential decrease in the concentration of paracetamol sulfate in outflow perfusate on stopping the infusion pump. Furthermore, given that paracetamol in the vascular space was in equilibrium with that in the liver (as assumed earlier), the rapid decrease in the outflow perfusate paracetamol concentration to undetectable concentrations (see Figure 4) indicates that the concentration of paracetamol in the liver also rapidly declined to undetectable concentrations.

Linear pharmacokinetics for paracetamol existed over the concentration range in the study. This assumption was based on the results of preliminary perfusion experiments, where similar steady-state pharmacokinetic parameters were determined for livers perfused with 10 μM paracetamol.

Based on these assumptions, a two-compartment venous equilibration model was proposed (Figure 1).

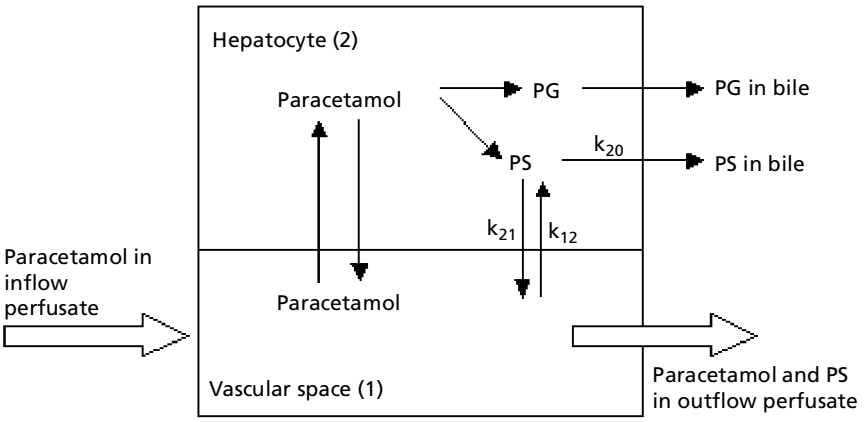

Figure 1 Proposed model for the hepatic disposition of paracetamol and its sulfate (PS) and glucuronide (PG) metabolites, based on the results of steady-state and loading–washout IPL experiments. k_{20} is the rate constant for PS transport from the hepatocyte into bile, k_{21} is the rate constant for PS transport from the hepatocyte into the vascular space and k_{12} is the rate constant for PS transport from the vascular space into the hepatocyte.

Using a similar model to that depicted in Figure 1 and the assumptions listed above, the following equations have previously been derived (Evans & Shanahan 1995):

$$R_{perf}{}^{PS}/R_{bile}{}^{PS} = k_{21}/k_{20} \quad (2)$$

$$\lambda_Z{}^{PS} = k_{21} + k_{20} \quad (3)$$

Since the relative rates of appearance of paracetamol sulfate (PS) in perfusate and bile can be accurately quantified at steady state and the terminal rate constant ($\lambda_Z{}^{PS}$) can be determined from the terminal slope of the natural log transformed concentration-versus-time profile for paracetamol sulfate in outflow perfusate during the washout phase, k_{21} and k_{20} (k_{20} is the rate constant for paracetamol sulfate transport from the hepatocyte into bile and k_{21} is the rate constant for paracetamol sulfate transport from the hepatocyte into the vascular space) can be determined (Evans & Shanahan 1995).

Statistical methods

Data are presented as arithmetic mean ± s.d. For the steady-state experiments, paired *t*-tests were used, within each experimental group, to compare the bile flow rate during the equilibrium phase with the value at steady state. Analysis of variance (SPSS for Windows 10.0.5, Chicago, IL) was used to test for differences among the experimental groups in bile flow and pharmacokinetic parameters at steady state. Where significant differences were identified, post-hoc analysis was performed using the least significant differences test.

For the loading–washout experiments, unpaired *t*-tests were used to test for differences in pharmacokinetic parameters between the two experimental groups.

In all cases, $P < 0.05$ was considered statistically significant.

Results

In all perfusions, the parameters reflecting liver viability remained within acceptance limits over the course of each perfusion. In the steady-state experiments, there was no difference in bile flow rate in control livers between initial (equilibration period) values and those at steady state. In the experiments with 1 μM isoflavone mixtures, there was no difference in the bile flow rate between the initial period values and those at steady state and there was no difference in the steady-state bile flow rate between these groups and the control group (Table 1). In contrast, the steady-state bile flow rate in livers perfused with the 10 μM isoflavone mixtures was higher than the initial bile flow rate in the same livers, and was also higher than the steady-state bile flow rate in the control livers (Table 1).

Paracetamol and paracetamol sulfate were recovered in outflow perfusate, and paracetamol sulfate and paracetamol glucuronide were recovered in bile; however, paracetamol glucuronide was not detectable in outflow perfusate and paracetamol was not detectable in bile. From 35 min after the start of the perfusion with paracetamol, the rate of recovery of paracetamol and paracetamol sulfate in perfusate, and paracetamol sulfate and paracetamol glucuronide in bile, was stable in all five experimental groups. Figures 2 and 3 show the data collected at 40, 45 and 50 min in perfusate and 35–50 min in bile, respectively, that were used to calculate pharmacokinetic parameters. In the control group, the liver cleared 43% of the paracetamol from the inflowing perfusate, almost all of which was accounted for by the formation of paracetamol sulfate (Table 1). The formation clearance to paracetamol sulfate was around 50 times greater than the formation clearance to paracetamol glucuronide and, as indicated by the small biliary extraction ratio for paracetamol sulfate, the majority of the paracetamol sulfate formed was recovered in outflow perfusate (Table 1).

Table 1 Hepatic viability and steady-state pharmacokinetic parameters for paracetamol and its metabolites (paracetamol sulfate (PS), paracetamol glucuronide (PG)) at an inflow paracetamol concentration of 5 μM.

	Control	+1 μM genistein/ biochanin A	+1 μM daidzein/ formononetin	+10 μM genistein/ biochanin A	+10 μM daidzein/ formononetin
Initial bile flow rate (μL min^{-1})	8.7 (1.8)	8.8 (2.0)	10.9 (2.7)	9.1 (1.1)	9.2 (1.8)
Steady-state bile flow rate (μL min^{-1})	8.0 (0.2)	8.0 (1.2)	9.4 (1.7)	11.3 (1.5)*†	11.8 (2.5)*†
E	0.43 (0.03)	0.40 (0.09)	0.37 (0.05)	0.22 (0.03)†	0.23 (0.09)†
CL (mL min^{-1})	12.8 (0.9)	12.0 (2.6)	11.0 (1.5)	6.6 (0.8)†	6.9 (2.6)†
E_B^{PS}	0.024 (0.006)	0.022 (0.002)	0.022 (0.004)	0.032 (0.007)†	0.030 (0.008)††
CL_f^{PS} (mL min^{-1})	12.2 (1.2)	11.5 (2.8)	10.3 (1.2)	6.7 (0.9)†	6.9 (2.7)†
CL_f^{PG} (mL min^{-1})	0.23 (0.04)	0.18 (0.05)	0.26 (0.07)	0.17 (0.04)	0.28 (0.18)
Mass balance (%)	98.8 (2.0)	98.9 (4.5)	98.7 (2.0)	101.0 (1.9)	101.2 (3.7)

Values are means (± s.d.). E is the hepatic extraction ratio of paracetamol, CL is the clearance of paracetamol, $E_B^{Metabolite}$ is the biliary extraction ratio of hepatically-generated metabolite and $CL_f^{Metabolite}$ is the formation clearance to a paracetamol metabolite. *$P < 0.05$, when compared with the initial bile flow rate for that experimental group; †$P < 0.05$, ††$P = 0.14$, when compared with control.

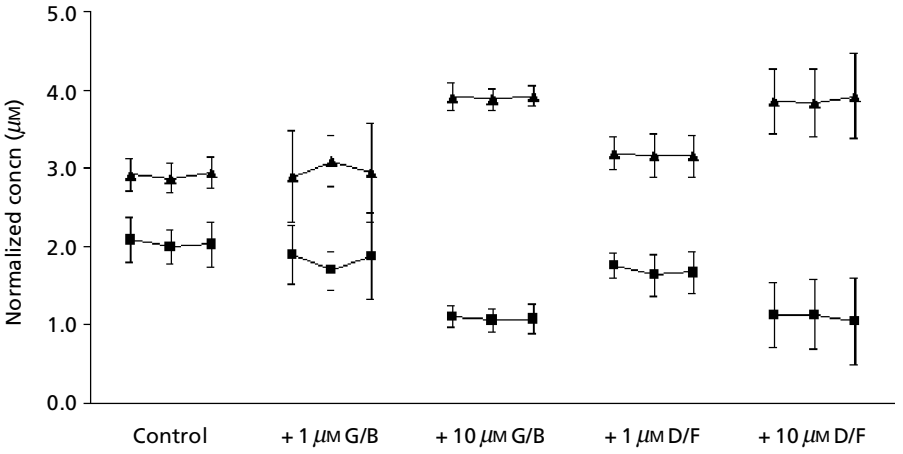

Figure 2 Mean concentration (± s.d.) of paracetamol (triangle) and PS (square) in outflow perfusate samples collected at 40, 45 and 50 min after starting the perfusion with paracetamol, when the rat liver was perfused in single-pass mode with albumin- and erythrocyte-free Krebs-Henseleit buffer. G/B = mixture of genistein and biochanin A; D/F = mixture of daidzein and formononetin. Outflow perfusate concentrations from each liver were normalized to the corresponding inflow paracetamol concentration of 5 μM.

At 1 μM, the isoflavones had no significant effect on any of the calculated steady-state pharmacokinetic parameters for paracetamol and its metabolites (Table 1). At 10 μM, both mixtures of isoflavones significantly decreased the hepatic clearance of paracetamol and the formation clearance to paracetamol sulfate, but only the mixture of genistein and biochanin A increased the biliary extraction ratio of paracetamol sulfate (Table 1). The isoflavones had no significant effect on the formation clearance to paracetamol glucuronide (Table 1).

In the loading–washout experiments, the rate of recovery of paracetamol and paracetamol sulfate in perfusate and bile were stable 25 min after the start of the paracetamol infusion. After the paracetamol infusion was stopped, paracetamol could be quantified in washout perfusate for 2 min and paracetamol sulfate in washout perfusate and bile for 10 and 25 min, respectively (Figures 4 and 5). When compared with control, the mixture of 10 μM genistein and biochanin A had no effect on the terminal rate constant for paracetamol in perfusate (1.49 ± 0.34 vs 1.50 ± 0.57 min^{-1}, $P = 0.98$) or k_{20} (0.0067 ± 0.0013 vs 0.0062 ± 0.0028 min^{-1}, $P = 0.78$), but did result in a decrease in k_{21} (0.277 ± 0.028 vs 0.210 ± 0.033 min^{-1}, $P < 0.05$).

Discussion

This study was designed to investigate the effects of phytoestrogenic isoflavones on the hepatic formation and transport of a sulfate metabolite. Although it is

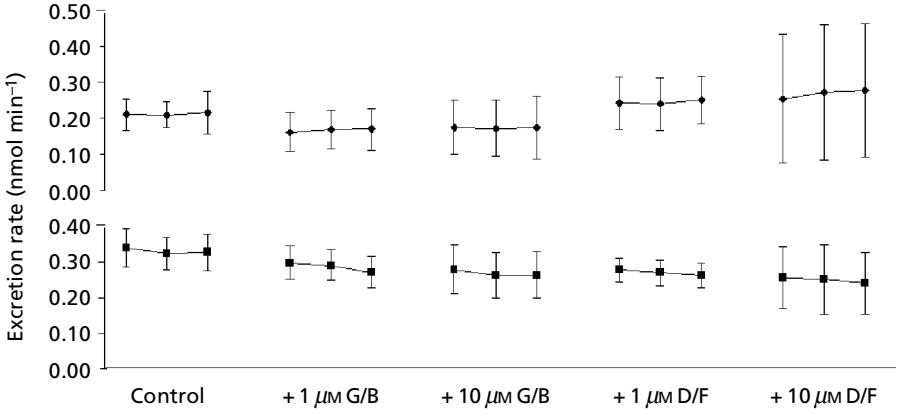

Figure 3 Mean biliary excretion rate (± s.d.) of paracetamol sulfate (square) and paracetamol glucuronide (diamond) in 5-min intervals from 35–50 min after commencing perfusion of the rat liver in single-pass mode with 5 μM paracetamol in an albumin- and erythrocyte-free Krebs-Henseleit buffer. G/B = mixture of genistein and biochanin A; D/F = mixture of daidzein and formononetin.

acknowledged that clinically relevant plasma concentrations of paracetamol are in the millimolar range (Howie et al 1977), the choice of 5 μM as an inflow concentration is justified because, at this concentration, the formation and transport of the sulfate metabolite of paracetamol can be studied with minimal interference from other paracetamol metabolites (Pang et al 1995).

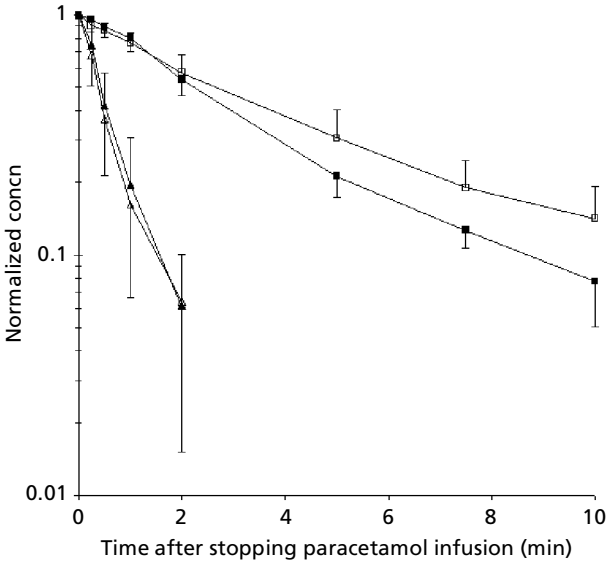

Figure 4 Mean normalized concentration (± s.d.) of paracetamol (triangle) and paracetamol sulfate (square) in outflow perfusate samples collected during the washout phase of the loading–washout experiments, after the rat liver was perfused in single-pass mode with 5 μM ^{14}C-paracetamol in albumin- and erythrocyte-free Krebs-Henseleit buffer in the absence (closed symbols) or presence (open symbols) of a 10 μM mixture of genistein and biochanin A. Outflow perfusate concentrations are normalized to the outflow concentration of that compound at steady state.

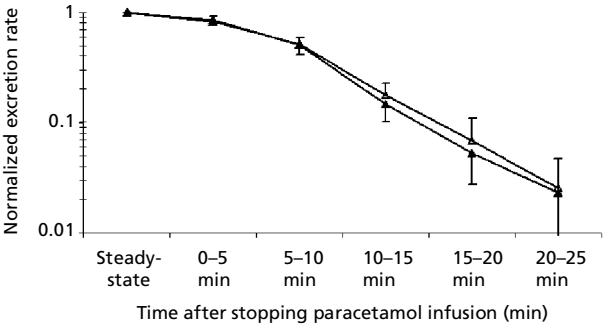

Figure 5 Mean normalized biliary excretion rate (± s.d.) of paracetamol sulfate during the washout phase of the loading–washout experiments, when the liver was perfused in single-pass mode with 5 μM ^{14}C-paracetamol in albumin- and erythrocyte-free Krebs-Henseleit buffer in the absence (closed symbols) or presence (open symbols) of a 10 μM mixture of genistein and biochanin A. Excretion rate is normalized to the excretion rate at steady state.

The total concentrations of isoflavone combinations (1 and 10 μM) were chosen because the maximum peripheral plasma genistein and daidzein concentrations in man after isoflavone supplementation are within the range 1–4 μM (King & Bursill 1998; Setchell et al 2001) and, with extensive hepatic extraction and enterohepatic cycling of the isoflavones (Sfakianos et al 1997), their concentration in portal blood is likely to be substantially greater than those in peripheral venous plasma.

The concentration-dependent increase in bile flow is consistent with a previous report where genistein increased the bile flow in the IPL, with half maximal stimulation at an inflow concentration of 4 μM (Jager et al 1997). The increase in bile flow has been linked to the transport of genistein metabolites into bile by the bile canalicular multispecific organic anion transporter, cMOAT (Jager et al 1997). Steady-state pharmacokinetic parameters in the control group (Table 1) are comparable with values in a previous report that used a similar inflow

paracetamol concentration in a similar perfusion medium (Pang et al 1995). Notably, almost all of the paracetamol cleared by the liver was accounted for by the formation of the sulfate metabolite and the majority of the paracetamol sulfate thus formed was recovered in outflow perfusate (97.6%).

Based on the proposed hepatic disposition model (Figure 1), the decrease in hepatic clearance of paracetamol and formation clearance to paracetamol sulfate during co-perfusion with 10 μM isoflavone mixtures could be due to inhibition of the sinusoidal uptake of paracetamol or to a reduction in the formation of paracetamol sulfate, or both. Multiple indicator dilution experiments with the IPL have shown that the sinusoidal membrane of the hepatocyte poses no substantial barrier to paracetamol (Pang et al 1995) and, given that the isoflavones in this study had no significant effect on the slope of the normalized washout profile for paracetamol, it can be concluded that the decrease in the hepatic clearance of paracetamol was not due to a decrease in the sinusoidal uptake of paracetamol.

The formation of paracetamol sulfate is mediated by phenolsulfotransferase enzymes (Falany 1991; Yasuda et al 1994, 1996) and requires 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as co-substrate (Klaassen & Boles 1997). A previous study found that formation of *p*-nitrophenol sulfate by a partially purified, P-form phenolsulfotransferase enzyme was inhibited by genistein (Eaton et al 1996) and since that same partially purified enzyme also metabolised paracetamol to paracetamol sulfate, it may therefore be reasonable to conclude that the isoflavones could also inhibit the formation of paracetamol sulfate by this enzyme. PAPS is synthesised from intracellular inorganic sulfate (Klaassen & Boles 1997), which is replenished from inflow perfusate to the IPL (Reinke et al 1981). The total rate of sulfation in this study equals the rate of sulfation of paracetamol (350 nmol (g liver)$^{-1}$ h^{-1}) and the isoflavones (approximately 22 nmol (g liver)$^{-1}$ h^{-1} (Jager et al 1997)) and given that the present inflow concentration of inorganic sulfate (1.2 mM) should support a sulfation rate of at least 580 nmol (g liver)$^{-1}$ h^{-1} (Bracht et al 1981; Reinke et al 1981), it is unlikely that the supply of inorganic sulfate in this study was rate limiting the formation of PAPS. It can therefore be concluded that the decrease in hepatic clearance of paracetamol and formation clearance to paracetamol sulfate was probably mediated by direct inhibition of phenolsulfotransferase by the isoflavones.

The sinusoidal and bile canalicular membrane transport of paracetamol sulfate is probably carrier mediated (Goresky et al 1992) and therefore the increase in the biliary extraction ratio of paracetamol sulfate during co-perfusion with the mixture of genistein and biochanin A (Table 1) could have been caused by inhibition of the sinusoidal efflux of paracetamol sulfate, stimulation of its bile canalicular transport, or both. However, the results of the loading–washout experiments demonstrated that the isoflavones had no effect on the bile canalicular transport of paracetamol sulfate, from which it may be concluded that the increase in biliary extraction ratio mediated by the mixture of genistein and biochanin A was due solely to inhibition of the sinusoidal efflux of paracetamol sulfate.

A number of sinusoidal efflux transporters have been identified (Hirohashi et al 1998; Li et al 1998), but, to the authors' knowledge, the transporter for paracetamol sulfate has not been established—although it is known that paracetamol sulfate is not a substrate for MRP3 (Xiong et al 2000). The major metabolite of genistein in the IPL is genistein glucuronide and, given that this is the only metabolite of genistein recovered in outflow perfusate (Jager et al 1997), it is possible that it competes with paracetamol sulfate for efflux transport into perfusate. It has previously been shown that genistein metabolites are predominantly transported into bile by cMOAT (Jager et al 1997) and, since paracetamol sulfate is thought not to be a substrate for cMOAT (Xiong et al 2000), it is not surprising that the 10 μM mixture of genistein and biochanin A did not affect the bile canalicular transport of paracetamol sulfate in this study.

Conclusion

In summary, this study confirms the potential for the phytoestrogenic isoflavones to be involved in pharmacokinetic interactions in the liver, by showing that they reduced the hepatic formation, and enhanced the biliary excretion, of a sulfate metabolite of a model substrate, paracetamol. Although it is proposed that these effects were mediated by inhibition of the phenolsulfotransferase enzyme that forms paracetamol sulfate from paracetamol and by inhibition of the carrier-mediated sinusoidal efflux transport of paracetamol sulfate, further studies are required to fully characterise the exact mechanisms of these observed effects, possibly by using hepatic microsome and hepatocyte isolated membrane models. Future studies are also clearly warranted to determine whether the interactions with paracetamol observed in this study are also observed in-vivo in man and to examine the effects of the isoflavones on the hepatic formation and transport of glucuronide metabolites.

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