British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01413.x
www.brjpharmacol.org

RESEARCH PAPER

Bile pigment pharmacokinetics and absorption in the rat: therapeutic potential for enteral administration

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Keywords
bile pigment; administration; kinetics; half-life; metabolism; excretion; inflammation; oxidative stress; antioxidant

Received
27 January 2011
Revised
2 March 2011
Accepted
17 March 2011

BACKGROUND AND PURPOSE

Bilirubin and biliverdin possess antioxidant and anti-inflammatory properties and their exogenous administration protects against the effects of inflammation and trauma in experimental models. Despite the therapeutic potential of bile pigments, little is known about their *in vivo* parenteral or enteral absorption after exogenous administration. This study investigated the absorption and pharmacokinetics of bile pigments after i.v., i.p. and intraduodenal (i.d.) administration in addition to their metabolism and routes of excretion.

EXPERIMENTAL APPROACH

Anaesthetized Wistar rats had their bile duct, jugular and portal veins cannulated. Bile pigments were infused and their circulating concentrations/biliary excretion were measured over 180 min.

KEY RESULTS

After i.v. administration of unconjugated bilirubin, biliverdin and bilirubin ditaurate, their plasma concentrations decreased exponentially over time. Subsequently, native and metabolized compounds appeared in the bile. When administered i.p., their absolute bioavailabilities equalled 14.0, 16.1 and 33.1%, respectively, and correspondingly 38, 28 and 34% of the same bile pigment doses were excreted in the bile. Administration of unconjugated bilirubin and bilirubin ditaurate i.d. increased their portal and systemic concentrations and their systemic bioavailability equalled 1.0 and 2.0%, respectively. Correspondingly, 2.7 and 4.6%, of the doses were excreted in the bile. Biliverdin was rapidly metabolized and these products were absorbed and excreted via the urine and bile.

CONCLUSIONS AND IMPLICATIONS

Bile pigment absorption from the peritoneal and duodenal cavities demonstrate new routes of administration for the treatment of inflammatory and traumatic pathology. Oral biliverdin administration may lead to the production of active metabolite that protect from inflammation/complement activation.

Abbreviations

AUC, area under the curve; C_{max}, maximal concentration at time = 0; C_{peak}, peak concentration; DMSO, dimethyl sulphoxide; i.d., intraduodenal; RM ANOVA, repeated-measures ANOVA; *t*#, concentration at # minutes; $t_{1/2\alpha}$, distribution half-life; $t_{1/2\beta}$, elimination half-life; V_d, volume of distribution

Introduction

Bile pigments, unconjugated bilirubin and biliverdin are formed in mammals via the catabolism of haem by haem oxygenase [EC 1.14.99.3] and chemical reduction by biliverdin reductase [EC 1.3.1.24] (Otterbein *et al.*, 2003). Until recently, bile pigments were generally considered harmful by-products (Rice and Shapiro, 2006); however, a shift in their perceived physiological importance has occurred recently (McGeary *et al.*, 2003; McCarty, 2007), supported by *in vitro* antioxidant (Stocker *et al.*, 1987; Frei *et al.*, 1988; Bulmer *et al.*, 2008b), anti-inflammatory (Nakagami *et al.*, 1993), anti-mutagenic (Bulmer *et al.*, 2008c), reports in addition to *in vivo* protection from ischaemia–reperfusion (Ceran *et al.*, 2001; Hammerman *et al.*, 2002), transplantation rejection (Nakao *et al.*, 2004) and experimental colitis (Berberat *et al.*, 2005) in animal models. Although investigations probing the physiological importance of bile pigments date back to the 1950s (Bernhard *et al.*, 1954), a substantial argument for their efficacy in the clinic has only recently been proposed (Scott *et al.*, 2007). Establishing the pharmacokinetics of bile pigment administration is essential for future research study design with the aim of revealing their therapeutic efficacy.

Lester and colleagues pioneered the early understanding of bilirubin metabolism (Lester *et al.*, 1961; Lester and Schmidt, 1963a), suggesting intestinal unconjugated bilirubin and bilirubin diglucuronide was absorbed from the gut and then excreted into the bile. However, later studies show that bacterial deconjugation of bilirubin glucuronide leads to unconjugated bilirubin re-absorption, demonstrating the enterohepatic circulation of bilirubin *in vivo* (Vitek and Carey, 2003). Impaired gastric motility (Kotal *et al.*, 1996), bacterial reductase activity (Vitek *et al.*, 2000) and bile salt malabsorption (Brink *et al.*, 1999) also facilitate intestinal re-absorption of unconjugated bilirubin leading to hyperbilirubinaemia.

Previous studies have not elucidated the kinetics of bilirubin absorption from the gut into the circulation *per se*, but measured absorptive flux of radiolabelled biliary excretion as an indirect marker of intestinal absorption (Lester and Schmidt, 1963a). Very few studies have sought to estimate the circulating bioavailability of exogenous bile pigment administration due to the lack of sufficiently sensitive measurement techniques. Thus, the application of HPLC has revealed that bile pigments are absorbed from the gut and peritoneal cavity into the circulation, in addition to demonstrating metabolite excretion via the liver. To assist in quantifying bile pigment intestinal absorption, we investigated their *in vitro* gastrointestinal permeability (Bulmer *et al.*, 2008a) and, subsequently, to quantify their bioavailability *in vivo*, we explored bile pigment absorption in Wistar rats.

The aim of this study was to obtain the kinetics of unconjugated bilirubin, biliverdin and bilirubin ditaurate absorption from the intestinal (and peritoneal) cavities into the systemic and portal circulations. The collection of bile, urine and intestinal contents (containing native and metabolized bile pigments) also described the fate of i.v., i.p. and intestinally administered pigments. These measurements revealed new sites and routes of bile pigment metabolism and excretion *in vivo* that will assist in realizing the clinical potential of these antioxidant, anti-inflammatory and anti-mutagenic compounds.

Methods

Drugs, chemical, reagents and other materials

All animal experiments were performed under low-light conditions. Bile pigment solutions were always protected from light using foil and administered within 5 min to minimize their photo degradation and autooxidation.

Unconjugated bilirubin was purified and supplied by BioPharma Pty Ltd (Brisbane, Qld., Australia; >98% spectrophotometry, TLC; 5% IIIα, 92% IXα, 4% XIIIα; HPLC). Lipid impurities that contaminate commercial unconjugated bilirubin preparations were removed by repeated ethanol washes of crude biliary extracts of unconjugated bilirubin, prior to supply (personal communication with Mr John Gyuran, CEO, BioPharma Pty Ltd). Bilirubin ditaurate disodium (>99.9%, diazo method) and biliverdin hydrochloride (>95%, TLC) were purchased from Frontier Scientific (Logan, UT, USA). The compounds isomeric composition could not be provided by the supplier. HPLC analysis of biliverdin and bilirubin ditaurate did not reveal the presence of IIIα and XIIIα isomers (data not shown) in these preparations, although this does not rule out the possibility of isomers existing. All other reagents and solvents were purchased from Sigma-Aldrich (Melbourne, Vic., Australia).

Formation of bile pigment sodium salts

A solution of 10 $g \cdot L^{-1}$ NaOH in ethanol was prepared. A volume of ethanol containing two equivalents of NaOH was added to a suspension of unconjugated bilirubin in ethanol. A volume of ethanol containing three equivalents of NaOH was added to a separate suspension of biliverdin hydrochloride (i.e. the bile pigments were titrated dropwise with NaOH), moderating any pH fluctuations. The ethanolic solutions were sonicated and stirred (5 min) until the bile pigments were completely dissolved and the solutions were lyophilized (–20°C), under reduced pressure (Savant Speed Vac; Thermo Scientific, Waltham, MA, USA). Formation of the sodium salts of unconjugated bilirubin and biliverdin did not significantly affect compound purity or result in the formation of by-products (as tested by HPLC; Figure S1). Bilirubin ditaurate, supplied as a disodium salt, was used as is. All bile pigments were stored under N_2 at –80°C. Validation of the bile pigments in plasma and bile samples are provided in Figure S2.

Test systems used

Male Wistar rats (320–430 g) were deprived of food for approximately 16 h and were allocated so that the average animal weight was similar in each group (370 ± 20 g). One milligram (2.7 $mg \cdot kg^{-1}$ body weight; i.v and i.p. administration) or 10 mg (27 $mg \cdot kg^{-1}$ body weight; i.d. administration) of the bile pigments were dissolved as super saturated solutions prior to use and were injected within 5 min (during which time solutions were protected from light using foil-covered containers). For the i.v. experiments, bile pigments were dissolved in PBS with sonication, filtered (0.45 μm

syringe filter) and 0.2 mL of this solution was added to 0.2 mL of the animals' own plasma collected previously (see below), and 0.2 mL of 0.9% sterile NaCl was added. Adding bile pigments to plasma ensured their dissolution, as the pigments bind to circulating albumin. For the i.p. and i.d. experiments, bile pigments were dissolved in 1 mL of PBS, sonicated and filtered prior to administration. Solubilizing the pigments in this manner resulted in the formation of optically clear solutions, without visible aggregation, which was also checked by inspecting the filters used. The i.p. dose was injected into the peritoneal cavity and the i.d. dose was injected into the duodenum 5 cm distal to the pyloric sphincter. The pH of the i.v. and i.p. solutions were 7.5 and the i.d. solutions were pH 8.0–8.6. Solutions were infused i.v. and i.p. in 15 s and i.d. in 120 s. The longer i.d. infusion time allowed the injected solution to fill the small intestine under minimal pressure.

To investigate the possible role of the intestinal contents on biliverdin metabolism, a 10 cm segment of the duodenum was removed from anaesthetized animals and the contents rinsed into a Petri dish using PBS, previously warmed to 37°C. The duodenal section was discarded and the animal killed. Ten milligrams of biliverdin (as for i.d. administration studies) was then added to the Petri dish (and to a control plate with PBS alone) and these plates were covered with foil and placed into an incubator (37°C) for 180 min. The bile pigment content of each plate was then analysed, using HPLC.

All animal care and experimental procedures complied with the Guidelines of the Australian National Health and Medical Research Council and were approved by the Ethical Committee of the University of Queensland.

Experimental design

Rats were anaesthetized with 1:1 zolitil : xylazine (50:10 mg·mL^{-1} final concentration; Virbac Pty Ltd, Milperra, NSW; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) with a final volume of 0.75 mL·kg^{-1}. The rats were prepared for surgery and placed on a heating pad to maintain a constant body temperature (37°C). The jugular vein was cannulated with flexible silastic tubing (1.19 OD × 0.64 ID mm; Dow Corning, Midland, MI, USA) and 0.4 mL·kg^{-1} of a 100 IU·mL^{-1} solution of sodium heparin (Pfizer, West Ryde, NSW, Australia) in sterile 0.9% NaCl (Baxter Healthcare Pty Ltd, Auckland, New Zealand) was infused. A mid-line laparotomy was performed, the common bile duct was identified and cannulated with 0.78 OD × 0.32 ID mm tubing (Microtube Extrusions, North Rocks, NSW, Australia). In some animals the portal vein was also cannulated (21 gauge Optiva; Medex Medical Pty Ltd, Rossendale, UK) and fixed using super glue (UHU, Smithfield, NSW, Australia). The portal cannula was elongated with an extension using 0.61 OD × 0.28 ID mm tubing (Microtube Extrusions, North Rocks, NSW, Australia) and glue. Vascular cannulas were kept patent with 10 IU·mL^{-1} heparin-treated saline. Bile flow was allowed to equilibrate for 15 min; thereafter, bile was collected for 15 min to approximate baseline bile pigment excretion. Bile was collected continuously and on ice in pre-weighed polypropylene 0.6 mL tubes (Molecular Bioproducts, San Diago, CA, USA). Bile volume was determined gravimetrically (assumed density 1.0). After the bile pigments or vehicle had been administered, the laparotomy wound was sutured closed, kept moist using 0.9% NaCl and gauze and the animal remained anaesthetized for 180 min with occasional i.p. administration (0.05 mL) of the previously described zolitil and xylazine solution. Subsequently, the animals were killed by removing the heart. The intestinal contents and urine were frozen for later analysis.

Bile was collected continuously, in aliquots, and stored on dry ice. Blood (150 µL) samples were also collected periodically, from the portal and/or jugular vein cannula, over a 180 min period. The blood and bile volumes removed were replaced with warm, sterile NaCl i.v. The blood samples were centrifuged (800× *g*; 5 min) and the supernatant (plasma) was aliquotted, frozen on dry ice and later transferred to a –80°C freezer, with the bile samples.

Measurements made

Plasma, bile, intestinal and urine samples were analysed for bile pigments using HPLC (Shimadzu, Kyoto, Japan). Unconjugated bilirubin (λmax 450 nm) and its isomers, bilirubin mono and diglucuronides (λmax 450 nm), bilirubin ditaurate (λmax 450 nm) and biliverdin (λmax 375 nm) were detected using a photodiode array (240–650 nm), in a single run. The bile pigments were separated using a reverse phase C18 column (Ultrasphere, 4.6 mm × 250 mm, 5 µm; Beckman Coulter, Fullerton, CA, USA) as described by McDonagh *et al.* (2002). The column was perfused isocratically with methanolic 0.1 M di-*n*-octylamine acetate (methanol : H_2O 95:5 v/v; 1 mL·min^{-1}; 19 min run time) mobile phase. One hundred and sixty microlitres of mobile phase was added to 40 µL of plasma or bile and this solution was vortexed briefly and centrifuged (10000× *g*, 5 min). Twenty (bile) or 50 µL (plasma) of the supernatant was injected. Exogenous unconjugated bilirubin [added in dimethyl sulphoxide (DMSO)] was quantitatively extracted from plasma proteins (>99%) to concentrations up to 60 µM (data not shown). Heparin-treated plasma samples with added sodium bilirubinate (10–60 µm) were vortexed and allowed to incubate at 37°C for 15 min (in the dark). HPLC analysis was then conducted as above and area under curve (AUC) integration was used to calculate the concentration of unconjugated bilirubin in samples. The extraction efficiency for the other bile pigments was not tested because their affinity for plasma albumin is less than that for unconjugated bilirubin. Unconjugated bilirubin and biliverdin hydrochloride served as standards for administered bilirubin and biliverdin sodium salts. Bilirubin ditaurate served as an external standard for bilirubin mono and diglucuronides in bile. Conjugated bilirubin standards can also be obtained by extraction from bile (Spivak and Carey, 1985) or by methanolysis of conjugated bilirubins (Muraca and Blanckaert, 1983), to improve stability, if necessary. All samples were analysed within 1 week of collection. The limit of detection for bile pigments approximated 40 nM. This concentration was determined by interpolating the concentration from a signal to noise ratio of >10, using peak height integration.

Data analysis and statistical procedures

Plasma and biliary bile pigment concentrations were calculated via AUC integration of HPLC peaks. Total bile pigment

excretion in bile was calculated by multiplying the bile pigment concentrations in each sample by the volume of excreted bile. Subtracting the total bile pigment excretion in the vehicle administration groups from that in the treatment groups approximated net bile pigment excretion (Gartner *et al.*, 1983). The peak (C_{peak}) and maximum (at theoretical time 0; C_{max}) plasma bile pigment concentrations, the area under the respective bile pigment concentration curves (AUC_{180}), volume of distribution (V_d), distribution ($t_{1/2\alpha}$) and elimination ($t_{1/2\beta}$) half-lives were calculated using Microsoft Excel Macro's supplied by the Foods and Drugs Administration. Bile pigment bioavailability was calculated by dividing the AUC_{180} for the i.p./intraduodenal groups by the AUC_{180} of the respective i.v. group, corrected for the different doses administered.

Changes, over time (plasma bile pigment concentrations and rates of total bile pigment excretion) were analysed using one-way repeated-measures ANOVA (RM ANOVA) (Bonferroni *post hoc*; Sigmastat, Ver. 3.10). If data sets lacked normal distribution and/or equal variance, Friedman repeated-measures ANOVA on ranks (Dunn's *post hoc*) determined which concentration time points were different from baseline (time 0 min, t_0). For significance of differences in circulating bile pigment concentrations and bile pigment excretion rates between treatment and vehicle groups *t*-tests or rank sum *t*-tests were applied. One-way ANOVA or its equivalent non-parametric procedure (Kruskal–Wallis ANOVA; Dunn's *post hoc*) tested for differences in the relative excreted bile pigment doses at 180 min. Data are presented as mean ± SEM. A $P < 0.05$ was considered significant.

Results

Vehicle (control) groups

The administration of the vehicle only was performed in each experimental model, i.v., i.p. and i.d., to assess the effect of the different surgeries on bile pigment excretion and the unconjugated bilirubin AUC over 180 min. Total bile pigment excretion, over the experimental period, was lower ($P < 0.05$) in the i.v. vehicle group (210 ± 16 nmol) compared to the i.d. without portal cannulation group (269 ± 19 nmol), i.d. with portal cannulation group (307 ± 23 nmol) and i.p. group (320 ± 14 nmol). Total bile pigment excretion in the i.v., i.p. and i.d. treatment groups was corrected by subtracting the total bile pigment excretion in the respective vehicle group. No significant differences in the unconjugated bilirubin AUC existed between any of the vehicle groups. The value from each control group was used to correct the respective treatment group's unconjugated bilirubin AUC. It was important to apply this correction because the pigment is naturally abundant in the circulation.

Intravenous administration

Sodium bilirubinate administration (1 mg; 2.7 mg·kg^{-1} body weight; same dose for all i.v. bile pigments) significantly increased the systemic unconjugated bilirubin concentration and this remained significantly elevated, versus 0 min (t_0), for 180 min ($P = 0.001$; Figure 1A). Figure 1B shows the peak rate of total bile pigment excretion (including bilirubin di-/mono-glucuronides and unconjugated bilirubin) after 10–15 min and this remained elevated (vs. vehicle) until 120 min.

Sodium biliverdinate administration increased the biliverdin and bilirubin concentration (Figure 1C, Table 1; $P < 0.001$). The rate of total bile pigment excretion increased significantly ($P < 0.001$) after 5–10 min, peaked at 10–15 min and remained elevated, versus t_0, until 90 min and versus vehicle administration, until 120 min ($P < 0.05$; Figure 1D).

Bilirubin ditaurate administration increased its systemic concentration after 2 min (Figure 1E, Table 1). The rate of total bile pigment excretion increased significantly (vs t_0) after 5 min and remained elevated until 15 min and 90 min (vs t_0 and vehicle respectively; $P < 0.05$; Figure 1F). Additional i.v. pharmacokinetic data for all pigments are presented in Table 1.

Intraperitoneal administration

Sodium bilirubinate administration (1 mg; 2.7 mg·kg^{-1} body weight; same dose for all i.p. bile pigments) increased its systemic concentration, peaking at 15 min (Table 1, Figure 2A; $P < 0.001$). The concentration remained significantly greater than at t_0, until 90 min ($P < 0.05$) and greater compared to the vehicle group, until 180 min (Figure 2A; $P = 0.004$). The rate of total bile pigment excretion peaked at 15–30 min (Figure 2B) and remained elevated (vs vehicle) there onwards ($P < 0.016$).

The systemic biliverdin concentration after sodium biliverdinate administration peaked after 15 min (Figure 2C, Table 1). Significant differences between the vehicle and treatment groups' bilirubin concentration existed from 15 to 150 min ($P < 0.05$). The plasma unconjugated bilirubin concentration in the treatment group peaked 60 min after administration (Figure 2C) and was mirrored by increases in the peak rate of total bile pigment excretion (60–90 min) that remained elevated ($P < 0.023$; vs vehicle; Figure 2D) from 30 to 180 min.

Bilirubin ditaurate administration increased its systemic concentration, peaking after 90 min (Figure 2E, Table 1). Bilirubin ditaurate administration significantly increased the rate of total bile pigment excretion, compared to t_0 ($P = 0.018$; RM ANOVA time effect) peaking after 30–60 min ($P < 0.05$) and remained elevated (vs vehicle) after 120 min ($P < 0.021$). The increased total bile pigment excretion was accompanied by a marked increase in bilirubin ditaurate excretion (Figure 2F). Additional i.p. pharmacokinetic data for all pigments are presented in Table 1.

Intraduodenal administration

Sodium bilirubinate administration (10 mg; 27 mg·kg^{-1} body weight; same dose for all i.d. bile pigments) significantly increased the systemic ($P < 0.001$) and portal ($P < 0.009$) unconjugated bilirubin concentration from t_0 (Figure 3A), both peaking at 15 min. Significant increases, versus t_0, in the systemic and portal unconjugated bilirubin concentration existed from 15 to 60 min (Figure 3A) and from 15 to 30 min (not denoted; Figure 3A), respectively ($P < 0.05$). The portal concentration was greater than the systemic concentration throughout the experimental period ($P < 0.036$, Figure 3A). The systemic unconjugated bilirubin concentration was significantly ($P < 0.007$) greater in the treatment group (vs

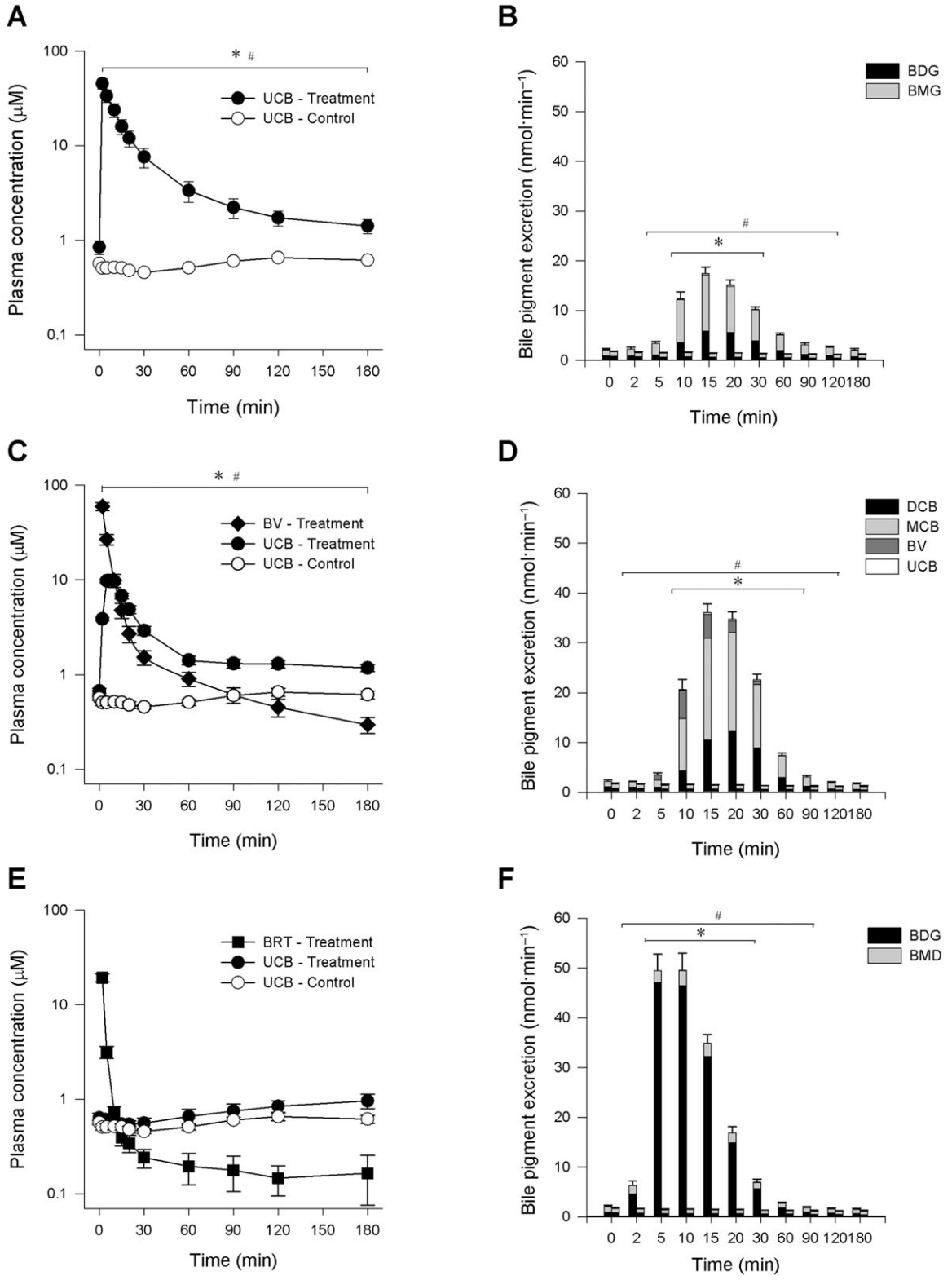


Figure 1

Effects of i.v. treatment with sodium bilirubinate (A, B), sodium biliverdinate (C, D) and bilirubin ditaurate (E, F) on the plasma concentration (left panels) of unconjugated bilirubin, biliverdin or bilirubin ditaurate; and rate of total bile pigment excretion (right panels; BDG, bilirubin diglucuronide; BMG, bilirubin monoglucuronide; UCB, unconjugated bilirubin; BV, biliverdin; stacked columns). Data points in in (A, C, E) indicate plasma concentrations at 0, 2, 5, 10, 15, 20, 30, 60, 90, 120, 180 min. *$P < 0.05$ versus t_0; #$P < 0.05$ treatment versus vehicle group (systemic unconjugated bilirubin concentration or rate of total bile pigment excretion) at that time point.

Table 1

Pharmacokinetic parameters in the systemic and portal circulations after i.v., i.p. and i.d. administration of different bile pigments

Compound	K distribution$_{\alpha}$ (nM·min^{-1})	$t_{1/2\alpha}$ (min)	K elimination$_{\beta}$ (nM·min^{-1})	$t_{1/2\beta}$ (min)	C_{max} (t_0, μM)	C_{peak} (μM)	AUC_{180} (μM·min)	V_d (L·kg^{-1})	Bioavailability (%)#	Biliary excretion (%)*
i.v. (2.7 mg·kg^{-1})										
UCB (n = 7)	76.8 ± 6.98	9.53 ± 0.95	6.46 ± 1.14	128 ± 21.3	54.7 ± 7.59	45.2 ± 5.78	969 ± 170	0.070 ± 0.002	–	29.1 ± 2.14
BV (n = 7)	261 ± 18.3	2.79 ± 0.21	9.51 ± 2.37	103 ± 25.5	97.3 ± 6.57	59.5 ± 5.87	454 ± 53.8	0.069 ± 0.003	–	57.3 ± 1.70
BRT (n = 6)	612 ± 39.5	1.16 ± 0.07	12.8 ± 4.11	74.7 ± 15.1	67.4 ± 9.46	19.3 ± 1.87	96.8 ± 12.3	0.055 ± 0.001	–	78.2 ± 4.98
i.p. (2.7 mg·kg^{-1})										
UCB (n = 5)	–	–	–	–	–	2.32 ± 0.20	250 ± 20.0	–	14.0	38.2 ± 4.74
BV (n = 4)	–	–	–	–	–	0.63 ± 0.14	73.0 ± 12.0	–	16.1	27.6 ± 3.17
BRT (n = 6)	–	–	–	–	–	0.23 ± 0.07	32.0 ± 7.29	–	33.1	34.1 ± 8.06
i.d. (27 mg·kg^{-1})										
UCB Systemic (n = 15)	–	–	–	–	–	1.52 ± 0.14	188 ± 14.7	–	1.0	2.72 ± 0.43
Portal (n = 6)	–	–	–	–	–	2.39 ± 0.32	306 ± 40.2	–	2.0	
BV Systemic (n = 9)	–	–	–	–	–	0.29 ± 0.09	29.7 ± 8.13	–	0.7	0.21 ± 0.31
Portal (n = 3)	–	–	–	–	–	0.33 ± 0.28	19.4 ± 14.0	–	0.4	
BRT Systemic (n = 10)	–	–	–	–	–	0.29 ± 0.26	21.8 ± 11.0	–	2.3	4.61 ± 1.13
Portal (n = 4)	–	–	–	–	–	1.12 ± 1.02	55.0 ± 42.6	–	5.7	

Data shown are mean ± SEM.
*Biliary bile pigment excretion in the treatment groups have been corrected by subtracting the endogenous bile pigment excretion in the respective vehicle group.
#The bioavailability of unconjugated bilirubin has been corrected for the endogenous unconjugated bilirubin AUC_{180} in the vehicle groups.
UCB, unconjugated bilirubin; BV, biliverdin; BRT, bilirubin ditaurate.

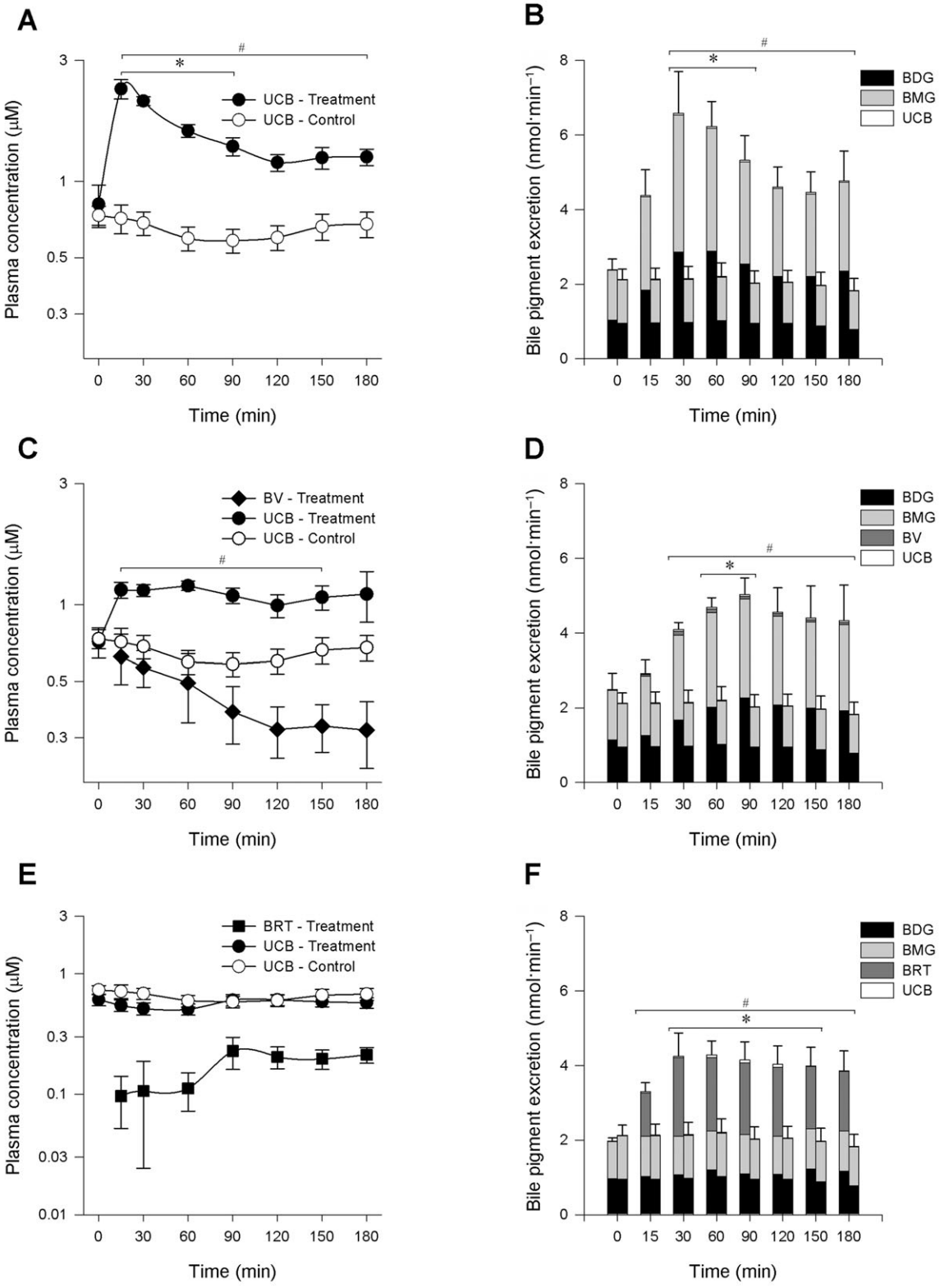


Figure 2

Effects of i.p. treatment with sodium bilirubinate (A, B), sodium biliverdinate (C, D) and bilirubin ditaurate (E, F) on the plasma concentration (left panels) of unconjugated bilirubin , biliverdin or bilirubin ditaurate; and rate of total bile pigment excretion (right panels; BDG, bilirubin diglucuronide; BMG, bilirubin monoglucuronide; UCB, unconjugated bilirubin; BV, biliverdin; stacked columns). Data points in (A, C, E) indicate plasma concentrations at 0, 15, 30, 60, 90, 120, 150, 180 min. *$P < 0.05$ versus t_0; #$P < 0.05$ treatment versus vehicle group (systemic unconjugated bilirubin concentration or rate of total bile pigment excretion) at that time point.

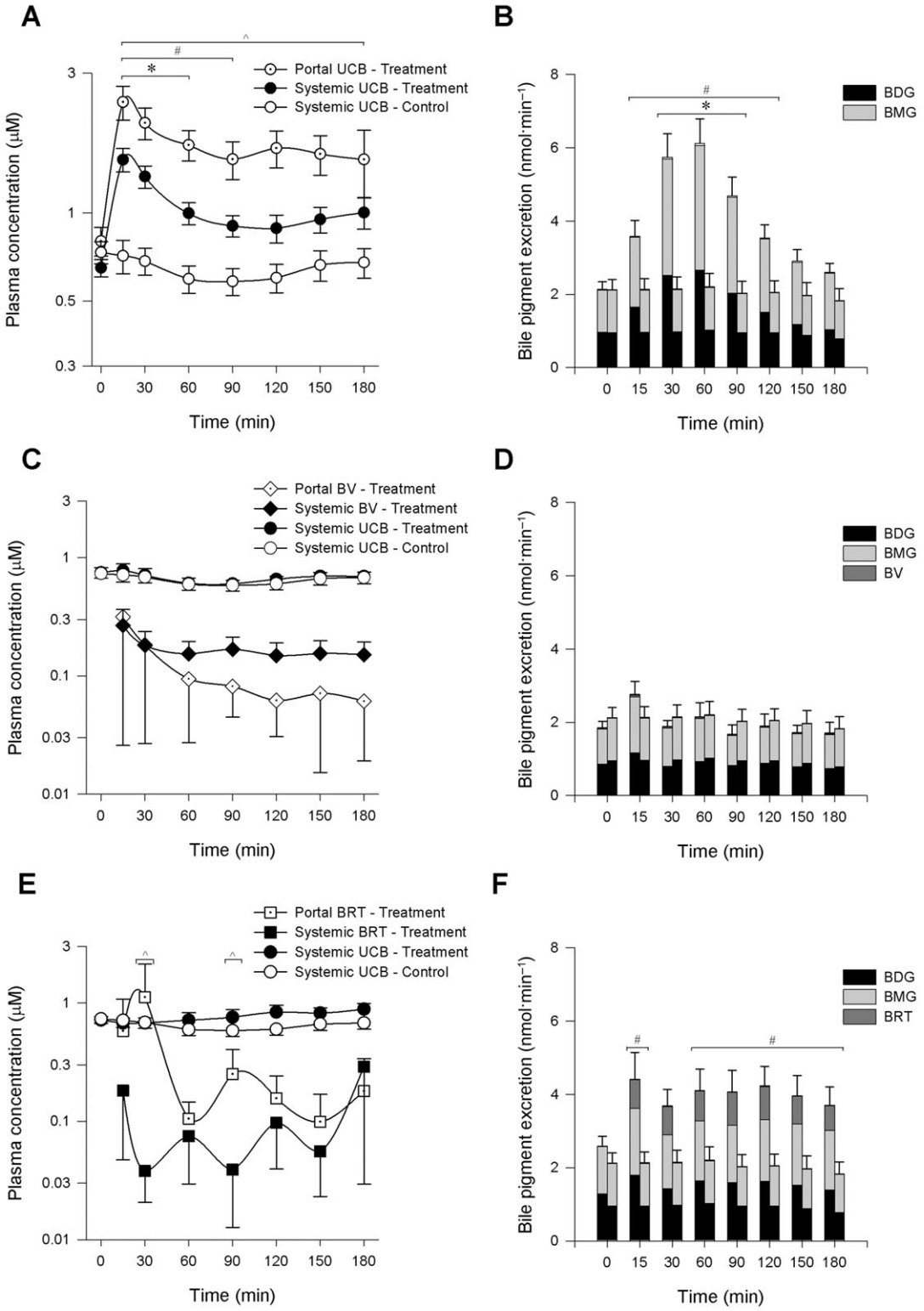
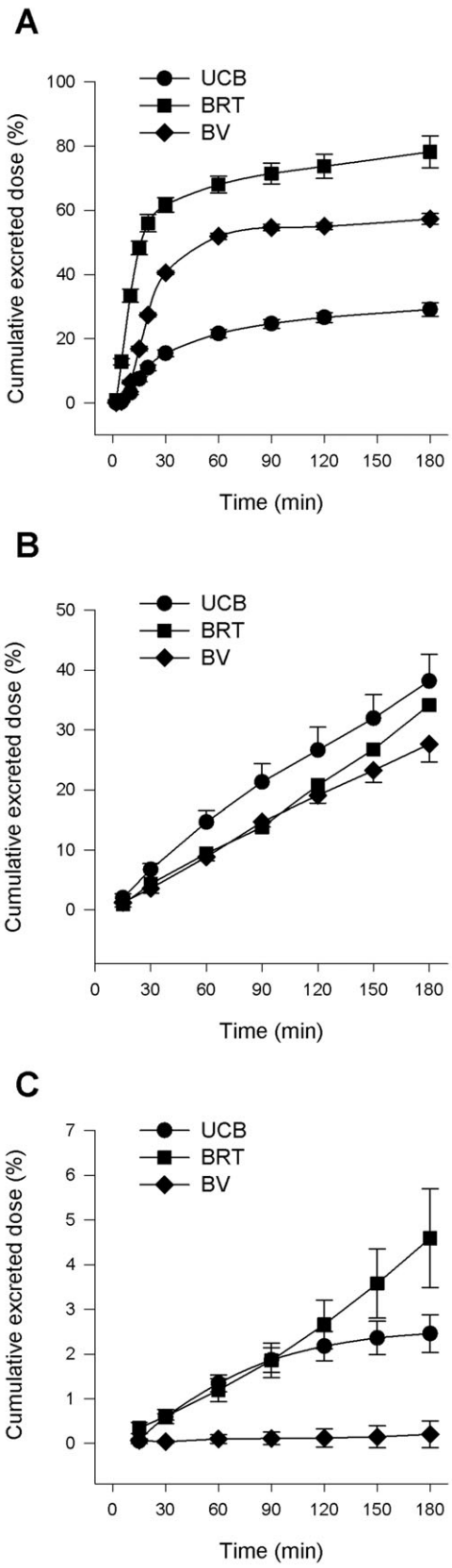


Figure 3

Effects of i.d. treatment with sodium bilirubinate (A, B), sodium biliverdinate (C, D) and bilirubin ditaurate (E, F) on the plasma concentration (left panels) of unconjugated bilirubin, biliverdin or bilirubin ditaurate and the rate of total bile pigment excretion (right panels; BDG; bilirubin diglucuronide, BMG; bilirubin monoglucuronide, UCB; unconjugated bilirubin; BV, biliverdin; stacked columns). Data points in panels (A, C, E) indicate plasma concentrations at 0, 15, 30, 60, 90, 120, 150, 180 min. *$P < 0.05$ versus t_0; #$P < 0.05$ treatment versus vehicle group (systemic unconjugated bilirubin concentration or rate of total bile pigment excretion) at that time point; ^$P < 0.05$ portal versus systemic concentrations in the treatment group.

Figure 4

Cumulative biliary excretion of sodium bilirubinate, sodium biliverdinate and bilirubin ditaurate after i.v. (A), i.p. (B) and i.d. (C) administration. For clarity, standard error bars on the cumulative excreted dose data after i.p. bilirubin ditaurate administration (B) have been omitted but were similar in magnitude to the other compounds.



vehicle) from 15 to 90 min (Figure 3A). The rate of total bile pigment excretion, versus t_0, increased significantly ($P < 0.001$), peaking after 30–60 min, and remained elevated until 90 min ($P < 0.05$; vs t_0) and from 15 to 120 min ($P < 0.036$; Figure 3B; vs vehicle).

Sodium biliverdinate administration increased its systemic and portal concentration that were at similar levels after 15 min ($P = 0.838$; Figure 3C, Table 1). The systemic unconjugated bilirubin concentration remained unchanged in the treatment and vehicle groups ($P = 0.653$), as did the rate of total bile pigment excretion (Figures 3D; $P = 0.367$). Surprisingly, very little biliverdin remained in the intestine after 180 min and three metabolites, absorbing in the 420–450 nm wavelength band (Figure S3) were present. These metabolites were also found in the plasma, bile and urine (Figures S4–S6 respectively). To establish whether the bacterial flora might be responsible for the biliverdin metabolism, fresh duodenal washes were incubated with biliverdin for 180 min at 37°C. After 180 min the same three metabolites were detected (Figure S7).

Bilirubin ditaurate administration increased its systemic and portal concentration, peaking at 180 and 30 min, respectively (Figure 3E, Table 1). The bilirubin ditaurate concentration was greater in the portal (vs systemic) circulation at 30 and 90 min ($P < 0.038$). The rate of bile pigment excretion, versus t_0, remained unchanged after i.d. bilirubin ditaurate administration ($P = 0.225$ RM ANOVA). However, significant increases versus the vehicle group (Figure 3F; $P < 0.05$) from 15 to 180 min were noted. Additional i.d. pharmacokinetic data are presented in Table 1.

Comparative analysis

Intravenous administration. The cumulative excreted doses, corrected for the excretion of bile pigments in the vehicle administration group, are presented in Table 1 (see biliary excretion) and Figure 4A. The cumulative excreted dose of bilirubin ditaurate was significantly greater than for sodium bilirubinate ($P < 0.05$) after 180 min.

Intraperitoneal administration. The i.p. bioavailability of sodium bilirubinate was similar to that of biliverdin but was less than that for bilirubin ditaurate (Table 1). Despite the greater bioavailability of bilirubin ditaurate, no significant differences in the cumulative excreted dose existed between the tested compounds after 180 min ($P = 0.592$; Figure 4B).

Intraduodenal administration. The systemic and portal bioavailabilities of bilirubin ditaurate were greater than for unconjugated bilirubin; however, biliverdin bioavailability remained very low. Table 1 (see biliary excretion) and Figure 4C show the cumulative excreted dose of the admin-

istered compounds. After 180 min the relative excreted dose of sodium bilirubinate and bilirubin ditaurate were significantly ($P < 0.05$) greater than that for sodium biliverdinate.

Discussion and conclusions

A comprehensive pharmacokinetic analysis of exogenous bile pigment administration in rats is presented here. Each pigment was clearly bioavailable from the peritoneal cavity; however, only bilirubin and bilirubin ditaurate possessed intestinal bioavailability. These data show enteral bilirubin administration increases circulating concentrations, and may provide systemic protection from inflammatory/oxidant insults. Biliverdin metabolism, and absorption of these metabolites, could explain the therapeutic efficacy of p.o. biliverdin administration.

Intravenous administration

The plasma disappearance of unconjugated bilirubin, after i.v. administration, has been well-documented in mammals (Berk *et al.*, 1969; Owens *et al.*, 1977; Gollan *et al.*, 1981; Rothuizen *et al.*, 1992). The volume of distribution (0.070 L·kg^{-1}) for unconjugated bilirubin, presented here, agrees well with that in humans (0.068 L·kg^{-1}), suggesting its distribution into the extracellular compartment (Owens *et al.*, 1977). The volume of distribution for unconjugated bilirubin and the low cumulative excreted dose is probably due to its strong affinity for vascular proteins and extracellular albumin, which has a sojourn time of 9.6 h (Cohen *et al.*, 1961; Owens *et al.*, 1977). The half-life of the bilirubin distribution and elimination phases compare favourably with that in the dog (Rothuizen *et al.*, 1992). The kinetics of plasma bilirubin ditaurate clearance agrees with that of exogenously administered bilirubin glucuronides in rats (Lester and Klein, 1966).

Little information on the plasma clearance of biliverdin after i.v. administration exists (Barrowman *et al.*, 1976; Gollan *et al.*, 1977; Mora *et al.*, 2003). The delivery of sodium biliverdinate increased the unconjugated bilirubin concentration (Figure 1C) suggesting reduction of biliverdin in the reticulo-endothelial system and builds upon previous findings. (Gollan *et al.*, 1977). Furthermore, our data showing that bile pigment excretion, after i.v. biliverdin administration, peaked within 30 min and abated after 90 min agree with previous findings in Wistar rats (Mora *et al.*, 2003). Also, in agreement with others (Barrowman *et al.*, 1976; Gollan *et al.*, 1977), we showed that ~57% of the biliverdin dose was excreted in the bile.

Intraperitoneal administration

The results obtained after i.p. administration suggest that unconjugated bilirubin diffuses through the peritoneum into the circulation and increases hepatic bile pigment excretion (Figure 2A,B). Biliverdin administration increased the circulating biliverdin and unconjugated bilirubin concentration and hepatic bile pigment excretion (Figure 2C,D). The vast majority of excreted bile pigments were mono/diglucuronide bilirubins, and not biliverdin. These observations suggest that biliverdin is reduced within the peritoneal cavity (Figure S8) or when diffusing across the peritoneal membrane. Bilirubin ditaurate administration increased its circulating concentration and hepatic excretion (Figure 2E,F). These findings, combined with the cumulative excreted dose and bioavailability data (Table 1; Figure 4B), demonstrate that i.p. bile pigment administration has clear bioavailability, resulting in sustained absorption for at least 3 h.

Intraperitoneal administration of bile pigments holds *in vivo* therapeutic potential in multiple models of infection, trauma and inflammation (Nakao *et al.*, 2004; Wang *et al.*, 2004; Berberat *et al.*, 2005; Overhaus *et al.*, 2006). Administration of 30 mg·kg^{-1} body weight of unconjugated bilirubin, in DMSO, has been shown to increase the resting levels of ~1.7 μM to 6.8 μM after 30 min in rats (Wang *et al.*, 2004). However, these results were probably compromised by the toxic effects of vehicle DMSO administered (~0.5 mL). When Vitek *et al.* (2005) administered a suspension of 40 mg·kg^{-1} unconjugated bilirubin to Wistar rats, daily for 7 days, the total bilirubin concentrations increased from 0.8 to 102 μM. Whereas in another study in Lewis rats, i.p. sodium biliverdinate (50 mg·kg^{-1}), increased the total bilirubin concentration from 0.7 to 18.3 μM after 30 min (Nakao *et al.*, 2004). Furthermore, administration of 40 mg·kg^{-1} of biliverdin i.p. twice in Gunn rats increased total serum bilirubin from 169 to 255 μM (Rice and Shapiro, 2006).

Intraduodenal administration

Investigations of i.d. bilirubin absorption were common 50 years ago (Lester *et al.*, 1961; Lester, 1963; Lester and Klein, 1966) when intestinal absorption was estimated by quantifying the proportion of an enterally administered radiolabelled bilirubin dose, excreted in the bile. However, the absorption of radiolabelled bilirubin into the portal and systemic circulation was not quantifiable at this time (Lester, 1963; Lester and Schmidt, 1963a). In the present study, the cumulative excreted doses of unconjugated bilirubin and bilirubin ditaurate were similar after 3 h (Table 1). Previous studies show that 2.5–28% of unconjugated bilirubin doses are excreted (as unconjugated and conjugated bilirubins) after ≤72 h in rats (Lester and Schmidt, 1963a; Gartner *et al.*, 1983; Alonso *et al.*, 1991) and humans (Lester and Schmidt, 1963b). Approximately, 15% and 2% of radiolabelled unconjugated bilirubin and bilirubin glucuronides, respectively, were excreted after 8 h in humans (Lester and Schmidt, 1963b). These data are supported by the relatively poor absorptive flux of bilirubin glucuronide in rats (Lester and Schmidt, 1963a).

In terms of therapeutic relevance, i.d. administration of unconjugated bilirubin and bilirubin ditaurate increased their portal and systemic concentrations. These data show that enteral bilirubin administration can result in appreciable absorption and thus provide a clinically relevant route of administration for efficacy studies. Although the bioavailability of unconjugated bilirubin was less than that for bilirubin ditaurate, circulating concentrations of unconjugated bilirubin increased two and threefold in the systemic and portal circulations respectively. The bioavailability result for unconjugated bilirubin in particular is probably an underestimate of its true value, due to its poor aqueous solubility. Strategies aimed at improving bilirubin solubility, including co-administration with bile salts, should facilitate unconjugated bilirubin absorption and increase bioavailability further

(Brink *et al.*, 1999). The substantial capacity for hepatic excretion of bilirubin ditaurate is clear when observing its i.v. clearance (Figure 1E) and rapid distribution and elimination half-lives (Table 1) and also help to explain the modest circulating concentrations after i.p. and i.d. administration. (Figures 2E and 3E) Therefore, although the bioavailability of bilirubin ditaurate was greatest, circulating concentrations were quite low and would be less likely to impart efficacy. These data show that 20–45% of the portal unconjugated bilirubin concentration was extracted from the liver/distributed throughout the vascular compartment and body. These data build upon findings published by Gartner *et al.* who showed that the first pass hepatic extraction of unconjugated bilirubin approximated 33% (Gartner *et al.*, 1997).

Very few studies have quantified bile pigment absorption, using circulatory measures, after i.d. administration. After administering unconjugated bilirubin to rats (2.9 or 5.4 $mg \cdot kg^{-1}$ in DMSO/intralipid), Kotal *et al.* (1996) reported that the systemic total bilirubin concentration increased from 0.86 to 1.52–1.72 μM after 180 min. The authors also administered a mixed glucuronide solution (~8.4 $mg \cdot kg^{-1}$) increasing the systemic bilirubin concentration from 0.85 to 1.5 μM, after 180 min. Concentrations from other time points and whether the absorbed bilirubin remained in an unconjugated or conjugated form were not reported. The data presented here add significantly to the findings of previous i.d. administration studies and describe, with sufficient fidelity, the absorption of different bile pigments into the portal circulation. The use of HPLC also showed that the bilirubin compounds are absorbed intact and that the individual pigment concentrations were quantifiable. The remarkable bioavailability of bilirubin ditaurate may be explained by its apparent paracellular absorption, as noted in our previous study of bile pigment *in vitro* intestinal permeability (Bulmer *et al.*, 2008a). Interestingly, when bilirubin ditaurate was administered i.d. a small amount appeared to be deaminated and absorbed as unconjugated bilirubin (Figure 3E). This conclusion is supported by elevated di- and mono-glucuronidated bilirubin in bile (Figure 3F).

Unexpectedly, biliverdin was poorly absorbed from the intestine. Bacterial (metabolism *in vivo*/*in vitro* duodenal washes) and/or cellular metabolism (similar metabolism profile, however, of minor effect within the peritoneum) may have accounted for this phenomenon. However, it should be noted that these hypotheses are purely speculative and further investigations are warranted to answer these questions. It should be noted that biliverdin is essentially resistant to modification by bilirubin reducing bacteria, *Clostridium perfringens* (Vitek *et al.*, 2006). Therefore, epithelial metabolism would seem a likely source of biliverdin metabolism. The results suggest that biliverdin was metabolized to three compounds that, based upon their spectral profiles, could possess rubinoid structure (absorbing at 420–450 nm) and suggest reduction or cleavage of biliverdin's C10 bridge. These compounds were subsequently absorbed into the circulation and excreted in to the bile (two compounds absorbing at 450 nm) or the urine (a single compound absorbing at 420 nm). Further characterization of these compounds is necessary and may reveal their structure to be that of dipyrroles (420 nm) or oxidized/reduced products of bilirubin (450 nm; McDonagh, 1979). These findings are of great interest as p.o. biliverdin administration protects from inflammation/complement activation (Nakagami *et al.*, 1993). Our findings show that intestinal biliverdin metabolism reduces its bioavailability; however, serendipitously show that metabolized products are potently absorbed and may be responsible for protection seen after p.o. biliverdin administration (Nakagami *et al.*, 1993).

Importantly, the bile duct was permanently cannulated in this study, completely interrupting the enterohepatic circulation of bile pigments *in vivo*. Therefore, circulating bile pigment concentrations, after administration, may be greater in conditions of uninterrupted biliary flow.

Bile pigments physicochemical properties, particularly of unconjugated bilirubin, should be carefully considered when evaluating the results of this study. Biliverdin and bilirubin ditaurate's pKas approximate 4–6 (Lightner *et al.*, 1996) and 1.5 (Ostrow, 1986) respectively. Estimates of unconjugated bilirubin's pKa vary widely from 4 to 9 (Hahm *et al.*, 1992; Boiadjiev *et al.*, 2004) and most recent estimates suggest the pKa of unconjugated bilirubin's two carboxyl groups equal 8.12 and 8.44 (Ostrow and Mukerjee, 2007). At the pH of solutions employed in this study (7.4–8.5) biliverdin and bilirubin ditaurate were ionized, water soluble and therefore, potentially absorbable. However, in the i.v. and i.p. solutions (pH ~7.4) unconjugated bilirubin existed (after dissociating from its salt), predominantly in its diacid form in addition to smaller amounts of mono/dianion. The diacid form has a low aqueous solubility (Mukerjee *et al.*, 2002), and tends to aggregate (Brodersen, 1979; Zucker *et al.*, 1999), promoting its diffusion from administered solutions into lipid membranes, allowing it to be transferred (in an ionized state) to circulating albumin/intercellular proteins. Even at a pH of 8.0–8.5 (as in the i.d. solution) it is possible that some unconjugated bilirubin existed as bilirubin diacid (albeit much less than at pH 7.4). The ionization state of the bile pigments would clearly have affected their bioavailability. When comparing routes of administration, however, it must be acknowledged that pH probably affected the relative bioavailability values, particularly of unconjugated bilirubin, due to the effects on ionization state and diffusibility of the molecule. Unfortunately, poor bilirubin solubility and pH-dependent ionization, both hindering absorption across lipid membranes, are inherent limitations when studying this compound. Clearly, the formation of unconjugated bilirubin visible/microscopic aggregates in the intestine/peritoneum would probably have hindered absorption and therefore, i.d. and i.p. bioavailabilities may have been underestimated. However, the formation of these aggregates occurs slowly (Bonnett *et al.*, 1976) and therefore, we are confident that this allowed for the various bilirubin species to be absorbed, particularly in the first 30 min, when greatest absorption was recorded. No precipitate was observed in the peritoneal cavities of animals administered sodium bilirubinate i.p.; however, this does not exclude the possibility of microscopic aggregates having formed. We hypothesize that after administration, unconjugated bilirubin (mono/dianion) would avidly bind to albumin in the peritoneal fluid (Barber *et al.*, 1990) and (diacid) the lipid membrane of the peritoneum. A slightly basic peritoneal pH would then facilitate shuttling of unconjugated bilirubin, via diffusion, from albumin to lipid membranes (Zucker *et al.*, 1995; Kamisako *et al.*, 2000).

Exploring the effects of co-administration of bile pigments with excipients was not an aim of this study. However, administering unconjugated bilirubin (in its dianionic form) i.d. with bile salts (Ostrow *et al.*, 1988; Rege *et al.*, 1988), may result in a different pharmacokinetic profile and an improved bioavailability, while making use of the micellar shuttle across the intestinal epithelium. Administering bilirubin in this manner would be possible up to a dose of ~10.5 $mg \cdot mL^{-1}$ in 50 mM taurocholate (pH 9.3; Nakama *et al.*, 1979; Wosiewitz and Schroebler, 1979).

Further limitations of our study include the effects of anaesthetics on hepatic bilirubin uridine diphosphate glucuronosyl transferase. However, the effects of barbiturates and ketamine on bile pigment excretion are minimal over 3 h (Gourley *et al.*, 1985). Permanently anaesthetizing the animals allowed for regular sampling of portal blood, which strengthened the experimental design and novelty of the results. We used a greater i.d. bile pigment dose versus i.v. and i.p. administration to maximize the likelihood of detecting bile pigments in the plasma and, like others, administered non-radiolabelled bile pigments (Gartner *et al.*, 1983; Kotal *et al.*, 1996). The synthesis and administration of radiolabelled bilirubin (Howe *et al.*, 1970) may further assist in quantifying bile pigment bioavailability and biodistribution. The use of HPLC discriminated between different bile pigments and had a low limit of detection (~40 nM).

This study describes complete plasma absorption/clearance and hepatic excretion profiles after i.v., i.p. and i.d. administration of bile pigments. After i.p. administration these pigments are clearly bioavailable and show a sustained elevation in plasma concentrations. Unconjugated bilirubin and bilirubin ditaurate were also absorbed from the intestine and provide a justification for p.o. administration studies in models of inflammatory and traumatic pathology.

Acknowledgements

AB is a 2001 Centenary Scholarship Recipient (Australian Commonwealth Government). The authors acknowledge Dr Russell Addison, Dr Karine Mardon, Mr Tyson Moore and Michael Morgan for their assistance and Prof. Anthony McDonagh for his methodological advice. Biopharma Pty Ltd provided the unconjugated bilirubin for this project.

Conflict of interest

ACB was provided with a stipend from BioPharma Pty Ltd during the course of his PhD studies (2004–2007).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Chromatograms showing the composition of supplied and sodium salt preparations of bile pigments. Chromatograms (A; 450 nm) of unconjugated bilirubin (green) and bilirubin disodium salt (blue). Chromatograms (B; 375 nm) of biliverdin hydrochloride (blue) and biliverdin disodium salt (green). Variability in the height of curves (<10%) is likely attributed to inherent inaccuracies in weighing small amounts of bile pigments (<3 mg) on analytical scales. Little, if any, impurities were detected (240–650 nm) in either bile pigment sodium salt preparation.

Figure S2 Validation chromatograms showing the elution of bilirubin ditaurate in DMSO/mobile phase [bilirubin ditaurate (BRT)] (A), BRT added to plasma (B) and BRT added to bile (C; bile in green; BRT added to bile in blue). BRT RT: 4.5 min @ 450 nm. Unconjugated bilirubin (UCB) (D; UCB in DMSO/mobile phase in green; UCB in plasma in blue), UCB in bile (E) UCB RT: 15.6 min @ 450 nm. Biliverdin (BV) (F; BV in DMSO/mobile phase in blue; small BV peak in bile in green), BV in plasma (G) BV RT: 6.15 min @ 375 nm.

Figure S3 HPLC chromatogram of the intestinal contents (450 nm) 180 min after i.d. vehicle (solid line) or sodium biliverdinate (dotted line) administration. I, III and IV, suspected metabolites of biliverdin; II, normal elution time of biliverdin; V, the normal elution time for unconjugated bilirubin. Absorbance spectra for compounds: I, λmax = 423 nm, RT 4.5 min; II, example absorbance spectrum of biliverdin, λmax = 375 nm, RT 5.9 min; III, λmax = 420–450, RT 6.7 min; IV, λmax = 450 nm, RT 9.7 min; example absorbance spectrum of unconjugated bilirubin, V, λmax = 450 nm, RT 15.5 min.

Figure S4 HPLC chromatogram of the plasma (450 nm) 180 min after i.d. vehicle (solid line) or sodium biliverdinate (dotted line) administration. I, III and IV, suspected metabolites of biliverdin; II, normal elution time for biliverdin; V, unconjugated bilirubin. Absorbance spectra for compounds: I, λmax = 423 nm, RT 4.5 min; III, λmax = 420–450, RT 6.7 min; IV, λmax = 450 nm, RT 9.7 min; V, unconjugated bilirubin, λmax = 450 nm, RT 15.5 min.

Figure S5 HPLC chromatogram of the bile (450 nm) 180 min after i.d. vehicle (solid line) or sodium biliverdinate (dotted line) administration. IV suspected metabolite of biliverdin. Absorbance spectra for compounds: IV, λmax = 450 nm, RT 9.7 min. Note that the retention time (9.7 min) and λmax of IV are similar to that found in the intestinal wash (Figure S8; IV) and plasma (Figure S9; IV).

Figure S6 HPLC chromatogram of the urine (450 nm) 180 min after i.d. vehicle (solid line) or sodium biliverdinate (dotted line) administration. Absorbance spectra for compounds: I: polar metabolite; note that the retention time (4.5 min) and λmax of I are similar to that found in the intestinal wash (Figure S8; I) and plasma (Figure S9; I).

Figure S7 Chromatogram showing the appearance of the metabolites (I, III and IV) in fresh intestinal washes from Wistar rats after 15 (solid line) and 180 (dotted line) min of co-incubation with sodium biliverdinate (450 nm). Absorbance spectra for compounds found in the dotted chromatogram: I, λmax = 423 nm, RT 4.5 min; II, biliverdin, λmax 375 nm, RT 5.9 min; III, λmax = 450 (arrow), RT 6.8 min (peak contains residual biliverdin λ 375 nm); IV, λmax = 450 nm, RT 9.8 min; V, absorbance spectrum of unconjugated bilirubin (an example of similar magnitude to IV is presented for comparison), λmax = 450 nm, RT 15.5 min. Note that the retention times and λmax of the metabolites I, III and IV are similar to those found in the intestinal wash 180 min after i.d. administration of biliverdin *in vivo* (Figure S8).

Figure S8 Chromatogram showing the appearance of the metabolites (I, III and IV) in peritoneal fluid in Wistar rats 180 min after delivery of biliverdin (solid line; 450 nm). Absorbance spectra for compounds found in the chromatogram: I, λmax = 423 nm, RT 4.5 min; II, biliverdin (BV), λmax = 375 nm, RT 5.9 min; III, λmax = 450, RT 6.8 min; IV, λmax = 450 nm, RT 9.8 min. Note that the retention times and λmax of the metabolites I, III and IV are similar (although much lower in concentration) to those found in the intestinal wash 180 min after i.d. administration of biliverdin *in vivo* (Figure S8).

Figure S9 HPLC chromatogram of the plasma (450 nm) 180 min after i.p. sodium biliverdinate administration. III and IV, suspected metabolites of biliverdin, absorbance spectra for compounds: III, λmax = 420–450, RT 6.7 min; IV, λmax = 450 nm, RT 9.7 min.

Figure S10 HPLC chromatogram of the bile (450 nm) 180 min after i.p. sodium biliverdinate administration. IV, suspected metabolite of biliverdin. Absorbance spectra for compounds: IV, λmax = 450 nm, RT 9.7 min. Note that the retention time (9.7 min) and λmax of IV are similar to that found in the peritoneal fluid (Figure S10; IV) and plasma (Figure S11; IV).

Figure S11 HPLC chromatogram of the urine (450 nm) 180 min after i.p. sodium biliverdinate administration. Absorbance spectra for compounds: I: polar metabolite; note that the retention time (4.5 min) and λmax of I are similar to that found in the peritoneal fluid (Figure S10; I).

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