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Canalicular and sinusoidal disposition of bilirubin mono- and diglucuronides in sandwich-cultured human and rat primary hepatocytes

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Canalicular and sinusoidal efflux of bilirubin glucuronides

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Abbreviations: B, bilirubin; BMG, bilirubin monoglucuronide, BDG, bilirubin di-glucuronide, pNP, p-nitrophenol, pNPG, p-nitrophenol glucuronide; MRP, multidrug resistance-associated protein; UGT, UDP-glucuronosyltransferase, UDPGA, UDP-glucuronic acid; EBSS, Earle's balanced salt solution; HBSS, Hanks' balanced salt solution; BEI, biliary excretion index; Q1 scan, the first quadrupole is scanning; MRM, multiple reaction monitoring

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Abstract

Due to cholestasis or adverse drug effects, the excretion of bilirubin conjugates can decrease therefore the level of bilirubin (B) and bilirubin glucuronides (BG) increases in the serum with the concomitant shift of bilirubin di- versus monoglucuronide (BDG/BMG) equilibrium. The aim was to utilize the collagen-sandwich culture of hepatocytes as an in vitro model for studying B conjugation and canalicular versus sinusoidal disposition of BG. Canalicular and sinusoidal efflux of BMG and BDG obtained in sandwich-cultured rat primary hepatocytes was compared with that measured in human hepatocyte cultures. The BMG and BDG were separated by HPLC and identified by MS. Biliary excretion index (BEI) was estimated by measuring disposition of BG into standard and Ca^{2+}, Mg^{2+} free medium. Significantly more BG were excreted into the canalicular networks than into the medium in 96-h sandwich culture of both human and rat hepatocytes (BEI: 62.5 and 80.6, respectively). The BDG/BMG ratio in the medium versus in the canalicular networks was 0.55/1.48, which is similar to the serum/bile values (0.6/1.5) observed in vivo by Mesa et al (1997). In contrast BEI for p-nitrophenol glucuronide (pNPG) was 5.2. The low BEI value is in agreement with empirical observations, which suggest that molecules with low molecular weight are preferably excreted by the kidney. In conclusion, sandwich-cultured primary hepatocytes provide a useful in vitro method to differentiate between sinusoidal and canalicular disposition of BG. Since the normal BDG/BMG ratio changes in hyperbilirubinemia, this model could be used to predict drug effects leading to hyperbilirubinemia.

Bilirubin (B), the breakdown product of haem is formed by degradation of haemoproteins. This toxic compound is cleared from circulation by the liver into the bile canaliculus mainly as bilirubin mono- (BMG) and diglucuronides (BDG) produced by UDP-glucuronosyltransferase (UGT1A1) (Burchell et al, 1997) via active transport processes (Jedlitschky et al, 1997). Hyperbilirubinemia can be classified into two types depending on whether B is conjugated or not. Unconjugated hyperbilirubinemia can be caused by mutations in UGTs as observed in Gilbert's and Crigler-Najjar syndromes in human and in Gunn rats, an animal model for these syndromes. Conjugated hyperbilirubinemia, such as Dubin Johnson syndrome, is caused by genetic defects in the canalicular BG transport mediated by MRP2 (König et al, 2003) or dysfunction of MRP2, e.g. due to cholestasis (Kamisako et al, 2000). Cholestatic expression pattern of sinusoidal and canalicular transporters highly differs from normal, consequently the kinetics of B uptake and efflux is altered (Rippin et al, 2001). Besides the genetic reasons drugs can also cause both kinds of hyperbilirubinemia either by competing for UGTs or transporters, or by causing cholestasis. In both kinds of hyperbilirubinemia the concentration and the duration of intracellular storage of B increase in hepatocytes, resulting in change of the amount and proportion of B, BMG and BDG both in the bile and in the serum.

Many reports have described the role of mrp1, mrp2 and mrp3 in the transport of BG under both normal and cholestatic conditions (Jedlitschky et al, 1997; Mesa, 1997; Keppler and König, 2000). However, these studies were all conducted either in vivo or using rather simplified systems such as membrane vesicles or expressed transporters.

Primary hepatocytes cultured in a sandwich configuration have been shown to be a special in vitro model in which cells reestablish their polarity, consequently the transport proteins are expressed specifically on the surface of the canalicular or sinusoidal membrane domain (Liu et al, 1999a; LeCluyse et al, 2000). The morphology resembles the natural state and allows differentiation of the canalicular efflux from the sinusoidal efflux of compounds. This suggests that sandwich cultures of hepatocytes can model their in vivo elimination into the bile and the serum, respectively. The sandwich culture of primary hepatocytes has been extensively characterized for use in estimating the biliary clearance of drugs in vitro. Following a substrate uptake period, extracellular Ca^{2+} depletion disrupts the tight junctions between adjacent cells, the content of the canalicular networks appears in the extracellular medium and can be analyzed (Liu et al, 1999b).

Many authors have proved the advantages of utilizing primary cultures of hepatocytes maintained in a collagen sandwich configuration in studying the hepatobiliary disposition of compounds mainly by using probe substrates, such as rhodamine 123, and different fluorescent mrp2 and mrp3 substrates (Liu et al, 1999b; Annaert et al, 2001; Kostrubsky et al, 2003). We used sandwich culture of primary rat and human hepatocytes with the aim of determining the role of basolateral and canalicular transport processes in B and BG disposition. In subsequent studies we are planning to investigate drug effects on B metabolism and transport. Drug interactions either with UGTs or transport proteins can alter biliary excretion of B leading to cholestasis and hyperbilirubinaemia in vivo. Sandwich culture of hepatocytes seems to be a suitable in vitro experimental model for studying these processes.

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Materials and Methods

Preparation of human and rat primary hepatocytes

Human liver tissues were obtained by a qualified medical staff from Semmelweis University of Budapest as rejected donor livers. Permission of Local Research Ethics Committee was obtained to use human tissues. All studies involving human tissue followed the tenets of the Declaration of Helsinki. Hepatocytes were prepared by a three-step perfusion procedure. Human liver samples were first flushed with Ca^{2+} free Earle's balanced salt solution (EBSS) containing EGTA, then with the same buffer without chelating agent and finally with EBSS containing Ca^{2+} and type IV collagenase (prepared from Clostridium histolyticum, Sigma-Aldrich, St. Louis, MO). Perfusions were carried out at 37^0C, pH 7.4 as described by Bayliss and Skett (1996). Rat hepatocytes were prepared from male Wistar rats (200-250g) (Charles River, Budapest) by in situ liver collagenase perfusion according to the method of Seglen (1976). Cell viability (> 90%) was determined by trypan blue exclusion. All procedures were approved by the Institutional Animal Care and Use Committee.

Sandwich-culture of primary human and rat hepatocytes

Hepatocytes were plated at a density of 1.8×10^5 cells/cm^2 in 30 mm dishes precoated with 0.15 ml of rat tail collagen type I solution (1.6 mg/ml) in Williams Medium E containing 5 % of fetal calf serum, 100 nM insulin, 2.5 μg/ml amphotericin B, 0.1 mg/ml gentamicin, 30 nM Na_2SeO_3, and 0.1μM dexamethasone (Sigma-Aldrich, St. Louis, MO). Calf serum and amphotericin B were present for the first 24 h then omitted. Cells were maintained at 37 ^{0}C in a humidified atmosphere of 95 % air-5 % CO_2. 4 h

after plating, and every day thereafter the medium was changed to Williams Medium E supplemented with insulin, gentamicin, dexamethasone, Na_2SeO_3. 24 h after plating the medium was aspirated and cells were overlaid with 200 µl of ice-cold, neutralized rat tail collagen type I solution (1.5 mg/ml, pH 7.4) to achieve sandwich configuration. 45 min after overlay, 1.5 ml of warm Williams Medium E supplemented with insulin, gentamicin, dexamethasone, Na_2SeO_3 was placed on the top of the gelled collagen layer. Rat tail collagen type I was isolated by the method of Koebe et al, 1994.

Bilirubin glucuronide and p-nitrophenol glucuronide efflux experiments

24-h, 72-h and 96-h sandwich culture of rat and 96-h sandwich culture of human hepatocytes were incubated with 25 µM of B (Merck, Darmstadt, Germany) for 60 min at 37^0C in a humidified atmosphere of 95 % air-5 % CO_2. B (60 mM) was dissolved in DMSO/1.0 M NaOH, 88/12 as a stock solution and diluted to 3 mM in Williams Medium E containing 20 mg/ml of BSA. pNP (Reanal, Hungary) was added to the medium at 0.1 mM concentration, the incubation lasted for 20 min. Biliary excretion of BG and pNP glucuronide was measured by the method of Liu et al, (1999b). Briefly, cells were loaded with B and pNP for 60 and 20 min, respectively in Williams Medium E as described above. Then the medium was removed, cells were rinsed twice with 2 ml of warm HBSS and incubated with 0.5 ml of standard or Ca^{2+}, Mg^{2+} free HBSS for 20 min. The Ca^{2+}, Mg^{2+} free HBSS was supplemented with 0.5 mM of EDTA. Aliquots of the incubation medium (withdrawn at the end of substrate loading phase) and that of the standard and Ca^{2+}, Mg^{2+} free HBSS were stored at -80 ^{0}C until analysis. The accumulation of conjugates in the canalicular networks was expressed by biliary excretion index (BEI) values. BEI was calculated as follows:

$BG_{Ca\ 2+\ Mg2+\ free\ HBSS}$ - $BG_{standard\ HBSS}$ / $BG_{Ca\ 2+Mg2+\ free\ HBSS}$ x 100.

All experiments were performed with hepatocytes from 3 independent preparations. The number of replicates within each experiment was 3.

Bilirubin glucuronide formation by human and rat hepatocyte suspension

Freshly isolated human and rat hepatocytes were suspended at a density of 2 x 10^6 cells/ml in warm HBSS and incubated with 25 μM of B for 60 min at 37^0C. Following incubation the cells were centrifuged at 50 g and the supernatant was stored at -80 ^{0}C until analysis.

Bilirubin glucuronide formation by human and rat hepatic microsomes

For the preparation of hepatic microsomes livers were homogenized in 0.1 M Tris-HCl buffer (pH 7.4) containing 1.15 % KCl and 1 mM EDTA. Microsomes were prepared by differential centrifugation according to van der Hoeven et al, (1974). Microsomal protein concentrations were determined by the method of Lowry et al, (1951) using bovine serum albumin as standard. The microsomal BG formation was measured according to the method of Burchell (1981). The incubation mixture contained 0.4 mM B, 4 mM of UDPGA (Sigma-Aldrich, St. Louis, MO) and 0.4 mg of protein in a final volume of 400 μl. Following 10 min of incubation at 37^0C reaction was stopped by adding 400 μl of ice-cold methanol. Samples were centrifuged at 10 000g for 10 min and the supernatant was stored at -80 ^{0}C until HPLC analysis. BG was confirmed by β-glucuronidase cleavage of the conjugates in separate incubations by the addition of 1000 U of β-glucuronidase (from bovine liver) in sodium acetate buffer (pH 4.5) in the presence or absence of 10 mM of D-saccharic acid-1,4-lactone. (Sigma-Aldrich, St. Louis, MO). Following incubation at 37^0C for 2 h reaction was stopped by adding ice

cold methanol, samples were centrifuged at 10 000g for 10 min and the supernatant was stored at -80 ^{0}C until HPLC analysis.

HPLC analysis of bilirubin and p-nitrophenol conjugates

The amount of conjugates was determined by HPLC analysis. Samples were filtered through a filter with 0.45 μm pore size, and 100 μl of sample was applied to HPLC. Chromatography was performed on a Merck Hitachi HPLC system using an L-6200 A pump, an L-4250 UV detector and a D-6000 A interface with a 100×4.6 mm Chromolith Performance RP 18e (Merck, Darmstadt, Germany) chromatographic column for analytical separation. To separate BG a gradient elution was employed. The solvents were composed of, A: 75 % 0.01 M sodium phosphate buffer pH 3.2 containing 150 μl/l triethylamine; 25 % acetonitrile, B: 20% sodium phosphate buffer pH 3.2 containing 1.5 ml/l triethylamine, 80 % acetonitrile. After 1 min of isocratic elution with solvent A the gradient started reaching 10 % of B in 6 min. Following separation of the conjugates, the column was rinsed of remaining substrate as well as other hydrophobic components by employing gradient reaching 100 % solvent B and then the column was re-equilibrated. BMG and BDG were monitored at 450 nm, the flow rate was 3 ml/min. pNP-glucuronide was analyzed isocratically using 93 % of 0.01 M sodium phosphate buffer pH 3.2, 7% acetonitrile as a mobile phase. The flow rate was 2 ml/min and the effluent was monitored with a spectrophotometric detector at 305 nm. Conjugates were quantified using calibration curves prepared with pNPG and B as standards.

Mass spectrometric analysis of bilirubin conjugates

Mass spectrometric experiments were run on an Applied Biosystem/MDS Sciex API-2000 tandem mass spectrometer in Turboionspray mode. Two Perkin Elmer Series

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200 HPLC pumps were coupled to the system. The samples were acquired in normal Q1 scan and in MRM scan modes. In the Q1 scan measurements the instrument was set in the mass range of 500 - 1000 using a scan time of 1 sec. In MRM mode 937-475 and 761-475 transitions were monitored using a scan time of 150 msec each. The collision energy was 30 eV in both transiting channels. The samples were injected into water: acetonitrile, 1:1 at a flow rate of 0.2 ml/min. Analyst 1.4 software was used for controlling the acquisitions and for data processing.

Statistics

Data are reported as mean ± S.D. Statistically significant differences were evaluated with two-tailed Student's *t* test. In all cases, $p < 0.05$ was deemed significant.

Results

HPLC analysis and identification of bilirubin glucuronides

Fig. 1 shows a typical HPLC chromatogram of bilirubin (B) and bilirubin mono- (BMG) and diglucuronides (BDG). The multiple peaks for BMG and BDG represent the isomers of each compound due to the endo and exo position of the vinyl groups reflecting the three isoforms of the parent B (IXα, XIIIα and IIIα) (Odell et al, 1990, Brower et al, 2001). The identity of the conjugates was confirmed by treatment with β-glucuronidase enzyme. The peak of all glucuronides disappeared due to enzymatic cleavage, while D-saccharic-acid-1,4-lactone inhibited the hydrolysis, consequently all peaks were preserved (data not shown). BDG and BMG were collected according to their peaks observed during HPLC and aliquots of the samples containing the separated conjugates were applied to MS analysis. The m/z for BMG was 761.2 and that of for BDG was 936.9. Calibration curve was prepared using B as standard. Since there is no significant difference between the absorbance spectra of B and BMG or BDG (Wu, 1983; Brower et al., 2001), this calibration was used for the determination of BMG and BDG concentration.

BG and pNPG biliary excretion index determination in sandwich-cultured human and rat hepatocytes

B and pNP was incubated with sandwich-cultured rat and human hepatocytes following 4 days of culture. After washing out the medium containing the substrates the cells were incubated in standard or Ca^{2+}, Mg^{2+} free HBSS. The amount of conjugates disposed into the medium was determined by HPLC analysis. Significantly more BG was observed in Ca^{2+}, Mg^{2+} free HBSS than in the standard medium both in human and rat

hepatocyte cultures (270 % and 500 % over the standard medium, respectively), showing that BG is predominantly excreted into the canalicular networks (Fig. 2). In contrast, although the amount of pNPG was significantly different in Ca^{2+}, Mg^{2+} free HBSS from that observed in the standard medium (84.6 ± 1.1 versus 80.2 ± 1.1 pmol/10^6 cells/min) the difference was much smaller than that in the case of BG.

BEI for BG was 62.5 in human and 80.6 in rat sandwich-cultured hepatocytes and 5.2 for pNPG in rat hepatocytes.

The ratio of BDG to BMG in the medium and in the canalicular networks

We compared the BDG/BMG ratio in the medium after 60 min of B loading period with that measured in the canalicular networks (Fig. 3). Sandwich-cultured rat hepatocytes were incubated with B for 60 min and the BG content of the medium was analyzed. Cells were rinsed twice with warm HBSS and 0.5 ml of standard or Ca^{2+}, Mg^{2+} free HBSS was added. The BG content of the canalicular networks was determined by the difference of BG concentration obtained in the Ca^{2+}, Mg^{2+} free HBSS and standard HBSS. The proportion of BDG and BMG in the medium at the end of the B loading phase was 35.5 ± 3 % and 64.5 ± 3 %, respectively. The proportion of BDG and BMG in the canalicular networks turned out to be 59.7 ± 2 % and 40.3 ± 3 %, respectively. These values were compared to the in vivo data published by Mesa et al (1997), who observed 38 ± 30 % of BDG and 62 ± 10 % of BMG in rat serum, and 60 ± 4 % of BDG and 38 ± 4 % of BMG in rat bile. The BDG/BMG ratio observed in the medium of sandwich-cultured rat hepatocytes and in the serum was almost the same (0.55, 0.6 respectively), while the reciprocal relationship was obtained for the conjugates in the canalicular networks (1.48) just like in the bile (1.5).

Shift of the equilibrium among conjugates towards BDG during culturing

In the medium the ratio of BDG to BMG produced by freshly isolated human hepatocytes was significantly different from that obtained with rat hepatocytes (36.5/63.5 and 6.9/93.1, respectively), suggesting that human hepatocytes produce more BDG than rat hepatocytes (Fig. 4). The equilibrium among conjugates in the medium of both human and rat hepatocyte cultures shifted towards BDG during culturing. The amount of BDG following 96 h of culture was about 2.5-fold greater than that of BMG in the medium of human hepatocyte cultures. In contrast, the medium of rat hepatocyte cultures contained significantly less BDG than BMG.

The ratio of BDG to BMG formed by human and rat hepatic microsomes

The UGT1A1 activities in the liver microsomes of five human donors were measured as total BG formation, and compared with that obtained in the liver microsomes pooled from fourteen donors (Fig.5). The ratio of BDG to BMG was also determined. The average of the UGT1A1 specific activities measured in the five livers was similar to that obtained with the pooled microsomes, 0.603 ± 0.232 and 0.670 nmol/mg prot./min, respectively, however almost three-fold differences were observed concerning the individual values. The UGT1A1 activity of the pooled liver microsomes prepared from five male Wistar rats was higher than that of the human microsomes (0.978 nmol/mg prot./min). The ratio of BDG to BMG formed by the pooled human microsomes was higher than that formed by rat microsomes (0.10 and 0.04, respectively).

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Discussion

During isolation by collagenase perfusion hepatocytes lose their polarity, consequently the membrane specific expression of transport proteins disappears. Previous studies using hepatocytes in sandwich culture have shown that the cells in this configuration reestablished dynamically, in 3 days after seeding bile canalicular networks between adjacent cells reformed (LeCluyse et al, 2000; Chandra et al, 2001). Several studies have demonstrated that three membrane domains were distinct after 5 days of culture, in addition canalicular transport proteins, such as mdr1, mrp2, bsep were localized to the specific membrane surface (Luttringer et al, 2002). Liu et al (1999a) demonstrated that long-term sandwich-cultured hepatocytes represent a useful in vitro model for studying biliary disposition of compounds. Extracellular Ca^{2+} depletion disrupted the tight junctions between adjacent cells, the content of the canalicular networks appeared in the extracellular medium and could be studied (Liu et al 1999b), consequently the BEI for a compound could be determined.

The aim of our work was to use the sandwich configuration of primary hepatocytes as an in vitro model in studying the basolateral versus canalicular disposition of bilirubin conjugates. Bilirubin conjugates are eliminated through basolateral (mrp1, mrp3) and canalicular (mrp2) transporters as well (Jedlitschky et al, 1997; Keppler and König, 2000; König et al, 2003), although under physiological conditions the bilirubin conjugates are primarily excreted from the body via the bile (Mesa et al 1997). In the present study the BEI values calculated for BG in 96 h sandwich culture of both rat and human hepatocytes clearly show that BG are preferably excreted into the canalicular networks, which suggest dominant role for mrp2 in BG elimination. This finding is in

good agreement with the in vitro data of Jedlitshky et al (1997) and the in vivo data of Clarke et al, (1997). In contrast to BG the low BEI value for pNPG suggests that this metabolite is preferably eliminated by the basolateral transporters, which is in line of the empirical observation that small molecules are mainly excreted via the urine.

Absence of or decrease in functionally active mrp2 localized to the canalicular membrane due to either genetic disorders, e.g. Dubin-Johnson syndrome or cholestasis prevents or diminishes the secretion of BG into the bile. The upregulation of mrp3 plays a compensatory role in these cases since the rate of basolateral efflux increases and prevents cell damage during impaired transport into the bile (Keppler and König, 2000). In Dubin-Johnson syndrome or Eisai transport deficient mutant rats the BG are excreted from hepatocytes by mrp3 resulting in conjugated hyperbilirubinaemia. Under such conditions the extended period for intracellular storage and metabolism of B may explain the shift of equilibrium of BDG to BMG towards BDG (Clarke et al, 1997; Mesa et al 1997). The BDG/BMG ratio is different in serum and bile in vivo, 0.6 and 1.5, respectively (Mesa, 1997), which is in good agreement with our data (medium and canalicular networks: 0.55 and 1.48, respectively). These results suggest that sandwich culture of hepatocytes is a useful model to distinguish between canalicular and basolateral transport of BG.

In freshly isolated rat hepatocytes the BDG/BMG ratio was much lower than that obtained in a 24 h culture. A reason for this BDG/BMG shift might be the change in either the transporter or UGT1A1 activity after seeding. The microsomal UGT1A1 activity towards bilirubin was found to be the same in rat liver, freshly isolated hepatocytes and hepatocytes after 5 days of culturing regardless of the culture

configuration (Richert et al, 2002). However, depending on the extracellular matrix and the medium used expression of transport proteins highly varies during culturing. Luttringer et al, (2002) described significant changes in the mRNA level of transport proteins during culturing even in sandwich configuration. These changes in the expression of transporters can be further modulated by the short-term regulation of the trafficking of these proteins from the intracellular pools to the membrane surface and vice versa (Kipp et al, 2001). Alterations in the regulation processes during both cell isolation and seeding might also explain the difference observed between freshly isolated hepatocytes and hepatocytes after attachment. The BDG/BMG value obtained from freshly isolated human hepatocytes is much higher than that observed with rat cells. This difference became more pronounced after 96 h of culturing, which is in agreement with reports indicating that the amount of BDG to BMG is significantly higher in human bile than in the rat bile (Clarke et al, 1997; Mesa et al, 1997). Although the hepatic UGT1A1 activities of the donors in the present study were in the normal range, we observed some variability among donors concerning the BDG/BMG ratio during microsomal metabolism. An explanation might be the inter-individual variations in the active transport processes across the endoplasmic reticulum membrane (Csala et al, 2004). The difference between BDG/BMG ratio obtained with freshly isolated hepatocytes from human and rat was retained on the microsomal level as well, BDG/BMG ratio was significantly lower in experiments with rat microsomes than with human microsomes.)

The present study demonstrates that sandwich culture of human and rat hepatocytes is a proper in vitro model for studying B conjugation and sinusoidal versus canalicular transport of BG. The BDG/BMG ratio obtained in the medium and

canalicular networks was very similar to that found previously in vivo in the serum and bile. In vitro determination of the shift in the BDG/BMG ratio brought about by drug interactions might be used for the prediction of insufficient in vivo activity of conjugating enzymes or transporters, the consequences of which can be impaired bile secretion and cholestasis.

References

Annaert PP, Turncliff RZ, Booth CL, Thakker DR and Bouwer KLR (2001) P-glycoprotein-mediated in vitro biliary excretion in sandwich-cultured rat hepatocytes. *Drug Metab Dispos* **29:**1277-1283.

Bayliss MK and Skett P (1996) *Human Cell Culture Protocols*. pp 369-389, Humana Press, New Jersey

Brower JO, Lightner DA and McDonagh AF (2001) Aromatic congeners of bilirubin: synthesis, stereochemistry, glucuronidation and hepatic transport. *Tetrahedron* **57:**7813-7827.

Burchell B (1981) Bilirubin UDPglucuronyltransferase, in *Methods in Enzymology, vol 77: Detoxification and Drug Metabolism: Conjugation and Related Systems* (Jakoby WB ed) pp 188-192, Academic Press, New York

Burchell B, McGurk K, Brierley CH and Clarke DJ (1997) UDP-Glucuronosyl-transferases, in *Comprehensive Toxicology, vol 3: Biotransformation* (Guengerich FP ed) pp 401- 435, Elsevier Science Ltd, Oxford

Chandra P, LeCluyse EL and Brouwer KLR (2001) Optimization of culture conditions for determining hepatobiliary disposition of taurocholate in sandwich-cultured rat hepatocytes. *In Vitro Cell Dev Biol* **37:**380-385.

Clarke DJ, Moghrabi N, Monagham G, Cassidy A, Boxer M, Hume R and Burchell B (1997) Genetic defects of the UDP glucuronosyltransferase-1 (UGT1) gene that cause familiar non-haemolytic unconjugated hyperbilirubinaemias. *Clin Chim Acta* **266:**63-74.

Csala M, Staines AG, Banhegyi G, Mandl J, Coughtrie MWH and Burchell B (2004) Evidence for multiple glucuronide transporters in rat liver microsomes. *Biochem Pharmacol* **68:**1353-1362.

Higuchi K, Kobayashi Y, Kuroda M, Tanaka Y, Itani T, Araki J, Mifuji R, Kaito M and Adachi Y (2004) Modulation of organic anion transporting polypeptide 1 and multidrug resistance protein 3 expression in the liver and kidney of Gunn rats. *Hepatol Res* **29:**60-66.

Hoeven van der TA and Coon MJ (1974) Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. *J Biol Chem* **249:**6302–6310.

Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B and Keppler D (1997) ATP- dependent transport of bilirubin glucuronides by the multidrug resistance protein mrp1 and its hepatocyte canalicular isoform mrp2. *Biochem J* **327:**305-310.

Kamisako T, Kobayashi Y, Takeuchi , Ishihara T, Higughi K, Tanaka Y, Garabazza EC and Adachi Y (2000) Recent advances in bilirubin metabolism research: mechanism of hepatocyte bilirubin transport and its clinical relevance. *J Gastroenterol* **35:**659-664.

Keppler D and König J (2000) Hepatic secretion of conjugated drugs and endogenous substances. *Seminars Liver Diseases* **20:**265-272.

Kipp H, Pichetshote N, and Arias IM (2001) Transporters on demand. *J Biol Chem* **276:**7218-7224.

Koebe HG, Pahernik S, Eyer P and Schildberg FW (1994) Collagen gel immobilization: a useful cell culture technique for long-term metabolic studies on human hepatocytes.

Xenobiotica. **24:** 95-107.

Kostrubsky VE, Strom SC, Hanson J, Urda E, Rose K, Burliegh J, Zocharsky P, Cai H, Sinclair JF and Sahi J (2003) Evaluation of hepatotoxic potential of drugs by inhibition of bile-acid transport in cultured primary human hepatocytes and intact rats. *Toxicol Sci* **76:**220-228.

König J, Nies AT, Cui Y and Keppler D (2003) MRP2 the apical export pump for anionic conjugates, in *ABC proteins: from bacteria to man* (Holland IB, Cole SPC, Kuchler K and C. F. Higgins eds) pp423-441, Elsevier Science Ltd. Oxford

LeCluyse EL, Fix JA, Audus KL and Hochman JH (2000) Regeneration and maintenance of bile canalicular networks in collagen-sandwiched hepatocytes. *Toxicol in Vitro* **14:**117-132.

Liu X, Chism P, LeCluyse EL, Brouwer KR and Brouwer KLR (1999a) Correlation of biliary excretion in sandwich-cultured rat hepatocytes and in vivo in rats. *Drug Metab Dispos* **27:**637-644.

Liu X, LeCluyse EL, Brouwer KR, Gan LSL, Lemasters JJ, Stieger B, Meier PJ and Brouwer KLR (1999b) Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. *Am J Physiol* **277:**G12-G21.

Liu X, LeCluyse EL, Brouwer KR, Lightfoot RM, Lee JI and Brouwer KLR (1999c) Use of Ca^{2+} modulation to evaluate biliary excretion in sandwich-cultured rat hepatocytes. *J Pharmacol Exp Ther* **289:**1592-1599.

Lowry H, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265-275.

Luttringer O, Theil FP, Lave T, Wernli-Kuratli K, Guentert TW and de Saizieu A (2002) Influence of isolation procedure, extracellular matrix and dexamethasone on the regulation of membrane transporters gene expression in rat hepatocytes. *Biochem Pharmacol* **64:**1637-1650.

Mesa VA, De Vos R and Fevery J (1997) Elevation of the serum bilirubin diconjugate fraction provides an early marker for cholestasis in the rat. *J Hepatol* **27:**912-916.

Odell GB, Mogilevsky WS and Gourley GR (1990) High-performance liquid chromatographic analysis of bile pigments as their native tetrapyrroles and as their dipyrrolic azosulfanilate derivatives. *J Chromatogr*.**529:**287-298.

Richert L, Binda , amilton G, Viollon-Abadie C, Alexandre E, Bigot-Lasserre D, Bars R, Coassolo P and LeCluyse E (2002) Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of cultured rat hepatocytes. *Toxicol in Vitro* **16:**89-99.

Rippin SJ, Hagenbuch B, Meier PJ and Stieger B (2001) Cholestatic expression pattern of sinusoidal and canalicular organic anion transport system in primary cultured rat hepatocytes. *Hepatology* **33:**776-782.

Seglen PO (1976) Preparation of isolated rat liver cells. *Meth Cell Biol* **13:**29-83.

Wu TW (1983) Delta bilirubin: the fourth fraction of bile pigments in human serum. *Israel J Chem* **23:**241-247.

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Legends for Figures

Fig. 1. A representative elution profile of the bilirubin conjugates from the supernatant of freshly prepared human hepatocytes incubated with 25 μM of bilirubin for 60 min at 37^0C. The elution peaks of bilirubin mono- and diglucuronides as well as the residual unconjugated bilirubin are indicated.

Fig. 2. Bilirubin glucuronide (BG) and p-nitrophenol glucuronide (pNPG) disposition into standard and into Ca^{2+}, Mg^{2+} free HBSS in sandwich cultures of primary human and rat hepatocytes. Data are expressed as % of conjugates excreted into the Ca^{2+}, Mg^{2+} free medium. The biliary excretion index (BEI) of BG and pNPG are reported above the individual bars. Means of 3 independent experiments ± S.D. *, $p < 0.05$, for standard versus Ca^{2+}, Mg^{2+} free HBSS; **, $p < 0.01$, for standard versus Ca^{2+}, Mg^{2+} free HBSS.

Fig. 3. The proportion of bilirubin di- and monoglucuronides (BDG and BMG) in the medium and in the canalicular networks of 96-h sandwich culture of rat hepatocytes. Data are expressed as % of total BG. The BDG/BMG ratio is indicated above the bars. Means of 3 independent experiments ± S.D.

Fig. 4. Change in the proportion of bilirubin mono- and diglucuronides (BMG and BDG) in the medium of rat and human hepatocyte suspensions and in primary hepatocyte cultures is presented. Data are expressed as % of total BG. The BDG/BMG ratio is indicated above the bars. Means of 3 independent experiments ± S.D. *, $p < 0.01$, for human hepatocyte suspension versus rat hepatocyte suspension, $^{\#}$, $p < 0.01$, for 96 h primary culture of human versus of rat hepatocytes.

Fig. 5. Formation of bilirubin mono- and diglucuronides (BMG and BDG) by human and rat hepatic microsomes. Bilirubin conjugating enzyme activity data of hepatic microsomes from five individual human donors are compared with the enzyme activity of hepatic microsomes pooled from 14 human donors, and with hepatic microsomes pooled from five Wistar rats. The BDG/BMG ratio is indicated above the bars. *, $p < 0.05$, for pooled hepatic human microsomes versus pooled hepatic rat microsomes.

HM - human microsomes; RM - rat microsomes

Figure 1

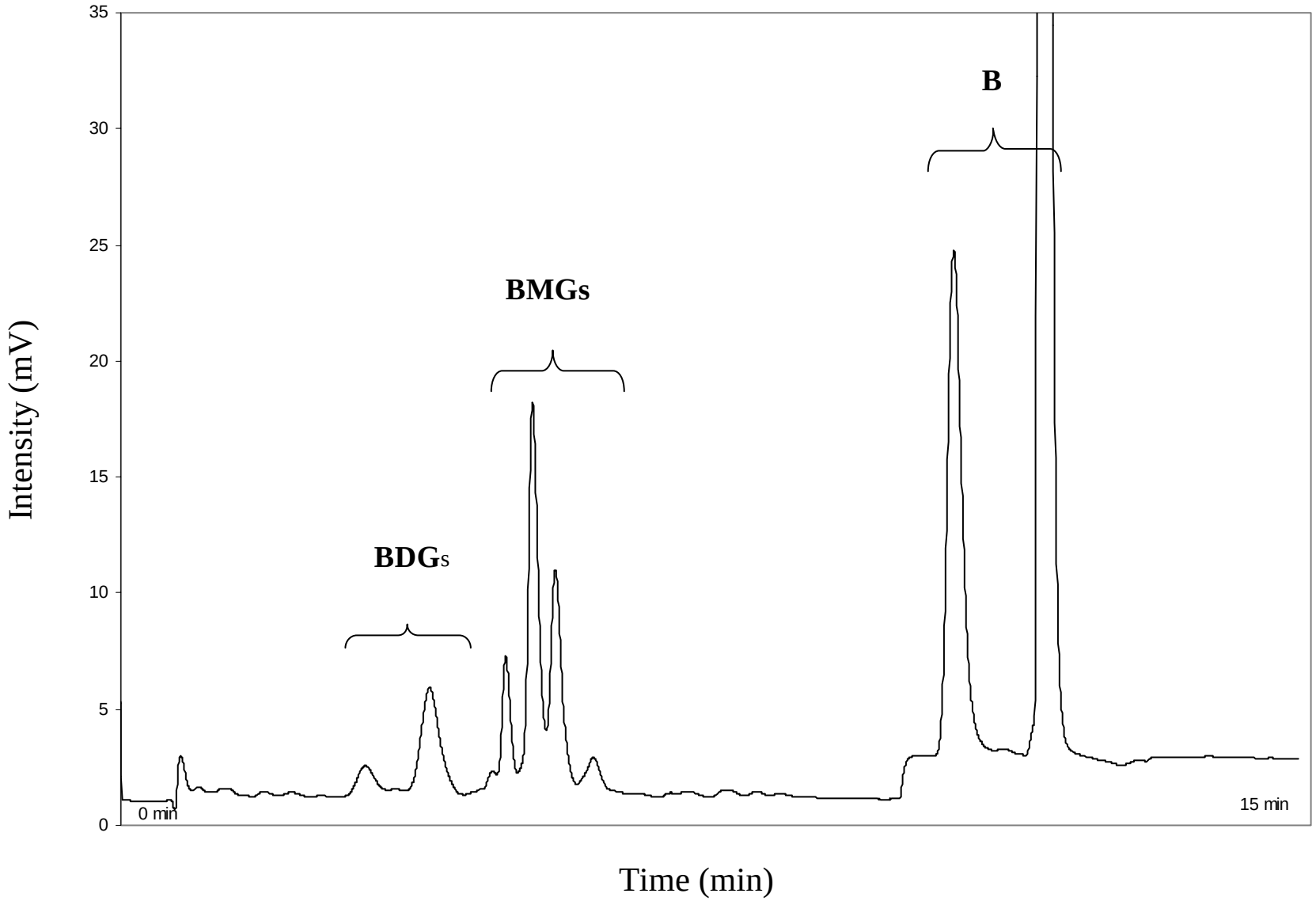

Figure 2

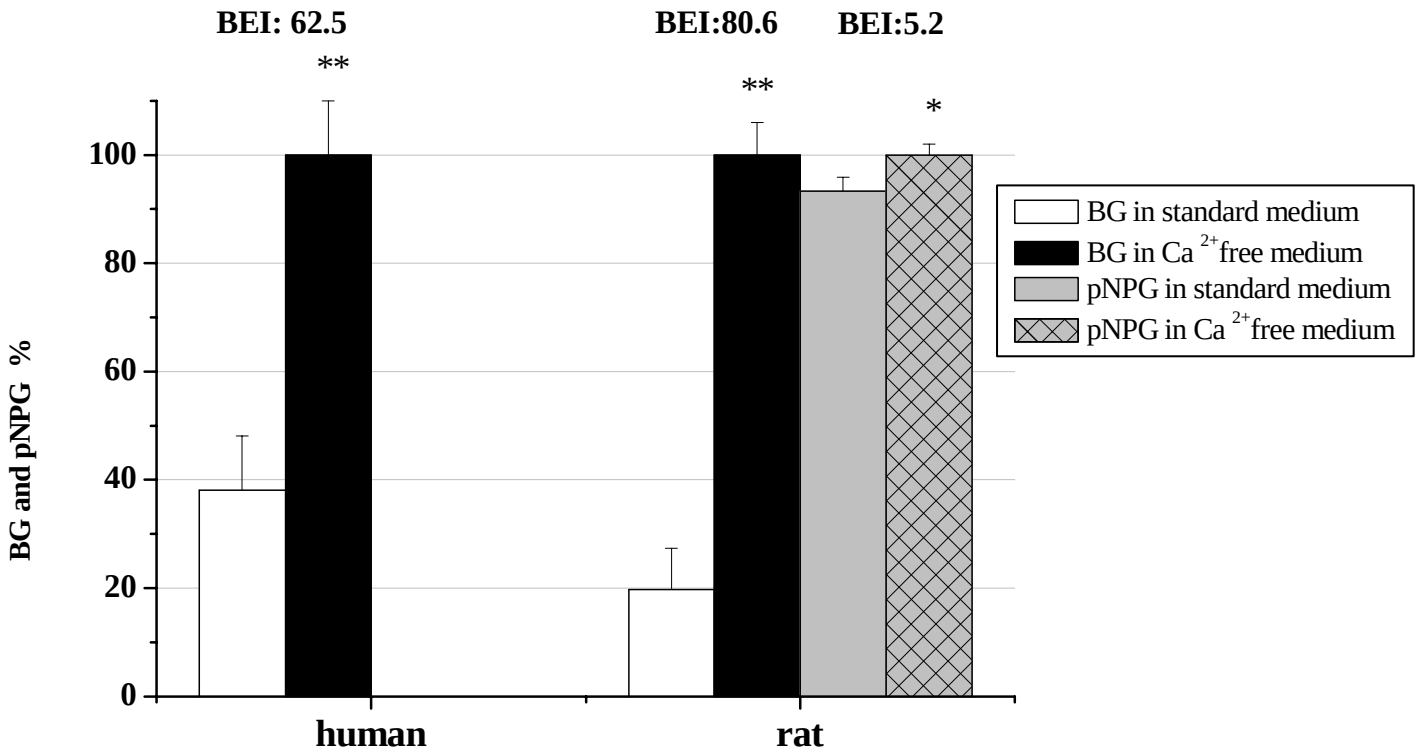

Figure 3

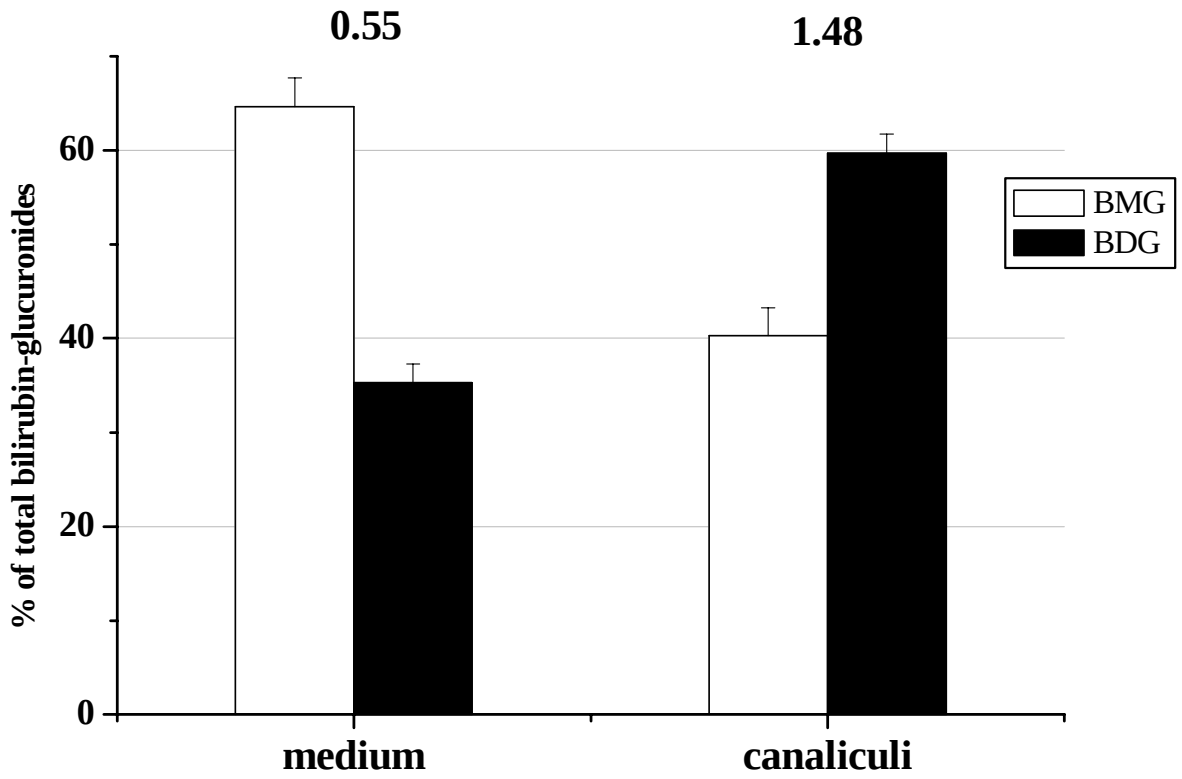

Figure 4

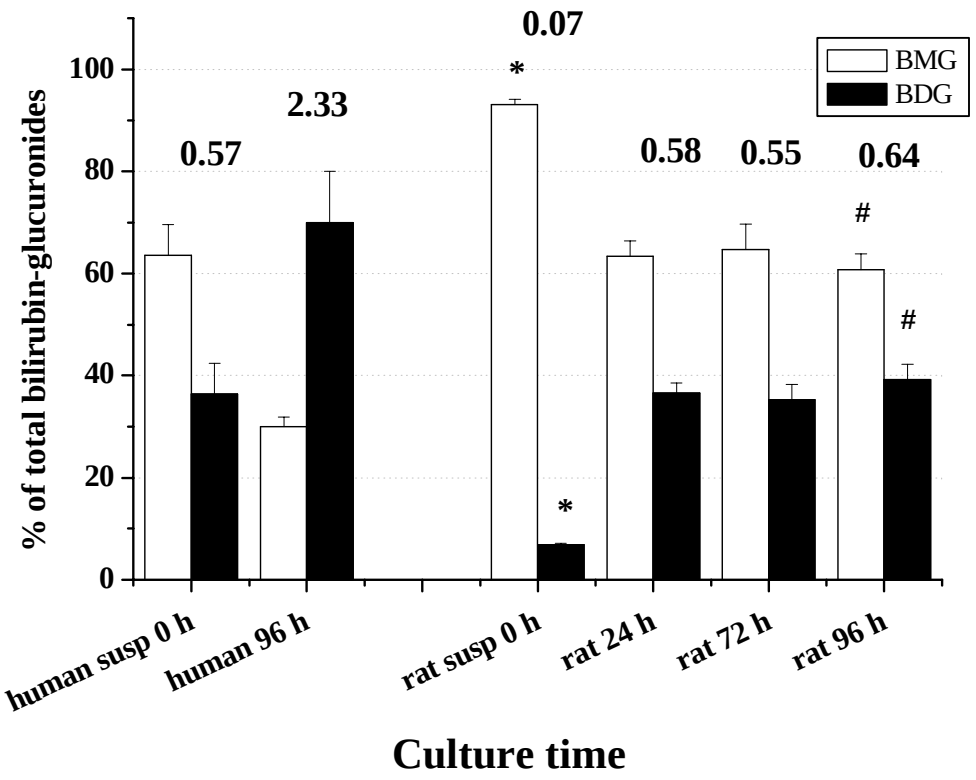

Figure 5