Journal of Pharmaceutical and Biomedical Analysis 92 (2014) 149–159

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Simultaneous determination of bilirubin and its glucuronides in liver microsomes and recombinant UGT1A1 enzyme incubation systems by HPLC method and its application to bilirubin glucuronidation studies

Guo Ma[*,1], Jiayuan Lin[1], Weimin Cai, Bo Tan, Xiaoqiang Xiang, Ying Zhang, Peng Zhang[**]

Department of Clinical Pharmacy, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, PR China

ARTICLE INFO

Article history:
Received 8 August 2013
Received in revised form 17 January 2014
Accepted 18 January 2014
Available online 27 January 2014

Keywords:
Bilirubin
Glucuronides
UGT1A1
Liver microsomes
HPLC

ABSTRACT

Bilirubin, an important endogenous substances and liver function index in humans, is primarily eliminated via UGT1A1-catalyzed glucuronidation. Instability of bilirubin and its glucuronides brings substantial technical challenges to conduct *in vitro* bilirubin glucuronidation assay. In the present study, we developed a simple and robust HPLC method for simultaneous determination of unconjugated bilirubin (UCB) and its multiple glucuronides, i.e. bilirubin monoglucuronides (BMGs, including BMG1 and BMG2 isomers) and diglucuronide (BDG) in rat liver microsomes (RLM), human liver microsomes (HLM) and recombinant human UGT1A1 enzyme (UGT1A1) incubation systems, and applied it to study *in vitro* bilirubin glucuronidation. UCB, BMG1, BMG2, BDG and their isomers in the incubation mixtures were successfully separated using a C18 column with UV detection at 450 nm and mobile phase consisted of 0.1% formic acid in water and acetonitrile by a linear gradient elution program. Assay linearities of bilirubin were confirmed in the range 0.01–2 μM. Precision of UCB, BMG1, BMG2 and BDG ($n = 5$) at low, medium and high concentration was within the range of RSD 0.4–3.7%, accuracy expressed in the mean assay recoveries of them ($n = 5$) ranged from 92.8 ± 1.5% to 104.3 ± 2.2% for intra- and inter-day assays and the mean extraction recoveries of them ($n = 5$) were above 91.5 ± 1.0%. Stability of bilirubin and its glucuronides was satisfactory at 37 °C in the incubation solutions during the reaction (30 min), 25 °C for 24 h and −70 °C for 7 d in the processed incubation samples with methanol. Furthermore, we established stable, reliable *in vitro* incubation systems and optimized the incubation conditions to characterize the kinetics of bilirubin glucuronidation by RLM, HLM and UGT1A1, respectively. The kinetic parameters of formation of total bilirubin glucuronides (TBG, the sum of BMG1, BMG2 and BDG) were as follows: K_m of 0.45 ± 0.016, 0.40 ± 0.022, 0.44 ± 0.018 μM, V_{max} of 2.65 ± 0.057, 1.86 ± 0.029, 2.95 ± 0.036 nmol/mg/min, CL_{int} of 5.92 ± 0.22, 4.70 ± 0.079, 6.72 ± 0.27 mL/mg/min by RLM, HLM and UGT1A1, respectively. Bilirubin glucuronidation obeyed the Hill equation by RLM and the Michaelis–Menten equation by HLM and UGT1A1 in the range of substrate concentration selected, respectively. In addition, the relative proportions between BDG and BMGs were in connection with enzyme sources (e.g. RLM, HLM and UGT1A1) and bilirubin concentration.

Abbreviations: UCB, unconjugated bilirubin; CB, conjugated bilirubin; BG, bilirubin glucuronides; BMGs, bilirubin monoglucuronides; BDG, bilirubin diglucuronide; TBG, total bilirubin glucuronides; UGT(s), UDP-glucuronosyltransferase(s); UGT1A1, UDP-glucuronosyltransferases1A1; RLM, rat liver microsomes; HLM, human liver microsomes; UDPGA, uridine diphosphoglucuronic acid; DMSO, dimethylsulfoxide; MRP2, multidrug resistance-associated protein 2; HPLC, high performance liquid chromatography; QC, quality control; LOD, limit of detection; LLOQ, lower limit of quantification; RSD, relative standard deviation; Conc., concentration(s); V, reaction velocity; K_m, Michaelis–Menten constant; V_{max}, maximum reaction velocity; CL_{int}, intrinsic clearances; R^2, residual sum of squares; *AIC*, Akaike information criterion; CDER, Center for Drug Evaluation and Research.

* Corresponding author. Tel.: +86 21 51980025; fax: +86 21 51980001.
** Corresponding author. Tel.: +86 21 51980024; fax: +86 21 51980001.
E-mail addresses: mg0328@126.com, mg0328@fudan.edu.cn (G. Ma), zhampm@126.com (P. Zhang).
[1] Co-first authors.

0731-7085/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jpba.2014.01.025

1. Introduction

Bilirubin is the principal constituent of mammalian bile pigment and end-product of heme catabolism. Approximately 250–300 mg of bilirubin is produced in a normal adult each day. Bilirubin is an important index of liver function and biomarker of hepatotoxicity, as well as an important clinical basis for determining jaundice. As an essential endogenous substance in humans and animals, bilirubin was long thought to be a non-functional and toxic waste product. Recent studies [1–3] have shown that bilirubin has multiple biological functions in animals and plants, for example, potent antioxidant and cytoprotective effects at physiological and mildly elevated concentrations, as well as activation of heme oxygenase, and can protect against cardiovascular diseases (e.g. atherosclerosis) and tumor development. However, it can cause apoptosis, cytotoxicity and neurotoxicity at markedly elevated plasma and tissue bilirubin levels, and result in severe, irreversible brain and neurological damage (e.g. kernicterus), especially in neonates [1,4–7].

Bilirubin is mainly metabolized by liver. Before it is transported into liver, bilirubin exists mostly in the form of unconjugated bilirubin (UCB) and binds highly to albumin in the blood. After hepatic uptake, UCB is extensively metabolized to bilirubin glucuronides (BG) by UDP-glucuronosyltransferases1A1 (UGT1A1) localized primarily in smooth endoplasmic reticulum of hepatocyte. In this glucuronidation reaction, a glucuronosyl moiety is conjugated to one of the propionic acid side chains, located on the C_8 and C_{12} carbons of the two central pyrrole rings of bilirubin, resulting in producing two bilirubin monoglucuronides (BMGs) isomers (i.e. BMG1 and BMG2). BMGs were further glucuronidated, and formed bilirubin 8,12-diglucuronide (BDG) [8] (Fig. 1). In adult humans, over 80% of the bilirubin conjugates are normally BDG [9], whereas BMGs predominate in newborns [10]. Finally, BG (i.e. BMGs and BDG) formed are secreted into bile by multidrug resistance-associated protein 2 (MRP2), and subsequently eliminated via feces and urine [11].

UGT1A1 is a critical enzyme responsible for metabolism and detoxification of bilurubin [12]. Glucuronidation by UGT1A1 is an essential step for bilirubin elimination [13]. Xenobiotics (e.g. SN-38 [14], atazanavir, indinavir [15,16], erlotinib [17], sorafenib [18]) inhibiting UGT1A1, and genetic variants resulting in partial or complete loss of UGT1A1 activity, can cause disorder of bilirubin metabolism, and lead to accumulation of bilirubin in blood and/or brain, which further result in jaundice, hyperbilirubinemia, kernicterus, Crigler–Najjar syndromes (Types I and II), Gilbert's syndrome, and even death [1,19–21]. As a result, in drug discovery, development and use settings, the *in vitro* ability of drug to inhibit bilirubin glucuronidation is commonly evaluated. In addition, some xenobiotics (e.g. phenobarbital, dexamethasone, rifampicin and herbal extracts Yin zhi huang) can also induce UGT1A1 gene expression and enhance UGT1A1 activity by a number of multifunctional nuclear receptors such as constitutive androstane receptor (CAR), pregnane X receptor (PXR), glucocorticoid receptor (GR), aryl hydrocarbon receptor (AhR), and hepatocyte nuclear receptor 1α (HNF1α) [22–25]. These factors contribute to promote bilirubin glucuronidation, and reduce serum UCB level. They might have important clinical application in preventing and treating unconjugated hyperbilirubinemia and neonatal jaundice.

It is not difficult to see that establishing a simple and robust assay method for accurate measurement of bilirubin and its glucuronides is vitally important for us to study bilirubin glucuronidation and its inhibition or induction, which has important clinical significance in diagnosis, prevention and treatment of bilirubin-related malady or toxic reaction, for example, jaundice, hyperbilirubinemia and kernicterus. However, as a weakly polar, poorly soluble compound, bilirubin is very labile. It is highly photosensitive and readily oxidized, rapidly degraded in both acidic and alkaline solutions, and high-affinity for proteins (e.g. serum albumin), as well as strong adsorption on experimental equipments and materials (e.g. nonspecific binding of bilirubin to walls of the plastic pipes, tips, vials and tubes, as well as the chromatographic channel and column) [19,26,27]. Equally as problematic is the instability of bilirubin glucuronides, especially BMGs. In aqueous media, BMGs was rapidly transformed into BDG and UCB by dipyrrole exchange mechanism [28]. Furthermore, bilirubin itself is composed of three isomers (i.e. bilirubin IX-α, XIII-α and III-α), and bilirubin glucuronidation involves a sequential reaction that produces multiple glucuronides (i.e. BMG1, BMG2, BDG and their isomers), resulting in difficult quantitation of glucuronidation assay and establishment of initial rate condition, if not given particular attention. All these factors, especially, *in vitro* instability, bring the substantial technical challenges in bilirubin glucuronidation [28,29]. These challenges have been manifested in significant disparities in estimated kinetic parameters and mechanism for bilirubin glucuronidation. Three groups [26,30,31] reported that bilirubin glucuronidation obeyed Michaelis–Menten kinetics. One group [32] reported it exhibited substrate inhibition kinetics, and the other group [33] reported it obeyed Michaelis–Menten kinetics at low protein concentration

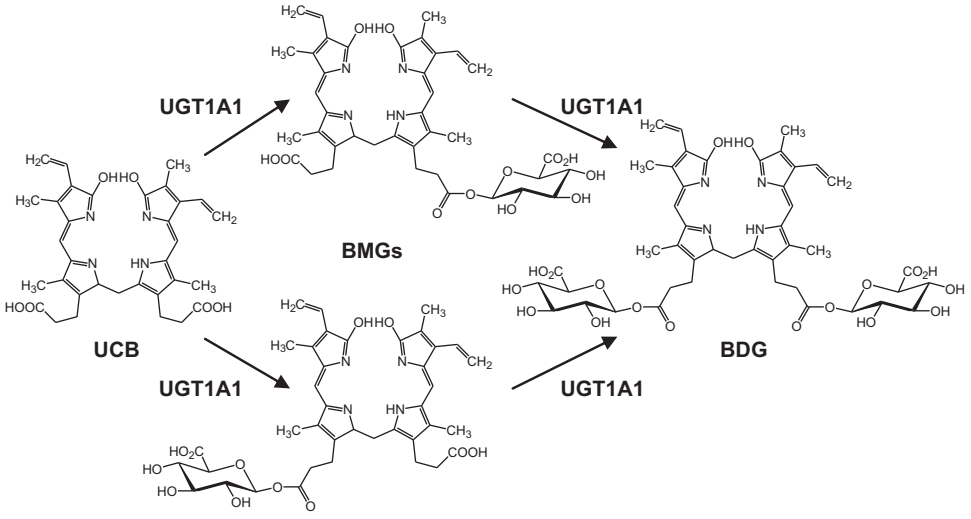

Fig. 1. The molecular structures of bilirubin and its glucuronides. UCB was metabolized to BMGs (including two isomers BMG1 and BMG2), and BMGs was further metabolized to BDG by UGT1A1. UCB, unconjugated bilirubin; BMGs, bilirubin monoglucuronides; BDG, bilirubin diglucuronide; UGT1A1, recombinant human UGT1A1 enzyme.