Clinica Chimica Acta 475 (2017) 28–35

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

Bile acid profiles in neonatal intrahepatic cholestasis caused by citrin deficiency

Ching-Hsuan Yang[a,1], Chiung-Yu Chen[b,1], Yen-Yin Chou[c], Hung-Chih Chiu[b], Wei-Lun Tsai[d], Shu-Chu Shiesh[a,d,*]

[a] Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[b] Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan
[c] Department of Pediatrics, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan
[d] Department of Pathology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan

ARTICLE INFO

Keywords:
Bile acid profile
Cholestasis
Citrin
Citrullinemia

ABSTRACT

Background: Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) is characterized by conjugated hyperbilirubinemia and increased plasma bile acid concentrations. However, the underlying mechanisms remain unclear. We established a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for simultaneously quantifying plasma bile acids and examined bile acid profiles in NICCD infants.
Methods: We measured 15 bile acids within 15 min and found a wide linear range for individual bile acids.
Results: The within-run and run-to-run CV of all bile acids was 1.2–10.9% and 3.1–10.8%, respectively, with a mean recovery of 90.5–112.6%. Compared to infants with citrullinemia without mutations in *SLC25A13* (non-NICCD), NICCD infants showed increased plasma total bile acid concentrations (mean: 201 vs. 42 μM, $p < 0.001$), with a distinct bile acid profile characterized by increased conjugated primary bile acid concentrations. The calculated ratios, including primary/secondary bile acid (714 vs. 235, $p < 0.05$) and conjugated/free bile acid (371 vs. 125, $p < 0.05$) ratios, were higher in NICCD infants. The area under receiver operating characteristic curve for conjugated/free bile acid ratio to identify infants with NICCD was 0.871 (95% confidence interval, 0.713–1.0).
Conclusions: Together, our findings indicated plasma bile acid profile as a potential noninvasive diagnostic biomarker for NICCD.

1. Introduction

Bile acids are synthesized by the conversion of cholesterol into 7α-hydroxycholesterol by microsomal cholesterol 7α-hydroxylase (CYP7A1) in the liver. These hepatic synthesized primary bile acids, namely cholic acid (CA) and chenodeoxycholic acid (CDCA), are dehydroxylated by microbes in the intestine, resulting in the formation of secondary bile acids deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA). Bile acids, traditionally known to facilitate digestion and lipid absorption, have been recently recognized as signaling molecules regulating various metabolic pathways, including glucose, lipid, and energy homeostasis, as well as innate immunity in the biliary tract [1–4]. Dysregulation of bile acids is associated with diabetes, atherosclerosis, non-alcoholic fatty liver, and other metabolic diseases [5].

Recent studies have revealed the distinct serum bile acid profiles in certain disease states. Patients with cholestasis of pregnancy show a predominant rise in taurocholic acid (TCA) and taurochenodeoxycholic acid (TCDCA) with a marked increase in the CA/CDCA ratio and decreased glycine-/taurine-conjugated bile acid ratio [6]. Ursodeoxycholic acid (UDCA) treatment has been shown to improve patient prognosis by decreasing serum TCA and TCDCA concentrations and reversing the glycine-/taurine-conjugated bile acid ratio to the normal concentrations [6]. Zhou et al. examined the serum bile acid profiles in

Abbreviations: NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; CYP7A1, cholesterol 7α-hydroxylase; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; ROC, receiver operating characteristic; AGC, aspartate-glutamate carrier; CTLN2, adult-onset type II citrullinemia; 23-norDCA, 23-nordeoxycholic acid; MRM, multiple reaction monitoring; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; INH, idiopathic neonatal hepatitis; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; UDCA, ursodeoxycholic acid; d_4-GCDCA, deuterated glycochenodeoxycholic acid; FXR, Famesoid X receptor

* Corresponding author at: Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, 1 University Road, Tainan 70101, Taiwan.
E-mail address: hsieh@mail.ncku.edu.tw (S.-C. Shiesh).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.cca.2017.10.005
Received 3 December 2016; Received in revised form 28 September 2017; Accepted 3 October 2017
Available online 04 October 2017

infants with biliary atresia and suggested the ratio of TCDCA/CDCA as a biomarker to distinguish biliary atresia from neonatal hepatitis syndrome with the area under receiver operating characteristic (ROC) curve of 0.923 [7]. Longitudinal profiles of serum bile acids in patients with cholestasis of pregnancy indicate increase in CA with either free or conjugated forms beginning at 24 weeks of gestation and reaching their peak concentrations at 40 weeks of gestation [8]. These data indicate that bile acid composition analyses might be useful for the differential diagnosis of diseases and for providing mechanistic insights into the pathogenesis of the diseases.

Citrin, encoded by the *SLC25A13* gene on chromosome 7q21.3, is localized in the mitochondrial inner membrane and is expressed predominantly in the liver, kidneys, and heart. Citrin functions as an aspartate-glutamate carrier (AGC2) for the exchange of matrix aspartate for cytosolic glutamate and hydrogen ions. Citrin deficiency is associated with neonatal intrahepatic cholestasis (NICCD) and adult-onset type II citrullinemia (CTLN2). Infants with NICCD may suffer from jaundice, fatty liver, hepatic fibrosis, and cholestasis. Symptoms in most NICCD infants usually self-resolve within the first year of life after treatment. Treatment with lactose (galactose)-free formulas containing medium-chain triglycerides and fat-soluble vitamins has been shown to improve most clinical presentations of the disease in many cases [9,10]. However, some infants progress into severe-type CTLN2 decades later and require liver transplantation [11–13]. NICCD is characterized by conjugated hyperbilirubinemia and markedly increased plasma bile acid concentrations. However, the bile acid profiles in NICCD and their longitudinal changes that occur with disease management remain unknown.

2. Materials and methods

2.1. Plasma samples

Patients < 1 y of age referred for the investigation of conjugated hyperbilirubinemia from 2007 to 2017 were considered eligible for this study. Conjugated hyperbilirubinemia was defined as serum direct bilirubin concentration > 1.0 mg/dl (17.1 μmol/l) if total bilirubin is < 5.0 mg/dl (85.5 μmol/l), or > 20% of the total if the serum total bilirubin > 5.0 mg/dl. Patients with homozygous or compound heterozygous *SLC25A13* mutation were defined as having NICCD (n = 5). Five NICCD patients harbored compound heterozygous mutations, of which 2 harbored the 851_854del/c.1638_1660dup mutation, two c.851_854del/c.615 + 5G > A, and one c.851_854del/c.1177 + 1G > A [14]. Patients with cholestasis not due to citrin deficiency were defined as non-NICCD (n = 20). Two of these non-NICCD patients were > 1-year-old and were therefore, excluded from the study. One diagnosed as classic citrullinemia type 1 was excluded from the study. Of the 17 non-NICCD patients, the diagnosis included progressive familial intrahepatic cholestasis type 2 (n = 1), hypothyroidism (n = 1), neonatal hepatitis (n = 1), urinary tract infection-related intrahepatic cholestasis (n = 2), hepatic failure (n = 2) and the others idiopathic neonatal cholestasis. *SLC25A13* mutations were not detected in these non-NICCD patients. Heparinized plasma samples were obtained from all the enrolled patients upon their first visit. One NICCD patient was followed up and his blood samples were drawn at several time points post-treatment. All plasma samples were aliquoted and stored at − 80 °C until analysis. This clinical study was conducted with the approval of the Ethics Committee of National Cheng Kung University Hospital and written informed consent was obtained from the parents of all the patients. Plasma samples of infant controls with no known liver disease were left-over from the routine analysis at the Department of Pathology of National Cheng Kung University Hospital (n = 13).

2.2. Analysis of plasma bile acid profiles by tandem mass spectrometry

2.2.1. Chromatographic conditions

Quantitative Bile acids were subjected to separation using an Agilent 1200 HPLC system (Agilent) with HTC PAL autosampler (CTC Analytics) on a Symmetry C18 column (150 × 2.1 mm with 3.5-μm particles, Waters). The column temperature was maintained at 40 °C. The elution of bile acids was under basic conditions (pH 8.6). The advantage of basic elution is the improvement of run-time and better resolution [15]. Mobile phase A consisted of 5 mmol/l ammonium acetate in water (87.5%) and 0.02% ammonium hydroxide (12.5%), and mobile phase B consisted of 5 mmol/l ammonium acetate in methanol (87.5%) and 0.02% ammonium hydroxide (12.5%). A gradient mode at a flow rate of 0.2 ml/min was set at 80% B, increased to 97% B over 11.5 min, and then brought back to 80% in 1 min. The column was re-equilibrated with 80% B for 2.5 min before the next injection.

2.2.2. Parameters of tandem mass spectrometry

The detection was carried out using an API 5000 tandem mass spectrometer (Sciex) equipped with an electrospray ionization source and operated with a negative ionization mode. The nebulizer gas, auxiliary gas, and curtain gas were set at 55 psi, 55 psi, and 25 psi, respectively. The voltage for the ion spray was − 4500 V and the temperature was 550 °C. The dwell time per transition was set at 80 ms. Mass parameters for individual bile acids and internal standards (IS) are listed in Supplementary Table 1.

2.3. Preparation of the standard solutions

Stock solutions (10 mmol/l) of individual bile acids were prepared in methanol. The stock solutions of the IS, 23-nordeoxycholic acid (23-norDCA), deuterated glycochenodeoxycholic acid (d_4-GCDCA), and deuterated lithocholic acid (d_4-LCA), were prepared in methanol at a concentration of 1 mmol/l. Stock solutions were aliquoted and stored at − 80 °C.

Stock solutions of the IS were mixed and diluted with acetonitrile as working IS solutions (2.5 μmol/l for 23-norDCA and d_4-GCDCA and 0.5 μmol/l for d_4-LCA). Working standards were prepared in methanol at concentrations of 0.02–10 μM for GCDCA, TCA, and TCDCA, 0.01–5 μM for GCA, GDCA, GUDCA, TDCA, TUDCA, CA, DCA and CDCA, and 2 nM to 1 μM for GLCA, TLCA, LCA, and UDCA. The samples were diluted 2-fold with water, and the diluted samples (100 μl) were added to 400 μl of working IS solution and vortexed for 1 min, followed by incubation for 10 min at − 20 °C. After centrifugation at 14,400 × *g* for 15 min, 400 μl of the supernatant was transferred to a clean glass tube. The supernatant was then dried under nitrogen gas and reconstituted with mobile phase A/B (1/1, v/v) and further transferred to a 96-well PVDF filter plate. After centrifugation at 760 × *g* for 10 min, 5 μl was injected for LC-MS/MS measurement.

2.4. Statistical analysis

Data are expressed as mean ± SE of the mean. The differences between 2 groups were compared by the Mann-Whitney test. All statistical analyses were performed using GraphPad Prism 6.0 software, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Quantitation of individual bile acid concentrations in plasma samples by LC-MS/MS

An LC-tandem mass spectrometry method for measuring 15 bile acids simultaneously in 15 min was established. Fig. 1 shows the representative chromatograms of individual bile acids with the multiple reaction monitoring (MRM) conditions. The calibration curves were