Journal of Chromatography B, 899 (2012) 135–145

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Bile acid profiling in human biological samples: Comparison of extraction procedures and application to normal and cholestatic patients

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ARTICLE INFO

Article history:
Received 2 December 2011
Accepted 9 May 2012
Available online 17 May 2012

Keywords:
Bile acids
Extraction
Liquid chromatography–mass spectrometry
Profiling
Routine analysis

ABSTRACT

The role of bile acids in cell metabolism, membrane biology and cell signaling is increasingly recognized, thus making necessary a robust and versatile technique to extract, separate and quantify a large concentration range of these numerous molecular species. HPLC–MS/MS analysis provides the highest sensitivity to detect and identify bile acids. However, due to their large chemical diversity, extraction methods are critical and quite difficult to optimize, as shown by a survey of the literature. This paper compares the performances of four bile acid extraction protocols applied to either liquid (serum, urine, bile) or solid (stool) samples. Acetonitrile was found to be the best solvent for deproteinizing liquid samples and NaOH the best one for stool extraction. These optimized extraction procedures allowed us to quantitate as much as 27 distinct bile acids including sulfated species in a unique 30 min HPLC run, including both hydrophilic and hydrophobic species with a high efficiency. Tandem MS provided a non ambiguous identification of each metabolite with a good sensitivity (LOQ below 20 nmol/l except for THDCA and TLCA). After validation, these methods, successfully applied to a group of 39 control patients, detected 14 different species in serum in the range of 30–800 nmol/l, 11 species in urine in the range of 20–200 nmol/l and 25 species in stool in the range of 0.4–2000 nmol/g. The clinical interest of this method has been then validated on cholestatic patients. The proposed protocols seem suitable for profiling bile acids in routine analysis.

1. Introduction

Bile acids are a group of end products of cholesterol catabolism bearing a pentanoic acid side chain and one to three hydroxyl groups at position α3, α7, and α12 of the cholane cycle. Two primary bile acids, cholic (CA) and chenodeoxycholic acid (CDCA) are primarily synthesized in hepatocytes from cholesterol and conjugated with glycine or taurine (shown in Fig. 1) [1]. Bile acids play a critical role in the digestion and absorption of dietary lipids within the intestinal lumen before their deconjugation and dehydroxylation by different bacterial phyla into secondary bile acids, deoxycholic (DCA) and lithocholic acids (LCA). In addition, CDCA is partially epimerized into α3 β7-OH ursodeoxycholic (UDCA) acid, the major tertiary bile acid. Dihydroxylated bile acids are then extensively reabsorbed through the ileal intestinal wall into the portal circulation. Due to their efficient uptake by the liver, bile acids remain at a low concentration in the peripheral blood circulation [2]. Besides amidation by glycine or taurine, bile acids can be also conjugated as 3α sulfated metabolites [3] or with sugars such as N-acetyl glucosamine, glucose or glucuronate. The sulfated species are water-soluble structures abundant in normal urine and consistently increased in cholestasis or intestinal dysfunctions.

Besides their role as natural detergents, some bile acids have been recently identified as signalling molecules interacting with two types of specific receptors: the G-protein-coupled transmembrane receptor TGR5, and the nuclear transcription factor Farnesoid X receptor (FXR). Consequently, bile acids appear as metabolic integrators involved in the regulation of cholesterol homeostasis [4]

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CV, coefficient of variation; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GC–MS, gas chromatography coupled to mass spectrometry; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glyco ursodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; HPLC–MS/MS, high pressure liquid chromatography coupled to tandem mass spectrometry; LCA, lithocholic acid; LOQ, limit of quantification; SPE, solid phase extraction; TCA, taurocholic acid; TCDCA, tauro chenodeoxycholic acid; TDCA, Tauro deoxycholic acid; THDCA, tauro hyodeoxycholic acid; TLCA, Tauro lithocholic acid; UDCA, ursodeoxycholic acid; -3S, 3 sulfate; RE, relative error.

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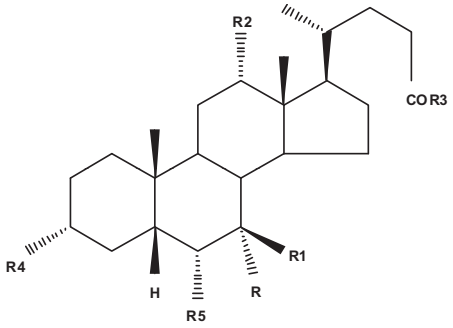
http://dx.doi.org/10.1016/j.jchromb.2012.05.015



	R	R1	R2	R3	R4	R5
CA	-OH	-H	-OH	-OH	-OH	-H
CDCA	-OH	-H	-H	-OH	-OH	-H
DCA	-H	-H	-OH	-OH	-OH	-H
LCA	-H	-H	-H	-OH	-OH	-H
UDCA	-H	-OH	-H	-OH	-OH	-H
HCA	-OH	-H	-H	-OH	-OH	-OH
HDCA	-H	-H	-H	-OH	-OH	-OH
Glycine Conjugated				$-NHCH_2CO_2^-$		
Taurine Conjugated				$-NH(CH_2)_2SO_3^-$		
Sulfated					$-SO_3O^-$	

Fig. 1. Chemical structure of bile acids and conjugates.

and of energy metabolism [5,6]. Because of the involvement of specific bile acids species in these various regulatory processes, an increasing attention has been given to their detailed profiling in various biomedical samples (serum, urine, bile and stool).

Bile acid analysis in serum and urine has been performed since many years to screen and follow up hepatobiliary and intestinal disorders [7], mainly to characterize cholestasis and to follow UDCA treatment of cholestatic liver diseases.

Various methods are currently used to identify and quantify bile acids in human serum (for review, see [8]). Gas chromatography coupled to mass spectrometry (GC–MS) is sensitive and specific but is time-consuming because of the multiple steps required for the processing of samples including the cleavage of amido- and sulfoconjugates and the methylation of carboxylic and hydroxyl groups [9]. However, GC–MS remains the reference method to ascertain bile acid structure and assign the position and stereochemistry of the hydroxyl groups on the cholane cycle. This method is still the reference to elucidate inborn errors in bile acid metabolism.

On the contrary, high pressure liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS) allows in a single step the measurement of both non-conjugated (free) and conjugated bile acids as native metabolites. For routine quantification of clinical samples, HPLC–MS/MS thus appears as the most suitable method to screen the bile acid profiles without tedious prior fractionation of conjugates (for review, see [8]).

Recent papers have described bile acid profiles in biological samples using various extraction methods followed by HPLC–MS/MS. However, bile acids, being amphipathic molecules dispersed in an aqueous medium are not evenly extracted by the methods described. Surprisingly, this central question has not been addressed until now and there is no available data that compare the critical efficiency for nanomolar and micromolar concentration of metabolites for the first step of the different techniques used.

In the present study, we compare four bile acids extraction techniques on liquid samples including, or not, a solid phase extraction (SPE) step and followed by HPLC–MS/MS. The aim of this comparative study is to select a simple and robust technique prior analysis with HPLC–MS/MS to profile bile acids in liquid samples such as human plasma, urine, and bile. We have also compared four extraction methods to extract bile acids from solid samples such as human stool. Finally we have set two techniques able to quantify 27 distinct human bile acids including sulfated species in the same run. These techniques were found the most efficients to measure simultaneously both hydrophobic and hydrophilic bile acid species in liquid or solid samples. The technique for liquid samples was applied to a group of healthy subjects and to another group of cholestatic patients. This new protocol can now be used to monitor bile acid markers of hepatobiliary and intestinal diseases which could be investigated in routine.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and solvents were of the highest purity commercially available. Bile acid standards CA, DCA, CDCA, UDCA, LCA, HCA, HDCA, and their corresponding glycine and taurine derivatives, as well as TLCA3S and GLCA3S were obtained from Sigma–Aldrich (Saint Quentin Fallavier, 38297, France). LCA3S was synthetized from LCA in the laboratory. GUDCA-3S and TUDCA-3S were a generous gift from Dr. J. Goto. The three internal standards were respectively 23-nor-5β-cholanoic acid-3α,12α diol from Steraloids Inc. (Newport, USA), ursodeoxycholic-2,2,4,4-d4 acid and lithocholic-2,2,4,4-d4 acid from CDN isotopes (Pointe-Claire, Quebec, Canada). Acetic acid, ammonium carbonate, ammonium acetate, trichloroacetic acid, acetonitrile and 2-propanol for HPLC were from Sigma–Aldrich (Saint Quentin Fallavier, 38297, France). Methanol (Chromanorm grade) was from VWR (Fontenay sous Bois, 94126, France). NaOH was from Merck (Darmstadt 64271, Germany).

2.2. Preparation of calibration standards

Stock solutions of the bile acids and the 3 internal standards were prepared in methanol (1 mg/ml) and stored in sealed vials at −20 °C. The 27 standard stock solutions were then pooled together to obtain a 30 μg/ml solution, further diluted in methanol to obtain an 6 levels in the calibration curve ranging from 0.006 to 30 μg/ml (corresponding to 0.01–80 μmol/l).

2.3. Extraction from liquid samples (serum, urine, bile)

Serum (500 μl), urine (2 ml of a 24 h urine pool) or bile (1 ml of a1/1000 dilution) were stored at −20 °C until measurement.

Four different methods adapted from previously published works [10–14] were compared. A comparative scheme of these methods is shown in supplementary data 1A. Five microliter of the stock solution of the three internal standards were added at the beginning of the extraction procedure to calculate the extraction yield.

2.3.1. Deproteinization with methanol (L1 protocol) or with trichloroacetic acid followed by methanol extraction (L2 protocol)

Proteins were precipitated by addition of methanol (80% final concentration (v/v)) or trichloroacetic acid (1% final concentration (v/v)). After mechanical stirring (1 min vortex) samples were incubated at room temperature for 20 min and clarified by centrifugation (4000 × *g*, 15 min). The supernatant was recovered and dried under a nitrogen stream at 50 °C. The residue was then dissolved in 150 μl methanol and 5 μl were injected into the HPLC–MS/MS system.