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Enzymatic Production of Bile Acid Glucuronides Used as Analytical Standards for Liquid Chromatography−Mass Spectrometry Analyses

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Abstract: The present study reports a novel method for the production and purification of analytical standards of glucuronide conjugates of bile acids, chenodeoxycholic (CDCA), lithocholic, (LCA) and hyodeoxycholic (HDCA) acids. CDCA−3G (CDCA−3-glucuronide) and −24G, LCA−3G and −24G, and HDCA−6G and −24G were enzymatically formed by using microsomes from human liver, purified by liquid chromatography, digested with recombinant β-glucuronidase, and quantified by liquid chromatography/electrospray ionization coupled to mass spectrometry (LC-ESI/MS). The position of the glucuronosyl moiety on the bile acids was determined by analyzing the susceptibility to hydrolysis under elevated pH and temperature conditions of the standards. By using the purified analytical standards, a LC-ESI/MS/MS method was developed for the determination of these glucuronide conjugates in in vitro assays. The linearity of the assay ranged from 0.5 to 40 ng/mL for the six glucuronides, and the limit of quantification (LOQ) was 0.5 ng/mL. Intra- and interday precisions and accuracy values were all lower than 10.2%. Furthermore, processed sample stability analyses revealed that the six standards were stable at 4 °C for more than 24 h. This method was successfully used for the quantification of CDCA, LCA, and HDCA glucuronides formed by human liver or hepatoma HepG2 cells. In conclusion, such a method allows the purification of high-quality analytical standards of glucuronide derivatives and may easily be used for the quantification of other endo- and xenobiotics that are glucuronidated.

Keywords: Enzymatic production; HPLC purification; glucuronide conjugates; bile acid; LC-ESI/MS/MS

Introduction

Bile acids (BAs) are biological detergents that subserve a number of important functions, including cholesterol excretion, intestinal uptake of fat-soluble vitamins, and the hepatic generation of bile flow.[1] BAs are synthesized in the liver by the conversion of cholesterol into the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA).[2] CDCA is subsequently converted into lithocholic acid (LCA) in the intestine, and LCA, which is highly cholestatic, is then detoxified into hyodeoxycholic acid (HDCA) back to the liver.[3] Due to their detergent properties, BAs are cytotoxic and perturbations in their normal synthesis, transport, or secretion are associated with a variety of pathophysiological conditions, including intrahepatic cholestasis.[4]

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One way of controlling BA levels consists of their conjugation to the glucuronosyl group. This reaction, called glucuronidation, facilitates BA secretion into the blood, followed by enhanced urinary excretion.[5−7] Glucuronidation consists of the transfer of the glucuronosyl group from UDP-glucuronic acid (UDPGA) to the acceptor molecule and is catalyzed by enzymes belonging to the UDP-glucuronosyltransferase (UGT) family.[8] These membrane bound enzymes of the endoplasmic reticulum produce glucuronidated products that are more hydrophilic and more easily excreted into bile or urine.[9]

An important consequence of BA glucuronidation is the introduction of an additional negative charge to the molecule, which allows their transport by conjugate transporters present in liver and intestine.[1] Glucuronidated BAs represent 7−8% of the BA pool in the plasma of cholestatic patients, while in urine, the proportion of these metabolites increases to up to 35% of total BAs.[10−12] The most abundant glucuronide conjugate reported in human plasma is CDCA glucuronide, followed by LCA glucuronide.[12−14] By contrast, the major glucuronide conjugate in urine corresponds to hyodeoxycholic acid (HDCA) glucuronide.[12−15] This 6α-hydroxylated metabolite of LCA is abundantly found in urine from patients suffering from cholestasis.[10,15−18] Furthermore, plasma levels of the glucuronide conjugates of LCA and CDCA are increased by 30- and 50-fold, respectively, in such patients.[12]

Glucuronide conjugation of BA involves either the 3α-hydroxyl group or the 24-carboxyl group of the steroid nucleus of CDCA and LCA, resulting in the formation of ether-type or acyl-type glucuronides, respectively.[19−21] (Figure 1). CDCA−3-glucuronide (CDCA−3G), CDCA−24G, LCA−3G, and LCA−24G were detected in human urine and plasma.[11,20,21] Interestingly, various studies demonstrated that the major urinary BA glucuronide, HDCA, is conjugated at both its 6α-hydroxyl and 24-carboxyl positions,[10,22−26]

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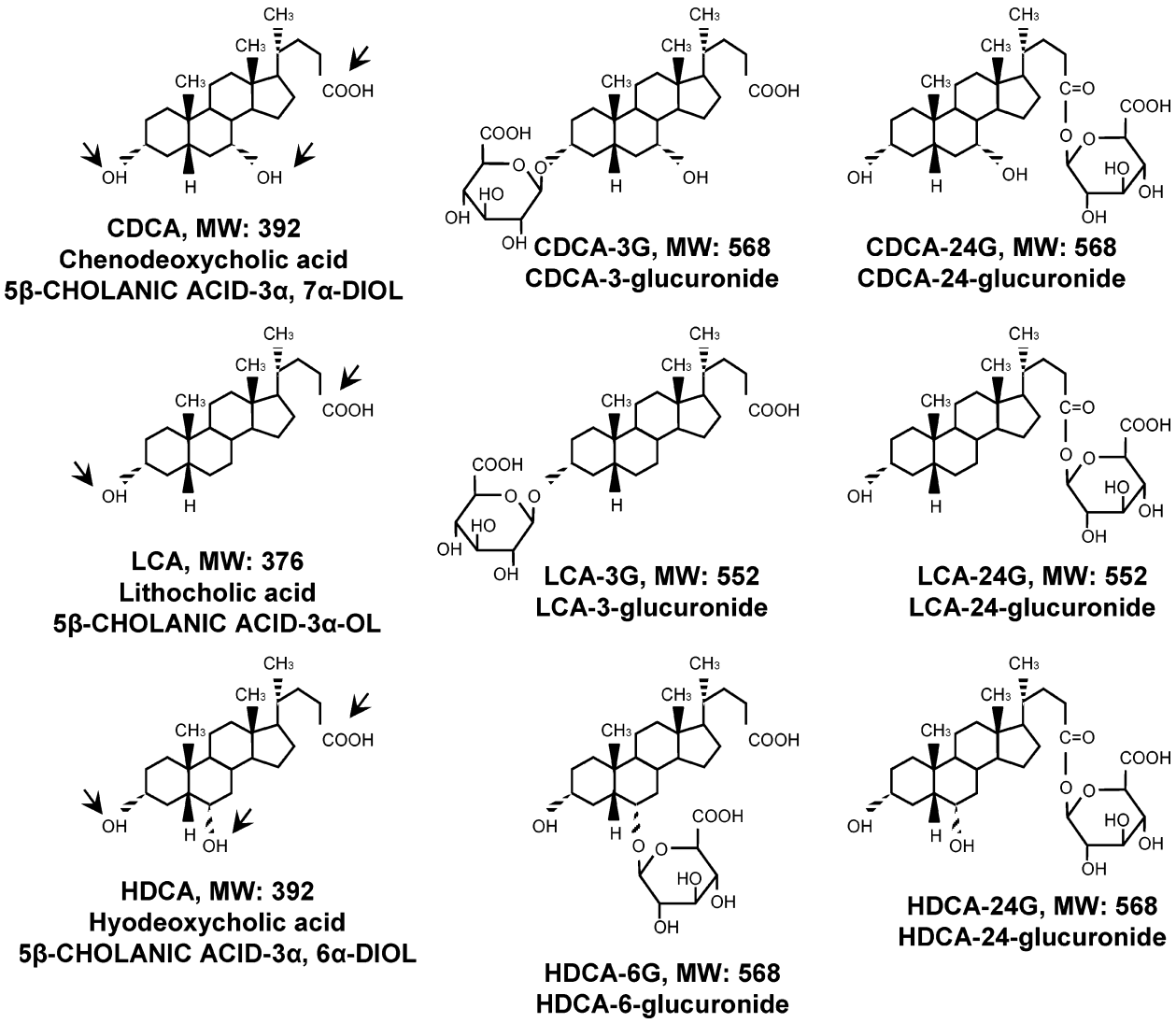

Figure 1. Structures of chenodeoxycholic, lithocholic, and hyodeoxycholic acids and of their glucuronide conjugates. Molecular weights (MW) of each compound are given in g/mol. Arrows indicate the potential site for glucuronide conjugation.

whereas the formation of HDCA−3G has never been reported. HDCA−6G is formed by the human UGT2B4 and UGT2B7 enzymes, whereas LCA−3G was reported as a product of the UGT2B7 enzyme.[13,22] Interestingly, human UGT1A3 plays a major role in the formation of 24-glucuronide conjugates of LCA and HDCA,[22] whereas the human enzymes glucuronidating CDCA are not yet identified.

Although these glucuronide conjugates have been identified several years ago, there are no commercial sources of CDCA−, LCA−, or HDCA−G. Therefore, investigators interested in the selective detection and sensitive quantification of these metabolites in either biological fluids or in vitro glucuronidation assays need to produce such analytical standards. Various studies reported chemical methods for the production and characterization of these bile acid glucuronides.[19−21,27−30] However, the present study reports a novel method for the purification of BA glucuronides, based on enzymatic assays using human liver microsomes (Figure 2). We demonstrate that these purified CDCA−3G, CDCA−24G, LCA−3G, LCA−24G, HDCA−

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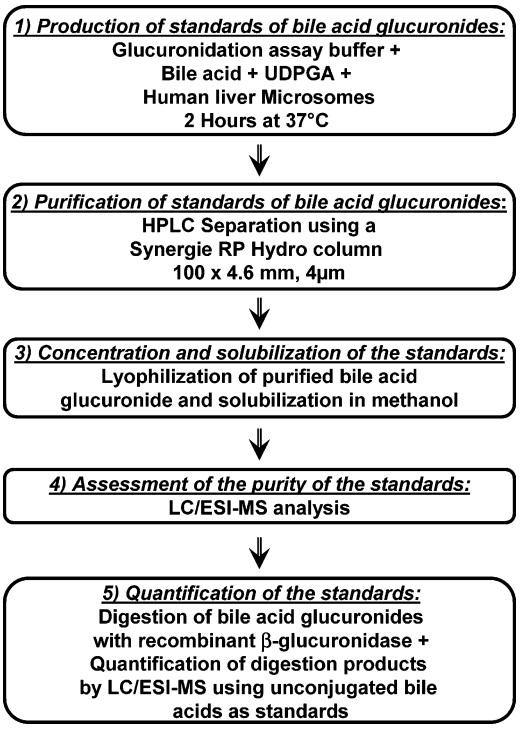

Figure 2. Method for the production, purification, and quantification of bile acid glucuronides. Ether and acyl glucuronides of CDCA, LCA, and HDCA were obtained by incubating the parent unconjugated molecules in the presence of human liver microsomes (step 1). Glucuronidated BAs were subsequently purified by HPLC (step 2) and lyophilized and dissolved in methanol (step 3). The purity of each BA standard was assessed by LC-ESI/MS analyses (step 4), and their concentrations were determined following β-glucuronidase digestion by using the corresponding unconjugated molecule as a standard for LC-ESI/MS quantification (step 5).

6G, and HDCA−24G are suitable for the simultaneous quantification of the corresponding metabolites formed in in vitro glucuronidation assays by liquid chromatography with electrospray ionization (LC-ESI) coupled to mass spectrometry (MS/MS).

Experimental Section

Materials. Chenodeoxycholic acid (CDCA), hyodeoxycholic acid (HDCA), and lithocholic acid (LCA) were purchased from Steraloids (Newport, RI). [$^2H_4$]-Chenodeoxycholic acid (CDCA-d_4) and [$^2H_4$]-lithocholic acid (LCA-d_4) were purchased from C/D/N Isotopes (Montréal, Canada). UDP−glucuronic acid and all aglycons were obtained from Sigma (St. Louis, MO) and ICN Pharmaceuticals, Inc. (Québec, Canada). Protein assay reagents were obtained from Bio-Rad Laboratories Inc. (Marnes-la-Coquette, France). Human liver microsomes were obtained from BD Biosciences Discovery Labware (Woburn, MA). Human hepatoma HepG2 cells were from the American Type Culture Collection (Rockville, MD). Cell culture reagents were from Life-Technologies (Burlington, ON). $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4, and β-glucuronidase type VII from *Escherichia coli* were purchased from Sigma (St. Louis, MO). Methanol (HPLC grade) was obtained from VWR Canlab (Montréal, Canada).

Cell Culture, Purification of Microsomes, and Glucuronidation Assays. HepG2 cell culture and microsome isolation were performed as previously described.[31,32] For glucuronidation assays, BAs (CDCA, LCA, or HDCA, as well as the corresponding deuterated CDCA-d_4 and LCA-d_4) were incubated in the presence of microsomes in a final volume of 100 μL of a glucuronidation assay buffer: 50 mM Tris-HCl, 10 mM $MgCl_2$, 8.5 mM saccharolactone (Sigma, St. Louis), 10 μg/mL phosphatidylcholine (Sigma), 25 μg of alamethicin (Sigma), 2.5 μg/mL pepstatin (Sigma), 0.5 μg/mL leupeptine (Sigma); added with 2 mM UDPGA, at 37 °C. Assays aimed at producing standards were performed for 2 h using 10 μg of a human liver microsome preparation composed of microsomal proteins isolated from livers of 19 different donors (BD Biosciences Discovery Labware). Analytic experiments were incubated for 1 h in the presence of 10 μg of microsome preparations from three human liver samples (BD Biosciences Discovery Labware) or from HepG2 cells. The glucuronidation reaction was stopped by the addition of 100 μL of methanol containing 2 nM butylated hydroxytoluene (BHT). Glucuronidation assays were then centrifuged at 13 000 rpm for 5 min to remove the precipitated proteins, and kept at −80 °C until further analyses.

β-Glucuronidase Digestion. The concentrations of purified BA glucuronides were determined after digestion with 5000 units/mL of *E.* coli β-glucuronidase type VII in a 25 mM phosphate buffer (pH = 6.5) for 1 h at 37 °C. The reaction was stopped by cooling on dry ice, and samples were subsequently acidified with acetic acid (pH ∼ 3) and extracted with a liquid−liquid extraction (ethyl acetate/chlorobutane, 50/50, v/v). The resulting unconjugated bile acids formed were quantified with LC-ESI/MS (see below).

Chromatographic Methods. Three different LC methods were used for the purification and/or quantification of unconjugated as well as glucuronide conjugates of BA. In all cases, the chromatographic system consisted of an Alliance 2690 (Waters, Milford, MA). Analytes were separated with a Synergie RP Hydro column, 100 × 4.6 mm, 4

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μm (Phenomenex,Torrance, CA). In all analyses solvent A corresponded to water−1 mM ammonium formate and solvent B to methanol−1 mM ammonium formate.

(1) Purification of Bile Acid Glucuronides. The purification of BA glucuronides was achieved using an isocratic separation (1.0 mL/min) for 7 min of 30% A and 70% B. For purification, glucuronidation assays were injected by batches of 40 μL, and elution of glucuronide conjugates was monitored by MS. Metabolite fractions were then collected and lyophilized at −80 °C for 16 h using a freeze dryer system (Labconco Corp. Kansas City, MI). The purified residues were dissolved in methanol and stored at −80 °C. These solutions were used as stock solutions for subsequent analyses.

(2) Quantification of Bile Acid Glucuronides. For quantitative analyses of BA glucuronides, the calibration curves were prepared in methanol:water (50:50, v:v), and 25 μL of the assays were injected in the HPLC system. Glucuronide conjugate separation was achieved as indicated above, and elution took place at a flow rate of 1.0 mL/min with a split ratio of 1:4. Initial conditions were 40% A and 60% B, followed by a linear gradient to 85% B in 3 min. This condition was maintained for 2 min, and the column was flushed with 95% B for 2 min and reequilibrated to initial conditions for an additional 2 min.

(3) Quantification of Unconjugated Bile Acids. Stock solutions of CDCA, LCA, and HDCA (Steraloids, Newport, RI) were prepared in methanol. Calibration curves were prepared in a 25 mM phosphate buffer (pH = 6.5), and extraction was carried out under the same conditions as for the samples. Under these conditions the method was linear from 1 to 400 ng/mL for all BAs. The chromatographic separation was achieved using the same column as for glucuronide conjugates, and elution took place at a flow rate of 1.0 mL/min. The initial conditions were 30% A and 70% B, followed by a linear gradient to 85% B in 2 min. This condition was held for 2 min, and the column was flushed with 95% B for 2 min and reequilibrated to initial conditions for an additional 2 min.

Mass Spectrometry. All analytes were detected with an API 3000 triple quadrupole mass spectrometer (Applied Biosystems-Sciex, Concord, Canada) equipped with a turbo ion-spray source. Unconjugated CDCA, LCA, and HDCA produced from β-glucuronidase digestion were monitored in a negative mode, and their quantification was conducted in single ion monitoring mode (SIM) at 391.5 *m*/*z* for HDCA and CDCA, 375.5 *m*/*z* for LCA, 395.5 *m*/*z* for CDCA-d_4, and 379.5 for LCA-d_4.

The glucuronide conjugates were also monitored in a negative mode, but quantification was achieved by the multiple reaction monitoring (MRM) mode. Negative-product MRM ion pairs were *m*/*z* 551.5 → 375.5 for LCA−G, 567.5 → 391.5 for HDCA−G and CDCA−G, 571.5 → 395.5 for CDCA-d_4−G, and 555.5 → 379.5 for LCA-d_4−G. The concentration of glucuronides produced in assays was obtained by reporting the peak area ratios to the standard curves established with the corresponding purified conjugate. Furthermore, integrities of detections were ensured by spiking samples containing CDCA−G or HDCA−G with CDCA-d_4−G, or by spiking those containing glucuronide conjugates of LCA with LCA-d_4−G. Mass spectrometry conditions were 500 °C for desolvation temperature, −4000 V spray voltage, −45 V OR, and −200 V RNG.

Results

Production and Purification of Bile Acid Glucuronides by Microsomes from Human Liver. Analytical standards for the quantification of BA glucuronides were produced with microsomes from human liver incubated in the presence of CDCA, LCA, or HDCA (200 μM). The glucuronide conjugates were subsequently purified by HPLC, quantified, and characterized (Figure 2). As previously reported,[11,20−22,26] incubation of CDCA, LCA, or HDCA with human liver microsomes led to the formation of two glucuronide conjugates for each BA. The two glucuronides of each BA shared the same ion of 567.5 *m*/*z* for CDCA− and HDCA− glucuronides and of 551.5 *m*/*z* for LCA−glucuronides. At that time, the low retention time metabolite was identified as G1, whereas the latter eluted glucuronide was labeled G2. These precursor ions were then selected and induced to fragments which were analyzed by the second mass analyzer (Q3) (Figure 3). For all G2 metabolites, tandem MS analyses resulted in the complete loss of the parent ions (except for HDCA−G2) and gave an almost unique product ion at 391.5, 375.2, and 390.8 *m*/*z* for CDCA−G2, LCA−G2, and HDCA−G2, respectively (Figure 3). These ions corresponded to the deprotonated aglycons formed from cleavage of the glycosidic bound (MH^- − 176) (reviewed in ref 33). Interestingly, G1 were less sensitive to fragmentation, since for all three bile acid derivatives, the parent ions are detected by the Q3 MS.

All six BA glucuronides were subsequently purified by HPLC as described in the Experimental Section (Figure 2). Furthermore, in order to create internal standards for further analyses, the deuterium labeled glucuronides of [$^2H_4$]-CDCA and [$^2H_4$]-LCA were also produced and purified. All purified glucuronides were reconstituted in methanol to form stock solutions, which were subsequently analyzed by LC-ESI/MS to ensure their purity. These analyses revealed that stock solutions were free from any unconjugated parent BA.

Under the indicated conditions (incubation of 10 μg of microsomes in the presence of 200 μM of BA for 2 h), the purification processes produced from 1 to 70 μg of conjugates per mg of microsomes, for CDCA−3G and HDCA−6G, respectively.

Identification of the Purified Bile Acid Glucuronides. The presence of only two nucleophilic acceptor groups for the glucuronosyl moiety on LCA (Figure 1) indicated that the two purified glucuronide conjugates correspond to LCA−3G (ether glucuronide) and LCA−24G (acyl glucuronide).

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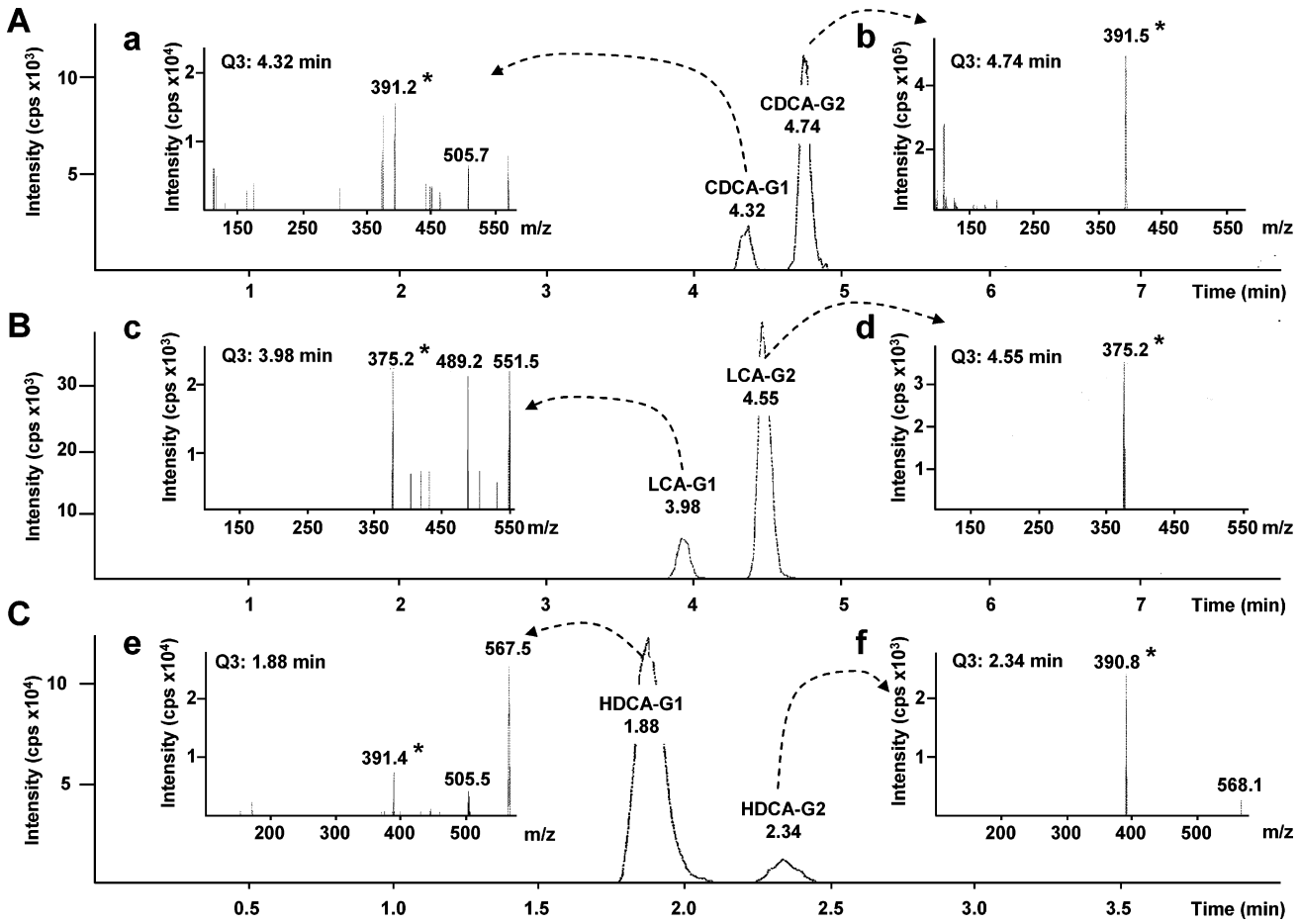

Figure 3. Multiple reaction monitoring of bile acid glucuronide conjugates formed in the presence of microsomes from human liver. (A−C) Chromatographic separation of the glucuronide conjugates formed by incubating BA (200 μM) in the presence of microsomal proteins of human liver (10 μg). (a−f) Spectra of product ions from low-energy collision (Q3) of the deprotonated ions of CDCA−G1 (a: eluted at 4.32 min), CDCA−G2 (b: eluted at 4.74 min), LCA−G1 (c: eluted at 3.98 min), LCA−G2 (d: eluted at 4.55 min), HDCA−G1 (e: eluted at 1.88 min), and HDCA−G2 (f: eluted at 2.34 min). Asterisks (*) indicate the *m*/*z* value corresponding to the unconjugated bile acids.

As shown in Figure 1, CDCA and HDCA contain three potential acceptor groups. In addition to their carboxyl group at position 24, CDCA contains two hydroxyl groups at positions 3 and 7, whereas the 3- and 6-OH groups of HDCA can be conjugated to the glucuronosyl moiety. However, previous studies demonstrated that incubation of these acids with human or rat liver microsomes produces only two glucuronide conjugates: the acyl 24 glucuronides, and the ether CDCA−3G and HDCA−6G conjugates, respectively.[19,22,26,34] Considering that acyl glucuronides are highly susceptible to hydrolysis under alkaline pH and elevated temperature conditions,[19,20,33,35−38] standards were incubated in the presence of increasing pH buffered solutions (10 mM ammonium formate buffered at pH 5−10 with 0.05 M HCl or 0.05 M NaOH) and at 60 °C (Figure 4). We observed that incubation of CDCA−G2, LCA−G2, and HDCA−G2 in high-pH buffered solution for 2 h drastically reduced the concentration of the detected glucuronides. The same conditions had no effect on CDCA−G1, LCA−G1, and HDCA−G1 (Figure 4A,C,E). In addition, incubation of the standards for 2 h at 60 °C resulted in the detection of only 12%, 10%, and 40% percent of the initial concentration of CDCA−G2, LCA−G2, and HDCA−G2, respectively (Figure 4B,D,F).

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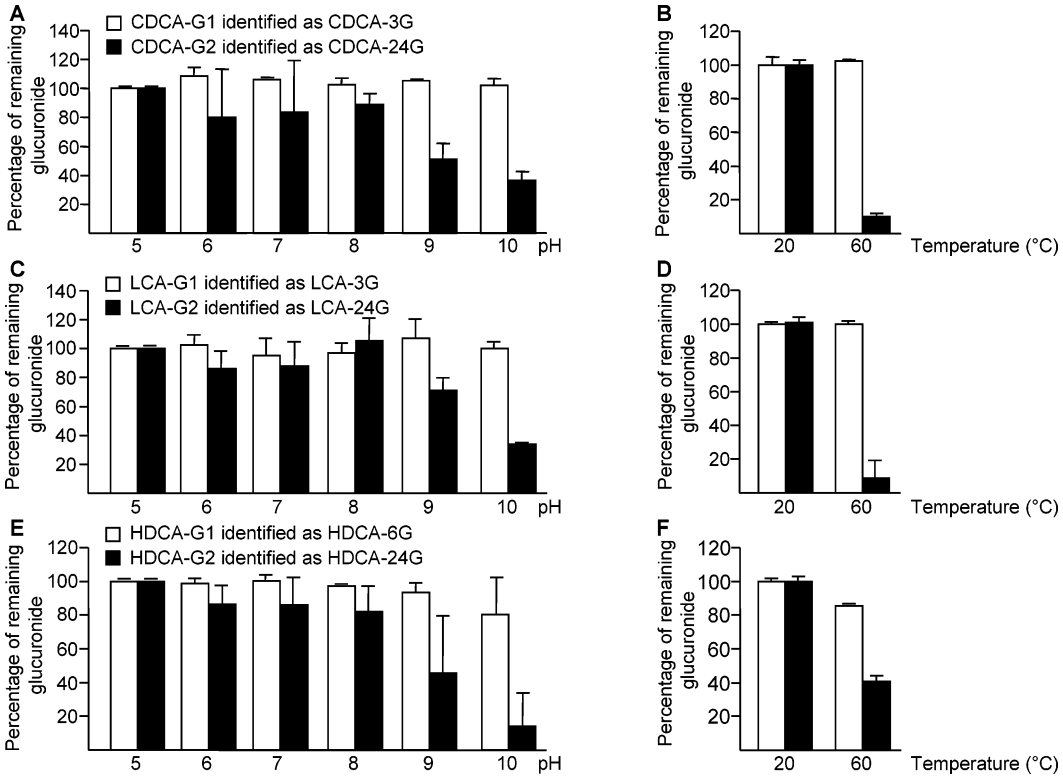

Figure 4. Effect of pH and temperature on bile acid glucuronides purified. Identification of the purified glucuronide conjugates was performed by incubating the purified glucuronides of CDCA (A, B), LCA (C, D), and HDCA (E, F) in the presence of increasing pH buffered solutions (A, C, E) or at 60 °C (B, D, F) for 2 h. These experiments identified CDCA−G1, CDCA−G2, LCA−G1, LCA−G2, HDCA−G1, and HDCA−G2 as CDCA−3G, CDCA−24G, LCA−3G, LCA−24G, HDCA−6G, and HDCA−24G, respectively. Values are means ± SD of two experiments performed in triplicate.

By contrast the elevated temperature had only minor effects on the amount of CDCA−G1, LCA−G1, and HDCA−G1. These experiments under extreme conditions were conducted to characterize the position of the glucuronide group on the bile acid molecules, and identified CDCA−G1, −G2, LCA−G1, −G2, HDCA−G1, and HDCA−G2 as CDCA−3G, −24G, LCA−3G, −24G, HDCA−6G, and −24G, respectively (Figure 4).

Quantification, Linearity Data, and Stability at 4 °C of the Purified Standards. Each purified standard was subsequently quantified by LC-ESI/MS after digestion with β-glucuronidase for 1 h at 37 °C. The complete deconjugation of each glucuronide derivative was assessed by comparing the level of glucuronide conjugates detected by LC-ESI/MS from digested and undigested samples (data not shown).

Seven point nonzero calibration graphs, ranging from 0.5 to 50 ng/mL for all purified glucuronides, were performed with samples prepared in either methanol:water (50:50, v:v) or glucuronidation assay buffer. The limit of quantification (LOQ) in incubation medium or methanol:water was 0.5 ng/mL for all analytes using a signal noise ratio of 3 (Figure 5). Furthermore, the peak area ratios versus concentrations over the working range (0.5−50 ng/mL) were linear (correlation coefficients r higher than 0.999) for all six standards in both methanol:H_2O and assay buffer (data not shown). Finally, variations in slopes or intercepts between the calibration graphs from methanol:H_2O and glucuronidation assays never exceeded 10%, thus indicating that the glucuronidation assay buffer does not alter the LOQ nor the working range of the method.

Processed sample stabilities of the purified glucuronides of CDCA, LCA, and HDCA were determined in the mobile phase for low-quality control (LQC, 1.5 ng/mL), medium-quality control (MQC, 25 ng/mL), and high-quality control (HQC, 40 ng/mL) samples to determine whether a loss of glucuronide occurs or not during the analytical run (data are summarized in Table 1). Stock solutions of each purified conjugate were diluted at the indicated concentration in methanol:water (50:50, v:v) and were incubated at 4 °C for 24 h. Results demonstrated that the analytical standards are stable at 4 °C for at least 24 h, with relative error (accuracy) values varying from −12.3% to 10.1% and coefficients of variation (precision values) ranging from 0.4% to 9.0% (Table 1). Similar experiments performed at 20 °C indicated that purified standards are also stable for 24 h at room temperature (data not shown).

Precision and Accuracy of the Method. Intra- and interday precisions and accuracy assays were performed in the glucuronidation assay buffer spiked with three concentrations of each bile acid glucuronide (1.5, 25, and 40 ng/mL). The intraday assay was performed by analyzing five aliquots of each concentration, whereas the interday data were realized by repeating the intraday assays on three different days. All precision values (CV) were lower than 10%,

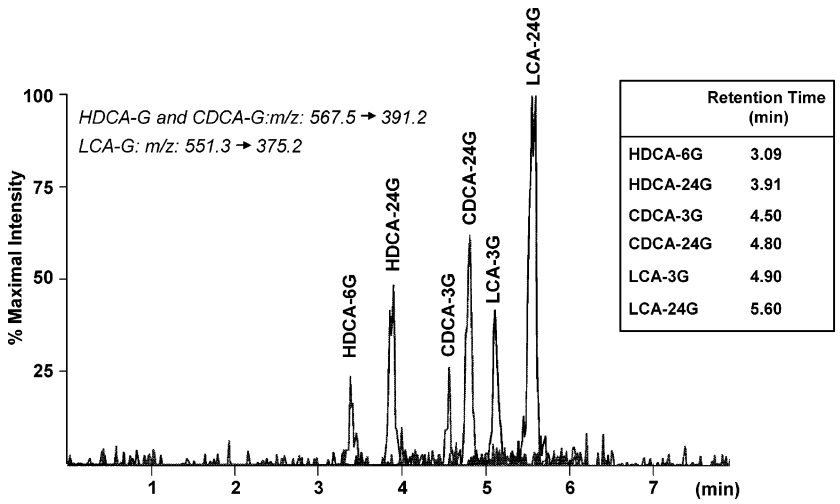

Figure 5. Chromatographic separation of the six bile acid glucuronides purified as analytical standards and diluted at the limit of quantification. All isolated glucuronide derivatives were pooled at the same concentration (0.5 ng/mL, which corresponds to the LOQ), prior to LC-ESI/MS/MS analysis.The chromatogram shown is representative of five experiments. The retention time for each conjugate is also indicated.

Table 1. Stability of Purified Bile Acid Glucuronides Conserved at 4 °C for 24 h[a]

	concn added (ng/mL)	RE[b] (%)	CV[c] (%)		concn added (ng/mL)	RE[b] (%)	CV[c] (%)
CDCA−3G	1.5[d]	6.0	0.9	LCA−24G	1.5[d]	8.4	4.8
	25[e]	−6.2	0.8		25[e]	1.0	2.1
	40[f]	−2.0	0.7		40[f]	−5.7	1.4
CDCA−24G	1.5[d]	7.0	2.4	HDCA−6G	1.5[d]	−12.3	9.0
	25[e]	2.1	0.5		25[e]	0.7	3.7
	40[f]	2.1	0.4		40[f]	−0.7	3.1
LCA−3G	1.5[d]	10.1	1.6	HDCA−24G	1.5[d]	−8.3	7.6
	25[e]	8.4	2.6		25[e]	−9.7	1.7
	40[f]	6.1	6.1		40[f]	5.6	3.5

[a] $n = 3$. [b] RE: Relative error. [c] CV: Coefficient of variation. [d] 1.5 ng/mL: Low-concentration quality control. [e] 25 ng/mL: Medium-concentration quality control. [f] 40 ng/mL: High-concentration quality control.

whereas accuracies were comprised in a −10% to +10% range (data not shown), thus demonstrating that the quantitative method is precise and accurate.

Formation of Bile Acid Glucuronides by Microsomal Proteins from Human Liver and Hepatoma HepG2 Cells. The purified analytical standards were used to quantify the glucuronidation activity of CDCA, LCA, and HDCA by microsomes from three human liver samples or from hepatoma HepG2 cells (Table 2). BAs (200 μM) were incubated with microsomal proteins (10 μg) for 1 h at 37 °C. The glucuronide conjugates were quantified in the glucuronidation assay buffer; however, when required, assays were diluted to reach the linearity zone of the method. In all samples, HDCA was the more effectively transformed into its 6-glucuronide conjugate, whereas CDCA and LCA were at least 5-fold more conjugated into 24-glucuronides than into 3-glucuronides (Table 2). HepG2 cells produced significantly lower amounts of all glucuronides, and CDCA−3G formation was below the limit of detection (Table 2).

Discussion

A comprehensive measurement of BA derivatives requires separation and analysis of each of the conjugated (and free) species. The advantages of being able to analyze conjugated species without lengthy physical separation and hydrolysis, as offered by LC/MS, have long been recognized.[39] Recently, improved sensitivity in bile acid quantification was obtained with the introduction of MS/MS instruments[40] and then with ESI.[41] However, these sensitive technologies require high-quality analytical standards. The lack of commercial sources of BA glucuronides requires investigators interested in the sensitive quantification of these conjugates to produce their own analytical standards. The present study reports a rapid and simple method for the production and purification of analytical standards for the simultaneous quantification of the glucuronide derivatives of CDCA, LCA, and HDCA.

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(41) Mills, K. A.; Mushtaq, I.; Johnson, A. W.; Whitfield, P. D.; Clayton, P. T. A method for the quantitation of conjugated bile acids in dried blood spots using electrospray ionization-mass spectrometry. *Pediatr. Res.* **1998**, *43* (3), 361−368.

Table 2. Formation of Glucuronide Conjugates of CDCA, LCA, and HDCA by Human Liver and Hepatoma HepG2 Cell Microsomes[a]

samples	CDCA−3G	CDCA−24G	LCA−3G	LCA−24G	HDCA−6G	HDCA−24G
A	13.67 ± 1.2	111.5 ± 9.2	14.1 ± 1.3	76.0 ± 0.9	11982 ± 758	300 ± 39
B	8.7 ± 0.7	65.7 ± 5.3	11.1 ± 0.9	52.3 ± 3.9	9150 ± 522	160 ± 13
C	19.6 ± 2.3	121.8 ± 11.4	18.0 ± 0.9	78.0 ± 2.5	12616 ± 447	264 ± 19
HepG2	BLD[b]	13.1 ± 1.6	1 ± 0.5	3.1 ± 0.5	357.2 ± 52.3	54.9 ± 3.6

[a] Ten micrograms of microsomes from three human liver samples or from HepG2 cells were incubated in the presence of 200 μM chenodeoxycholic (CDCA), lithocholic (LCA), or hyodeoxycholic (HDCA) acids and UDP-glucuronic acid (UDPGA, 2 mM) for 1 h at 37 °C in the glucuronidation assay buffer described in the Experimental Section. Glucuronide conjugates were quantified by using the LC-ESI/MS/MS method described. All values are expressed in ng/h/mg proteins and are means ± SD of three determinations. [b] BLD: below the limit of detection.

These compounds were formed from unconjugated bile acids by classical glucuronidation assays. The same approach was also efficient to produce internal standards of glucuronide conjugates with deuterated molecules.

The limiting step for such a method concerns the identification of the glucuronide conjugate products. Previous studies reported proton nuclear magnetic resonance (NMR) of such conjugates.[26−28,42] In the present study, the position of the glucuronosyl group on the steroid nucleus of each BA was determined by comparing the resistance of the six compounds to elevated pH and temperature conditions. Indeed, a large number of studies definitely established the high susceptibility of carboxyl-linked glucuronide derivatives to hydrolysis under alkaline pH and elevated temperature conditions.[19,20,35−38] This approach allowed the characterization of the 24-acyl glucuronide conjugates of CDCA, LCA, and HDCA.

The stability of the second glucuronide of CDCA, LCA, and HDCA suggested that they corresponded to hydroxyl-linked glucuronide conjugates. The presence of only one OH group at position C3 of LCA revealed that the stable conjugate corresponds to LCA−3G, a metabolite generally formed in the presence of human liver microsomes.[22,27,43] By contrast, both CDCA and HDCA molecules present two potential OH acceptor groups at positions 3 and 7 (CDCA) or 3 and 6 (HDCA). However, previous reports clearly established that bile acids carrying a 6α-hydroxyl group, such as HDCA, are glucuronidated at C6,[10,22−26] in contrast to BAs lacking a hydroxyl group at this position, i.e., CDCA, which are glucuronidated at C3.[10,11,21] Overall, these observations indicate that the purified BA glucuronides were CDCA−3G and −24G, LCA−3G and −24G, and HDCA−6G and −24G.

Analyses of low energy collision induced dissociation of the six analytical standards revealed the formation of various product ions. Interestingly, the low-energy fragmentation of all six glucuronides resulted in abundant losses corresponding to the removal of the sugar moiety, which is a characteristic loss for sugar-conjugated bile acids.[33] Tandem MS analyses also revealed that fragmentation of acyl glucuronides resulted in a greater loss of the parent ions than that of hydroxyl-linked glucuronides (Figure 3). Furthermore, in CDCA−, LCA−, and HDCA−24G, fragmentation only led to the loss of the sugar group, thus confirming the lower stability of the carboxyl linked glucuronides, as previously reported.[33] By contrast, dissociation of the ether-type glucuronides failed to completely break the parent ions and resulted in the formation of various product ions in addition to the conjugated molecule. These observations are in accordance with the pH and temperature sensitivity-based differentiation between carboxyl- and hydroxyl-linked glucuronides.

The LC-ESI/MS/MS method developed in the present study was used to quantify the formation of the glucuronide derivatives by human liver and hepatoma HepG2 cells samples. With the exception of proteins from HepG2 cells that were unable to form quantifiable levels of CDCA−3G, all microsomal preparations produced the assayed glucuronide conjugates. Furthermore, in accordance with previous reports, a predominant formation of CDCA−24G and LCA−24G was observed in all samples, whereas HDCA was predominantly conjugated at position 6α.[11,20−22,26] On the other hand, glucuronide conjugates of BAs are easily purified from human urine and plasma by using solid-phase extraction;[20,21,39,44] therefore, the quantification method developed here could be extended to the measurement of circulating and urinary levels of these conjugated metabolites.

In conlusion, this five-step method which allows the production of high-purity analytical standards may easily be used for the production of standards for a variety of endo- or xenobiotics detoxified as glucuronide conjugates, including other BAs such as cholic acid, deoxycholic acid, hyocholic acid, or ursodeoxycholic acid glucuronides. In addition, the same method also allows an easy production of internal standards, just by using the deuterated analogue of the analyzed molecule. In addition, replacing human liver microsomes by recombinant UGT enzymes could further ensure the identity of the standard produced and enhance the yield of the method. However, since the human CDCA-conjugating UGT enzymes have not been yet identified, such an approach could not be used in the present study.

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Nevertheless, the standards of CDCA glucuronides produced here constitute unique analytical tools to successfully conduct future experiments aimed at identifying the human UGT enzyme(s) that form(s) CDCA glucuronide derivatives.

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