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Identification of a novel conjugate in human urine: bile acid acyl galactosides

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Abstract

We report a novel conjugate, bile acid acyl galactosides, which exist in the urine of healthy volunteers. To identify the two unknown peaks obtained in urine specimens from healthy subjects, the specimens were subjected to solid phase extraction and then to liquid chromatographic separation. The eluate corresponding to the unknown peaks on the chromatogram was collected. Following alkaline hydrolysis and liquid chromatography (LC)/electrospray ionization (ESI)-mass spectrometric (MS) analysis, cholic acid (CA) and deoxycholic acid (DCA) were identified as liberated bile acids. When a portion of the alkaline hydrolyzate was subjected to a derivatization reaction with 1-phenyl-3-methyl-5-pyrazolone, a derivative of galactose was detected by LC/ESI-MS. Finally, the liquid chromatographic and mass spectrometric properties of these unknown compounds in urine specimens were compared to those of authentic specimens and the structures were confirmed as CA 24-galactoside and DCA 24-galactoside. These results strongly imply that bile acid 24-galactosides, a novel conjugate, were synthesized in the human body.

Keywords: Bile acid; Galactose; Glycoside; Atmospheric pressure chemical ionisation; Electrospray ionisation; Liquid chromatography/mass spectrometry; Tandem mass spectrometry

1. Introduction

Bile acids are major cholesterol metabolites in the liver, and are excreted into the small intestine via the bile duct. In the intestinal lumen, bile acids assist in lipolysis and the absorption of fats by forming mixed micelles and then return to the liver upon absorption in the ileum and proximal colon. Because of efficient hepatic uptake, there are low concentrations of bile acids in the peripheral blood. Both primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), and secondary bile acids, deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA), which are produced by the action of enzymes existing in the microbial flora of the colonic environment, undergo various phase II reactions such as amino acid, sulfuric acid, and glycosidic conjugation, in the liver before excretion into the bile duct and peripheral blood.

Bile acid glucosides, such as glucuronides [1–3], glucosides [3,4], and *N*-acetylglucosaminides [5–7], have been identified in human biological fluids. These glycosides have ether linkage between the hydroxy group on the steroid nucleus of bile acids and the anomeric hydroxy group of sugar moiety. More recent publications indicate that bile acids

Abbreviations: APCI, atmospheric pressure chemical ionization; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; ESI, electrospray ionization; HDCA, hyodeoxycholic acid; LC, liquid chromatography; LCA, lithocholic acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PMP, 3-methyl-1-phenyl-5-pyrazolone; R*k*s relative retention factors; UGT, UDP–glucuronosyltransferase; UDCA, ursodeoxycholic acid

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and their short-chain analogs are metabolized into acyl glucuronides by the action of rat liver microsomal glucuronosyltransferases [8–12]. In addition, bile acid 24-glucuronides have been identified in human urine [13].

These previous findings readily led us to anticipate the existence of bile acid acyl glucosides in human biological fluids, and we therefore chemically synthesized five types of authentic bile acid acyl glucosides, where the 24 carboxylic acid group of the bile acids are bound to the anomeric hydroxy group of glucose, as potential phase II metabolites [14]. We have analyzed the urine of healthy volunteers and searched for bile acid acyl glucosides using liquid chromatography (LC)/atmospheric pressure chemical ionization (APCI) mass spectrometry (MS). Unfortunately, no peaks corresponding to acyl glucosides were detected on the chromatogram. However, as shown in Fig. 1, two intense peaks at *m*/*z* 697 and 713, corresponding to dihydroxylated bile acid acyl glycoside and trihydroxylated bile acid acyl glycoside, respectively, were found with different retention times than those of authentic specimens of bile acid acyl glucosides. In this study, we have identified these unknown compounds by means of LC/MS and tandem mass spectrometry (MS/MS) analysis coupled with microchemical and derivatization reactions.

2. Experimental

2.1. Materials

Cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, octanoic acid, and 3-methyl-1-phenyl-5-pyrazolone (PMP) were purchased from Nacalai Tesque (Kyoto, Japan). Ursodeoxycholic acid was kindly provided by Mitsubishi Pharma Corporation (Tokyo, Japan). Bile acid 24-glucosides (CA 24-glucoside, UDCA 24-glucoside, CDCA 24-glucoside, DCA 24-glucoside and LCA 24-glucoside) [14] and stable isotope labeled bile acids [15] were synthesized in our laboratory according to previously reported methods. An OASIS HLB cartridge (60 mg) was purchased from Waters (Milford, MA, USA). All other chemicals and solvents were analytical grade. All glassware was silanized with trimethylchlorosilane.

1-*O*-(24-Cholyl)-β-D-galactopyranose (CA 24-galactoside), and 1-*O*-(24-deoxycholyl)-β-D-galactopyranose (DCA 24-galactoside) were synthesized by condensation of 2,3,4,6-tetra-*O*-benzyl-D-galactopyranose (Sigma, St. Louis, MO, USA) with the corresponding bile acid in the presence of 2-chloro-1,3,5-trinitrobenzene (Tokyo Kasei Kogyo, Tokyo) and subsequent hydrogenesis of the resulting benzyloxy-protected bile acid 24-β-D-galactopyranoside over 10% palladium on charcoal [16]. Proton (^{1}H) nuclear magnetic resonance (NMR) spectra were obtained on a ECA-600 Fourier transform spectrometer (JEOL, Tokyo) operating at 600 MHz with CD_3OD as the solvent. High-resolution mass (HR-MS) analyses were carried out using a JMS-LCmate (JEOL) double focusing mass spectrometer equipped with an APCI probe in the negative ion detection mode and the resolution was set at 3000. CA 24-galactoside: ^{1}H NMR, δ: 0.71 [3H, singlet (s), 18-CH_3], 0.91 (3H, s, 19-CH_3), 1.01 [3H, doublet (d), J = 6.6 Hz, 21-CH_3], 3.36 [1H, broad multiplet (brm), 3β-H], 3.52 [1H, double doublet (dd), J = 10.2, 8.4 Hz, 4′-H], 3.61 [1H, triplet (t), J = 6.6 Hz, 5′-H], 3.66 (1H, dd, J = 10.2, 8.4 Hz, 2′-H), 3.71 (2H, d, J = 6.6 Hz, 6′-H), 3.79 [1H, multiplet (m), 7β-H], 3.87 (1H, d, J = 2.4 Hz, 3′-H), 3.94 (1H, m, 12β-H), 5.43 (1H, d, J = 8.4 Hz, 1′-H). HR-MS, calculated for $C_{30}H_{49}O_{10}$ $[M-H]^-$: 569.3325; found, *m*/*z* 569.3326. DCA 24-galactoside: ^{1}H NMR, δ: 0.70 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 1.00 (3H, d, J = 6.6 Hz, 21-CH_3), 3.51 (1H, brm, 3β-H), 3.53 (1H, dd, J = 9.6, 3.6 Hz, 4′-H), 3.62 (1H, t, J = 6.6 Hz, 5′-H), 3.66 (1H, dd, J = 9.6, 8.4 Hz, 2′-H), 3.71 (2H, d, J = 6.6 Hz, 6′-H), 3.87 (1H, d, J = 2.4 Hz, 3′-H), 3.94 (1H, m, 12β-H), 5.43 (1H, d, J = 8.4 Hz, 1′-H). HR-MS, calculated for $C_{30}H_{49}O_9$ $[M-H]^-$: 553.3376; found, *m*/*z* 553.3358.

2.2. Apparatus

The LC/MS analyses were carried out using a JMS-LCmate double focusing mass spectrometer equipped with an APCI probe or an electrospray ionization (ESI) probe in the negative ion detection mode, combined with LC-10AD pumps (Shimadzu, Kyoto). Nitrogen gas was employed as a nebulizing gas at a flow rate of 1.25 L/min. The resolution of the mass spectrometer was set at 750. In the APCI mode, the needle voltage was set at −2.5 kV, and the temperatures of orifice-1 and the vaporizer were set at 150 and 500 °C, respectively. In the ESI mode, the needle voltage was set at −2.5 kV, and the temperatures of orifice-1 and the desolvating plate were set at 150 and 250 °C, respectively.

The sample solution was injected into the LC/APCI-MS system coupled with a trapping column (Inertsil ODS-3 cartridge column, 5 μm, 10 mm × 4.0 mm i.d., GL Science, Tokyo) for retaining hydrophobic compounds, and was then washed with a solution of 20 mM ammonium acetate (pH 7.0 adjusted with ammonia) and acetonitrile (19:1, v/v) for 1 min at a flow rate of 1 mL/min. By switching the six-port valve to the other position, the retained compounds were transferred to the analytical column (Symmetry C18, 5 μm, 150 mm × 4.6 mm i.d.; Waters), and were separated under the linear gradient mode at a flow rate of 0.9 mL/min; the mobile phase consisted of 1 mM ammonium acetate solution (pH 7.0 adjusted with ammonia)/acetonitrile (A) (5:2, v/v) and (B) (5:6, v/v). The mobile phase B content was changed from 0 to 100% over 30 min. A water–acetonitrile solution (8:5, v/v) containing 200 mM octanoic acid and ammonia was introduced to the eluate under a post-column mode at a flow rate of 0.1 mL/min. Monitoring for the ions corresponding to the octanoate adducts was set at *m*/*z* 713, 697 and 681 for tri-, di-, and mono-hydroxylated