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Evidence for bile acid glucosides as normal constituents in human urine

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A glucosyltransferase catalysing formation of bile acid glucosides was recently isolated from human liver microsomes. In order to investigate the potential occurrence of such bile acid derivatives in vivo, a method was devised for their isolation and purification from urine. Conditions were established with the aid of glucosides of radiolabelled, unconjugated glycine and taurine conjugated bile acids prepared enzymatically using human liver microsomes. Analysis by gas chromatography and mass spectrometry of methyl ester trimethylsilyl ether derivatives indicated the excretion of glucosides of nonamidated hyodeoxycholic, chenodeoxycholic, deoxycholic, ursodeoxycholic and cholic acids and of glycine and taurine conjugated chenodeoxycholic and cholic acids. Additional compounds were present giving mass spectral fragmentation patterns typical of di- and trihydroxy bile acid glycosides. Semiquantitative estimates indicated a total daily excretion of about 1 μmol.

Bile acid; Glucoside; Microsome; (Human liver, Urine)

1. INTRODUCTION

Formation of bile acid glucosides was recently shown to be a novel reaction of bile acids [1] in addition to the known conjugation with amino acids [2], sulphuric acid [3] and glucuronic acid [4,5]. A glucosyltransferase catalyzing the transfer of glucose from lipophilic alkyl-β-D-glucopyranosides or dolichol monophosphate glucose to bile acids could be isolated and further characterized from human liver microsomes [1]. Evidence for the existence of these novel bile acid conjugates in vivo was lacking. The present paper describes the detection of glucosides of several unconjugated bile acids, and provides evidence for the occurrence of glucosides of glycine and taurine conjugated bile acids in urine of healthy humans.

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2. MATERIALS AND METHODS

2.1. *Materials*

Human liver samples were obtained from organ donors within 30 min after cessation of life support and were perfused with cold 0.9% NaCl. The samples were stored, homogenized and microsomes were prepared as described [6]. Only tissues with apparently normal histology were used.

All labelled and unlabelled bile acids were obtained as described [7], except for [24-^{14}C]hyodeoxycholic acid (1.2 μCi/μmol) which was a kind gift of Dr M. Parquet (Paris, France). Lipidex-DEAP and Lipidex 5000 were from Packard-Becker (Groningen, The Netherlands), Sepralyte C18 from Analytichem (Harbor City, USA), and Sep-Pak C_{18} cartridges from Waters Associates (Milford, USA). All other chemicals and conditions used for determination of radioactivity by liquid scintillation counting and thin-layer scanning were the same as described [7–9].

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2.2. *Preparation of bile acid glucosides*

Using preparations of human liver microsomes [6], ^{14}C-labelled glucosides of chenodeoxycholic (CDCAgluc), hyodeoxycholic (HDCAgluc), deoxycholic (DCAgluc) and cholic (CAgluc) acids and ^{3}H-labelled ursodeoxycholic acid glucoside (UDCAgluc) were synthesized as reference compounds by enzymatic conjugation of the respective bile acid with octyl-β-D-glucopyranoside as described [1]. Using the same conditions, [^{14}C]glycocholic acid glucoside (GCAgluc) and tauro-[^{14}C]cholic acid glucoside (TCAgluc) were also synthesized from the respective amino acid conjugates. Hyodeoxycholic acid [^{14}C]glucuronide (HDCAglucA) was prepared for reference purposes by enzymatic conjugation of hyodeoxycholic acid with UDP-[^{14}C]glucuronic acid [7]. The bile acid conjugates were purified by ion-exchange chromatography on Lipidex-DEAP [8] and, after methylation, by straight-phase chromatography on Lipidex 5000 (see analysis of urine) [9]. GCAgluc and TCAgluc were treated with cholylglycine hydrolase [10], and rechromatographed on Lipidex-DEAP before methylation and chromatography on Lipidex 5000. Purification steps were monitored by gas-liquid chromatography (GLC) after methylation with diazomethane and conversion into trimethylsilyl (TMS) ether derivatives [8].

2.3. *Analysis of urine*

Six different 24 h urine samples from healthy humans were analysed. After centrifugation and filtration, 1 l of each urine sample was extracted with Sep-Pak C_{18} using one cartridge for each 125 ml. Bile acids were eluted with 10 ml of 80% methanol. The eluates were combined, evaporated and dissolved in 30 ml water. This solution was extracted with a Sep-Pak C_{18} cartridge, and the 80% methanol eluate was reextracted in the same way twice after evaporation of the methanol. The bile acids were eluted with 5 ml of 70% ethanol in the third extraction. This eluate was directly subjected to chromatography on Lipidex-DEAP [8]. Fractions containing glycine, taurine and sulphate conjugates were treated with cholylglycine hydrolase [10] and, after extraction with Sep-Pak C_{18}, rechromatographed on Lipidex-DEAP. The unconjugated bile acids and bile acid glucosides were eluted with 0.1 M acetic acid in 70% ethanol and the glucuronides with 0.25 M formic acid in the same solvent [11]. After extraction with Sep-Pak C_{18} and methylation, glucosides and glucuronides were finally purified by chromatography on Lipidex 5000. Unconjugated bile acid methyl esters and other contaminants were eluted with chloroform/hexane 1:4–1:1 (v/v) [9], and methyl esters of bile acid glucosides or glucuronides were then recovered with methanol.

2.4. *Gas-liquid chromatography and mass spectrometry*

GLC was performed using a Carlo-Erba HRGC instrument with a fused silica capillary column (Quadrex, 25 m × 0.32 mm, cross linked methyl silicone, 0.25 μm film thickness) and a flame ionization detector. After injection at 60°C, the temperature was increased at 30°C/min to 300°C. Helium carrier gas pressure was ~100 kPa. Appropriate *n*-hydrocarbons were added for determination of retention indices (RI).

A VG 7070E double focussing mass spectrometer and a DS 2350 data system was used for fast atom bombardment mass spectrometry (FABMS) and gas chromatography-mass spectrometry (GC/MS). FAB spectra were recorded in the negative ion mode. In GC/MS, spectra were recorded by scanning the range m/z 750–20 at a rate of 2 s/decade, using the same column as described above. The electron energy was 22.5 eV.

3. RESULTS AND DISCUSSION

3.1. *Characterization of bile acid glucosides prepared in vitro*

Enzymatically prepared bile acid glucosides were eluted from Lipidex-DEAP together with unconjugated bile acids using 0.1 M acetic acid in 70% methanol. They were separated from bile acid glucuronides, which were eluted with 0.15 M ammonium acetate, pH 6.6, together with taurine conjugated bile acids [8], or with 0.25 M formic acid [11]. GCAgluc and TCAgluc were eluted in the fractions containing glycine and taurine conjugates, respectively [8]. After treatment with cholylglycine hydrolase [10], the resulting CAgluc was obtained in the 0.1 M acetic acid fraction.

The conjugation of chenodeoxycholic and hyodeoxycholic acids with glucose and glucuronic acid, respectively, was established by negative ion FABMS. Quasi molecular ions, $[M-1]^-$, were ob-

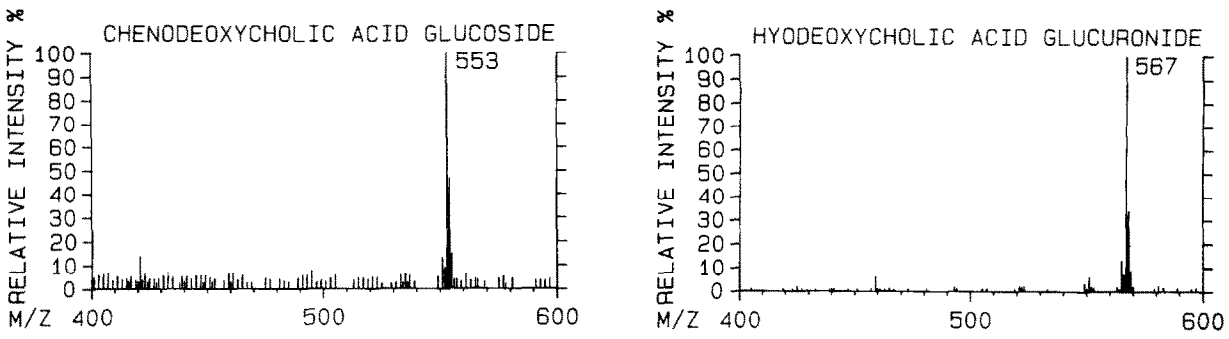

Fig.1. Upper mass region of the negative ion FAB mass spectra of chenodeoxycholic acid glucoside (left) and hyodeoxycholic acid glucuronide (right).

tained at the expected masses: *m/z* 553 for CDCAgluc and *m/z* 567 for HDCAglucA (fig.1). The chromatographic behaviour on Lipidex-DEAP shows that the sugar moiety of all the glucosides prepared was attached at a ring position and not at C-24.

The retention indices of the methyl ester TMS ether derivatives of the enzymatically prepared compounds were 4238 for HDCAgluc, 4296 for UDCAgluc, 4354 for DCAgluc, 4377 for CDCAgluc, and 4433 for CAgluc. The mass spectra of these derivatives showed characteristic fragmentation patterns. In each case, ions at *m/z* 204 and 217 derived from the glucose moiety were intense. The derivatives of the glucosides of CDCA, HDCA, DCA and UDCA showed ions at *m/z* 371 and 461, and that of CA at *m/z* 549, 459 and 369. These are fragment ions typical of the derivatives of di- and trihydroxy bile acid glycosides formed by losses of the sugar moiety and trimethylsilanol [12].

3.2. *Characterization of bile acids in urine*

Potential glucosides of unconjugated bile acids in urine were purified by chromatography on Lipidex-DEAP and, following methylation, on Lipidex 5000 using conditions established with the reference bile acid glucosides. GLC of the methyl ester TMS ether derivatives from each urine sample showed a typical pattern of at least nine peaks with retention indices between 4198 and 4538 (fig.2). GC/MS analysis showed that each peak gave a fragmentation pattern typical of derivatives of di- or trihydroxy bile acid glucosides. Comparison of fragmentation patterns and retention indices indicated the presence of glucosides of CDCA, DCA, HDCA, UDCA and CA in normal urine. However, the major peaks did not correspond to any of the known bile acid glucosides,

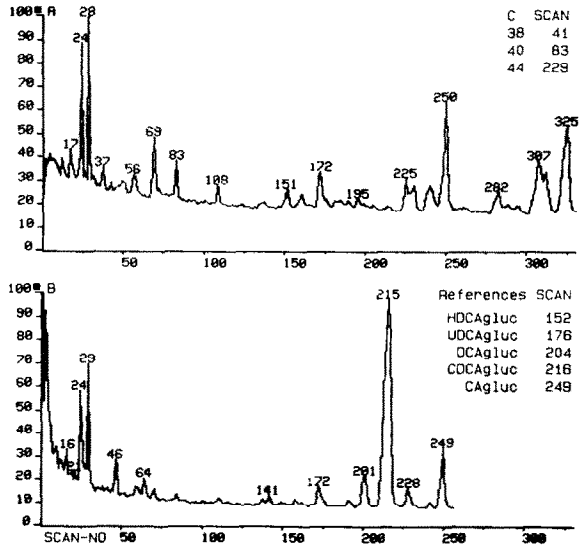

Fig.2. Total ion current chromatograms of methyl esters TMS ether derivatives obtained in the GC/MS analysis of a urine sample after purification (cf. text). Upper panel (A), derivatives of glucosides of unconjugated bile acids; lower panel (B), derivatives of bile acid glucosides obtained after hydrolysis of the glycine-conjugate fraction from Lipidex-DEAP with cholylglycine hydrolase. No further peaks appeared after scan 330 (A) and 255 (B). For comparison of retention indices, scan numbers are given for three *n*-hydrocarbon standards (A) and for the enzymatically prepared reference compounds (B).

and not all of them were found in each urine sample.

Fractions from Lipidex-DEAP containing glycine and taurine conjugated bile acids were treated with cholylglycine hydrolase, followed by purification and derivatization as described for glucosides of the unconjugated bile acids. GLC and GC/MS showed the presence of bile acid glucosides in the glycine (fig.2), and to a lesser extent in the taurine conjugate fractions. CDCAgluc and CAgluc were the major compounds. Thus, bile acid conjugates containing both glucose and amino acid (preferably glycine) moieties seem to be normal constituents in human urine.

The total area of the GLC peaks identified by retention indices and fragmentation patterns to be derivatives of bile acid glucosides was compared with the peak areas of known amounts of the enzymatically prepared reference compounds. The excretion of bile acid glucosides was estimated to be about 1 μmol/day, i.e. in the same range as that of bile acid glucuronides in healthy subjects [12].

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