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Low Molecular Weight Copper-Binding Ligands in Human Bile[1] (42249)

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Abstract. The aim of this study was to detect and identify major low molecular weight (<10,000) copper-binding ligands in human bile. Modified gel chromatography was used as the method of ligand detection because it ensures the detection of labile as well as inert metal–ligand complexes. Conjugated bilirubin, peptides, and amino acids, primarily glycine, were isolated as the major ligands. In contrast to the other copper-binding ligands, the peptides were poor zinc binders, suggesting the possibility that they may confer necessary specificity to trace metal elimination.

Bile is normally the major excretory medium of copper in man (1, 2). It is important to identify the organic-binding ligands which normally transport copper in bile since these ligands may be essential for the copper elimination process, and thus may be crucially involved in metal homeostasis. Previous publications have reported several potentially relevant biliary ligands (3–8). However, since the concentrations of competing ligands vastly exceed the concentration of copper, it is not clear which of the reported ligands are dominant. Furthermore, some of the methodologies previously used to identify biliary copper-binding ligands are questionable due to the lability of the complexes. For instance, identification of metal–ligand complexes by conventional gel permeation chromatography is dangerous since the rather loose complex of metal and ligand may be dissociated during the experiment. The technique of modified gel chromatography (MGC) was designed to address these problems. MGC reliably ensures the simultaneous detection of all major ligands in a biological sample. In this paper, we describe the identification of the major low molecular weight copper-binding ligands in human gall bladder bile detected by the technique of MGC.

MGC involves the gel filtration of a biological specimen on a column equilibrated with a chosen metal ion (9, 10). Ligands in the chromatographed sample are separated by molecular weight on the gel matrix and the column is monitored for metal binding peaks by atomic absorption spectrophotometry. An attribute of MGC in ligand detection is that it supplies excess metal ions to the ligands in the sample, thus permitting all of the major metal binders to be visualized. Also, the equilibration of the column with metal ions prevents any molecular weight-based separation of ligands from metal during the migration down the gel column, a phenomenon which can occur in normal gel filtration chromatography. Hence, the detection of all the major exchangeable as well as nonexchangeable complexes is ensured. The detection limit for some common ligands is 0.1 μmole in the chromatographed sample (10).

Materials and Methods. *Chemicals.* Water used in all experiments was deionized by a Millipore water purification system (Continental Water Systems, El Paso, Tex.). Copper and zinc for MGC were analytical reagents purchased as nitrate salts from Mallinckrodt Chemical Works, St. Louis, Missouri, and J. T. Baker Chemical Company, Philipsburg, New Jersey, respectively. Sodium Nitrate (A.C.S.) was purchased from Matheson Coleman and Bell, Norwood, Ohio. Glacial acetic acid as well as nitric acid was purchased from Mallinckrodt Chemical Works. Sodium

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borohydride and tris(hydroxymethyl)aminomethane (Trizma) were reagent grade from Sigma Chemical Company, St. Louis, Missouri. Chemicals used in ninhydrin testing were ninhydrin (1,2,3-indantrionehydrate) (reagent grade, Nutritional Biochemicals Corp., Cleveland, Ohio) and *n*-butyl alcohol (analytical reagent, Mallinckrodt Chemical Works). Other chemicals required for the development and visualization of thin-layer chromatographs were potassium dichromate (analytical reagent, Mallinckrodt Chemical Works), potassium iodide (analytical reagent, Mallinckrodt Chemical Works), sodium hypochlorite (Curtin Matheson Scientific, Elk Grove Village, Ill.), *o*-tolidine (Matheson Coleman and Bell, Norwood), and pyridine (analytical reagent, Mallinckrodt Chemical Works). Assays for reduced and oxidized glutathione required monopotassium phosphate (analytical reagent, Mallinckrodt Chemical Works), nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma Chemical Co.), ethylenediaminetetraacetic acid (EDTA) (disodium salt, Sigma Chemical Co.), glutathione reductase (EC 1.6.4.2) Type III (Sigma Chemical Co.), dithionitrobenzoic acid (Sigma Chemical Co.) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes) (Research Organics, Inc., Cleveland, Ohio). Formic acid (analytical reagent, Mallinckrodt Chemical Works) was used in anion exchange experiments. A large number of known reference standards were used in chromatography against which unknown binding substances, detected in biological specimens, were compared. All reference ligands were of analytical grade.

Bile. Human gall bladder bile was obtained during cholecystectomy, from United Hospital, Grand Forks, North Dakota.

Preparation of bile. Bile was stored frozen at −20°C until use. Normally, pools of 100 ml of bile were used (approximately 5–6 patients). Throughout this study, involving the bile from a total of 80 individuals, no marked variations in biliary metal-binding characteristics were observed. Human bile was centrifuged at 12,000*g* for 45 min. The pellet was discarded and the supernatant fraction was diluted 1:1 with water and subjected to sequential ultrafiltration through Amicon XM300, YM10, and UM05 membranes. In some instances the YM10 filtrate was used without UM05 filtration and at other times the UM05 retentate was saved. Finally, the ultrafiltrates were lyophilized.

Modified gel chromatography. The copper solvent system consisted of pH 5.7, 25 m*M* acetate, 5 ppm Cu as nitrate, and 0.5 *M* $NaNO_3$, and the zinc system consisted of pH 7.4, 25 m*M* Trizma, 10 ppm Zn as nitrate and 0.5 *M* $NaNO_3$. A pH of 5.7 in the copper system was optimal for peak detection and solubility. The columns consisted of sealed Sephadex G-10 or G-15 gel (Sigma Chemical Co.), approximately 0.9 × 60 cm. The sealing procedure reduces the carboxyl end groups of the Sephadex polydextran chains with sodium borohydride. This technique, devised by Lönnerdal and Låås (11) and Lönnerdal and Hoffman (12), serves to prevent metal–gel binding during MGC. All columns were equilibrated by the elution of 3–4 column volumes of the metal-containing solvent before the sample was applied. Prior to all chromatography experiments the lypophilized bile fractions were dissolved in the appropriate elution solvent and immediately applied to the column. The solvent was eluted through the column at a flow rate of approximately 0.5 ml/min, maintained by a peristaltic pump, and 1.0-ml fractions were collected. The fractions were diluted with 4.0 ml of 1% HNO_3, mixed, and the metal content was determined by atomic absorption spectrophotometry with a Perkin–Elmer Model 560 spectrophotometer (Perkin–Elmer Corp., Norwalk, Conn.).

Specific chemical tests. A ninhydrin test for amino acids and peptides consisted of spotting an unknown on filter paper, spraying the spot with a ninhydrin solution (0.4 g ninhydrin in 100 ml *n*-butyl alcohol), oven heating for 5–10 min at 90°C, and visually examining for coloration.

An assay for reduced glutathione was used with Ellman's reagent (13) to determine the presence of sulfhydryl groups in the peptide fraction of human bile. The assay consisted of reacting 0.8 ml buffer (0.1 *M* monopotassium phosphate, pH 8.0), 0.18 ml sample (or water blank), and 0.02 ml dithionitrobenzoic acid (39 mg/1.0 ml, 0.1 *M* KH_2PO_4, pH 7.0), for 2.0 min, and the change in absorption at 412 nm was recorded. This assay was quantitatively accurate to 1.8 nmole of glutathione. In

addition, an assay was used for oxidized glutathione with glutathione reductase, accurate to 1 nmole in the sample tested. This assay mixture contained 0.8 ml buffer (0.1 *M* Hepes, 0.1 *M* NADPH, 0.5 *M* EDTA, pH 7.5), 0.20 ml sample (or water blank), and 4 μg of glutathione reductase for a total volume of 1.0 ml. The change in absorption due to the oxidation of NADPH was read at 340 nm.

A clinical test kit (Bilistrate, General Diagnostics, Morris Plains, N.J.) was used to detect conjugated and unconjugated bilirubin in bile fractions. The manufacturer's procedures were followed.

Peptide thin-layer chromatography. Two-dimensional silica gel G (20 × 20 cm, Fisher Scientific Co., Pittsburgh, Penn.) thin-layer chromatography (TLC) was employed to detect peptides and amino acids. The TLC was developed in *n*-butyl alcohol:acetic acid:pyridine:water (15:10:3:2, by volume) in the first dimension, and *n*-butyl alcohol:acetic acid: water (4:1:1, by volume) in the second dimension. The developed chromatograms were visualized by three methods. Ninhydrin staining was first used to detect linear, nonprotected peptides and any amino acids. Following the ninhydrin test, the chlorine/tolidine reaction was carried out which, in addition to detecting compounds also visible in the ninhydrin reaction, is able to detect cyclic and derivatized peptides (14). Finally, the chromatograph was sprayed with chromic sulfuric acid solution to detect compounds present other than amino acids and peptides (15, 16).

Amino acid analyses and peptide sequencing. Free amino acids were analyzed by Nat Suwarnasarn and Charles Vomacka in the University of North Dakota Chemistry Department with a Durrum amino acid analyzer (Dionex Corp., Sunnyvale, Calif.).

Peptide amino acid analyses and sequencing were performed at the Protein Sequencing Facility in the Department of Biochemistry, University of Iowa.

Results. *Detection of metal-binding peaks.* Copper MGC of human bile ultrafiltrates is shown in Figs. 1A–C. The chromatograph of the Amicon YM10 bile ultrafiltrate (mol wt nominally less than 10,000) (Fig. 1A) contains three peaks of V_e/V_0 = 1.02, 1.26, and 1.49 with a Sephadex G-10 column, and V_e/V_0 = 1.21, 1.50, and 1.86 on Sephadex G-15. The

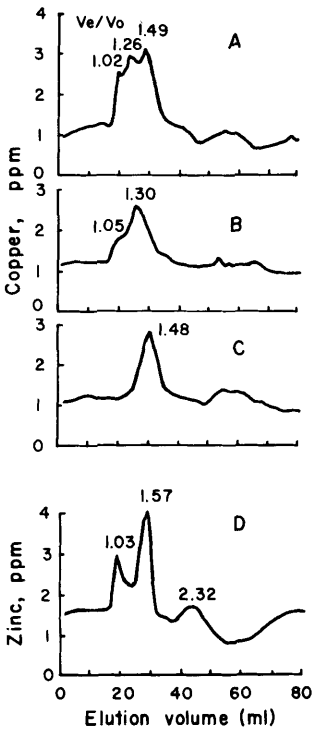

FIG. 1. Copper and zinc MGC of human bile ultrafiltrates. The chromatographs are of various bile ultrafiltrates chromatographed on sealed Sephadex G-10. Solvent system for A–C consisted of 25 m*M* acetate, pH 5.7, 5 ppm Cu (as nitrate), and 0.5 *M* $NaNO_3$. The solvent system for D was 25 m*M* Trizma, pH 7.4, 0.5 *M* $NaNO_3$, and 10 ppm Zn (as nitrate). (A) Sample: 15 mg lyophilized YM10 ultrafiltrate dissolved in 1.0 ml solvent, column: V_0 = 19.5 ml, 0.9 × 59 cm. (B) Sample: 15 mg lyophilized UM05 retentate dissolved in 1.0 ml solvent, column: V_0 = 20.0 ml, 0.9 × 60 cm. (C) Sample: 10 mg lyophilized UM05 ultrafiltrate dissolved in 1.0 ml solvent, column: V_0 = 21.0 ml, 0.9 × 61 cm. (D) Sample: 30 mg lyophilized YM10 ultrafiltrate, column: V_0 = 18.5 ml, 0.9 × 61 cm. Fractions HB1, HB2, and HB3 show the V_e/V_0 values of 1.02, 1.26, and 1.49 as seen in A. C shows only HB3, with fractions HB1 and HB2 retained by the UM05 membrane.

statistical variation in the V_e/V_0 estimates was ±0.06. These three peaks are designated HB1, HB2, and HB3 as seen from left to right. Only one of these peaks (HB3, V_e/V_0 = 1.48 on Sephadex G-10) passes through an Amicon UM05 ultrafiltration membrane (nominal mol wt cutoff of ~500) as is shown in Fig. 1C. The peaks of V_e/V_0 = 1.02 and 1.26 (HB1 and HB2, respectively) do not appreciably penetrate the Amicon UM05 membrane and are

consequently concentrated in the UM05 retentate (Fig. 1B).

The zinc MGC of the YM10 filtrate (30-mg sample as opposed to 15 mg for copper MGC) shows only two peaks with V_e/V_0's of 1.03 and 1.57 on Sephadex G-10 (Fig. 1D). Presumably, HB2 (V_e/V_0 = 1.26) binds zinc poorly. The two zinc peaks behave similarly to the corresponding copper peaks with regard to UM05 ultrafiltration.

Table I presents copper MGC elution volumes for standards of previously reported biliary metal-binding ligands as well as other ligands possibly present in bile, such as small peptides.

Purification of metal-binding peak contents. The ligands present in the metal-binding peaks were isolated for identification. During purification, the presence of HB1 and HB2 was monitored by MGC.

The contents of HB1 (V_e/V_0 = 1.02 on Sephadex G-10) were isolated in the following manner. Whole human gall bladder bile (100 ml) was centrifuged at 12,000*g* for 45 min.

TABLE I. MGC ELUTION VOLUMES FOR BILE STUDIES WITH COPPER ON SEPHADEX G-15

Standard compound or human bile peak	V_e/V_0 (pH 5.7)
HB1 peak	1.21
HB2 peak	1.50
HB3 peak	1.86
Bilirubin (unconjugated)	Insoluble
Gly-Gly-His	1.54
Gly-Gly-His-CH_3-amide	1.65
Asp-Ala-His-CH_3-amide	1.33
Gly-His-Lys	1.47
Taurocholate	Nonbinder
Na choleate	Nonbinder
Deoxycholate	Insoluble
Glycocholate	Nonbinder
Glutathione (reduced)	1.14 (1.00)[a]
Glutathione (oxidized)	1.14
Alanine	1.82
Threonine	1.82
Histidine	2.20
Glycine	1.84
Glutamate	1.56
Glutamine	1.86
Isoleucine	1.96
Serine	1.82
Tyrosine	2.56
Valine	1.82
Taurine	Nonbinder
Chloride	1.92

[a] Occasionally two peaks would form.

The supernatant fraction was then ultrafiltered at 4°C under N_2 (50–60 psi) through Amicon XM300 and YM10 membranes. This filtrate was lyophilized and the ultrafiltrate was then chromatographed as given above on a sealed Sephadex G-25 column (1.5 × 90 cm), particle size: 10–40 μm, void volume of 23.5 ml. The solvent system was buffered to pH 5.7 with 25 m*M* acetate. The early eluting noncolored fractions were discarded and the later brown-colored fractions were pooled and ultrafiltered through an Amicon UM05 ultrafiltration membrane. The UM05 retentate was reconstituted with water and ultrafiltered again. The twice-ultrafiltered, or "dialyzed," retentate was then lyophilized. The lyophilized material was chromatographed on Sephadex G-10 (0.9 × 60 cm column) with a solvent system of 25 m*M* acetate, pH 5.7. The colored metal-binding fractions (HB1) were pooled and lyophilized. The yield was approximately 1–2 mg from 100 ml of bile.

To purify HB2 (V_e/V_0 = 1.26 on Sephadex G-10), human bile was pooled to 100 ml and centrifuged at 12,000*g* for 45 min. The supernatant fraction was diluted 1:1 with deionized water and ultrafiltered through an Amicon XM300 membrane and then through an Amicon YM10 membrane. The YM10 ultrafiltrate was passed through an Amicon UM05 membrane, the retentate was reconstituted, and again ultrafiltered through an Amicon UM05 membrane to dialyze away the lowest molecular weight components of bile. The filtrates were discarded and the retentate was lyophilized. The lyophilized retentate was applied in samples of 150 mg to an anion exchange column (Bio-Rad AG1X-8, 200–400 mesh, 0.9 × 30 cm, formate form). The samples were eluted with 180 ml water followed by 120 ml 0.01 *M* formate. The water wash was discarded and the 0.01 *M* formate wash, containing the HB2 fraction, was saved. The 0.01 *M* formate fraction was pooled, lyophilized, and chromatographed on a sealed Sephadex G-15 (1.5 × 90 cm, 5 m*M* acetate, pH 5.7). The eluent flow from the column was monitored at a wavelength of 210 nm. The HB2 fraction, which absorbed at this wavelength, was pooled and lyophilized.

The HB3 fraction (V_e/V_0 = 1.48 on Sephadex G-10) was not purified beyond ultrafiltration through an Amicon UM05 mem-

brane, which separated it from all other binding peaks (Fig. 1).

Identification of biliary copper-binding ligands. The intermediate molecular weight range of HB1 (500–10,000, by ultrafiltration), its red-brown color when isolated (virtually identical to that of purchased bilirubin), its negative reaction with ninhydrin, and a previous report of bilirubin–copper complexes in bile (5) indicated that HB1 may be bilirubin. A clinical test for bilirubin (based on the van den Bergh test) was performed which demonstrated that isolated HB1 contained bilirubin diglucuronide but not unconjugated bilirubin.

The purification procedure for fraction HB2 was based on the supposition that HB2 is either a small peptide or a combination of coeluting small peptides. The evidence for this was a positive ninhydrin reaction and a molecular weight range which was crudely estimated by ultrafiltration to be greater than 500 and by gel filtration (Sephadex G-10) to be less than 700. When HB2 was chromatographed by two-dimensional TLC, five spots were visualized by all three detection methods; ninhydrin, chlorine/tolidine, and chromic sulfuric acid reactions (Fig. 2). This is interpreted to mean that no cyclic or derivatized peptides and no non-amino acid or nonpeptide compounds were present in HB2 to any appreciable extent. Four of the five spots were quite faint (spots 1–4 in Fig. 2) and one spot (spot 5) appeared to be the major constituent based on its dark color in the visualization methods. The multiple spots seen with TLC indicate the presence of more than one peptide in HB2. However, since its elution profile matches that of glycine, spot 4 may be the result of minor copurification of this amino acid, which is highly concentrated in bile (see below). The HB2 fraction was shown to contain multiple peptides by an Edman degradation sequencing procedure. The HB2 fraction was also acid hydrolyzed and subjected to automatic amino acid analysis. The resultant molar ratio composition of amino acids is shown in Table II. As suspected, there is a large number of different amino acids present, suggesting multiple small peptides in HB2. Also, the high amount of glycine suggests copurification of this amino acid, which is highly concentrated in bile.

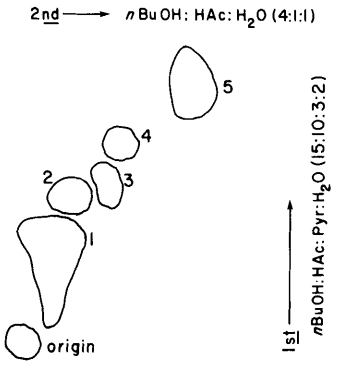

FIG. 2. Two-dimensional thin-layer chromatography of purified HB2. The TLC plate used was silica gel G, 20 × 20 cm, 250 μm thick. The solvent used in the first dimension was *n*-butyl alcohol:acetic acid:pyridine:water (15:10:3:2) and the solvent used in the second dimension was *n*-butyl alcohol:acetic acid:water (4:1:1). Visualization with ninhydrin, chlorine/tolidine, and chromic sulfuric acid reactions all gave similar results. Spots 1–4 were quite faint. Spot 4 matched the elution pattern of glycine.

TABLE II. MOLAR RATIO COMPOSITION OF PEPTIDE AMINO ACID RESIDUES IN HB2[a]

Amino acid	Molar ratio
Asp	2.053
Thr	0.895
Ser	0.932
Glu	2.889
Pro	1.323
Gly	6.837
Ala	2.127
Val	0.977
Met	0.340
Ile	1.013
Leu	2.118
Tyr	0.486
Phe	0.989
His	1.821
Arg	0.626
Lys	1.821

[a] Analysis was performed at the Protein Structure Facility in the Department of Biochemistry, University of Iowa.

The HB3 peak (V_e/V_0 = 1.48 on Sephadex G-10, 1.78 on Sephadex G-15) was the sole metal-binding fraction in the Amicon UM05 filterable fraction of bile. This peak was found to give a positive reaction with ninhydrin and had a similar elution volume to a number of free amino acids (Table I). An automatic amino acid analysis indicated that the UM05

filtrate of bile did indeed contain large amounts of amino acids with similar elution volumes to this peak (Table III). Glycine makes up 61% of the total molar concentration of the free amino acids in this fraction.

Detection of glutathione in biliary ultrafiltrate. Both reduced and oxidized forms of glutathione are reportedly present in bile at approximately 1 m*M* concentrations (17–19) and thus may be important biliary ligands. Both reduced and oxidized forms of glutathione shared elution patterns to spots seen in the HB2 two-dimensional TLC described above. However, the MGC elution volumes differed when HB2 and either form of glutathione were chromatographed separately or together with bile, indicating that glutathione was not present in HB2 (Table I). Since glutathione is reportedly present in bile and is a metal-binding ligand, further comparison to the peptide fraction of bile, HB2, was warranted.

An assay was used with Ellman's reagent to determine the presence of sulfhydryl groups in HB2. Standards of purchased reduced glutathione demonstrated that this assay was accurate to 1.8 nmole. However, no glutathione was detected in 1 mg purified HB2.

An assay for the oxidized form of glutathione using glutathione reductase was used. This assay which was accurate with glutathione standards to 1 nmole demonstrated the absence of the disulfide form of glutathione in 1 mg of purified HB2.

Discussion. In previous studies, biliary copper has been associated with peptides (6,7) and amino acids (7) in rats, and with proteins (3,4), peptides (6), conjugated bile pigments (5), and taurochenodeoxycholate (8) in man. This report establishes which of these ligands are most prominent in human bile and gives an indication of their metal-binding capacities relative to each other. The three types of low molecular weight ligands found in human bile by MGC are conjugated bilirubin, small peptides, and amino acids (Fig. 1A shows their respective V_e/V_0's to be 1.02, 1.26, and 1.49 on Sephadex G-10).

Since bile is the major medium of copper excretion (1), some of the ligands visualized by MGC may play a role in the elimination of copper from the body. Copper entering the bile *via* hepatic lysosomal processing may do so in association with the detected biliary copper-binding peptides and amino acids, since these ligands themselves are quite likely products of lysosomal processing, arising from normal protein degradation. It is not clear whether the detected peptides are entirely random cleavage products of protein degradation or if specific peptides are present. It is doubtful that the detected peptides are artifacts of protein decomposition during sample preparation since their MGC metal-binding peak was constant in relative size and position among many different bile samples, and over time.

Of the three types of detected ligands, the peptide fraction appears to be the most specific in that it binds copper much better than zinc (Fig. 1). Such specificity may be required in the copper elimination process to prevent the indiscriminate elimination of other trace metals such as zinc. Thus, the peptide fraction appears to be a more promising candidate for physiological biliary copper transport than anything else in the low molecular weight fraction.

An anticipated biliary peptide ligand, glutathione, was not detected in the low molecular weight fraction of bile. Glutathione is reported to be present in bile in millimolar concentrations (17–19) and was shown by MGC to bind copper (Table I). The reason it escaped detection may be due to conjugation of glutathione to high molecular weight fractions (20) or to the apparent aggregation of glutathione molecules often seen in MGC of pure glutathione (Table I). These phenomena are probably due in part to the oxidizing conditions of the laboratory, as reported by others (19, 20). Also, trace metals can rapidly oxidize thiols (20).

TABLE III. AMINO ACID ANALYSIS OF LYOPHILIZED HUMAN BILE AMICON UM05 ULTRAFILTRATE

Amino acid	Molar content
Tau	1.23×10^{-5}
Thr	8.64×10^{-7}
Ser	1.14×10^{-5}
Gly	5.96×10^{-5}
Ala	1.14×10^{-5}
Leu	1.94×10^{-6}
Other amino acids detected	0

Note. Molarity based on 1.2 mg bile isolate dissolved in 2.0 ml and injected onto automatic amino acid analyzer column.

The ligands found in bile should also be considered in the study of the intestinal absorption of trace metals such as copper and zinc. Biliary copper (excreted copper) is poorly reabsorbed (4, 21, 22). However, it is feasible that bile may contain ligands which facilitate metal absorption from the intestinal lumen as well as tight-binding ligands used in copper elimination. This idea is supported by the fact that bile contains high levels of free amino acids, which are ligands believed to enhance copper absorption (1).

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