Fluorescent Bile Acid Derivatives: Relationship Between Chemical Structure and Hepatic and Intestinal Transport in the Rat

FERNANDO HOLZINGER, CLAUDIO D. SCHTEINGART, HUONG-THU TON-NU, SABINE A. EMING, MARIA J. MONTE, LEE R. HAGEY, AND ALAN F. HOFMANN

Studies were performed to characterize hepatic and intestinal transport, as well as biotransformation during transport, of a spectrum of fluorescent bile acids containing a fluorophore attached to the side chain. The following two classes of compounds were studied: 1) aminofluorescein (amF) coupled directly to the carboxylic group of a bile acid which was cholic, ursodeoxycholic, or cholylglycine; and 2) nitrobenzoxadiazolyl (NBD) coupled to the ε-amino group of a lysine conjugated bile acid, which was cholic or ursodeoxycholic. Fluorescein, a cholephilic organic anion, was studied as a control. Fluorescent bile acids were synthesized and their structures confirmed by nuclear magnetic resonance and mass spectrometry. Using the biliary fistula rat, hepatic transport, biotransformation, and choleretic activity were defined; intestinal absorption was assessed by jejunal or ileal perfusion studies. All fluorescent bile acids had hepatic transport maxima about one-sixth that reported for cholyltaurine, but derivatives of cholylglycine were transported best. Bile acids underwent little (<5%) biotransformation during hepatocyte transport. Only the amF conjugate of cholylglycine had normal choleretic activity; other compounds were hypocholeretic or cholestatic. In contrast, fluorescein was well transported, was partly glucuronidated, and had normal choleretic activity. NBD-tagged, but not amF-tagged, bile acids were actively transported by the intestine (ileum > jejunum), and no fluorescent bile acid had passive intestinal permeability; NBD-tagged bile acids were biotransformed during intestinal transport (jejunum > ileum). We conclude that the structure of the fluorophore as well as that of the bile acid influences transport by the hepatocyte and enterocyte. These fluorescent bile acids differ from fluorescein in being impermeable to cell membranes and undergoing little biotransformation during hepatocyte transport. Of these fluorescent bile acids, cholylglycylamF has hepatocyte transport and choleretic properties most closely resembling those of a natural bile acid. (HEPATOLOGY 1997;26:1263-1271.)

Bile acids tagged with a fluorophore have proved useful for characterizing aspects of hepatocyte,[1-13] enterocyte,[14,15] and cholangiocyte[16] transport. Several types of fluorescent bile acids have been prepared. The laboratory of G. Kurz[4,5,17] described a number of bile acid derivatives in which a hydroxy group on the steroid nucleus was replaced by nitrobenzoxadiazolyl (NBD), a well-known fluorophore. Earlier, Sherman and Fisher[1] reported the coupling of aminofluorescein (amF) to cholylglycine (glycocholate) and proposed that the amF was coupled to the oxygen atom at C-3. Subsequently, Schteingart et al.[18] showed that the assigned structure was not correct, and that in the compound prepared by Sherman and Fisher[1] the amF was coupled to the carboxyl group at the end of the side chain. Mills and his colleagues[6] coupled fluorescein to the ε-amino group of cholyllysine, thus preparing compounds in which fluorescein was coupled by an amino-*n*-butyl tether to the α-carbon of cholylglycine. More recently, our laboratory has prepared analogous derivatives by coupling NBD to the ε-amino group of cholyllysine or chenodeoxycholyllysine.[10]

If fluorescent bile acids are to be used as surrogate molecules for bile acids or other hepatophilic organic anions, detailed characterization of their hepatic handling is needed. It is also useful to know whether these molecules are transported actively or passively by the small intestine, since molecules that are well absorbed by the small intestine may undergo enterohepatic cycling.

We report here studies on the hepatic and intestinal transport of five fluorescent bile acid derivatives using the anesthetized biliary fistula rat. In the compounds that were used, the fluorophore (NBD or fluorescein) was present on the side chain. Three different bile acid moieties were used—cholic acid (C), ursodeoxycholic acid (UDC), and cholylglycine (CG)—to test whether the chemical structure of the

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Abbreviations: NBD, nitrobenzoxadiazolyl; amF, aminofluorescein; C, cholic acid; UDC, ursodeoxycholic acid; CG, cholylglycine; C-taurine, cholyltaurine; UDC-taurine, ursodeoxycholyltaurine; T_{max}, maximal hepatic transport rate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; ACA, apparent choleretic activity; CGamF, cholylglycylamidofluorescein; CamF, cholylamidofluorescein; UDCamF, ursodeoxycholylamidofluorescein; C-L-NBD, cholyl-(Nε-NBD)-lysine; UDC-L-NBD, ursodeoxycholyl-(Nε-NBD)-lysine.

From the Division of Gastroenterology, Department of Medicine, University of California, San Diego, CA 92093-0813.

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Dr. Holzinger is a Visiting Postdoctoral Fellow from the Department of Visceral and Transplantation Surgery, Inselspital, University of Bern, Bern, Switzerland.

Dr. Schteingart's present address: Ferring Research Institute Inc., San Diego, CA 92121.

Ms. Ton-Nu's present address: La Jolla Pharmaceuticals, San Diego, CA 92121.

Dr. Eming's present address: Department of Dermatology, University of Cologne, Germany.

Dr. Monte's present address: Department of Physiology and Pharmacology, University of Salamanca, Spain.

Dr. Hagey's present address: Center for Reproduction of Endangered Species, San Diego Zoological Society, San Diego, CA 92134.

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Address reprint requests to: Alan F. Hofmann, M.D., Ph.D., Department of Medicine 0813, University of California, San Diego, La Jolla, CA 92093-0813. Fax: (619) 543-2770.

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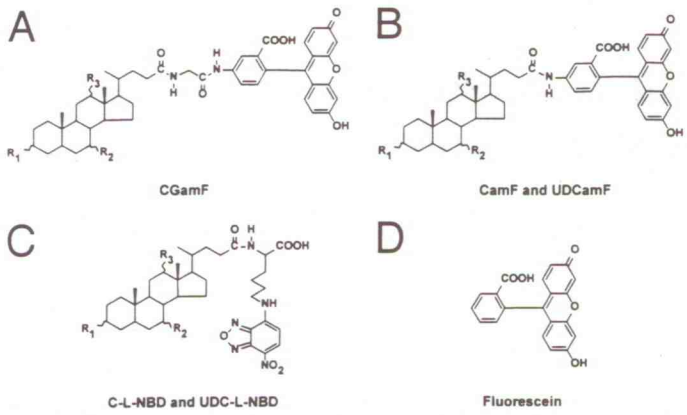

FIG. 1. Chemical structures of fluorescent bile acid derivatives and fluorescein.

steroid moiety influenced hepatic or intestinal handling. Since three of the molecules were tagged with fluorescein (as amF), we also characterized the hepatic handling of fluorescein to factor out the effect of the bile acid moiety on the amF-tagged bile acids. Results with these organic anions were compared with literature values for cholyltaurine (C-taurine) and ursodeoxycholyltaurine (UDC-taurine), common conjugated bile acids, whose transport characteristics by the rat liver[19-21] and intestine[22-25] have been well defined.

MATERIALS AND METHODS

Experimental Design

Experiments were performed using the anesthetized, biliary fistula rat because we wished to characterize the disposition of these molecules in the whole animal and because our laboratory has had considerable experience with hepatic transport[20,26,27] and intestinal transport[22] of bile acids in the biliary fistula rat. Two types of studies were performed. In the first set of studies, hepatocyte transport was assessed by measuring the maximal transport rate (T_{max}). Bile acid biotransformation [by thin-layer chromatography (TLC) and HPLC] and bile flow in relation to bile acid recovery [apparent choleretic activity (ACA)] were measured at the same time. In the second, intestinal absorption was assessed by perfusing the jejunum or ileum and equating biliary recovery with intestinal absorption.

Chemicals: Fluorescent Bile Acids and Fluorescein

Five fluorescent bile acids were used in this study, three tagged with amF [cholylglycylamidofluorescein (CGamF), cholylamidofluorescein (CamF), and ursodeoxycholylamidofluorescein (UDCamF)] and two with NBD [cholyl-(Nϵ-NBD)-lysine (C-L-NBD) and ursodeoxycholyl-(Nϵ-NBD)-lysine (UDC-L-NBD)]. Fluorescein sodium salt was purchased from Sigma Chemical Co. (St. Louis, MO); it was found by HPLC to have a purity >98% and was used as received. Chemical structures of all compounds are shown in Figure 1. Trivial names, abbreviations, orientation of nuclear substituents, molecular weights, and HPLC retention times are given in Table 1.

All amF-tagged bile acids were synthesized by conjugation of commercially available 5-aminofluorescein (Sigma Chemical Co., St. Louis, MO) with the carboxylic group of C, UDC, CG. Conjugation was performed using the coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in a pyridine-hydrochloric acid buffer. This reaction couples the amino group of the fluorescein molecule to the carboxyl group of the bile acid side chain by an amide bond. Details of synthetic methods have been published elsewhere.[18] The Nϵ-NBD–tagged bile acid derivatives were prepared by coupling the relatively smaller NBD fluorophore to the ϵ amino group of cholyl- and ursodeoxycholyl-lysine using a series of reactions similar to the ones described by Schneider et al.[17]

Compounds were purified by adsorption chromatography using silica gel column chromatography as previously described.[28] The final preparations were >98% pure by HPLC.[29]

Physiological Experiments

Animal studies were approved by the Animal Subjects Committee of the University of California, San Diego.

Anesthetized Biliary Fistula Rat. Unfasted adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250 to 330 g were anesthetized by intraperitoneal injection of pentobarbital, 7.5 mg/100 g body wt (Nembutal®, Abbott Laboratories, North Chicago, IL). Anesthesia was maintained by repeated intramuscular doses (10 mg) of pentobarbital given every 2 to 2.5 hours. Abdominal temperature was maintained throughout the experiment at 38 ± 0.5°C with a temperature-controlled heating lamp connected to a rectal temperature probe (Yellow Springs Instrument Co., Yellow Springs, OH). The jugular vein was cannulated using PE-10 polyethylene tubing (Clay Adams, Parsippany, NJ; inner diameter, 0.28 mm; outer diameter, 0.61 mm). 0.9% sodium chloride (Baxter, Deerfield, IL) was infused at a rate of 2.2 mL/h using a Harvard syringe pump (Harvard Apparatus Co., Millis, MA). The abdomen was then opened by a midline incision and an external biliary fistula was constructed using PE-10 polyethylene tubing. Bile was collected every 15 minutes in preweighed plastic vials. After a 2 hour control period, the fluorescent bile acids or fluorescein (dissolved in 0.9% sodium chloride and 0.05 N NaOH, pH ≈ 8.0) were infused. Fluorescent bile acids were infused over a dose range from 0.3 to 8 μmol/ kg · min, and usually one to three doses were studied per animal. Each dose was infused for 90 minutes before proceeding to the next dose. (This procedure gives a value for T_{max}, but not for recovery, because of the confounding effect of toxicity at higher doses.)

Higher doses of fluorescent bile acids were not administered either because of toxic effects (see "Results") or lack of compound (an infusion of a fluorescent bile acid at a rate of 8 μmol/ kg · min requires ≈ 150 mg of the compound). However, an infusion rate of 8 μmol/ kg · min exceeds the usual flux of bile acids to the liver in the healthy rat.[30] Fluorescein was infused over a dose range from 0.5 to 14 μmol/ kg · min. At the end of the infusion period of the tested compound, saline was infused for an additional 2 hours.

To test for renal excretion of infused compounds and their metabolites, the urinary bladder was aspirated at the end of each experiment, and urine was analyzed by TLC.

Intestinal Perfusion. Adult male Sprague-Dawley rats were fasted overnight before the experiment. After completion of the biliary fistula as described above, a jejunal segment distal to the ligament of Treitz (≈20 cm in length) or a segment of the terminal ileum (≈20 cm in length) was surgically isolated. A PE-90 polyethylene tube (inner diameter, 0.86 mm; outer diameter, 1.27 mm) was introduced into the proximal lumen and tied in place. A large-bore polyethylene tube (Tygon R-3603, Fisher Scientific Co., Pittsburgh, PA; inner diameter, 1.60 mm; outer diameter, 3.20 mm) was inserted into the distal part of the isolated segment. (In the case of the ileum the tube was carefully pushed through the ileocecal valve for about 5 mm and tied in place.) The isolated segment was then rinsed with 20 to 40 mL 0.9% sodium chloride at 38°C using a syringe in order to remove intestinal contents. After the tube placements, the abdominal temperature was maintained at 38° ± 0.5°C by means of a temperature-controlled heating lamp. The perfusate, which was kept at 38°C in a water bath, consisted of isotonic TRIS buffer (TRIS, 25 mmol/L; NaCl, 130 mmol/L, pH 8.0). Fluorescent bile acids or fluorescein were dissolved in the perfusate to a concentration of 1 mmol/L.

A 60-minute control period, during which buffer was perfused, was followed by a 60 minute perfusion of the test compound. A peristaltic pump (Harvard Apparatus, Millis, MA) was used to per-

TABLE 1. Chemical Properties of Fluorescent Bile Acid Derivatives and Fluorescein

Trivial Name	Abbreviation	Nuclear Substituents			Molecular Weight (free acid form)	RRT by HPLC*
		R_1 3-OH	R_2 7-OH	R_3 12-OH		
Cholylglycylamidofluorescein	CGamF	α	α	α	794.9	2.46
Cholylamidofluorescein	CamF	α	α	α	737.9	5.79
Ursodeoxycholylamidofluorescein	UDCamF	α	β		721.9	2.98
Cholyl-(Nϵ-NBD)-lysine	C-L-NBD	α	α	α	699.8	2.44
Ursodeoxycholyl-(Nϵ-NBD)-lysine	UDC-L-NBD	α	β		683.8	1.44
Fluorescein	F				332.3	0.36

* Relative retention time by HPLC to C-taurine. These values are pH-dependent.[29]

fuse the intestinal segment in single pass fashion with a pulsatile flow rate adjusted to 4 mL/min. This perfusion rate has been shown to eliminate unstirred layer effects and to maintain an unchanged bile acid concentration in the perfused segment in previous studies of this type.[22] Little distension of the isolated intestine was observed at this flow rate. Bile samples were collected at 10 minute intervals. The intestine was perfused with isotonic saline for 120 minutes following the 60 minute infusion of the test compound. At the end of each experiment, the intestinal segment was removed and the length of perfused segment was measured. Each compound was studied one to three times per isolated jejunal or ileal segment. Since the observed biliary excretion rates during intestinal perfusion were far below hepatic T_{max} values, intestinal uptake was equated with biliary recovery.

Bile Analysis

The concentrations of all compounds in bile were determined spectrophotometrically (Shimadzu UV-12016, Shimadzu Scientific Instruments, Japan) rather than fluorometrically because of possible effects of pH and microenvironment (quenching) on fluorescence emission.[31,32] The absorbance of studied compounds did not vary between pH 7.0 and pH 8.0. A standard calibration curve from 0 to 1 mmol/L in the appropriate buffer (see below) was obtained for each compound. Bile samples were diluted 100- to 5,000-fold with buffer in order to adjust the concentration to the calibration range, depending on compound and biliary concentration.

Bile from experiments using amF-tagged bile acids was diluted with isotonic TRIS buffer, pH 8.0 (composition given above), and its absorbance at 492 nm determined. These compounds were not biotransformed during hepatic transport (see results), and hydrolysis with β-glucuronidase was therefore unnecessary.

Bile from experiments using NBD-tagged bile acids was diluted with sodium phosphate buffer (25 mmol/L sodium phosphate; 125 mmol/L NaCl, pH 7.4), and its absorbance at 478 nm determined.

Bile samples from experiments with fluorescein were diluted with TRIS buffer, pH 8.0 (composition given above), and their absorbance at 492 nm was measured. Because about half of the fluorescein was converted to its glucuronide (see results), which is known to have a different absorbance, samples were hydrolyzed with β-glucuronidase before measurement.[33-35] To do this, 100 μL sodium acetate buffer (100 mmol/L, pH 5.0) containing 100 units β-glucuronidase Type H-5 (from *Helix pomatia*) (Sigma Chemical Co., St. Louis, MO) were added to an aliquot of bile (10 μL), and the mixture was incubated for 30 minutes at 37°C. Using this procedure, hydrolysis was complete.

For the determination of hepatic and intestinal biotransformation, bile aliquots (2-6 μL) were examined by TLC using silica gel plates (Silica Gel 60 F-254, 20 × 20 cm, thickness 0.25 mm; E Merck, Darmstadt, Germany). When fluorescent bile acids and/or their metabolites were present in bile in low concentrations, the compound was isolated from bile using a hydrophobic column (bonded octadecyl; PrepSep™-C_{18}; Fisher Scientific, Fair Lawn, NJ). The adsorbed compound was eluted with methanol and analyzed by TLC, using a solvent system for conjugated bile acids (isoamylacetate: propionic acid: *N*-propanol: water = 4:3:2:1, vol:vol).[36] Fluorescent compounds and their metabolites before and after enzymatic deconjugation were visualized with UV light (at 366 nm).

Biotransformation was also assessed by HPLC using a modification[37] of the technique described by Rossi et al.[29] The method uses an octadecylsilane column (RP C-18) with isocratic elution at 0.75 mL/min. The eluting solution is composed of a mixture of methanol (67.4% by volume) and 0.01 mol/L KH_2PO_4, adjusted to apparent pH 5.4. Fluorescent bile acids and their metabolites were detected in the column effluent by monitoring absorbance at both 204 and 230 to 250 nm (for the fluorescent compounds); the same procedure

TABLE 2. Hepatic Transport Properties of Fluorescent Bile Acid Derivatives and Fluorescein: Calculated and Observed Values for T_{max}, J_{50}, and Dose Causing Toxicity in the Anesthetized Biliary Fistula Rat

	CGamF	CamF	UDCamF	C-L-NBD	UDC-L-NBD	Fluorescein
No. of animals	8	8	8	7	8	11
T_{max}* [μmol/kg · min]	3.73 ± 0.79	2.79 ± 0.79	3.43 ± 0.67	3.68 ± 0.98	1.52 ± 0.19	2.80 ± 0.33
J_{50}* [μmol/kg · min]	2.95	3.09	4.19	3.17	1.33	3.08
T_{max}/J_{50}†	1.26	0.90	0.82	1.16	1.14	0.91
Observed T_{max}‡ [μmol/kg · min]	2.53 ± 0.09	2.00 ± 0.04	1.93 ± 0.04	2.53 ± 0.04	1.10 ± 0.02	2.42 ± 0.09
Toxic dose§ [μmol/kg · min]	8.0§	6.0§	8.0§	8.0§	5.0§#	>14.0

Abbreviations: T_{max}, maximal transport rate; J_{50}, infusion rate that produced half-maximal transport rate.

* Mean values ± SE calculated by nonlinear regression for best fit curve of pooled data omitting the datapoints for toxic doses. For C-taurine, a major endogenous bile acid in the rat, the T_{max} is 14.6 ± 1.2 μmol/kg · min.[20] For UDC-taurine, the T_{max} is 32.4 ± 2.5 μmol/kg · min.[19]

† The quotient (T_{max}/J_{50}) permits comparison of transport rates in relation to infusion rates well below the J_{50}.

‡ Mean values ± SD of maximal biliary recovery observed in all experiments (size per group = 6).

§ Bile flow and/or recovery decreased during infusion time.

Rat died during infusion time.

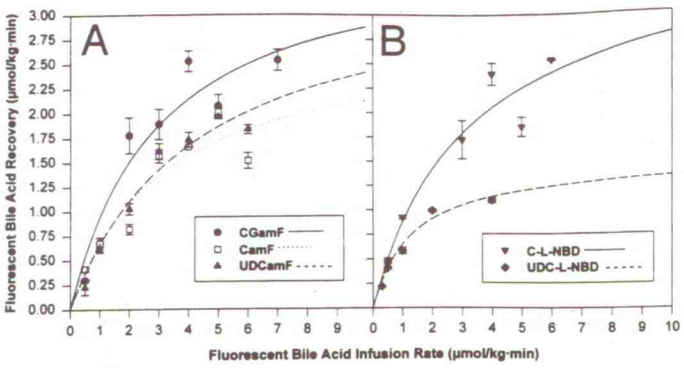

FIG. 2. Relationship between infused dose and biliary recovery of fluorescent bile acid derivatives in the anesthetized biliary fistula rat. The points represent means ± SE and the lines are the best fit to the Michaelis–Menten equation. Recovery values for toxic doses were omitted before curve fitting and calculation of the T_{max} values (Table 2). All fits were converged, tolerance was satisfied, and parameter dependencies was shown to be >0.84. (A) amF-tagged fluorescent bile acids. (B) NBD-tagged fluorescent bile acids.

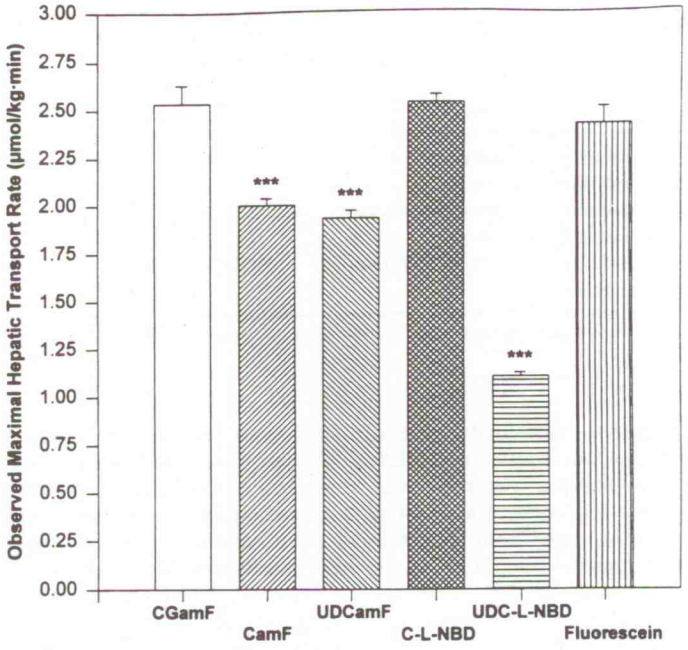

FIG. 4. Observed values for T_{max} of fluorescent bile acid derivatives and fluorescein (and its glucuronide). Data of all experiments were pooled. The values shown are means ± SD of the six highest biliary secretion rates. CamF, UDCamF, and UDC-L-NBD showed significantly lower T_{max} values compared to CGamF, C-L-NBD, and fluorescein (***$P < .0005$ by unpaired Student's t test).

was used for fluorescein and its metabolites. HPLC retention times of fluorescent bile acids were also determined in this manner.

Renal excretion of infused fluorescent bile acids and their metabolites was assessed only by TLC because urinary recovery of infused bile acids was negligible. Renal excretion of fluorescein and its glucuronide was not quantified, because this has been well defined by others.[34]

Biliary volume was determined gravimetrically assuming a density for bile of 1.00 g/mL.

Data Analysis

Data are expressed as means ± SD unless stated otherwise. Where appropriate, differences between groups were tested for significance using unpaired Student's t test. A P value < .05 was considered significant. Best-fit coefficients of the biliary dose-recovery curves for each compound were obtained by pooling data from all animals studied. The last three collections of all experiments (both intravenous infusion and intestinal perfusion of tested compound) were averaged, because approximate steady-state biliary secretion was obtained during this time period. Hepatic transport kinetics (T_{max} and J_{50}) were calculated using nonlinear regression (SigmaPlot, Jandel Scientific, San Rafael, CA). A K_m could not be calculated because the unbound concentration of compound accessible to the canalicular transport system was not known. Differences between curves were tested for statistical significance using an analysis of covariance procedure.

For intestinal perfusion experiments, the absorption rate was also expressed in relation to the length of perfused intestinal segment (in nmol/cm · min) to allow comparison with published data previously obtained for the intestinal absorption of natural bile acids.[22]

The apparent choleretic activity of each compound [Δ bile flow/Δ biliary recovery of infused compound (and its metabolites)] was determined by linear regression (SigmaPlot, Jandel Scientific, San Rafael, CA). Extrapolation of this line to the ordinate is defined as bile acid–independent bile flow.

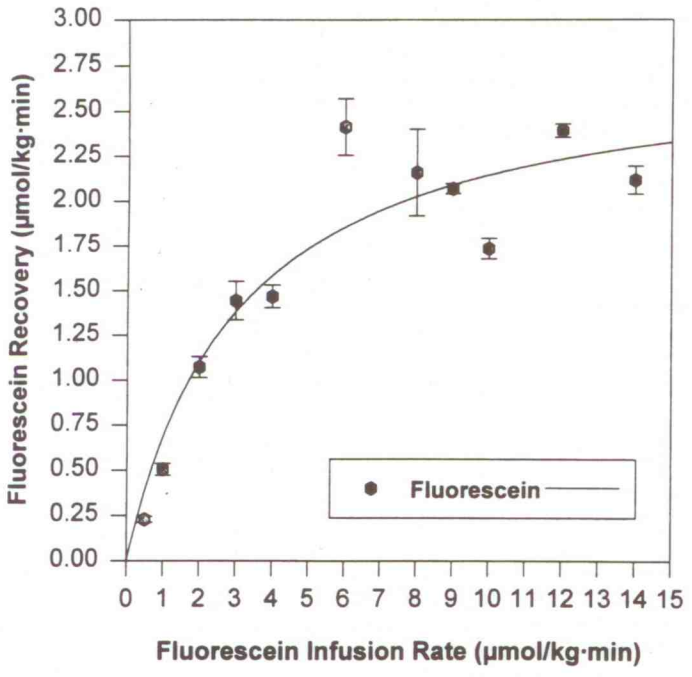

FIG. 3. Relationship between infused dose and biliary recovery of fluorescein (and its glucuronide) in the anesthetized biliary fistula rat. For details, see legend to Fig. 2.

RESULTS

Route of Excretion

Fluorescent bile acids were recovered solely in bile after intravenous administration or infusion into the intestine, as no fluorescence was detected in urine. In contrast, fluorescein and its metabolites were recovered in both bile and urine, based on TLC findings, and in agreement with the detailed studies of Chen et al.[33,34]

Hepatic Transport and Biotransformation

Hepatic transport properties of fluorescent bile acids and fluorescein are given in Table 2. Table 2 also includes pub-

TABLE 3. Biotransformation of Fluorescent Bile Acid Derivatives and Fluorescein Determined by HPLC of Bile: Percent Unchanged Compound and Metabolites Recovered in Bile After Intravenous Infusion and Intestinal Perfusion

Route of Infusion Chemical Form	Intravenous*		Jejunal		Ileal	
	Unchanged	Metabolites	Unchanged	Metabolites	Unchanged	Metabolites
CGamF	98.8%	1.2%	#	#	#	#
CamF	99.3%	0.7%	#	#	#	#
UDCamF	96.8%	3.2%	#	#	#	#
C-L-NBD	99.7%	0.3%	<0.1%	>99.9%	64.8%	35.2%
UDC-L-NBD	96.1%	3.9%	<0.1%	>99.9%	66.3%	33.7%
Fluorescein	45.7%	54.3%†	39.2%	60.8%	62.3%	37.7%

* For the intravenous infusion data, bile samples from the experiments with 2 μmol/kg · min infusion dose were used.

† Out of the total fluorescein metabolite fraction, 53.7% was identified as fluorescein glucuronide. A second, unknown metabolite was present in a proportion of <0.6%.

Because absorption was < 0.01% of infused bad, biotransformation could not be assessed.

lished values for the T_{max} of C-taurine and UDC-taurine. The relationship between infused dose and biliary recovery is shown in Figures 2 and 3. The observed T_{max} values are shown in Figure 4.

The observed transport maximum for all fluorescent bile acids was found to be <2.6 μmol/ kg · min, a value less than one-fifth that of C-taurine (T_{max} = 14.6 μmol/kg · min) and less than one-twelfth that of UDC-taurine (T_{max} = 32.4 μmol/kg · min). CGamF was transported significantly better than CamF (2.53 ± 0.09 vs. 2.00 ± 0.04; $P < .0005$), suggesting that the presence of a second amide bond enhanced biliary excretion. The two fluorescent bile acids linked to C (CGamF and C-L-NBD) were transported at the same rate (2.53 ± 0.09 and 2.53 ± 0.04). Fluorescent bile acids linked to UDC (UDCamF and UDC-L-NBD) were transported more slowly (1.93 ± 0.04 and 1.10 ± 0.02, respectively; $P < .0005$).

The T_{max} values were confounded by toxicity for UDC-L-NBD, which at an infusion rate of ≥ 5 μmol/kg · min caused a sharp drop in bile flow and biliary recovery, with pulmonary distress and death. For other fluorescent bile acids, bile flow decreased at an infusion rate of 8 μmol/kg · min (6 μmol/kg · min for CamF). In contrast, bile flow did not decrease when fluorescein was infused, even at a dose of 14 μmol/kg · min.

None of the amF- or NBD-tagged bile acids underwent appreciable biotransformation during hepatocyte transport based on TLC and HPLC findings (Table 3, Column 1). By HPLC, at least 96% of each compound was recovered as the unchanged compound in bile. By two-dimensional TLC, there were 1 to 2 small, weakly fluorescent metabolites with low R_f values that were formed from all fluorescent bile acids. These spots were resistant to β-glucuronidase and solvolysis, indicating that they were not glucuronides or sulfates; a glutathione conjugate remains a possibility.

In contrast, fluorescein underwent considerable glucuronidation. About half of the fluorescein was recovered in bile as fluorescein glucuronide; the remainder was largely unchanged compound.

Choleretic Properties

The relationship between bile flow and biliary recovery of the infused compounds is summarized in Table 4. Experimental results are shown in Figure 5. Of all fluorescent bile acids, only CGamF had a normal choleretic activity (ACA = +7.3 (μL/μmol). Other fluorescent bile acids were either hypocholeretic (C-L-NBD) or inhibited bile formation as indicated by a negative value for the apparent choleretic activity (CamF, UDCamF, and UDC-L-NBD).

Biliary secretion of fluorescein and its glucuronide induced a greater choleresis than that induced by any of the fluorescent bile acids. The ACA for fluorescein was +16.3 μL/μmol (see "Discussion").

TABLE 4. Apparent Choleretic Activity of Intravenously Infused Fluorescent Bile Acid Derivatives and Fluorescein in the Anesthetized Biliary Fistula Rat

Compound	n	ACA* [μL/μmol]	Intercept (BAIF)† [μL/kg · min]	r
CGamF	15	+7.3	79	0.74
CamF	18	−1.9	70	0.34
UDCamF	15	−4.6	90	0.62
C-L-NBD	17	+2.2	111	0.37
UDC-L-NBD	21	−39.5	90	0.97
Fluorescein	16	+16.3	72	0.98

Abbreviations: n, number of collection periods; BAIF, bile acid independent flow; r, correlation coefficient; ACA, apparent choleretic activity.

* Δ bile flow/Δ recovery of infused compound (and its metabolites). For C-taurine, the apparent choleretic activity has been reported to be +7.5 μL/μmol[20]; for UDC-taurine, a well transported bile acid in the rat, the apparent choleretic activity has been reported to be +7.1 μL/μmol.[27]

† Bile flow at zero bile acid output (by extrapolation) is defined as the bile acid–independent bile flow.

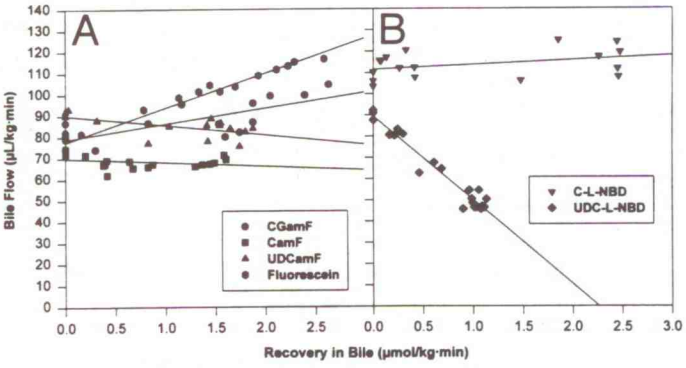

FIG. 5. Relationship between bile flow and biliary recovery of fluorescent bile acid derivatives and fluorescein in the anesthetized biliary fistula rat. (A) Data for amF-tagged bile acids and fluorescein. (B) Data for NBD-tagged bile acids. The slope of the regression lines is defined as apparent choleretic activity (ACA) of the infused compounds (and its metabolites); values are given in Table 4.

TABLE 5. Intestinal Absorption Rates and Recovery of Fluorescent Bile Acid Derivatives (and Metabolites), Natural Bile Acids, and Fluorescein by the Isolated Jejunum or Ileum During Single Pass Perfusion With 1 mmol/L Solution at High Flow Rate*

	n	Jejunal Absorption [nmol/cm · min]	Ileal Absorption [nmol/cm · min]	Recovery (% Dose) Jejunum	Recovery (% Dose) Ileum
Fluorescent bile acids					
CGamF	2	0.01 ± 0.007	0.01 ± 0.002	<0.01%	<0.01%
CamF	2	0.01 ± 0.005	0.01 ± 0.003	<0.01%	<0.01%
UDCamF	2	0.01 ± 0.006	0.01 ± 0.007	<0.01%	<0.01%
C-L-NBD	4	0.07 ± 0.006†	2.12 ± 0.099	0.07%	1.67%
UDC-L-NBD	4	0.07 ± 0.012†	1.05 ± 0.027	0.08%	0.68%
Natural bile acids					
C-taurine‡		0.11 ± 0.013	7.5 ± 0.4	0.09%	5.88%
UDC-taurine‡		0.13 ± 0.20	4.3 ± 0.3	0.10%	3.37%
Fluorescein	6	1.32 ± 0.125§	1.32 ± 0.225§	0.82%	1.00%

Abbreviation: n, number of animals.

* Intestinal absorption was equated with biliary recovery of the perfused compound (and metabolites) since biliary recovery was far below hepatic transport maxima.

† During jejunal perfusion with NBD-tagged fluorescent bile acids a delay in biliary recovery was observed. The values given are the absorption rate during the last 30 min of the perfusion.

‡ Intestinal absorption values for natural bile acids were obtained from the literature (23, 25). Recovery was then calculated applying the same model conditions as for the fluorescent bile acids.

§ During jejunal and ileal perfusion with fluorescein, steady-state conditions of biliary recovery were not obtained within perfusion time. The values given are the absorption rate during the last 30 minutes of the 60-min perfusion.

Intestinal Absorption and Biotransformation

Intestinal absorption rates for an intraluminal concentration of 1 mmol/L as well as total biliary recovery are given in Table 5. Figures 6 and 7 show the time course of absorption of fluorescent bile acids. Table 3 (columns 2 and 3) summarizes the biotransformation of fluorescent bile acids in the intestinal perfusion studies.

Bile acids tagged with a fluorescein moiety underwent little intestinal absorption in the jejunum or ileum. The NBD-tagged bile acids, in contrast, were transported by both the jejunum and the ileum. The rate of ileal absorption was 15 times greater than jejunal absorption for the UDC derivative and 30 times greater for the C derivative. Nonetheless, in both the jejunum and the ileum, the NBD-tagged bile acids were transported more slowly than the corresponding natural taurine conjugates.

During jejunal perfusion with both NBD derivatives, biliary recovery (and therefore intestinal absorption) continued for the 2 hours following the infusion of the test compound (Fig. 6). In contrast to intravenous infusion, when fluorescent bile acids were recovered largely unchanged in bile,

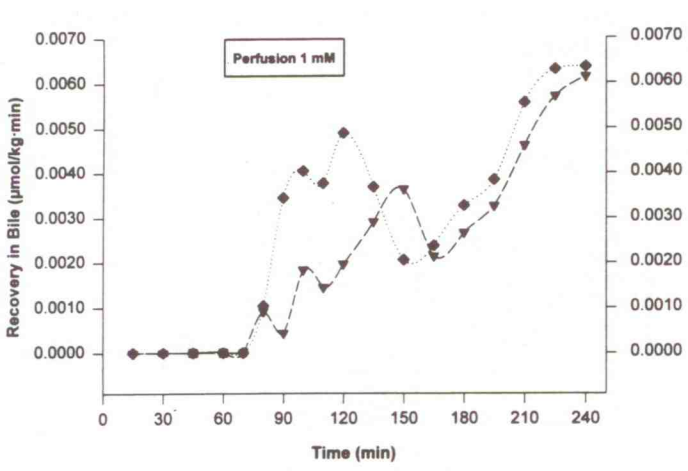

FIG. 6. Time-course of biliary recovery of fluorescent bile acids after jejunal perfusion in the anesthetized biliary fistula rat. Data for amF-tagged bile acids are omitted because absorption from the jejunum was negligible (<0.001 µmol/kg · min). NBD-tagged bile acids were recovered entirely as a metabolite when infused into the jejunum (see text and Table 3). In contrast to Table 5, recovery in bile is given in µmol/kg · min to allow comparison with hepatic transport maxima. (▼) C-L-NBD; and (◆) UDC-L-NBD.

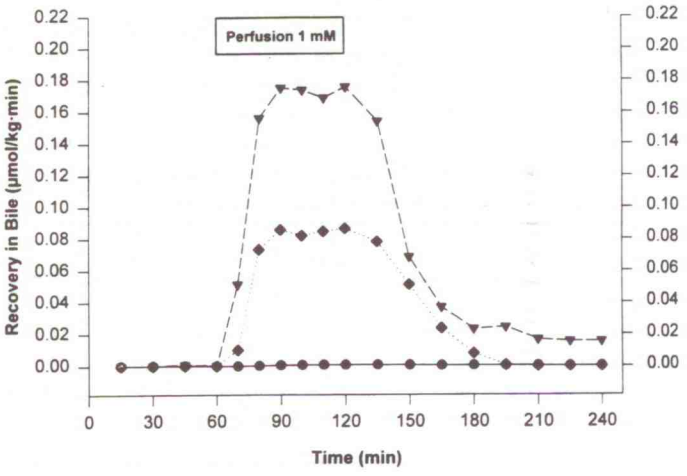

FIG. 7. Time-course of biliary recovery of fluorescent bile acids after ileal perfusion in the anesthetized biliary fistula rat. In contrast to the jejunum, NBD-tagged bile acids were recovered as such (60%-70%) and as the same metabolite observed during jejunal perfusion (30%-40%). As in the jejunal perfusion experiments, amF-tagged bile acids were not absorbed from the ileum as shown by the datapoints for CGamF. In contrast to Table 5, recovery in bile is given in µmol/kg · min to allow comparison with hepatic transport maxima. (●) CGamF; (▼) C-L-NBD; and (◆) UDC-L-NBD.

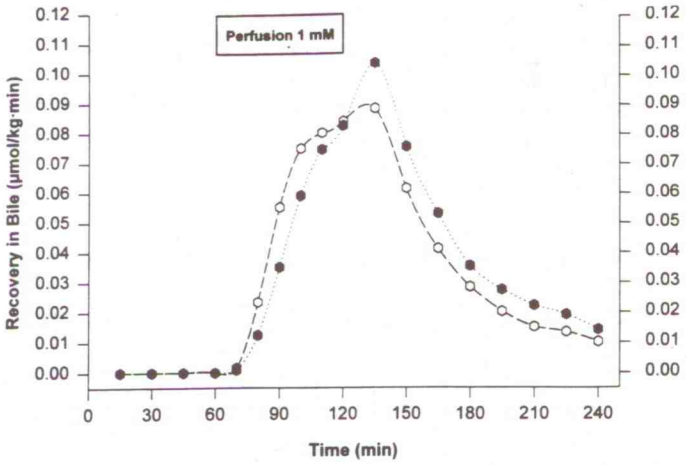

FIG. 8. Time-course of biliary recovery of fluorescein and its glucuronide after jejunal and ileal perfusion in the anesthetized biliary fistula rat. In contrast to Table 5, recovery in bile is given in µmol/kg·min to allow comparison with hepatic transport maxima. (○) Jejunum; and (●) ileum.

extensive biotransformation was observed when bile acids were absorbed from the jejunum, since by HPLC, no unchanged compound was present in bile (see Table 3). A single peak containing a hydrophilic fluorescent metabolite with a relative retention time by HPLC to C-taurine of 0.57 was detected. The compound had a lower molecular weight by mass spectrometry than the infused compound, suggesting that some or all of the bile acid moiety had been lost. The compound was unchanged after incubation with β-glucuronidase, indicating that it was not a glucuronide.

In the ileum, delayed biliary recovery of NBD-tagged bile acids was not observed (Fig. 7). Nonetheless, about one-third of the NBD derivatives was converted to a more polar metabolite which, by HPLC, was identical to the metabolite during the jejunal perfusion. In contrast to the jejunum, the polar metabolite was secreted rapidly.

Figure 8 shows the time course of fluorescein absorption. Jejunal uptake of fluorescein was much greater than that of any fluorescent bile acid, being in the range of 1.32 ± 0.12 nmol/cm·min. In contrast to NBD-tagged fluorescent bile acids, which were absorbed preferentially by the ileum, fluorescein was absorbed at the same rate from both the jejunum and the ileum.

The pattern of biotransformation in bile was identical after intravenous or enteral administration of fluorescein. However, the rate of glucuronidation was greater after jejunal perfusion than ileal perfusion. The pattern of biotransformation in urine was identical to that in bile.

Analysis of bile by TLC demonstrated the presence of a trace proportion (<1%) of a second metabolite in addition to the fluorescein-glucuronide. The second metabolite was resistant to both β-glucuronidase and sulfatase treatment. It was also different from fluorescin (a reduced congener of fluorescein) and fluorescin monoglucuronide, which have been identified as metabolites of fluorescein in the urine of the rabbit.[33] The unknown metabolite was again transformed into fluorescein when incubated at alkaline pH (NaOH 0.1 N, 120 minutes at 40°C). One possible metabolite is again the glutathione conjugate of fluorescein. Since recovery of the metabolite in bile was only 0.6% by HPLC, no further attempts to identify this metabolite were undertaken.

DISCUSSION

These results provide new information on the transport and choleretic properties of a group of fluorescent bile acids in which the fluorophore is present on the side chain. They indicate that such compounds are transported by the hepatocyte at a rate far slower than that of C-taurine, irrespective of the nature of the steroid moiety. In agreement with previous studies,[4,6,18] the compounds were shown to undergo little biotransformation during hepatocyte transport. Failure of the compounds to be absorbed by the jejunum indicates that the compounds have little passive membrane permeability, in contrast to fluorescein. The fluorescent bile acids were recovered solely in bile, again in contrast with fluorescein, which was recovered as such together with its glucuronide in both bile and urine. To the best of our knowledge, this is the first detailed study on the handling of these fluorescent bile acids in the intact animal. Mills et al.[6] reported that cholyllysylfluorescein is efficiently extracted in the isolated perfused liver and efficiently transported into bile. More recently, this group has reported in abstract form that the plasma disappearance of intravenously injected cholyllysylfluorescein in humans is quite similar to that of natural conjugated bile acids.[38]

Structure-Transport Relationships

The number of compounds studied was too few to permit any extensive generalizations. Among the amF-tagged compounds, the cholylglycyl derivative (CGamF) was transported best, that is, its transport rate exceeded that of CamF and UDCamF, whose transport rates did not differ. For the two L-NBD derivatives, the UDC derivative was transported far more slowly than the cholyl derivative, a finding that was astonishing, considering the excellent transport of UDC-taurine in the rat.[19] The type of fluorescent tag had no consistent effect on transport. C-L-NBD was transported more rapidly than CamF; in contrast, UDCamF was transported better than UDC-L-NBD.

The high transport rate of cholyllysylfluorescein reported by Milkiewicz et al.[38] was astonishing to us. Their finding indicates that both the basolateral and canalicular carriers efficiently transport a bile acid derivative containing a (bulky and charged) fluorescein moiety, even when this is attached to an *n*-butyl tether, rather than directly to the carboxyl group of glycine. In addition to the obvious and significant differences in molecular shapes, it is important to note that our aminofluorescein conjugates have two ionizable acidic groups, whereas cholyllysylfluorescein has three. In aminofluorescein conjugates the acidic functions are the carboxyl group and the phenol group of fluorescein. We have determined the two pK_a values for CGamF to be 5.3 and 6.5 (the two ionizing groups of fluorescein have been estimated to have pK_a values of 4.2 and 6.3[39]). Cholyllysylfluorescein has the same two acidic groups on the fluorescein moiety (probably with similar pK_a values plus the terminal carboxyl of the lysine moiety. This is likely to have a pK_a value close to those of glycine conjugated bile acids, namely, pK_a of 3.9. Whether the presence of the more acidic carboxyl group of cholyllysylfluorescein is the major factor responsible for its rapid transit rate through the hepatocyte is not known.

Identification of the carriers involved in the hepatocyte or enterocyte transport of these molecules was not an aim of this study. Uptake by the hepatocyte of CGamF and CDC-L-

NBD has been shown to involve basolateral Na^+/taurocholate cotransporting polypeptide (of the hepatocyte), based on the study of Boyer et al.[11] in which the basolateral Na^+/taurocholate cotransporting polypeptide was transfected into COS-7 cells. No information is available on the transport of these fluorescent bile acids by the basolateral Na^+-independent organic anion transporting polypeptide (of the hepatocyte), the sodium-independent basolateral transporter (A. Wolkoff, personal communication, January 1997). Canalicular secretion of all fluorescent bile acids is likely to involve the putative canalicular carrier(s) for bile acids, since L-NBD–tagged bile acids (R. Oude Elferink, personal communication, October 1992) as well as amF-tagged bile acids[3] are well transported in the multispecific organic anion transporter–deficient (TR^-) rat.

The modest but unequivocal uptake of L-NBD derivatives from the perfused jejunum is consistent with a previous study in the guinea pig[40] that provided evidence for a jejunal bile acid transporter differing in its transport properties from those of the cloned ileal bile acid transporter.[41] The lack of any measurable absorption of the amF-tagged bile acids indicates that these molecules are not substrates for this carrier(s) or the ileal bile acid tranporter and are not absorbed passively, either paracellularly or transcellularly, at least at a luminal pH of 8.0. The rapid absorption of fluorescein by the jejunum is presumed to result from transcellular transport that begins with passive membrane permeation by nonionic diffusion (of the protonated species). Such passive permeation is in turn explicable by the hydrophobic nature of the multi-ring structure of fluorescein and its being a relatively weak acid, which should undergo protonation at the acidic microclimate present at the luminal face of the enterocyte apical membrane.[42] Based on current concepts,[43] the fluorescein molecule is likely to be too large to be absorbed by the paracellular route in the rat jejunum. The hepatocyte has also been shown to take up fluorescein in a passive manner.[44]

Rapid ileal transport of the lysyl-NBD derivatives is consistent with their being satisfactory substrates for the ileal bile acid tranporter as well as the basolateral transporter of the ileal enterocyte. The ileal bile acid tranporter is known to transport cholyl conjugates more rapidly than dihydroxy conjugates.[22] In agreement, C-L-NBD was better absorbed by the ileum than UDC-L-NBD, a dihydroxy bile acid. In the jejunum, these two fluorescent bile acids were transported at similar rates, in agreement with the transport rates for natural conjugated bile acids reported by Amelsberg et al.[40]

Structure-Biotransformation Relationships

None of the fluorescent bile acids was appreciably biotransformed during hepatocyte transport. In contrast, during jejunal transport, the NBD derivatives were biotransformed extensively, and none of the infused compound was recovered unchanged. Recovery of the metabolite was greatly delayed. The most reasonable explanation for these findings is that the infused compound could not be transported out of the jejunal enterocyte and was retained in the jejunal enterocyte until it had been biotransformed to a metabolite that was a substrate for basolateral anion transporters. Impaired hepatocyte transport of the metabolite cannot be the explanation for the prolonged biliary excretion after jejunal infusion, as the metabolite was rapidly recovered in bile during ileal perfusion. C-L-NBD has been shown to undergo carrier-mediated transport across the basolateral membrane of the cholangiocyte.[16] Therefore, the basolateral conjugated bile acid transporter of the ileal enterocyte and cholangiocyte appears to be capable of transporting L-NBD derivatives of bile acids, whereas the basolateral membrane transporter of the jejunal enterocyte is not.

Choleretic Properties

Among the fluorescent bile acids, only CGamF had a choleretic activity similar to that of C-taurine and UDC-taurine. The remaining bile acids either had little choleretic activity (C-L-NBD) or actually caused decreased bile flow. The simplest explanation for the diminution in bile flow is that fluorescent bile acids other than CGamF inhibit transport by a multispecific organic anion transporter or other canalicular carriers whose substrates generate bile acid–independent flow. The toxicity of UDC-L-NBD was not anticipated, as UDC[45] and its taurine conjugate[19] are quite nontoxic when infused intravenously. In unpublished experiments using the isolated perfused rat liver, we have found that UDC-L-NBD, although well taken up by the liver, is poorly excreted into bile, explaining its cholestatic effect. We interpret the pulmonary toxicity of UDC-L-NBD to result from a decrease in the hepatic uptake occuring after hepatocyte accumulation. As a result, a much greater load of UDC-L-NBD will be delivered into the pulmonary circulation. Toxicity of high doses of UDC-taurine have also been noted in the bile duct–ligated animal.[46]

The greater choleretic activity of the mixture of fluorescein and its glucuronide was anticipated, since both compounds are likely to be divalent anions at canalicular pH. Divalent anions are accompanied by two cations, so that if ACA is expressed in relation to recovery of only the anion, a greater ACA value will be observed whenever a divalent anion is excreted into bile.[47]

Conclusions

We conclude that in this spectrum of bile acids tagged with a fluorophore on the side chain, both the bile acid structure and the structure of the fluorophore influence hepatocyte and enterocyte transport, enterocyte biotransformation, and choleretic properties. In our studies, the compound with the most satisfactory physiological properties (lack of toxicity and normal choleretic activity) was the amF conjugate of cholylglycine, CGamF. This compound and cholyllysylfluorescein[6,38] appear to have transport properties not too dissimilar to those of natural conjugated bile acids. Such compounds may prove to be useful for characterizing hepatocyte transport function in health and disease.

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