Journal of Chromatography, 562 (1991) 697–712
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5571

Pre-column derivatization of free bile acids for high-performance liquid chromatographic and gas chromatographic–mass spectrometric analysis

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ABSTRACT

Pentachlorophenyl (PCP) esters of five free bile acids (FBA) were obtained by reacting the FBA and Kovacs' complex (KC) in a 1:8 molar ratio in acetone at 65°C, and were purified by column chromatography on silica gel. The esters were crystallized from benzene–hexane, derivatized as trimethylsilyl ethers for gas chromatography on a DB-1 capillary column and for gas chromatography mass spectrometry with a DB-5 column, and mass spectrometry (MS) in the electron-impact (EI) positive-ion mode at 70 eV. The reaction is specific for FBA even in the presence of glycine and taurine conjugates of bile acids. The PCP esters were treated with benzylamine in chloroform or methanol to produce N-benzyl derivatives of FBA. The N-benzylamides were separated by high-performance liquid chromatography (HPLC) on a 4-μm Nova-Pak C_{18} column, studied by thermospray–LC–MS, and in the direct insertion probe–EI positive-ion mode.

INTRODUCTION

The clinical importance of the determination of bile acids in biological fluids is well documented [1–5]. However, bile acids have no UV chromophores detectable at 254 or 366 nm [useful filters in cheaper UV detectors for high-performance liquid chromatography (HPLC)], so they have been detected and determined largely using differential refractometers [6,7]. The poor sensitivity of these devices, especially with the very low concentrations of bile acids in some biological fluids, has prompted a search for alternative methods. A number of procedures [6,7] have utilized a UV detector set at 193–215 nm, but the limit of detection with this system is in the 10–30 pmol range. Pre- and post-column derivatization procedures using HPLC [8–23] and pre-column derivatization procedures for gas chromatography (GC) [24–27] have been proposed.

0378-4347/91/$03.50

During the course of studies designed to extend current methods of detection, identification and determination of bile acids to the femtomole level, pentachlorophenyl (PCP) esters of five free bile acids (FBA) were prepared and found to be useful intermediates in the preparation of amides, some of which are useful for analysis. This paper describes the preparation of N-benzylamides of FBA (but not those conjugated with glycine or taurine) and their identification and determination by HPLC, electron impact (EI) mass spectrometry (MS) in the positive-ion mode and thermospray–MS. A preliminary account of these studies has appeared [28].

EXPERIMENTAL

Chemicals

The five FBA were obtained from Sigma (St. Louis, MO, U.S.A.) and had purities of 98% or higher.

Kovacs' complex (KC) was prepared from pure pentachlorophenol (Dow Chemical, Midland, MI, U.S.A., of >99% purity, obtained from Fluka, Buchs, Switzerland) and N,N'-dicyclohexylcarbodiimide (99% purity, Janssen Chimica, Beerse, Belgium) according to Kovacs [29]. Owing to its toxicity, the PCP used in this work was carefully recycled. All crystalline products were dried under vacuum for at least 12 h at 25°C. Melting points were then determined in duplicate with a Fisher–Johns apparatus and were uncorrected [30]. Infrared spectra were acquired on a Perkin-Elmer Model 21 double-bean IR spectrometer using potassium bromide pellets.

Inorganic acids and bases were of analytical-reagent grade and organic solvents were of HPLC grade. Freshly distilled pyridine was passed through microcolumns of Sep-Pak silica until it was free from primary amines. Ninhydrin (0.1% in acetone, Sigma) was used to detect primary amines.

Column and thin-layer chromatography

Preparative column chromatography was performed in a glass tube (20 cm × 2.5 cm I.D.) packed with silica gel (100–200 mesh). The columns were washed with the most polar solvent to be used to avoid interferences from contaminants.

Analytical thin-layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (0.25 mm) (Merck, Darmstadt, F.R.G.) sensitive to UV light at 254 nm. Elution of the plates was carried out with chloroform–methanol (97.5:2.5, v/v) for the separation of the PCP esters. The corresponding N-benzylamides were eluted with chloroform–methanol (95:5, v/v). Both types of compounds were first detected under UV light and then developed with molybdophosphoric acid spray [15% (w/v) in 95% ethanol] by gently warming to 70 °C.

HPLC instrumentation

HPLC (analytical and semi-preparative) was performed with a Waters Assoc.

(Milford, MA, U.S.A.) system including a Model 6000A pump, a U6K 2-ml loop injector, a Lambda-Max M480 variable-wavelength UV detector and a Data Module M730 recorder–integrator. Semi-preparative HPLC was conducted with a μBondapak C_{18} stainless steel column (30 cm × 7.8 mm I.D.) (Waters Assoc.) at a flow-rate of 2 ml/min. A stainless-steel guard column (2.4 cm × 4 mm I.D.) containing the same packing was also used. Analytical HPLC was performed on a Nova-Pak C_{18} column (10 cm × 8 mm I.D., 4-μm particle size) (Waters Assoc.) contained in an RCM-100 radial compression module, at a flow-rate of 1 ml/min. Aliquots of reaction mixtures injected into the HPLC system were removed from vials (3-ml internal volume) fitted with Mininert valves (Supelco, Bellefonte, U.S.A.).

Gas chromatography

GC was conducted on a Hewlett-Packard Model 5890A gas chromatograph equipped with an on-column injector and a DB-1 capillary column (100% methylsilicone, 30 m × 0.25 mm I.D., 0.25-μm film thickness), both from J&W Scientific (Folsom, CA, U.S.A.), a flame ionization detector and an HP3390A recorder. The column was heated at 0.7°C/min from 200 to 300°C and the detector was kept at 310°C. The gas chromatograph used for GC–MS was equipped as above except for a splitless injector and a DB-5 capillary column (95% methylsilane + 5% phenylsilane, 30 m × 0.32 mm I.D., 0.5-μm film thickness, J&W Scientific) maintained at 265°C for a 45-min run. High-purity helium was used as the carrier gas at a pressure of 0.35 bar.

Mass spectrometry

GC–MS in the positive-ion mode with EI and chemical ionization (CI) (with isobutane) was performed on a VG TRIO-2 instrument featuring a quadrupole mass analyser, an optical signal amplifier and a DEC microPDP 11/73 computer equipped with a multi-task disk operating system (RX 11M Plus version 3.0) and software needed for spectral data manipulation. Direct insertion probe (DIP)–EI positive-ion experiments were carried out on an LKB 9000 instrument described previously [31]. Thermospray–LC–MS positive- and negative-ion experiments were carried out with a Shimadzu HPLC system connected to a Vestec thermospray–LC–MS system [32]. The experimental conditions were comparable to those described by Setchell and Vestal [33]. The following temperatures were used in the thermospray unit: at the entrance to the vaporizer, 124°C; at the vaporizer, 230°C; at the source block, 301°C; at the tip heater, 313°C; and in the vapour, 300°C. The filament was kept on in both positive- and negative-ion runs.

UV spectral studies

UV spectra were obtained with a Beckman DU-70 spectrophotometer connected to an Epson FX-80 dot-matrix printer. Solutions of each of the five FBA PCP esters in ethanol (1 mg in 25 ml) were introduced into 0.5-ml quartz cuvettes

(0.5-ml internal volume, 1-cm optical path length); spectra were recorded from 200 to 340 nm.

Syntheses of PCP esters

Initial syntheses were undertaken with 100 mg of FBA to provide the desired esters and amides in high purity, not high yields. FBA and KC in a 1:8 molar ratio and 50 ml of acetone were refluxed in a suitable flask for 30 min. When FBA could no longer be detected by TLC, the reaction was halted by addition of 1 ml of concentrated hydrochloric acid and the solvents were removed using a rotavapor (Buchi). The residue was taken up in chloroform (100 ml) and the solution was washed four times with 100 ml of 0.1 *M* hydrochloric acid and 0.1 *M* sodium hydroxide solution. The chloroform phase was dried, chromatographed on a silica gel column and eluted with chloroform–acetone in various ratios (Table I). Those fractions containing only PCP esters (as assayed by TLC) were crystallized from benzene–hexane. Fractions containing small amounts of contaminants were further purified by semi-preparative HPLC (Table I).

Study of yields in the formation of PCP esters of FBA

Studies to establish the optimum conditions for the maximum yield of cholic acid PCP (CPCP) ester were carried out with 1 mg of cholic acid and KC in a 1:8 molar ratio contained in a Mininert vial with the following solvents and temperatures: methanol–chloroform (1:1, v/v) at room temperature (RT); acetone at RT; acetone at 40°C and 65°C; and acetonitrile at 65°C. The electronic peak height of CPCP, as reported by the M730 Data Module integrator, was measured by HPLC for each of four samples containing CPCP at levels equivalent to 133, 100, 66 and 33% yield and the peak heights were plotted against those yields. Reactions in the solvents and temperatures given above were carried out with removal of 5-μl aliquots of medium after 5 min and every 15 min thereafter to a total of 155 min. From a plot of these data, the optimum yield of CPCP was obtained. A double reciprocal plot prepared with a spreadsheet computer program (Quattro, Borland) was used to adjust the data [34]. After 155 min, analysis by TLC of the reaction mixture provided an insight into the nature of by-products of the reaction. Analytical HPLC of the PCP esters was carried out using a mobile phase containing 2-propanol–acetonitrile–water (65:10:25, v/v/v) at a flow-rate of 1 ml/min. Detection was accomplished at 230 nm (0.050 a.u.f.s.).

Formation of TMS ethers of PCP esters of FBA

Samples of 100 μg of each of the five PCP esters were derivatized for GC and GC–MS analysis as trimethylsilyl (TMS) ethers by adding 100 μl of a freshly prepared solution containing hexamethyldisilazane, recently purified pyridine and trimethylchlorosilane (42:32:26, v/v/v). The internal standard, 7α, 12α-dihydroxy-5β-cholanic acid (Calbiochem, San Diego, CA, U.S.A.), was added to facilitate reporting of relative retention times (RRT). The PCP ester, the internal standard and the reagent were then heated to 65°C for 60 min, prior to analysis.

TABLE I

PROPERTIES OF PENTACHLOROPHENYL (PCP) ESTERS OF FIVE FREE BILE ACIDS

Parameter	Cholic (C)	Ursodeoxycholic (UDC)	Chenodeoxycholic (CDC)	Deoxycholic (DC)	Lithocholic (LC)
Silica gel column[a], solvent for purification					
Acetone (%)	10	5	5	5	2.5
Chloroform (%)	90	95	95	95	97.5
C_{18} semi-prep HPLC[b], solvent for purification					
Isopropanol (%)	52.6	58.5	58.5	58.5	65
Acetonitrile (%)	8.1	9.0	9.0	9.0	10
Water (%)	39.3	32.5	32.5	32.5	25
Yield of product (%)[c]	54	53	55	45	62
Melting point (°C)[d]	129–130	109–110	111–113	148–150	100–102
Relative retention time in GC[e]	1.113	1.136	1.081	1.048	0.955
R_F in TLC[f]	0.07	0.33	0.28	0.31	0.78
Mobility in HPLC[g]					
k'	6.71	6.00	12.00	12.43	26.71
rk'	0.54	0.48	0.97	1.00	2.15
UV maxima[h]					
Wavelength (nm)	212.5	213.0	213.0	213.5	213.5
ε_{max}	$4.99 \cdot 10^4$	$5.04 \cdot 10^4$	$4.92 \cdot 10^4$	$4.98 \cdot 10^4$	$4.92 \cdot 10^4$

[a] Silica gel column (100–200 mesh), 30 cm × 2.5 cm I.D.; initially packed with $CHCl_3$.

[b] μBondapak C_{18} stainless-steel column, 10.μm particles, 30 cm × 7.8 mm I.D., flow-rate 2 ml/min; UV detection at 230 nm (2.0 a.u.f.s.). Mobile phase adjusted for peak collection at 40 min.

[c] Crystallized from benzene–*n*-hexane and/or acetonitrile. Emphasis on high purity, not high yields.

[d] Melting points obtained with a Fisher–Johns instrument are not corrected.

[e] Operational details are given with the chromatograms. All PCP esters were converted to PCP ester–TMS ethers for GC studies. Relative retention time(s) are related to the internal standard (7α,12α-dihydroxy-5β-cholanic acid) as the TMS ester–TMS ether.

[f] TLC plates [20 cm prepacked aluminium-backed silica plates (Merck)] were developed with methanol–chloroform (2.5:97.5, v/v). The dried plates were sprayed with molybdophosphoric acid, 15% in 95% ethanol, and warmed to 70°C.

[g] Details of operation are given with the chromatograms.

[h] Solvent, 95% ethanol.

Syntheses of N-benzylamides of FBA

The N-benzylamides of the five FBA were initially obtained by adding FBA (100 mg), KC and benzylamine to methanol in molar ratios of 1:10:90 and incubating at 65°C for 15 min. The reaction mixture was treated as described for FBA PCP esters and purified by TLC with chloroform–methanol (95:5, v/v) as eluent. Silica containing the compound of interest was extracted with acetonitrile, concentrated at 65°C and crystallized at RT in *ca.* 20% yield.

A mixture of the five N-benzylamides of FBA was separated by analytical HPLC on a Nova-Pak C_{18} column with 10 m*M* ammonium acetate–2-propanol–acetonitrile (50:33:17, v/v/v) as eluent and was detected by UV measurement at 215 nm (0.050 a.u.f.s.). Mass spectra were acquired using DIP–EI at 70 eV.

Study of yields in the formation of N-benzylamides of FBA from their PCP esters

The formation of the N-benzylamide of cholic acid from CPCP was followed by analytical HPLC to obtain optimized reaction conditions. Equimolar amounts of CPCP and the N-benzylamide of ursodeoxycholic acid (UDCNB) were each dissolved in methanol (0.24 μmol), added to a known amount of benzylamine and the volume made up to 2 ml with methanol in a Mininert vial that was incubated at a known constant temperature. Aliquots of 10 μl were taken from the microreactor after 5 min, and every 20 min thereafter until 65 min, and injected directly into the HPLC system already described for N-benzylamides of FBA. Two series of experiments were carried out. In the first series, the temperature was kept at 65°C and six experiments were done with increasing molar ratios of CPCP to benzylamine (1:19, 1:38, 1:57 and 1:76). In the second series, the molar ratio of CPCP to benzylamine was maintained at 1:76 and the temperature was increased from RT to 100°C (RT, 50, 65 and 100°C). As the electronic peak heights of equimolecular amounts of UDCNB and CNB were shown to be identical, the ratios of the peak heights of CNB and UDCNB were recorded as percentage yields. The data obtained were adjusted by the method already described for the formation of CPCP.

RESULTS AND DISCUSSION

Fig. 1 shows the structural features of FBA, the reactions to prepare the five PCP esters and the N-benzylamides obtained from them. The experimental conditions to produce the highest yields are also given in Fig. 1.

Table I summarizes the data obtained from the analysis of PCP esters of FBA. The CPCP calibration graph obtained by HPLC had a correlation coefficient of 0.9922, with a slope of 0.9386 and an intercept of 1.036. Fig. 2 shows the results obtained by measuring the yields of CPCP under various sets of experimental conditions. The correlation coefficients obtained for the double reciprocal plots [34] necessary to adjust the data by the Lineweaver–Burk method were over 0.9500. Acetone at 65°C for 30 min provided 100% yields (not shown). Whenever

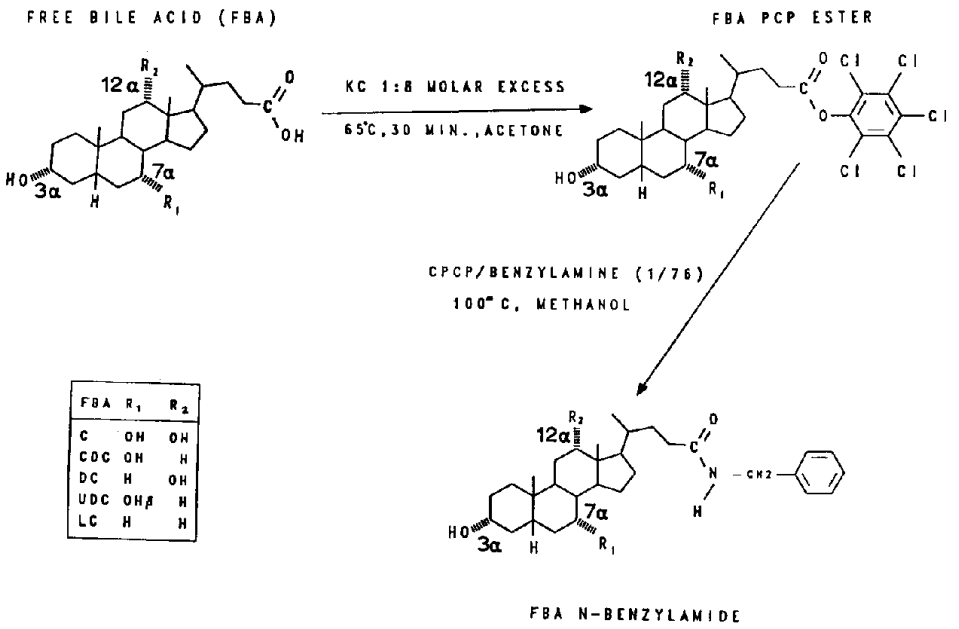

Fig. 1. Reactions involved in the synthesis of PCP esters and N-benzylamides of FBA.

the synthesis of CPCP was conducted in methanol or acetonitrile, by-products of a steroidal nature where found in TLC. With methanol the by-product was identified by GC as methyl cholate by comparison with a standard. No steroidal by-products were observed when the reaction was conducted in acetone. A 1:8

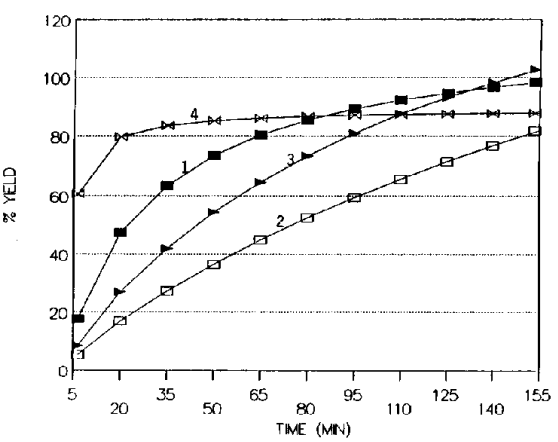

Fig. 2. Formation of CPCP monitored by HPLC. Four sets of experimental conditions were used: 1 = methanol–chloroform (1:1, v/v) at room temperature; 2 = acetone at room temperature; 3 = acetone at 40°C; 4 = acetonitrile at 65°C.

molar excess of KC was found to be optimum; larger amounts of KC did not improve the yields or the rate of the reaction. Complete separation of all five PCP esters of FBA by TLC was accomplished when the amounts of ester applied to the plates did not exceed the equivalent of 5 μg of FBA (Table I).

GC of the five PCP esters of FBA and 7α,12α-dihydroxy-5β-cholanic acid as their TMS derivatives is shown in Fig. 3. Table II shows the results obtained by GC–EI-MS in the positive-ion mode at 70 eV of the same TMS derivatives. The main fragmentation pathway involves the loss of 264 dalton, corresponding to pentachlorophenol, followed by up to three consecutive losses of 90 dalton, one for each silylated hydroxyl group. The results obtained from GC–MS-CI with positive-ion detection of the same five TMS ethers–PCP esters of FBA (Table III) show fragmentation patterns comparable to those of GC-EI-MS in the positive-ion mode. The ion at m/z 291 corresponds to PCP + carbonyl; the ions M − 305 and M − 319 correspond to loss of successive methylene groups of the side-chain (*e.g.*, C-23 and C-22). The loss of 347 units corresponds to the complete loss of the side-chain.

Fig. 4 gives an HPLC trace showing baseline resolution of each of the five N-benzylamides of FBA. The component that controls the resolution of the five compounds was found to be acetonitrile. When the water concentration was maintained and the proportions of 2-propanol and acetonitrile were varied, lower concentrations of acetonitrile provided shorter runs but with lower resolution. Substitution of ammonium acetate buffer for a phosphate buffer of the same molarity or water alone did not produce a significant change in retention times. An average detection limit of 10 ng was found for the five N-benzylamides of FBA. The commercially available KC was found to contain impurities with retention times similar to those observed for several of the N-benzylamides of FBA. The use of reagents of the highest purity is imperative.

Figs. 5 and 6 show the results obtained when the production of the N-benzylamide of cholic acid (CNB) from CPCP was monitored by HPLC. In Fig. 5 the

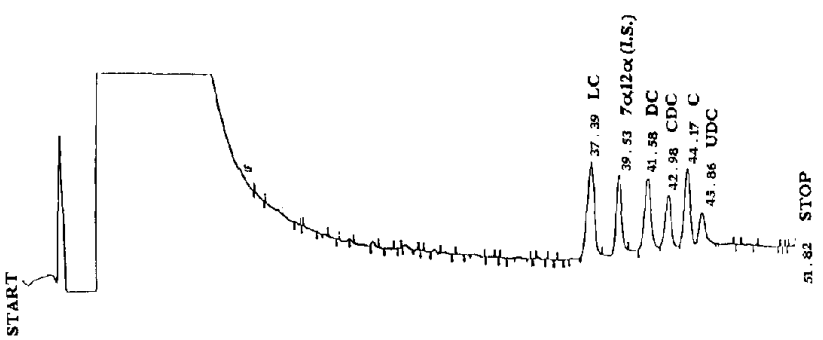

Fig. 3. GC separation of five PCP esters of FBA. Conditions: 30 m × 0.25 mm I.D. DB-1 capillary column; oven temperature raised from 250 to 300°C at 0.7°C/min; helium carrier gas at 0.35 bar; internal standard 7α,12α-TMS ester TMS ether. Numbers at peaks indicate retention times in min.

TABLE II

MASS SPECTRAL DATA: ELECTRON IMPACT [GC MS OF PCP–$(TMS)_x$ DERIVATIVES]

Major fragment ions as mass (m/z) and % abundance.

Cholic (C)		Ursodeoxycholic (UDC)		Chenodeoxycholic (CDC)		Deoxycholic (DC)		Lithocholic (LC)		Structural assignment
m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	
870	–	782	–	782	–	782	–	694	–	M^+ (Not seen)
590	0.6	503	2.0	503	0.4	503	*25.6*	415	3.8	M − (264 + 15)
–	–	439	*24.5*	–	–	–	–	–	–	M 343
–	–	428	2.8	428	0.3	428	2.5	340	0.4	M − (264 + 90)
426	13.6	338	9.1	338	**100.0**	338	4.2	–	–	M − (264 + 2 × 90)
411	2.4	323	6.3	323	35.0	323	3.3	–	–	M − (264 + 3 × 90 + 15)
336	20.4	–	–	–	–	–	–	–	–	M − (264 + 3 × 90)
321	10.1	–	–	–	–	–	–	–	–	M − (264 + 3 × 90 + 15)
										Retain rings:
253	53.3	255	3.6	255	10.5	255	*92.5*	257	9.8	A + B + C − 3(H_2O) (C)
										A + B + C − 2(H_2O) (UDC)
										A + B + C − 2(H_2O) (CDC)
										A + B + C − 2(H_2O) (DC)
										A + B + C − (H_2O) (LC)
243	6.4	243	28.5	243	26.1	243	–	–	–	
211	6.4	213	3.6	213	13.0	213	4.0	215	13.4	
129	15.6	129	40.3	129	27.0	129	8.8	131	**100.0**	

TABLE III

MASS SPECTRAL DATA: CHEMICAL IONIZATION (ISOBUTANE) [GC–MS OF PCP-(TMS)$_x$ DERIVATIVES]

Major fragment ions as mass (m/z) and % abundance.

Cholic (C)		Ursodeoxycholic (UDC)		Chenodeoxycholic (CDC)		Deoxycholic (DC)		Lithocholic (LC)		Structural assignment
m/z	%	m/z	%	m/z	%	m/z	%	m/z	%	
870	–	782	–	782	–	782	–	694	–	M^+ (Not seen)
–	–	–	–	–	–	–	–	447	4.4	M − (pentachlorophenyl)
–	–	–	–	–	–	–	–	430	47.8	M − (264)
–	–	–	–	–	–	–	–	396	33.7	Unknown
–	–	428	3.0	428	2.2	428	3.5	340	**100**	M − (264 + 90)
–	–	338	**100**	338	**100**	338	**100**	–	–	M − (264 + 2 × 90)
426	18.5	–	–	–	–	–	–	–	–	M − (264 + 2 × 90)
336	**100**	–	–	–	–	–	–	–	–	M − (264 + 3 × 90)
–	–	–	–	–	–	–	–	–	–	M − (264 + 90 + 15)
–	–	323	17.8	323	10.9	323	23.9	–	–	M − (264 + 2 × 90 + 15)
321	22.2	–	–	–	–	–	–	–	–	M − (264 + 3 × 90 + 15)
–	–	40.1	1.4	401	0.7	–	–	313	80.9	M − (291 + 90)
–	–	–	–	–	–	–	–	315	38.6	Unknown
399	2.0	311	36.3	311	24.3	311	33.0	–	–	M − (291 + 2 × 90)
309	20.9	–	–	–	–	–	–	–	–	M − (291 + 3 × 90)
–	–	–	–	–	–	–		299	72.5	M − (305 + 90)
385	3.3	297	32.0	297	16.8	297	38.3	–	–	M − (305 + 2 × 90)
295	17.6	–	–	–	–	–	–	–	–	M − (305 + 3 × 90)
–	–		–	–	–	–	–	285	52.6	M − (319 + 90)
371	3.3	283	20.7	283	10.7	283	16.7	–	–	M − (319 + 2 × 90)
281	23.2	–	–	–	–	–	–	–	–	M − (319 + 3 × 90)
–	–	–	–	–	–	–	–	257	67.0	M − (347 + 90)
–	–	255	14.5	255	10.3	255	20.8	–	–	M − (347 + 2 × 90)
253	27.5	–	–	–	–	–	–	–	–	M − (347 + 3 × 90)
243	16.9	243	19.8	243	7.5	243	17.6	243	29.6	Unknown

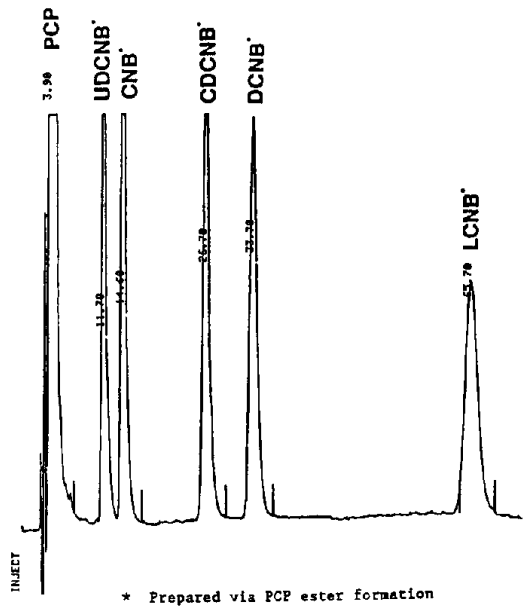

Fig. 4. HPLC separation of five N-benzylamides of FBA. Column, 10 cm × 8 mm I.D., 4-μm Nova-Pak C_{18}; flow-rate, 1 ml/min; mobile phase, 10 mM ammonium acetate–2-propanol–acetonitrile (50:33:17, v/v/v); detection, UV at 215 nm (0.050 a.u.f.s); amount injected, 5 μg each. Retention times (min): PCP, 3.90; UDCNB, 11.70; CNB, 14.60; CDCNB, 26.70; DCNB, 33.70; LCNB, 65.70.

temperature was maintained at 65°C and the various curves were obtained with CPCP–benzylamine in different ratios. A ratio of 1:76 produced the highest yield in a shorter reaction time. In Fig. 6 the ratio of CPCP to benzylamine was maintained at 1:76 and the reaction temperature was varied. A temperature of 100°C was shown to produce a yield of over 95% in 30 min.

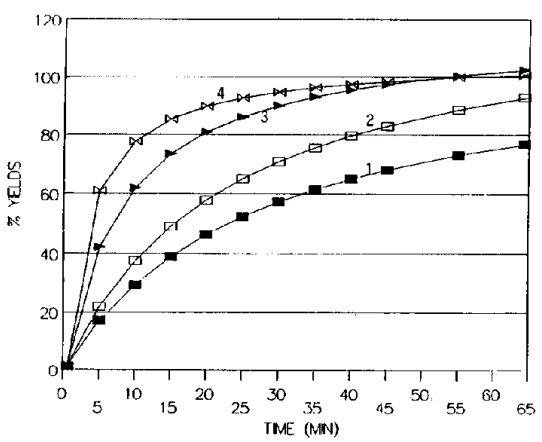

Fig. 5. Formation of CNB monitored by HPLC. Temperature of the reaction mixture maintained at 65°C and CPCP to benzylamine molar ratio varied from 1:19 to 1:76: 1 = 1:19; 2 = 1:38; 3 = 1:57; 4 = 1:76.

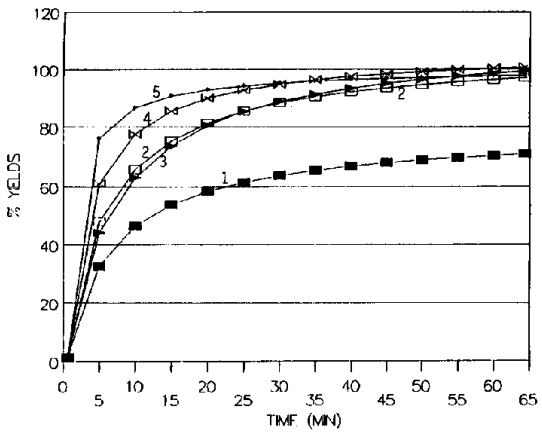

Fig. 6. Formation of CNB as monitored by HPLC. CPCP to benzylamine molar ratio maintained at 1:76 and temperatures varied from RT to 100°C: 1 = RT; 2 = 37°C; 3 = 50°C; 4 = 65°C; 5 = 100°C.

Table IV gives the data concerning the properties of the five N-benzylamides of FBA and summarizes the data obtained by thermospray–LC–MS. The major ions in the negative-ion mode were $[M - H]^-$ and $[M + CH_3COO]^-$. In positive-ion mode the major ions were $[M + H]^+$ and those corresponding to the loss of one molecule of water (18 dalton) for each of the hydroxyl groups present in the molecule.

Table V collates all the data obtained by MS with DIP and EI positive-ion detection of each of the five N-benzylamides of FBA. Molecular ion peaks of less than 1% intensity could be seen for each compound. The main fragmentation pathway was the loss of up to three molecules of water, followed by the loss of a 149 dalton fragment containing C-23 and C-24, the amide bond and the benzyl moiety. The tropylium cation (*m/z* 91) was noted in each spectrum. Ions at *m/z* 190 (positive ion including the complete side-chain) and 149 (McLafferty rearrangement fragment involving C-23 and C-24, the amide bond and the benzyl moiety) were also intense.

PCP esters of the glycine and taurine bile acid conjugates were not obtained. The formation of PCP esters is specific for FBA even in the presence of the conjugated bile acids. In the case of glycine conjugated bile acids a reaction takes place but does not produce a PCP ester. The identification of the sodium hydroxide-extractable product containing no PCP moiety is in progress. Preliminary results suggest that an azlactone was formed. As expected, taurine conjugated bile acids do not react with KC under the conditions described above. The formation of amides from PCP derivatives may be useful in the determination of FBA by HPLC in the presence of their conjugated counterparts, thus avoiding potential losses of free bile acids caused by the need to isolate them after extraction from biological fluids.

TABLE IV

PROPERTIES OF N-BENZYLAMIDES OF FREE BILE ACIDS

Property	Cholic (C)	Ursodeoxycholic (UDC)	Chenodeoxycholic (CDC)	Deoxycholic (DC)	Lithocholic (LC)	Structural assignment
Melting point (°C)[a]	233–236	108–110	177–180	177–179	177–180	
Mobility in HPLC[b]						
k'	3.75	2.81	7.70	9.98	20.40	
rk'	0.38	0.28	0.77	1.00	2.04	
m/z in thermospray–LC–MS[c]						
Positive-ion mode						
	498	482	482	482	466	[M + H]
	480	464	464	464	448	[M + H − 18]
	462	446	446	446	–	[M + H − 18 × 2]
	444	–	–	–	–	[M + H − 18 × 3]
Negative-ion mode						
	556	540	540	540	524	[M + CH_3COO]
	496	480	480	480	464	[M − H]
Infrared[d]	All five N-benzylamides presented bands at:					
	1037 cm^{-1}	Hydroxyl group at 3α-position				
	< 900 cm^{-1}	Aromatic ring				
	1680–1655 cm^{-1}	Secondary amide				
	1550–1530 cm^{-1}					
	1300–1260 cm^{-1}					

[a] Melting points obtained in duplicate with a Fisher–Johns instrument are uncorrected.

[b] Nova-Pak C_{18} column, 4-μm particles, 10 cm × 8 mm I.D., flow-rate 1 ml/min. Mobile phase: 0.01 *M* ammonium acetate –2-propanol–acetonitrile (50:33:17, v/v/v). Detection, UV at 215 nm (0.050 a.u.f.s.). Retention time for DCNB was 33.70 min.

[c] Vestec instrument with filament on. Temperatures: entrance vaporizer, 124°C; vaporizer, 230°C; source block, 301°C; tip heater, 313°C; in vapour, 300°C.

[d] Spectra obtained from KBr pellets.

TABLE V

MASS SPECTRAL DATA OBTAINED BY DIRECT INSERTION PROBE WITH 70-eV ELECTRON IMPACT IN THE POSITIVE-ION MODE OF FIVE N-BENZYLAMIDES OF FREE BILE ACIDS

Cholic (C)		Ursodeoxycholic (UDC)		Chenodeoxycholic (CDC)		Deoxycholic (DC)		Lithocholic (LC)		Structural assignment
m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	
497	5.5	481	10.4	481	0.2	481	5.6	465	0.8	M^+
479	2.8	463	2.2	463	10.1	463	8.9	447	0.3	M − 18
461	5.1	445	2.1	445	11.9	445	7.5	–	–	M − (18 × 2)
443	4.1	–	–	–	–	–	–	–	–	M − (18 × 3)
312	3.2	296	2.0	296	1.4	296	2.1	–	–	M − (149 + 18 × 2)
253	36.7	255	3.3	255	12.0	255	37.5	–	–	M − (190 + 18 × 2)
149	85.4	149	**100.0**	149	**100.0**	149	**100.0**	149	**100.0**	MacLafferty rearrangement
190	15.4	190	6.5	190	6.2	190	14.7	190	5.9	Side-chain
91	**100.0**	91	71.2	91	78.0	91	85.7	Not recorded		Tropylium ion

In conclusion, the formation of N-benzylamides of FBA through the formation of PCP esters followed by aminolysis is accomplished rapidly and produces stable and well characterized final products. Experiments using a fluorescent primary amine, instead of benzylamine, showed that less than 25 pmol of the corresponding amide can be detected, thus making possible the determination of FBA in less than 1 ml of human serum. Further, the interpretation of the highly complex fragmentation patterns observed in mass spectra obtained from these fluorescent amides of FBA would have been difficult without the knowledge gained by the studies reported here.

ACKNOWLEDGEMENTS

This work was supported in part by NIH Grant HL-07878 and the Portuguese Navy. We thank Dr. Gordon Lindenblad for valuable assistance with GC, Anthony Thomas with GC–MS and Mrs. Ping H. Patrick with HPLC. Thermospray–LC–MS data were kindly obtained by Dr. Fong-Fu Hsu at Washington University Mass Spectrometry Resource (St. Louis, MO, U.S.A.) and supported by NIH grant RR00954.

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