Determination of plasma bile acids by capillary gas-liquid chromatography-electron capture negative chemical ionization mass fragmentography

F. Stellaard, S. A. Langelaar, R. M. Kok, and C. Jakobs

Department of Pediatrics, Free University Hospital, Amsterdam, The Netherlands

Summary Combined capillary gas-liquid chromatography-electron capture negative chemical ionization mass spectrometry of pentafluorobenzyl ester-TMSi ether derivatives of bile acids and isotope dilution using deuterated internal standards are introduced as a sensitive and selective analysis technique for plasma bile acids. As a result of the high ionization efficiency of pentafluorobenzyl derivatives under electron capturing conditions and minimal fragmentation, the detection limit of this technique is low: 1 pg for each bile acid. The high sensitivity enabled the detection and quantitation of atypical bile acids in 200-μl aliquots of plasma from fasting healthy adults as exemplified by trihydroxycoprostanic acid (0.002 ± 0.001 μmol/l) and dihydroxycoprostanic acid (0.013 ± 0.002 μmol/l). —**Stellaard, F., S. A. Langelaar, R. M. Kok, and C. Jakobs.** Determination of plasma bile acids by capillary gas-liquid chromatography-electron capture negative chemical ionization mass fragmentography. *J. Lipid Res.* 1989. **30:** 1647–1652.

Supplementary key words *pentafluorobenzyl ester* • TMSi ether derivatives

The bile acid pattern in biological fluids may show distinct differences in diseases involving the enterohepatic circulation of bile acids. The relative concentrations of the normal bile acids cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) may change significantly under conditions such as cholestasis, cirrhosis, or bile acid malabsorption. Furthermore, atypical bile acids have been detected such as hyocholic acid (HCA), 3β-hydroxychol-5-enic acid, and tetrahydroxylated bile acids in plasma and urine of patients with cholestatic liver disease (1, 2), but also physiologically in fetal bile (3), and in urine (4) and meconium (5) of the neonate. Dihydroxy- and trihydroxycoprostanic acid (DHCA and THCA) are detected in patients with genetic defects in bile acid biosynthesis caused by the absence or abnormality of hepatic peroxisomes (6–8). Atypical bile acid metabolites can be detected in bile, blood, urine, or feces. However, bile is difficult to obtain, bile acids in feces are usually converted by intestinal bacterial enzymes, and the kidneys may discriminate between the various bile salts. Therefore, serum or plasma bile acids may best reflect abnormalities in bile acid metabolism. The ease of detecting bile acids in plasma, in particular atypical bile acids, depends largely on the degree of cholestasis, which may be the primary disease or secondary to another metabolic disease. Since most of the atypical bile acids cannot be detected in plasma of healthy adults, the following hypotheses may be considered. *1*) Atypical bile acids are formed also in healthy controls and are present in control plasma in concentrations below the detection limits of existing analytical techniques; and *2*) atypical bile acids are synthesized only as a response to the cholestatic condition or as the result of a metabolic defect.

In order to get a better insight into bile acid metabolism and also to detect milder elevations of atypical bile acids, we applied a method sufficiently sensitive to detect atypical bile acids in small volumes (200 μl) of plasma from fasting adults. The analytical technique used is combined capillary gas-liquid chromatography-electron capture negative chemical ionization mass spectrometry (GLC-ECNCIMS) applied to pentafluorobenzyl (PFB) ester-TMSi derivatives as recently described for bile acids by Goto et al. (9).

MATERIALS AND METHODS

Materials

The reference compounds cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, hyodeoxycholic acid, ursodeoxycholic acid, hyocholic acid, and 3β-hydroxychol-5-enic acid were obtained from Steraloids, Inc, Wilson, NH. Ursocholic acid was donated by Prof. G. Paumgartner, Munich, F. R. G. Trihydroxycoprostanic acid was a kind gift from Prof. G. Parmentier, Leuven, Belgium. [2,2,4,4-D_4]Cholic acid (isotopic purity 98%) and [11,12-D_2] chenodeoxycholic acid (isotopic purity 95%) were purchased from Merck Sharp and Dohme, Montreal, Canada. Cholylglycine hydrolase from *Clostridium perfringens* (welchii) was purchased from Sigma Chemicals, St. Louis, MO as the partially purified product. Pentafluorobenzyl bromide was obtained from Pierce Chemical Company, Rockford, IL. All other chemicals were of analytical grade and used without prior treatment.

Sample collection

Plasma was obtained from 10 healthy fasting adults

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; HDCA, hyodeoxycholic acid; UCA, ursocholic acid; HCA, hyocholic acid; THCA, trihydroxycoprostanic acid; DHCA, dihydroxycoprostanic acid; 3βOHΔ^5, 3β-hydroxychol-5-enic acid; PFB, pentafluorobenzyl; BSTFA, bis-trimethylsilyl-trifluoro acetamide; TMSi, trimethylsilyl; DMESi, dimethylethylsilyl; GLC, gas-liquid chromatography; MS, mass spectrometry; EI, electron impact; CI, chemical ionization; ECNCI, electron capture negative chemical ionization; SIM, selected ion monitoring.

(44–70 yr) who did not have any hepatobiliary or gastrointestinal disease.

Sample preparation

Two hundred μl plasma from fasting adults or 100 μl plasma from children were added to 0.25 nmol [2,2,4,4-D_4]CA and 0.25 nmol [11,12-D_2]CDCA as internal standards. Bile acid conjugates were hydrolyzed enzymically as follows. The plasma was diluted with 500 μl distilled water and 200 μl acetate buffer (pH 5.6). The pH was then adjusted to 5.6–6.0 with 0.2 M acetic acid. Thereafter, 100 μl 0.2 M EDTA, 100 μl 0.2 M mercaptoethanol, and 20 U cholylglycine hydrolase were added and the mixture was incubated at 37°C overnight in a waterbath. After adjustment of the pH to above 10 with 200 μl 1 N NaOH, the mixture was incubated at 64°C for 30 min to disrupt bile acid-protein binding (10). After cooling to room temperature, 400 μl 1 N HCl was added and the free bile acids were extracted two times with 6 ml diethylether after saturating the aqueous phase with sodium chloride. This step was preferred over extraction on C18 cartridges (11) as solvolysis of any sulfates that might be present is included in the ether extraction (12). Moreover, the recovery was lower when small plasma samples were extracted with C18 cartridges. After evaporation of the combined diethylether layers, the sample was treated with 100 μl of a 10% pentafluorobenzyl bromide solution in acetonitrile for 20 min at 30°C after addition of 20 μl triethylamine. The PFB esters were extracted into 4 ml ethyl acetate after addition of 0.5 ml 1 N HCl. The ethyl acetate was evaporated and the residue was treated with 100 μl bis-trimethylsilyl-trifluoro acetamide (BSTFA)-pyridine 1:1 (v/v) for 1 h at 80°C to form the TMSi ether derivatives. Immediately prior to the analysis, the sample was evaporated and taken up into 25 μl hexane. One to two μl was analyzed by capillary gas–liquid chromatography–mass spectrometry.

Gas–liquid chromatography–mass spectrometry

The PFB ester TMSi ether derivatives of bile acids were separated by capillary GLC using a 25 m × 0.25 mm OV-1701 coated fused silica column (CP Sil 19CB, Chrompack Int., Middelburg, The Netherlands). The column was connected to an uncoated fused silica capillary inserted into the ion source of a VG 30-253 quadrupole mass spectrometer. Helium was used as carrier gas at a flow rate of 1.5 ml/min. The sample was introduced by splitless injection at 320°C and the compounds of interest were trapped at the beginning of the column at an oven temperature of 150°C. After 1 min the oven temperature was raised to 320°C at 30°/min and kept at 320°C for 10 min. Under these conditions the PFB-TMSi derivatives of the various bile acids studied eluted between 11 and 15 min. The capillary interface to the ion source and the ion source itself were maintained at 320°C. This high ion source temperature was necessary since the peak shapes of the higher boiling compounds were broadened and disturbed at lower source temperatures. Electron capture negative chemical ionization was achieved using methane as moderating gas at a source pressure of 10^{-4} mbar. Mass spectra of authentic standards were obtained by scanning over the mass range *m/z* 50–800 with a 1-sec scan time. The biological samples were analyzed in the Selected Ion Monitoring mode using a 50-msec acquisition time at the base peak of each mass spectrum $(M\text{-}PFB)^-$ (see Results). Calibration curves for CA, CDCA, DCA, LCA, HCA, UDCA, 3β-hydroxychol-5-enic acid, and THCA were established for starting amounts ranging from 5 to 200 pmoles. The areas obtained for unlabeled CDCA, DCA, UDCA, LCA, and 3β-hydroxychol-5-enic acid were related to the area obtained for [11,12-D_2]CDCA, whereas the areas for unlabeled CA, HCA, and THCA were related to the area of [2,2,4,4-D_4]CA. No reference compound for DHCA could be obtained. Therefore, this compound in plasma was tentatively identified by analysis of plasma from Zellweger patients. In such plasma one compound was clearly elevated and appeared at a retention time just after that of THCA. The NCI mass spectrum showed just the $(M\text{-}PFB)^-$ ion as expected for DHCA.

RESULTS

The mass spectra of PFB-ester TMSi-ether derivatives of bile acids consist mainly of the $(M-181)^-$ ion as a result of loss of the PFB group yielding the very stable carboxylate anion (**Fig. 1**). Cleavage of TMSiOH as usually seen

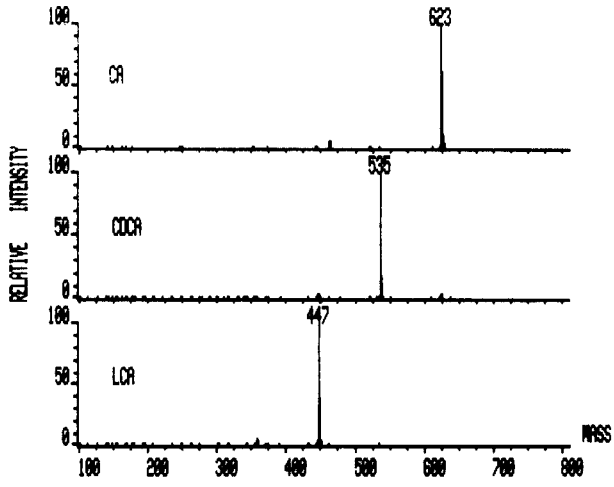

Fig. 1. ECNCI mass spectra of PFB-TMSi derivatives of CA, CDCA, and LCA.

in electron impact or positive chemical ionization mass spectrometry occurs only to a minor extent. The $(M-181)^-$ ion represents 50–60% of the total ion current. This lack of fragmentation creates a set of characteristic ions determined by the length of the side chain and the number of TMSi groups, oxo groups, and double bonds (**Table 1**). The high ionization efficiency initiated by electron capture negative ionization combined with the low degree of fragmentation results in high sensitivity. Detection limits for all bile acids investigated were 1 pg (2.5 fmol) injected onto the column. Separation of isomeric bile acids having the same characteristic $(M-181)^-$ ion was obtained by the CP Sil 19CB column at the conditions used (**Fig. 2**). Calibration curves for all bile acids subjected to the sample work-up procedure were linear in the range 5–200 pmol. The linear regression correlation coefficients varied from 0.991 to 0.999, and the slopes varied from 0.83 to 1.32 when the measured isotope ratios were plotted against the calculated ratios. Differences in slope for the various bile acids can be explained by different mass spectrometric responses, due mainly to possible differences in ionization efficiency and/or mass discrimination due to the large mass range involved: *m/z* 447 for monohydroxy C_{24} bile acids (LCA) to *m/z* 665 for trihydroxy C_{27} bile acids (THCA). The reproducibility of the method is demonstrated in **Table 2** which shows the results of eight repetitive analyses of one fasting plasma. At the highest concentration studied (CDCA, mean 0.71 μmol/l) the coefficient of variation was 2.5%, whereas this value increased to 21.1% at the lowest concentration level (THCA, mean 0.004 μmol/l). **Fig. 3** shows a set of representative mass chromatograms for dihydroxy and trihydroxy C_{24} bile acids obtained from plasma from a fasting healthy adult. **Fig. 4** and **Fig. 5** show 3β-hydroxychol-5-enic acid, THCA, and DHCA in the same plasma sample. Concentrations of the various bile acids studied in adult fasting plasma are in **Table 3**. All primary (CA, CDCA), secondary (DCA, LCA, UDCA), and atypical bile acids (3β-hydroxychol-5-enic acid, HCA, THCA, DHCA) were consistently found. THCA and DHCA represented 0.9 and 1.6% of their corresponding C_{24} metabolites CA and CDCA.

TABLE 1. Characteristic NCI mass fragments for PFB-TMSi derivatives of known bile acids

Number of C-atoms	Number of TMSi-Groups	Number of Oxo-Groups	Number of Double Bonds	*m/z*
24	1	0	0	447
24	2	0	0	535
24	3	0	0	623
24	4	0	0	711
24	0	1	0	373
24	0	2	0	387
24	0	3	0	401
24	1	1	0	461
24	1	2	0	475
24	2	1	0	549
24	1	0	1	445
27	2	0	0	577
27	3	0	0	665
27	4	0	0	753
27	2	1	0	591
27	3	1	0	679
27	2	0	1	575
27	3	0	1	663

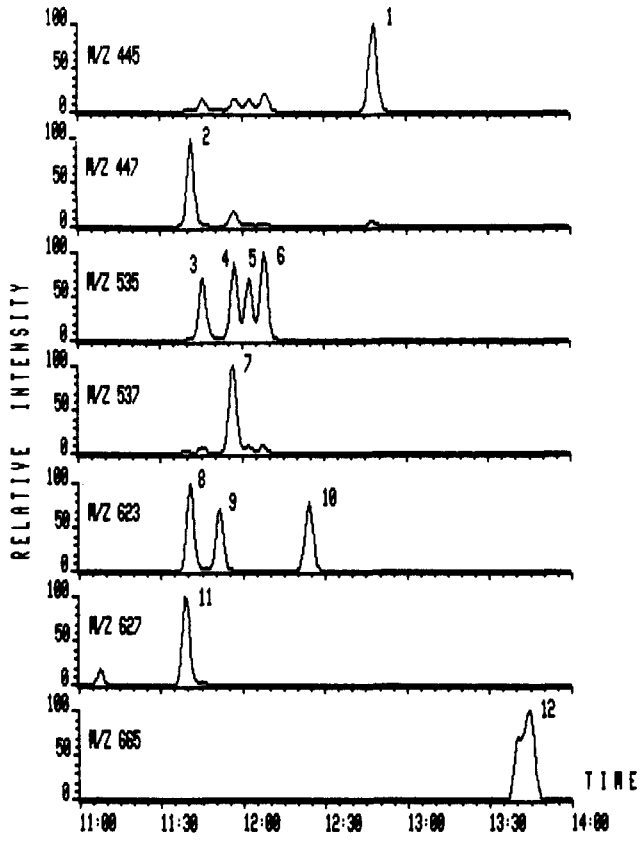

Fig. 2. Mass chromatograms of PFB-TMSi derivatives of bile acid standards. 1: 3βOH Δ^5, 2: LCA, 3: DCA, 4: CDCA, 5: HDCA, 6: UDCA, 7: [11,12-D_2]CDCA, 8: CA, 9: UCA, 10: HCA, 11: [2,2,4,4-D_4]CA, 12: THCA.

DISCUSSION

In this study we applied negative chemical ionization mass spectrometry as a very sensitive means of detecting plasma bile acids after separation by capillary gas-liquid chromatography. Similar techniques have been used successfully for other acids (13–18) and have recently been applied to bile acid standards by Goto and coworkers (9). Goto et al. (9) reported optimal results with PFB ester dimethylethylsilyl (DMESi) ether derivatives and isobutane as moderating gas. The data presented in this paper

TABLE 2. Reproducibility of total analysis determined with 200-μl aliquots of fasting adult plasma (n = 8)

	Mean Concentration	Coefficient of Variation
	μmol/l	%
CDCA	0.71	2.5
CA	0.29	2.3
DCA	0.41	3.2
DHCA	0.01	18.4
THCA	0.004	21.1

indicated two advantages of PFB-DMESi over PFB-TMSi derivatives. First, a better chromatographic resolution was achieved, and second, more advantageous ECNCI mass spectra were obtained showing the $(M-181)^-$ ion as base peak and only little fragmentation. In their hands (9) the PFB-TMSi derivatives of bile acids exhibited the $(M-181-TMSi_n)^-$ ion (n = number of TMSi groups) as the base peak. This type of unusual fragmentation was not observed under our experimental conditions. Goto et al. (9) also reported a detection limit of 2 fg for dihydroxy C_{24} bile acids when the $(M-181)^-$ ion was monitored continuously. The discrepancy with our detection limits of 1 pg may, at least partly, be explained by the fact that our data were obtained by monitoring at least 10 ions simultaneously. Thus, our procedure to define the detection limit closely resembles the actual situation in which bile acid profiles are determined using stable isotope-labeled internal standards.

In addition to being extremely sensitive, the ECNCI technique is also selective, since only negative ion-producing compounds are detected and the specific mass ions for the bile acid PFB-TMSi derivatives are very high

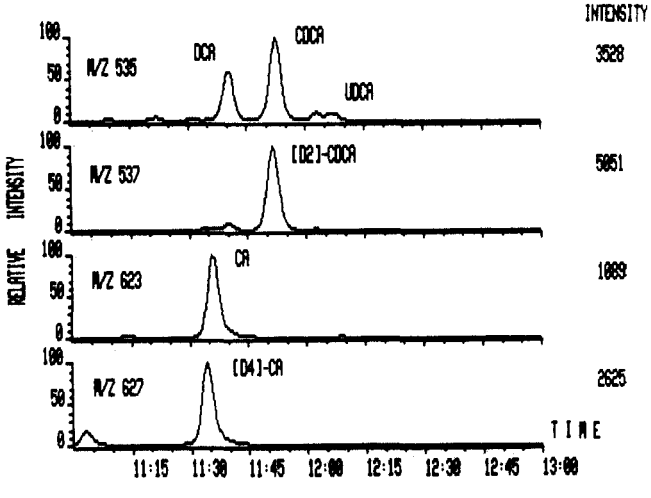

Fig. 3. Mass chromatograms of PFB-TMSi derivatives of dihydroxy- and trihydroxy-C_{24} bile acids from plasma of a healthy adult and of the labeled internal standards.

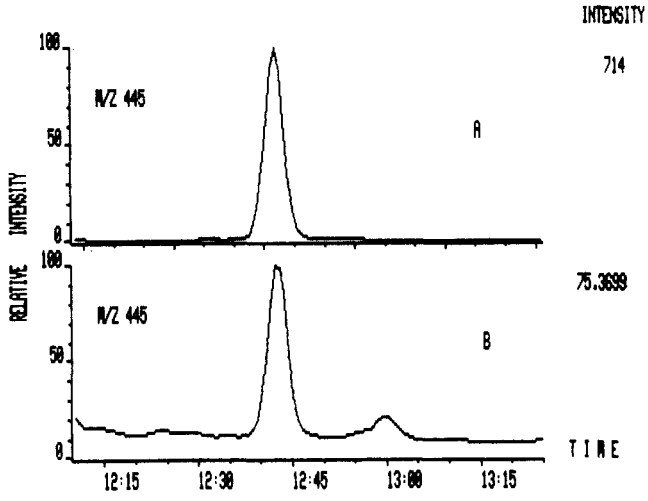

Fig. 4. Mass chromatograms of the PFB-TMSi derivative of the standard 3β-hydroxychol-5-enic acid (A) and its presence in fasting plasma (B). Ninety pmol standard and 200 μl plasma were carried through the sample work-up procedure.

(*m/z* 445-665). The technique is also selective in such a way that bile acids are separated by mass according to the number of TMSi groups, oxo-groups, double bonds, and the length of the side chain (C_{24}, C_{27}). PFB-TMSi derivatives of isomeric homologs have similar NCI spectra and need to be resolved by GLC retention time. For the purpose of structural information on atypical bile acids, EI-MS of methyl ester-TMSi derivatives remains the method of choice. However, for this technique larger sample volumes and higher concentrations are required. The GLC peak obtained for THCA is much broader than the ones for the C_{24} bile acids. Lowering the final GLC oven temperature leads to separation of the peak into two components. This observation is in accordance with the observation of Batta and coworkers (19, 20). Their studies with X-ray crystallography on THCA revealed two diastereoisomers at position C-25.

The sample work-up procedure is composed of enzymatic hydrolysis, extraction, and derivatization. The techniques have been used extensively by many investigators. The only new aspect is the use of enzymatic deconjugation in diluted plasma without prior extraction of the bile acid conjugates. Evidence for the validity of the method is provided by the good agreement found when our control values for the major bile acids CA, CDCA, and DCA are compared with literature data on serum bile acid concentrations determined by GLC-isotope dilution mass spectrometry (21-23). These data were obtained with enzymatic hydrolysis after extraction or with alkaline hydrolysis with and without prior extraction. Furthermore, comparison of sample work-up of adult plasma with and without hydrolysis showed a 2- to 3-fold increase in bile acid concentration (data not shown) after hydroly-

sis, which is consistent with the 30–50% contribution of unconjugated bile acids in adult plasma (24).

Reproducibility studies showed coefficients of variation between 2.3% and 3.2% for the major bile acids CDCA, CA, and DCA when 200-μl aliquots of fasting plasma were used. The minor bile acids present in this plasma sample showed much larger variations due to two factors. First, their concentrations are one to two orders of magnitude lower. Second, their concentrations are quantitated using deuterated analogs of CDCA and CA added as internal standards at a concentration of 1 μmol/l. Reduction of the amount of the internal standards added to the sample to an appropriate level would improve the quantitation of THCA and DHCA, but cause a dramatic increase in isotope ratio for CA and CDCA. Ideally, THCA and DHCA require their own labeled internal standards added to plasma at a concentration of approximately 0.01 μmol/l. However, these labeled compounds are not commercially available. Although we are aware of this problem, we are still convinced that the THCA and DHCA concentrations in normal plasma determined in this study represent the concentration range in which these compounds exist in plasma.

The presence of atypical bile acids in plasma of healthy adults supports the hypothesis that metabolic routes for the formation of 3β-hydroxychol-5-enic acid and HCA are still used to some extent in the adult healthy state. The contribution of these routes may be enhanced under cholestatic conditions. The presence of THCA and DHCA proves that these compounds are normal intermediates in the biosynthesis of CA and CDCA.

The main advantage of this technique is its superior sensitivity. Therefore its application is most useful in those cases where the bile acid concentrations are low and/or the available sample volume is very small. Small samples are most likely to be met in pediatric studies when atypical bile acids are to be detected within the first days of life. The method will enable quantitative measurement of mild elevations of atypical bile acids due to physiological cholestasis in newborn infants or due to metabolic dysfunction even at an age where the plasma bile acid concentration is no longer elevated. The data obtained so far suggest that for the analysis of the major bile acids CDCA, CA, and DCA the sample volume may easily be reduced further to 20–50 μl even in the fasting state. For the quantitative analysis of THCA and DHCA in healthy subjects, 200 μl plasma is required. The technique also offers an opportunity to determine the pool sizes and turnover rates of CDCA and CA in newborn infants using in vivo isotope dilution of stable isotope-labeled bile acids and isotope ratio measurements in plasma (25, 26). For this purpose repeated blood sampling is required.

In order to support the prenatal diagnosis of genetic disorders involving bile acid biosynthesis, it has been a main goal to adapt the technique presented for measurements in amniotic fluid in which the concentrations of peroxisomal bile acid intermediates are very low. Preliminary results from our laboratory confirm the suitability of the ECNCI-MS determination of THCA in amniotic fluid as a tool for prenatal diagnosis of Zellweger syndrome (27). Recently, two other defects involving bile acid synthesis have been described showing characteristic metabolites in urine (28–30). It may be expected that more inborn errors in bile acid biosynthesis will be discovered in the near future, emphasizing the need for postnatal and prenatal detection of various intermediates in bile acid synthesis. For this purpose combined capillary GLC-ECNCI mass spectrometry may play an important role.

TABLE 3. Concentrations of bile acids and the ratios THCA/CA and DHCA/CDCA in adult fasting plasma (n = 10)

Bile Acid	Concentration
	μmol/l ± SD
CDCA	0.90 ± 0.46
CA	0.23 ± 0.12
DCA	0.43 ± 0.37
LCA	0.071 ± 0.045
UDCA	0.053 ± 0.010
3βOH Δ^5	0.014 ± 0.002
HCA	0.015 ± 0.011
THCA	0.002 ± 0.001
DHCA	0.013 ± 0.002
Total	1.68 ± 0.71
	Ratio
THCA/CA	0.009 ± 0.009
DHCA/CDCA	0.016 ± 0.007

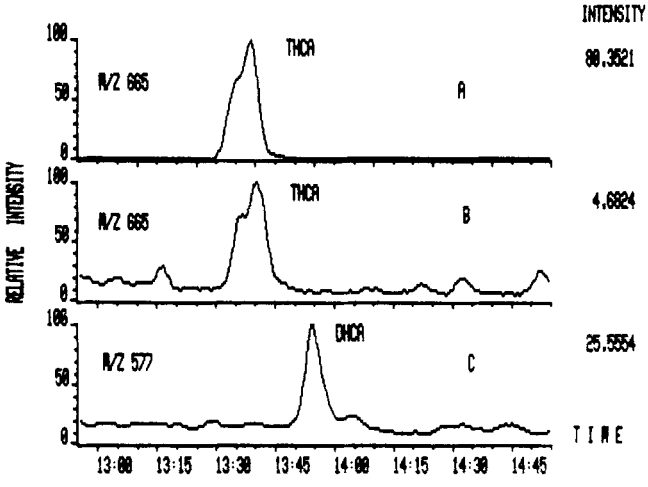

Fig. 5. Mass chromatograms of the PFB-TMSi derivatives of the standard THCA (A) and the presence of THCA and DHCA in fasting adult plasma (B and C). Ninety pmol standard and 200 μl plasma were carried through the sample work-up procedure.

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