[Chem. Pharm. Bull. 28(10)2941—2947(1980)]

Electrochemical Detector for High Performance Liquid Chromatography. IV.[1] Analysis of Fatty Acids, Bile Acids and Prostaglandins by Derivatization to an electrochemically Active Form

SATORU IKENOYA, OSAMU HIROSHIMA, MASAHIKO OHMAE, and KIYOSHI KAWABE

Analytical Research Laboratory, Eisai Co., Ltd.[2]

(Received April 3, 1980)

A high performance liquid chromatography (HPLC) procedure with an electrochemical detector (ECD) has been developed for the analysis of fatty acids, bile acids and prostaglandins. These carboxylic acids were converted to *p*-hydroxyanilides by reaction with *p*-aminophenol in the presence of 2-bromo-1-methylpyridinium iodide and triethylamine. The anilides were oxidized in a two-electron process and analyzed by reversed-phase HPLC–ECD. The detection limits were 0.5, 2 and 2 ng for stearic acid, chenodeoxycholic acid and prostaglandin $F_{2\alpha}$, respectively. The method was applied to the determination of free fatty acids in guinea pig plasma and bile acids in human bile.

Keywords——high performance liquid chromatography; electrochemical detector; *p*-hydroxyanilide derivatives; fatty acids; bile acids; prostaglandins; plasma; bile

Chemical derivatization techniques for high performance liquid chromatography (HPLC) have generally been used to permit the detection of otherwise hard-to-detect compounds, to enhance detectability and to improve resolution. Fatty acids have been derivatized with *p*-bromo phenacyl bromide,[3] 1-benzyl-3-*p*-tolyltriazine,[4] *R* or *S*-α-methyl-*p*-nitrobenzylamine,[5] *p*-methoxyaniline[6] and 4-bromo-7-methoxycoumarin,[7] in order to increase the ultraviolet absorption and fluorescence intensity. Bile acids have been separated as nitrophenacyl[8] and phenacyl[9] esters. Furthemore, chromophore and fluorophore derivatives of prostaglandins have been obtained by reaction with *p*-nitrobenzyl-,[10] *p*-nitrophenacyl-,[11] *p*-bromophenacyl-bromide(halide)[12] and 4-bromo-7-methoxycoumarin.[13] These methods facilitated HPLC analysis of trace amounts of carboxylic acids. However, few applications to biological samples have been reported.

We have tried to develop a convenient and sensitive method for the determination of fatty acids and other biologically important acidic compounds, and found that a combination of derivatization to an electrochemically active form with the use of an electrochemical detector (ECD) is suitable for the analysis of these compounds in biological samples.

The present paper describes the analysis of fatty acids, bile acids and prostaglandins as *p*-hydroxyanilide derivatives by HPLC–ECD, and its application to the biological samples.

1) Part III: O. Hiroshima, S. Ikenoya, M. Ohmae, and K. Kawabe, *Chem. Pharm. Bull.*, **28**, 2512 (1980).
2) Location: *4-6-10, Koishikawa, Bunkyo-ku, Tokyo, 112, Japan.*
3) H.D. Durst, M. Milano, E.J. Kikta, S.A. Connelly, and E. Grshka, *Anal. Chem.*, **47**, 1797 (1975).
4) I.R. Politzer, G.W. Griffin, B.J. Dowty, and J.L. Laseter, *Anal. Lett.*, **6**, 539 (1973).
5) D. Valentine, K.K. Chan, C.G. Scott, K.K. Johnson, K. Toth, and G. Saucy, *J. Org. Chem.*, **41**, 62 (1976).
6) N.E. Hoffman and J.C. Lias, *Anal. Chem.*, **48**, 1104 (1976).
7) W.D. Dunges, *Anal. Chem.*, **49**, 442 (1977).
8) J. Goto, M. Hasegawa, H. Kato, and T. Nambara, *Clin. Chem. Acta.*, **87**, 141 (1978).
9) F. Stellaard, D.L. Hachey, and P.D. Klein, *Anal. Biochem.*, **87**, 359 (1978).
10) W. Morozowich, Abst, 119th Meeting A. Ph. A. Academy of Pharmaceutical Science, April 1972.
11) W. Morozowich and S.L. Douglas, *Prostaglandins*, **10**, 19 (1975).
12) F.A. Fitzpatrick, *Anal. Chem.*, **48**, 499 (1976).
13) J. Yurk, S.J. Weiss, J.E. Davis, and P. Needleman, *Prostaglandins*, **16**, 291 (1978).

Experimental

Apparatus——The HPLC system and ECD were similar to those described previously.[14] The column was 25 cm × 4.6 mm I.D. stainless steel tubing packed with Nucleosil C-18 (Machery-Nagel Co., 10 μm). HPLC measurements were performed at 25 ± 0.1°. Controlled potential electrolysis was carried out using a Hokuto Denko HA-101 potentiostat. The ultrasonic cleaner bath (40 kHz) used was a Branson type 220.

Reagents——All saturated and unsaturated fatty acids and prostaglandins E_2 and $F_{2\alpha}$ were purchased from Sigma Chemical Co., U.S.A. Bile acids were obtained from BDH Chemical, England. *p*-Aminophenol (AP) was purchased from Tokyo Kasei Co. and recrystallized from ethanol. 2-Bromo-1-methylpyridinium iodide (dec. 219—220°) was prepared according to the procedure of Mukaiyama *et al.*[15] Other chemicals of reagent grade were used without further purification.

Derivatization——Procedure A: Carboxylic acids (10—200 μg of each), AP (1 mg) and triethylamine (150 μl) were mixed with dry dichloromethane (1 ml) in a 5 ml Reacti-Vial (Pierce Chemical Co., U.S.A.). 2-Bromo-1-methylpyridinium iodide (at least a 3-fold molar excess with respect to the carboxylic acids) was then added, and the vial was sealed with a Teflon-lined screwcap, and placed in an oil bath at 60° for 30 min. After cooling the vial, the solution was concentrated under a stream of nitrogen. The residue was shaken vigorously with a mixture of 0.1 N HCl (1 ml) and ethyl acetate (1 ml) and centrifuged at 2500 rpm for 5 min. The supernatant was subjected to HPLC.

Procedure B: Carboxylic acids (50—200 μg of each), AP (1 mg) and triethylamine (150 μl) were mixed with dry dichloromethane (1 ml) in a 5 ml Reacti-Vial, and the mixture was dissolved in an ultrasonic bath at 25° under a nitrogen atmosphere. 2-Bromo-1-methylpyridinium iodide (at least a 3-fold molar excess with respect to the carboxylic acids) was added and the vial was capped. The vial was placed in an ultrasonic bath at 25° until the reaction was completed (the solution became cloudy). The solution was then treated as described in procedure A.

Preparation of *p*-Hydroxystearanilide——Stearic acid (150 mg) and oxalyl chloride (200 mg) were refluxed for 30 min in dichloromethane (10 ml). The solvent and excess oxalyl chloride were removed at 40° under reduced pressure. The crude stearyl chloride was dissolved in ethylmethylketone (10 ml) and cooled in an ice bath. A solution of AP in ethylmethylketone (60 mg/3 ml) was added gradually and the mixture was stirred at 0—5° for 1 hr. The solution was diluted with 200 ml of dichloromethane, washed successively with 1 N HCl, saturated $NaHCO_3$ and water, and dried over anhydrous $MgSO_4$. The dried solution was concentrated under reduced pressure and the residue was recrystallized from ethanol.

Pretreatment of Free Fatty Acids in Guinea Pig Plasma——Plasma (0.5 ml) was placed in a glass centrifuge tube, then 20 ml of chloroform–methanol (2: 1) was added, and the tube was shaken on a Vortex shaker for about 30 sec. Four ml of water was added and the mixture was shaken for 5 min, then centrifuged at 2500 rpm for 5 min. The chloroform layer was removed and the aqueous layer was extracted twice with 10 ml of chloroform–methanol (2: 1). The chloroform layers were combined, dried over anhydrous Na_2SO_4 and passed through a glass filter. The filtrate was evaporated to dryness under a stream of nitrogen at 40°. The residue and authentic fatty acids were treated according to procedure A, and subjected to HPLC.

Pretreatment of Bile Acids in Human Bile——A healthy volunteer was given orally 200 mg b.i.d. after breakfast and dinner for 3 weeks. Bile was collected by duodenal drainage after intravenous injection of ceruletide diethylamine. Bile (200 μl) was hydrolyzed with 4 N NaOH–methanol (1: 1) in a screw-capped Pyrex glass tube at 80° for 16 hr. The solution was adjusted to pH 1.5 with 6 N HCl and extracted with three 10 ml portions of ethyl acetate.[16] The ether layers were evaporated to dryness under a stream of nitrogen. The residue and authentic bile acids were treated according to procedure A, and subjected to HPLC.

Results and Discussion

Derivatization of Carboxylic Acids

In selecting the reagent for the present derivatization, the following factors are important: (1) the reagent for the derivatization should be oxidized at low potential; (2) the derivatization reaction should be specific, quantitative, free from side reaction and completed in a short time under mild conditions; (3) it should be easy to eliminate excess reagent from the reaction solution. *p*-Aminophenol(AP) was considered to meet these requirements. The excess AP

14) S. Ikenoya, T. Tsuda, Y. Yamano, Y. Yamanishi, K. Yamatsu, M. Ohmae, K. Kawabe, H. Nishino, and T. Kurahashi, *Chem. Pharm. Bull.*, **26**, 3530 (1978).
15) K. Saigo, M. Usui, K. Kikuchi, E. Shimada, and T. Mukaiyama, *Bull. Chem. Soc. Jpn.*, **50**, 1863 (1977).
16) M. Shino, Y. Nezu, T. Tateyama, K. Katayama, J. Tsutsumi, and K. Kawabe, *Yakugaku Zasshi*, **99**, 421 (1979).

in the reaction mixture could be eliminated easily by washing with acid solution, such as 0.1 N HCl, and its anilide derivative was oxidized at low potential. Other amines such as 3,4-dihydroxybenzylamine and *p*-anisidine could not be used because of their lower activity or higher oxidation potential than AP.

In general, amides are prepared from free carboxylic acids and amines by means of coupling reagents such as 1,3-dicyclohexylcarbodiimide,[17] N,N′-carboxydiimidazole,[18] diethylphosphorylcyanide,[19] diphenylphosphoryl azide,[20] triphenylphosphine[21] and polystyryldiphenyl phosphine.[22] These reactions require a high temperature to remove water formed in the reaction mixture.

Recently, Mukaiyama *et al.*[23] utilized the onium salt, *e.g.* the 2-bromo-1-methylpyridinium iodide (BMP), for the preparation of amides, since the carboxylic acid is activated in the presence of a weak base such as triethylamine. In the present reaction, the carbonyl carbon atom seems likely to undergo nucleophilic attack by the amino group of AP rather than the hydroxy group in the presence of BMP and triethylamine, because of the stronger basicity of the former.

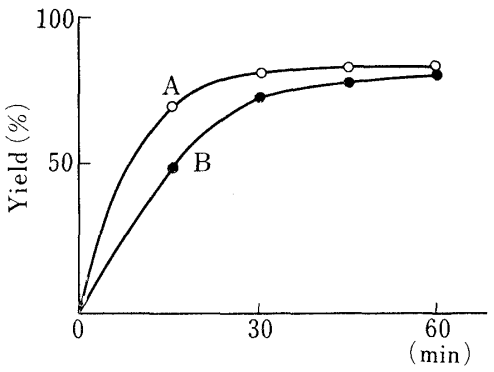

Fig. 1. Reaction of Stearic Acid with *p*-Aminophenol

A; at 60° (procedure A).
B; at 25° (procedure B).

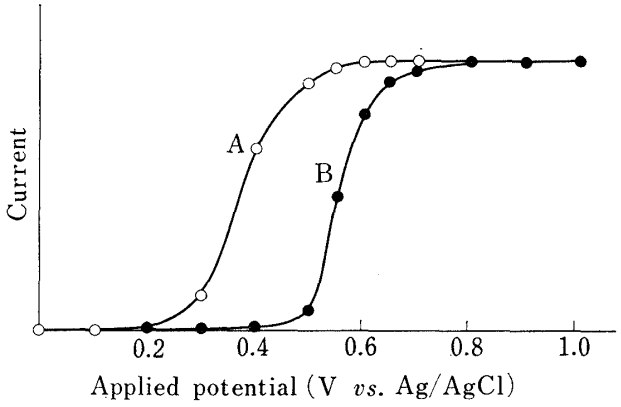

Fig. 2. Current–Potential Curves for *p*-Hydroxystearanilide

A; addition of 0.1% pyridine.
B; addition of 0.1% $HClO_4$.

Table I. Anilide Formation from Carboxylic Acids with *p*-Aminophenol

Carboxylic acid	Procedure[a]	Reaction time (min)	Yield[b] (%)
Lauric acid (C_{12})	A	45	84
	B	45	77
Stearic acid (C_{18})	A	30	82
	B	45	78
Cholic acid	A	60	75
	B	100	65

a) Procedure A and B were carried out at 60° and 25°, respectively.
b) The yield was estimated using 50 μg of acid.

17) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
18) H.A. Staab, *Angew. Chem. Int. Ed. Engl.*, **1**, 351 (1962).
19) S. Yamada, Y. Kasai, and T. Shioiri, *Tetrahedron Lett.*, **1973**, 1595.
20) T. Shioiri, K. Ninomiya, and S. Yamada, *J. Am. Chem. Soc.*, **94**, 6203 (1972).
21) L.E. Barstow and V.J. Hruby, *J. Org. Chem.*, **36**, 1305 (1971).
22) P. Hodge and G. Richardson, *Chem. Commun.*, **1975**, 622.
23) E. Bald, K. Saigo, and T. Mukaiyama, *Chem. Lett.*, **1975**, 1163.

The reaction product of stearic acid with AP was identified as *p*-hydroxystearanilide (HSA) by mass spectrometry. The effect of AP concentration on this reaction was studied, using 100 μg of stearic acid and 150 μl of triethylamine. The peak height of HSA formed became constant at concentrations above 0.5 mg/ml of AP. The amount of BMP added to the reaction mixture was also varied to maximize the HSA peak height. A constant yield was obtained when more than 0.5 mg of BMP was used in a test solution containing 100 μg of stearic acid and 1 mg of AP. The reaction of stearic acid with AP is shown in Fig. 1. The yield of HSA was 82% in 30 min at 60°, determined with a known amount of authentic HSA, whereas the yield at 25° was 78% in 45 min. Similar results were obtained for the reaction of lauric acid and cholic acid at 25° and 60°, as shown in Table I. Linearity of the calibration graphs for stearic acid, lauric acid and cholic acid was excellent over the range of 10 nmol to 1 μmol in the final reaction mixture.

On the basis of these results, it was decided that fatty acids and bile acids would be derivatized according to procedure A, and prostaglandins, which are unstable to heat, according to procedure B. In procedure B, the use the ultrasonic bath accelerates the reaction, probably by increasing the solubility. *p*-Hydroxyanilide derivatives of these compounds were stable for more than one week at 4°.

Electrochemical Studies

Fig. 2 shows a plot of the peak current *versus* applied potential for HSA. When 0.1% pyridine or 0.1% $HClO_4$ was added to the mobile phase (methanol–H_2O, 900: 100), the limiting current of HSA reached a plateau at about 0.6 or 0.7 V *vs.* Ag/AgCl, respectively. Similar results were obtained for other fatty acids, bile acids and prostaglandins. Addition of pyridine to the mobile phase resulted in a low oxidation potential for HSA, which was ascribed to partial deprotonation of HSA, and an increase of the retention of the fatty acids in comparison with addition of 0.1% $HClO_4$. Electrolysis of HSA in methanol–H_2O–70% $HClO_4$ (900: 100: 1, containing 0.05 M $NaClO_4$) mixture resulted in consumption of 2 Faradays per mole, and the anodic oxidation products were identified as stearylamide and *p*-benzoquinone by thin-layer chromatography and infrared spectroscopy. A similar oxidation process was suggested for the anodic oxidation of *p*-hydroxybenzanilide.[24] Thus, the following scheme is proposed for the anodic oxidation of HSA; BMP used as the catalyst was not detected by ECD in the anodic mode.

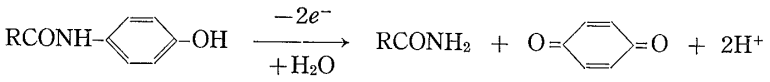

Chart 1

Chromatographic Separation

First, we examined the separation of fatty acids, bile acids and prostaglandins using several reversed-phase packing materials (Lichrosorb RP-18, Nucleosil C-18 and Nucleosil C-8) for HPLC. Among these packing materials, Nucleosil C-18 was definitely superior to the others in resolution. Fig. 3 shows the isocratic separation of fatty acid derivatives. The retention times of the saturated fatty acids increased with the number of carbon atoms, while the retention time of the unsaturated homologs, such as oleic acid, linolenic acid, decreased with increasing unsaturation. This could be ascribed to the increment of polarity in the fatty acids. The detection limit of HSA was 0.5 ng, and the linear dynamic range of current for this compound was approximately 10^4 (10 ng—2 μg).

The chromatographic separation of authentic bile acid derivatives is shown in Fig. 4. Bile acids were eluted in the other of decreasing number of hydroxy groups on cholanic acid. Addi-

24) S. Ikenoya, M. Masui, H. Ohmori, and H. Sayo, *J. Chem. Soc., Perkin II*, **1974**, 571.

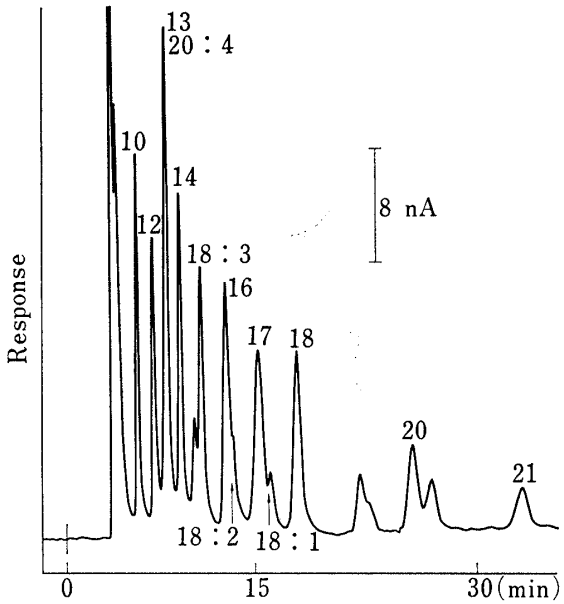

Fig. 3. Chromatogram of *p*-Hydroxyanilides of Fatty Acids

Column; Nucleosil C-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; methanol–H_2O–pyridine (900: 100: 1, containing 0.05 M $NaClO_4$).
Applied potential; 0.7 V *vs.* Ag/AgCl.
Flow rate; 1.2 ml/min.
Injection amount; C_{10}–C_{21} acids, 30—110 ng.
The numbers in the figure represent the ratio (number of carbon atoms): (number of double bonds) for fatty acids.

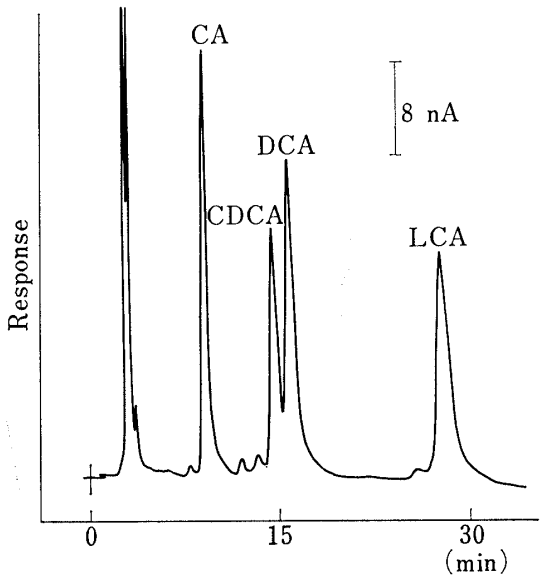

Fig. 4. Chromatogram of *p*-Hydroxyanilides of Bile Acids

Column; Nucleosil C-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; methanol–H_2O–$HClO_4$ (750: 250: 1, containing 0.05 M $NaClO_4$).
Applied potential; 0.7 V *vs.* Ag/AgCl.
Flow rate; 0.95 ml/min.
CA; cholic acid (180),[1] CDCA; chenodeoxycholic acid (150), DCA; deoxycholic acid (160), LCA; lithocholic acid (170).
1) The amount(ng) injected is shown in parentheses.

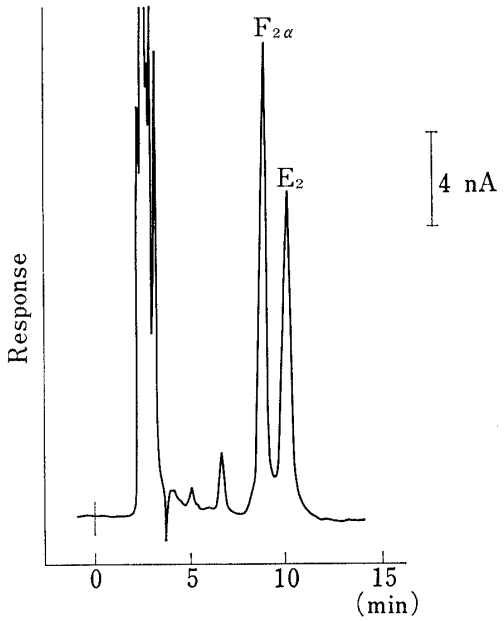

Fig. 5. Chromatogram of *p*-Hydroxyanilides of Prostaglandins $F_{2\alpha}$ and E_2

Column; Nucleosil C-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; methanol–H_2O–$HClO_4$ (600: 400: 1, containing 0.05 M $NaClO_4$).
Applied potential; 0.7 V *vs.* Ag/AgCl.
Flow rate; 1.1 ml/min.
Amount injected (ng): $F_{2\alpha}$; 95, E_2; 85.

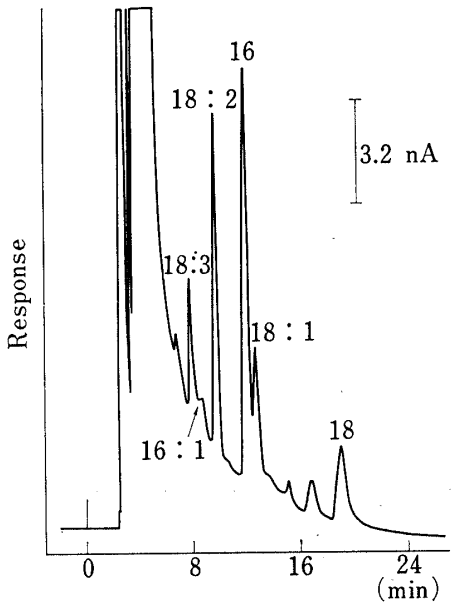

Fig. 6. Chromatogram of Free Fatty Acids in Guinea Pig Plasma

Column; Nucleosil C-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; methanol–H_2O–$HClO_4$ (880: 120: 1, containing 0.05 M $NaClO_4$).
Applied potential; 0.75 V *vs.* Ag/AgCl.
Flow rate; 1.2 ml/min.

tion of 0.1% $HClO_4$ to the mobile phase (methanol–H_2O, 750: 250) was effective for the separation of anilides of bile acids. The detection limit of authentic *p*-hydroxychenodeoxycholanilide was 2ng, and the linear dynamic range of current for this compound was approximately 10^4 (20 ng—1.5 μg).

The separation of prostaglandins is shown in Fig. 5. The order of elution reflects the increasing polarity of prostaglandins. The detection limit of prostaglandin $F_{2\alpha}$ was about 2 ng, which is almost the same as that of *p*-nitrophenacyl ester[11] using an ultraviolet detector (UV).

Application to Biological Samples

In order to investigate the applicability of the present methods, free fatty acids in guinea pig plasma and bile acids in human bile were determined.

D'Amboise has reported an HPLC–UV method for the determination of free fatty acids as phenacyl esters, but the present method offers improved sensitivity.

Fig. 6 shows a chromatogram of the free fatty acids in guinea pig plasma. The mobile phase contained 0.1% $HClO_4$ to improve the separation of the solvent peak and the fatty acid peaks of interest. The concentration of each fatty acid in the plasma was estimated from calibration curves of peak height against concentration of fatty acid, obtained using authentic fatty acids treated in the same manner as the sample. The extraction recovery of stearic acid from plasma was 93% and the composition of the free fatty acids in guinea pig plasma is shown in Table II.

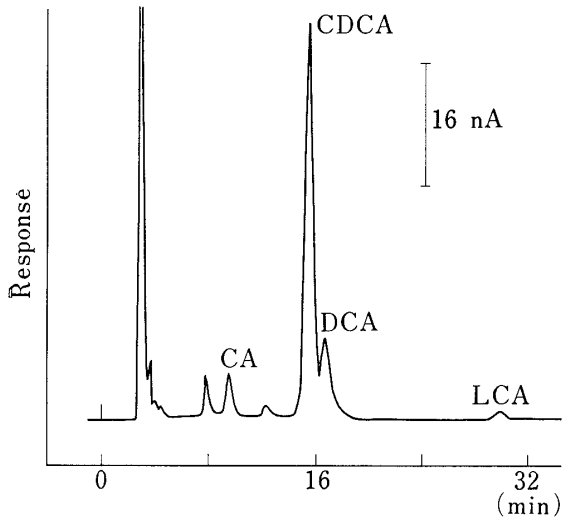

Fig. 7. Chromatogram of Human Bile after Oral Administration of Chenodeoxycholic Acid

Column; Nucleosil C-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; methanol–H_2O–$HClO_4$ (750: 250: 1, containing 0.05 M $NaClO_4$).
Applied potential; 0.75 V *vs.* Ag/AgCl.
Flow rate; 0.9 ml/min.

Table II. Free Fatty Acid Composition of Guinea Pig Plasma

Fatty acid	Concentration (μg/ml)	Relative ratio (%)
Palmitic acid (C_{16})	27.3	32.9
Palmitoleic acid (C_{16-1})	2.0	2.4
Stearic acid (C_{18})	9.1	11.0
Oleic acid (C_{18-1})	7.8	9.4
Linoleic acid (C_{18-2})	27.8	33.5
Linolenic acid (C_{18-3})	9.0	10.8

Table III. Concentration of Bile Acids in Human Bile determined by Two Methods[a]

Bile acid	HPLC (mg/ml)	GC–MS (mg/ml)
Cholic acid	2.4	—
Chenodeoxycholic acid	30.2	33.9
Deoxycholic acid	5.6	5.3
Lithocholic acid	1.2	—

a) A volunteer was given orally 400 mg/day of chenodeoxycholic acid for 3 weeks.

A chromatogram of the bile acids in human bile after oral administration of chenodeoxycholic acid is shown in Fig. 7. The conjugated bile acids in bile were hydrolyzed and the peak height with that of the corresponding authentic sample treated in the same manner as the bile sample. The extraction recovery of chenodeoxycholic acid from bile was 85%. The results of determination of the bile acids in a human bile sample are given in Table III, together with the data obtained by gas chromatography–mass fragmentgraphy.[16] No significant difference is apparent between the two results.

Derivatization of carboxylic acids with AP is simple, relatively rapid and can be applied to unstable compounds such as prostaglandins by using BMP as a catalyst. *p*-Hydroxyanilides of carboxylic acids are oxidized at low potential and, therefore, can be specifically detected by ECD. In addition, the method described here is more sensitive and rapid than conventional methods. Consequently, the present method should prove to be useful in the fields of clinical chemistry and biological chemistry.