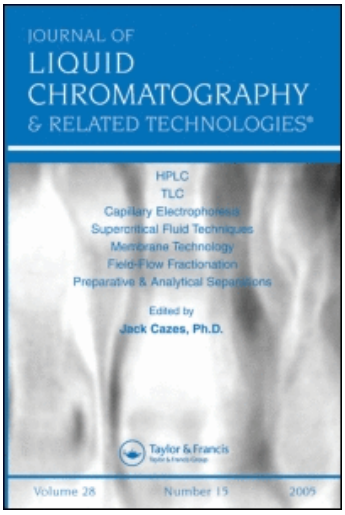

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DETERMINATION OF FATTY ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION USING A FERROCENE DERIVATIZATION REAGENT

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ABSTRACT

New derivatization method using ferrocene reagents has been developed for the determination of fatty acids by high-performance liquid chromatography with electrochemical detection. Condensation of fatty acids with 3-bromoacetyl-1,1'-dimethylferrocene was effected in the presence of 18-crown-6 and potassium fluoride. The resulting esters showed the satisfactory sensitivity at +0.60 V vs. an Ag/AgCl reference electrode with a detection limit of 0.5 pmole. Also the high selectivity was obtained by using a twin electrode electrochemical detector. The proposed derivatization method was found applicable to the determination of fatty acids in human serum.

INTRODUCTION

High-performance liquid chromatography (HPLC) with electrochemical detection (ECD) is a useful tool for the trace analysis

of various compounds in biological fluids. In recent years, pre- and post-column labeling methods have been developed to extend its applicability [1-5]. In the previous papers of this series we proposed novel ferrocene reagents for pre-column labeling of amines [2], hydroxyl compounds [4] and glucuronides [5] in HPLC/ECD. As the ferrocene derivative undergoes facile oxidation and the product is in turn readily reduced, it can be detected selectively in the presence of electroactive compounds such as phenols, catechols, and aromatic amines.

The present paper deals with the development of a derivatization method using ferrocene as an electrophore for the determination of carboxylic acids by HPLC/ECD. In addition, the application of this method to the determination of principal fatty acids in serum is described.

MATERIALS AND METHODS

Materials

Bromoacetylferrocene (I), 3-bromoacetyl-1,1'-dimethylferrocene (II), and N-(4-dimethylaminophenyl)maleimide were synthesized in these laboratories as described in the previous papers [5,6]. 2-Hydroxyestrone was prepared by the method reported by Stubenrauch et al. [7]. Fatty acids were supplied by Tokyo Kasei Kogyo Co. (Tokyo, Japan). Silica gel 60 HF_{254} (E. Merck AG, Darmstadt, F.R.G.) was used for preparative thin layer chromatography (TLC). All other reagents and chemicals were obtained from Nakarai Chemicals Ltd. (Kyoto, Japan) and purified by recrystallization or distillation prior to use.

Instruments

Mass (MS) spectral measurements were run on a Hitachi M-52 spectrometer (Hitachi Ltd., Tokyo, Japan). HPLC was carried out on a Toyo Soda 803A chromatograph (Toyo Soda Co., Tokyo, Japan)

equipped with a Yanagimoto VMD-501 electrochemical detector having twin electrode in series system (Yanagimoto Co., Kyoto, Japan). The applied potential of the detector was set vs. an Ag/AgCl reference electrode. A TSKgel ODS-80TM (5 μm) column (15 cm x 0.4 cm i.d.) (Toyo Soda Co.) and a LiChrosorb RP-18 (5 μm) column (15 cm x 0.4 cm i.d.) (E. Merck AG) were used under ambient condition.

Preparation of Standard Samples

To a solution of stearic acid (5 mg) in methanol (1 ml) was added KOH (5 mg) in methanol (0.5 ml) and the solvent was evaporated off under reduced pressure. A suspension of the residue in benzene (2 ml) was treated with 18-crown-6 (10 mg) and each derivatization reagent (5 mg) at 80°C for 30 min. After evaporation of the solvent, the residue was purified by preparative TLC to give the desired compound as yellow oily substance (ca. 2 mg). The esters formed from I: TLC (benzene) Rf 0.36, MS m/z 510; from II: TLC (benzene) Rf 0.33, MS m/z 538.

Determination of Fatty Acids in Serum by HPLC/ECD

Margaric acid (internal standard: IS) (0.5 μg) in methanol (50 μl) was added to human serum (50 μl) in 0.5 M phosphate buffer (pH 6.5, 200 μl), and the whole was extracted twice with hexane-chloroform (1:1, 2 ml). The organic layer was washed with H_2O and evaporated off in vacuo below 50°C. To the residue were added reagent II (30 μg) in dimethylformamide (50 μl), 18-crown-6 (100 μg) in dimethylformamide (100 μl), and KF (ca. 1 mg), and the whole was vortex-mixed, kept at 80°C for 60 min, and then subjected to HPLC/ECD.

Recovery Test for Fatty Acids added to Control Serum

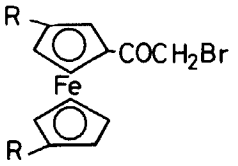
The spiked samples were prepared by adding 0.1 μg, 0.5 μg, and 2 μg each of fatty acids to the hexane-chloroform treated

control serum (50 µl) in 0.5 M phosphate buffer (200 µl). A test sample was subjected to extraction with hexane-chloroform followed by derivatization with II in the manner described above.

RESULTS AND DISCUSSION

In the previous paper [5] we reported the syntheses of bromoacetylferrocene (I) and 3-bromoacetyl-1,1'-dimethylferrocene (II) as derivatization reagents for carboxylic acids (Fig. 1). The reactivities of these reagents and electrochemical properties of the products were investigated using stearic acid as a model compound. The half-wave potential ($E_{1/2}$) of stearic acid derivative obtained with II was +0.56 V, which was somewhat lower than that (+0.67 V) of the ester derived from I (Fig. 2a). This would be ascribable to the negative inductive effect of methyl groups in II. The detection limit of the derivative obtained with II at +0.60 V was 0.5 pmole (signal to noise ratio=5 at 4 nA full scale). Among usual electroactive compounds, such as phenol (estriol), catechol (2-hydroxyestrone), and aromatic amine (N-(4-dimethylaminophenyl)maleimide-N-acetylcysteine adduct), only catechol exhibited anodic response at this applied potential (Fig. 2a). The oxidation product of stearic acid ester obtained with II was reduced at the downstream electrode (+0.2 V) while that of the catechol showed no cathodic response at this applied potential. In consequence, the use of a twin electrode system could detect the ferrocene derivative with high selectivity (Fig. 2b).

Based upon these results derivatization with II was further investigated. The applied potentials of the detector were set at +0.6 V and +0.2 V for the upstream and downstream electrodes, respectively. Condensation of stearic acid with II was effected in the presence of 18-crown-6 and potassium fluoride in dimethylformamide at 80°C [8]. The yielded amount of the derivative was estimated by comparison with the peak height of the standard



I: R=H II: R=CH_3

FIGURE 1. Structures of Derivatization Reagents.

TABLE 1
K' Values of Fatty Acid Derivatives Relative to Margaric Acid Derivative

Fatty Acid Derivative	Column	
	TSKgel ODS-80TM*	LiChrosorb RP-18**
Myristoleic acid ($C_{14:1}$)	0.38	0.35
Linolenic acid ($C_{18:3}$)	0.48	0.38
Myristic acid (C_{14})	0.51	0.52
Palmitoleic acid ($C_{16:1}$)	0.55	0.48
Arachidonic acid ($C_{20:4}$)	0.59	0.43
Linoleic acid ($C_{18:2}$)	0.64	0.50
Palmitic acid (C_{16})	0.80	0.77
Oleic acid ($C_{18:1}$)	0.86	0.70
Stearic acid (C_{18})	1.27	1.25
Margaric acid (C_{17}) (IS)	1.00 (17.3 min)	1.00 (11.0 min)

Solvent system: * 0.1 M $NaClO_4$ in methanol-H_2O (13:1), ** 0.1 M $NaClO_4$ in acetonitrile. Flow rate: 1 ml/min.

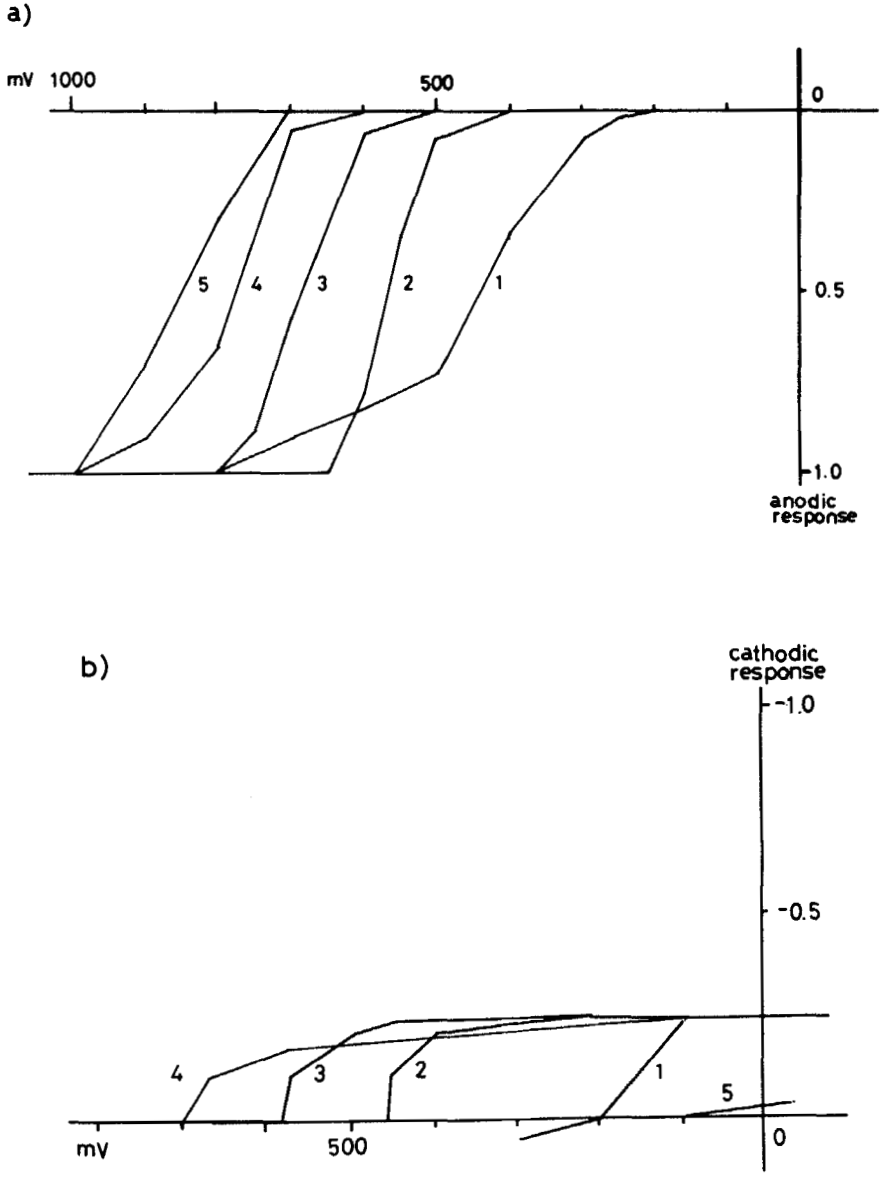


FIGURE 2. Hydrodynamic Voltammograms of Stearic Acid Derivatives and Electroactive Compounds.
1, 2-Hydroxyestrone; 2, stearic acid derivative with II; 3, stearic acid derivative with I; 4, N-(4-dimethylaminophenyl)maleimide-N-acetylcysteine adduct; 5, estriol.
The maximum response of each compound was taken as 1.0.
a) Anodic response, b) cathodic response: upstream electrode +0.8 V (1,3), +0.65 V (2), and +1.0 V (4,5).

sample. The reaction rate increased with reaction time up to 30 min and then reached a plateau.

The utility of II was then tested for the determination of fatty acids in serum. The authentic fatty acids including margaric acid (C_{17}) (IS), which is known to be absent in human serum [9], were derivatized with II for 60 min and the resulting esters were subjected to HPLC/ECD on two different columns (Table 1). The ferrocene derivatives of linoleic acid ($C_{18:2}$), palmitic acid (C_{16}), oleic acid ($C_{18:1}$), and stearic acid (C_{18}), which are principal fatty acids in human serum, were distinctly separated on a TSKgel ODS-80TM column within 25 min. When the amount ratio of each fatty acid to the IS (0.5 μg) was plotted against the peak height ratio, a linear relationship was observed in the range 0.25 - 2 μg (per tube) of the fatty acid, the regression equations being $y = ax + b$ where a=2.50, 1.41, 1.10, and 0.76 for $C_{18:2}$, C_{16}, $C_{18:1}$, and C_{18}, respectively, and b=0. In addition, fatty acids added to the control serum at three levels (0.1, 0.5, 2 μg/50 μl) were recovered at the rate of $\geq$ 80.6% (C.V. $\leq$ 4.8, n=8). It is evident from these data that the proposed analytical procedure is satisfactory with respects to the accuracy and precision.

Fatty acids in 50 μl of human serum were extracted with hexane-chloroform and then derivatized with the reagent as described above. A typical chromatogram showed five peaks corresponding to $C_{18:2}$, C_{16}, $C_{18:1}$, C_{17} (IS), and C_{18} (Fig. 3). These peaks were identified by comparison with authentic samples in the chromatographic behaviour on two different columns (Table 1) and collection efficiency (the ratio of the current at the downstream detector to that at the upstream detector): the value being 0.25. The concentrations of principal fatty acids in serum specimens taken from seven healthy subjects are listed in Table 2.

A variety of derivatization methods have been developed for the determination of fatty acids by HPLC with ultraviolet and

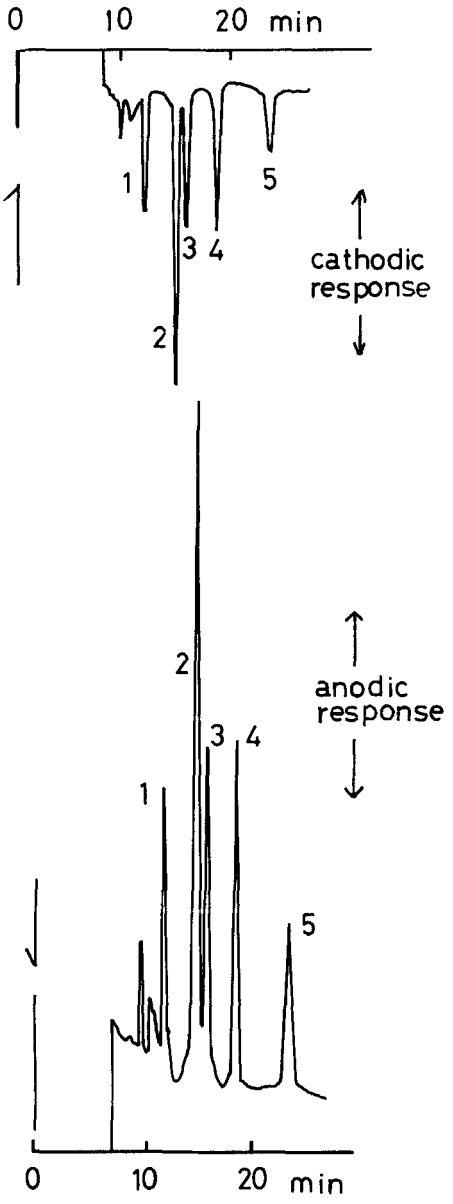


FIGURE 3. High-Performance Liquid Chromatogram of Fatty Acids in Human Serum.
Conditions: TSKgel ODS-80TM, 0.1 M $NaClO_4$ in methanol-H_2O (13:1), 1 ml/min. Upstream electrode +0.6 V, downstream electrode +0.2 V.
1, $C_{18:2}$; 2, C_{16}; 3, $C_{18:1}$; 4, C_{17} (IS); 5, C_{18}.
Interferences of excess reagents due to saturation of the output of the detector were overcome by taking off the connector for ca. 10 min after injection.

TABLE 2
Serum Levels of Principal Fatty Acids in Healthy Subjects

Subject		Serum Concentration (nmole/ml)			
Age (years)	Sex	$C_{18:2}$	C_{16}	$C_{18:1}$	C_{18}
43	M	18.5	78.8	55.9	19.0
26	M	28.5	68.6	73.3	9.8
24	M	30.0	98.3	97.0	22.5
23	M	13.6	42.9	38.9	10.5
22	M	14.3	49.1	24.1	11.2
22	F	22.1	59.3	53.8	12.7
22	F	12.1	38.2	20.5	10.5
Mean		19.9	62.2	51.9	13.7
S.D.		7.2	21.4	27.2	5.0

Abbreviations used are the same as in Table 1.

fluorescence detection [10], but few methods using ECD are available for this purpose [11]. The proposed method using a twin electrode ECD has proved to be highly selective for the determination of carboxylic acids in biological fluids. Further application to the related compounds is being conducted in these laboratories and the details will be reported elsewhere.

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