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2-(4-Hydrazinocarbonylphenyl)-4,5-diphenylimidazole as a versatile fluorescent derivatization reagent for the high-performance liquid chromatographic analysis of free fatty acids

Kenichiro Nakashima*, Youko Taguchi, Naotaka Kuroda and Shuzo Akiyama

School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-Machi, Nagasaki 852 (Japan)

Gengli Duan

School of Pharmacy, Shanghai Medical University, 138 Yi Xue Yuan Road, Shanghai 200032 (People's Republic of China)

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ABSTRACT

A new fluorescent derivatization reagent with a lophine skeleton, 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole (HCPI), has been synthesized and applied to the assay of saturated free fatty acids. The HCPI derivatives with five representative saturated fatty acids [caproic acid (C_6), lauric acid (C_{12}), palmitic acid (C_{16}), stearic acid (C_{18}) and arachidic acid (C_{20})] were synthesized to examine their fluorescence properties. The fluorescence spectra (λ_{ex} *ca.* 335 nm, λ_{em} *ca.* 455 nm) of the derivatives were almost identical in methanol, *n*-heptane and acetonitrile. For the high-performance liquid chromatographic assay, fatty acids were derivatized with HCPI using a condensing agent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and pyridine at room temperature. The HCPI derivatives were separated on a C_{18} column with a gradient elution of methanol–water, and the eluates were monitored at 455 nm using an excitation wavelength of 335 nm. By this method, seven kinds of free fatty acid (C_6, C_{10}, C_{12}, C_{14}, C_{16}, C_{18} and C_{20}) could be determined using margaric acid (C_{17}) as an internal standard. Calibration curves were linear over the range 0.2–400 pmol per 20-μl injection (r = 0.994–1.000). Relative standard deviations of the peak-height ratio for five replicate measurements of fatty acids (100 pmol per injection) were 0.7–2.6%. Detection limits were 7–57 fmol at a signal-to-noise ratio of 3. The method could be successfully applied to the determination of four saturated fatty acids (C_{12}, C_{14}, C_{16} and C_{18}) in normal human serum.

INTRODUCTION

The sensitive determination of fatty acids is very important in biological or biomedical chemistry. Therefore, various fluorescent derivatization reagents have been developed for the determination of fatty acids by high-performance liquid chromatography (HPLC): *e.g.* 4-bromomethyl-7-methoxycoumarin (Br-Mmc) [1–3], 9-anthryldiazomethane (ADAM) [4,5], 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ) [6,7], 3-bromomethyl-6,7-methylenedioxy-1-methyl-2(1*H*)-quinoxalinone (Br-MMEQ) [8], monodansyl cadaverine (MDC) [9,10], 2-(2,3-naphthalimino)ethyl trifluoromethanesulphonate (NE-OTf) [11], 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide [12] and 4-substituted 7-aminoalkylamino-2,1,3-benzoxadiazoles [13,14].

* Corresponding author.

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Generally, these reagents react with fatty acids to give highly intense fluorescent derivatives. Among these, Br-MMEQ is the most sensitive fluorescent derivatization reagent for fatty acids and gives sub-femtomole detection limits. However, the reaction of Br-MMEQ with fatty acids requires heating to complete the derivatization. On the other hand, MDC, NE-OTf, 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide and 4-substituted 7-aminoalkylamino-2,1,3-benzoxadiazole react with fatty acids at room temperature to give fluorescent derivatives.

Lophine (2,4,5-triphenylimidazole) is one of the well known classical chemiluminescent compounds [15], but there are a few examples of its analytical applications [16–19]. Thus we were interested in the application of lophine derivatives as analytical reagents. In the previous study, we found that compounds with a lophine skeleton fluoresce intensely, and we synthesized some lophine derivatives to examine them as fluorescent substrates for lipase [20].

This paper describes the use of a lophine derivative, 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole (HCPI), as a versatile fluorescent derivatization reagent for fatty acids. HCPI has a hydrazinocarbonyl group as a reactive group, as well as 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide, and reacts with fatty acids to give intensely fluorescent derivatives at room temperature by using a condensing reagent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and pyridine. This derivatization was used for the HPLC assay of saturated free fatty acids in human serum.

EXPERIMENTAL

Apparatus

Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650-10S fluorescence spectrophotometer (Tokyo, Japan) in 10-mm quartz cells with 10 nm bandwidths for both the excitation and emission monochromators. Infrared spectra were measured on a Jasco IRA 810 spectrophotometer (Tokyo, Japan). Melting temperatures were measured on a Yanagimoto MP-53 melting-temperature apparatus (Tokyo, Japan) and uncorrected.

Chemicals

Saturated fatty acids (valeric acid, C_5; caproic acid, C_6; caprylic acid, C_8; capric acid, C_{10}; lauric acid, C_{12}; myristic acid, C_{14}; palmitic acid, C_{16}; margaric acid, C_{17}; stearic acid, C_{18}; arachidic acid, C_{20}) and unsaturated fatty acids (palmitoleic acid, $C_{16:1}$; oleic acid, $C_{18:1}$; linoleic acid, $C_{18:2}$; linolenic acid, $C_{18:3}$, arachidonic acid, $C_{20:4}$) were purchased from Sigma (St. Louis, MO, USA). Stock solutions of the acids ($1 \cdot 10^{-3}$ *M*) were prepared in N,N-dimethylformamide (DMF) and diluted with DMF to obtain the appropriate concentrations for the working solutions. EDC was obtained from Nacalai Tesque (Tokyo, Japan). Water was deionized and distilled once. All other chemicals used were reagent grade.

Synthesis of HCPI and its derivatives with saturated free fatty acids (Fig. 1)

4-(4,5-Diphenyl-1*H*-imidazol-2-yl)benzoic acid methyl ester (**1**) was synthesized according to Ito's method [21]. To ammonium acetate (10.0 g) in 30 ml of acetic acid were added benzil (3.15 g, 0.015 mol) and terephthalaldehydic acid methyl ester (2.46 g, 0.015 mol), and the mixture was heated at 80°C for 9 h under stirring. After cooling to room temperature, the reaction mixture was poured into cold water. The resulting precipitates were filtered, washed with water, and recrystallized from ethanol to give pale yellow crystals: yield, 4.31 g (81%), m.p., 245–248°C. Elemental analysis: calculated for $C_{23}H_{18}N_2O_2$: C, 77.95%; H, 5.12%; N, 7.91%; found: C, 77.94%; H, 5.23%; N, 7.90%. IR λ_{max} (KBr, cm^{-1}): 1700 (C=O), 3350 (NH).

For the synthesis of HCPI (**2**), compound **1** (1.47 g, 4.15 mmol) in 100 ml of ethanol was treated with 80% hydrazine hydrate (15 ml) and refluxed for 4 h. After cooling to room temperature, the mixture was poured into cold water. The resulting precipitate was filtered, washed with water, and recrystallized from ethanol–ben-

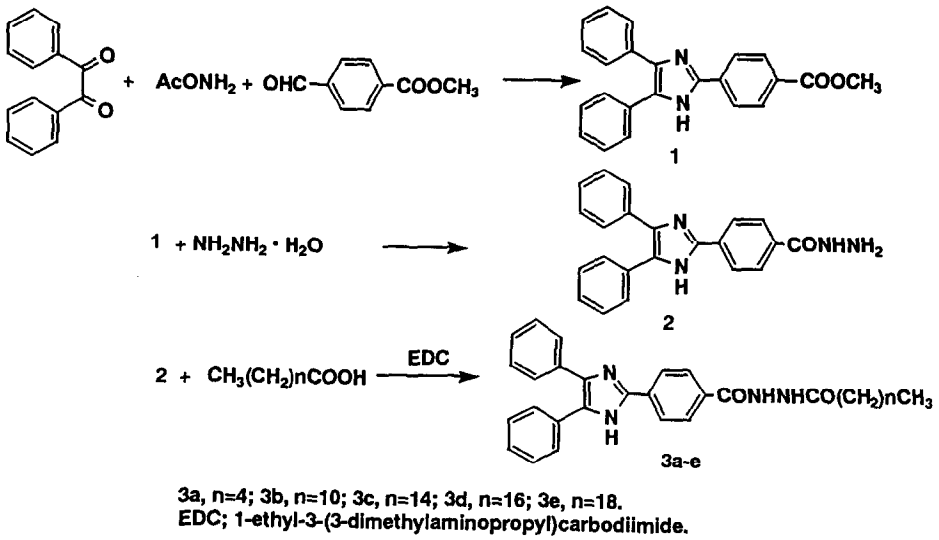

Fig. 1. Synthesis of HCPI and its derivatives with saturated free fatty acids.

zene (1:1, v/v) to give colourless powders: yield, 0.9 g (61%); m.p., > 300°C. Elemental analysis: calculated for $C_{22}H_{18}N_4O$: C, 74.55%; H, 5.12%; N, 15.81%; found: C, 74.65%; H, 5.26%; N, 15.64%. IR λ_{max} (KBr, cm^{-1}): 1610 (C=O), 3050–3200 (broad, NH).

For the synthetis of the HCPI derivatives of fatty acids, HCPI (0.1 g, 0.28 mmol), EDC (0.07 g, 0.28 mmol) and the fatty acid (0.28 mmol) were dissolved in 20 ml of ethanol containing 2% pyridine, and stirred for 3 h at room temperature. The reaction mixture was evaporated to dryness and the residue was recrystallized from ethanol.

HCPI-caproic acid (**3a**) was obtained as colourless crystals: yield, 59%; m.p., 264–267°C. Elemental analysis: calculated for $C_{28}H_{28}N_4O_2 \cdot H_2O$: C, 71.47%; H, 6.43%; N, 11.91%; found: C, 71.21%; H, 6.37%; N, 11.87%. IR λ_{max} (KBr, cm^{-1}): 1650 (C=O), 3230 (NH).

HCPI-lauric acid (**3b**) was obtained as colourless crystals: yield, 48%; m.p., 239–241°C. Elemental analysis: calculated for $C_{34}H_{40}N_4O_2$: C, 76.09%; H, 7.51%; N, 10.44%; found: C, 76.12%; H, 7.52%; N, 10.42%. IR λ_{max} (KBr, cm^{-1}): 1605 (C=O), 3200 (NH).

HCPI-palmitic acid (**3c**) was obtained as colourless crystals: yield, 12%; m.p., 230–232°C. Elemental analysis: calculated for $C_{38}H_{48}N_4O_2$: C, 76.99%; H, 8.16%; N, 9.45%; found: C, 76.57%; H, 8.16%; N, 9.34%. IR λ_{max} (KBr, cm^{-1}): 1605 (C=O), 3200 (NH).

HCPI-stearic acid (**3d**) was obtained as colourless crystals: yield, 23%; m.p., 229–231°C. Elemental analysis: calculated for $C_{40}H_{52}N_4O_2$: C, 77.38%; H, 8.44%; N, 9.03%; found: C, 77.15%; H, 8.41%; N, 9.09%. IR λ_{max} (KBr, cm^{-1}): 1605 (C=O), 3180 (NH).

HCPI-arachidic acid (**3e**) was obtained as colourless crystals: yield, 71%; m.p., 225–227°C. Elemental analysis: calculated for $C_{42}H_{56}N_4O_2$; C, 77.73%; H, 8.70%; N, 8.63%; found: C, 77.28%; H, 8.75%; N, 8.46%. IR λ_{max} (KBr, cm^{-1}): 1605 (C=O), 3150 (NH).

Derivatization procedure

To 100 μl of a working solution of fatty acids were added 50 μl each of 1.0 *M* EDC in water, 10% pyridine in water and 100 μl of 30 m*M* HCPI in DMF. The mixture was mixed well and allowed to stand for 45 min at room temperature, and a 20-μl portion was injected into the HPLC column. For the reagent blank, 100 μl of DMF were used instead of a working solution.

Preparation of serum sample

To 50 μl of serum in a 10-ml screw-capped test-tube were added 10 μl of $1 \cdot 10^{-4} M$ (or $1 \cdot 10^{-3} M$) margaric acid in DMF as an internal standard and 200 μl of 0.5 *M* phosphate buffer (pH 6.5). After mixing, 2 ml of chloroform–*n*-heptane (1:1, v/v) were added to the mixture, vortex-mixed for 1 min, and centrifuged at 1500 *g* for 5 min. A 1.5-ml aliquot of the organic layer was evaporated and dissolved in 200 μl of DMF, and a 100-μl sample of the resulting DMF solution was used for the derivatization procedure described above.

HPLC apparatus and conditions

The HPLC system consisted of two pumps (LC-6A, Shimadzu, Kyoto, Japan), a 7125 injector with a 20-μl loop (Rheodyne, Cotati, CA, USA), a Shim-pack CLC-ODS separation column (150 mm × 6.0 mm I.D., 5 μm, Shimadzu), a Shimadzu RF-550 fluorescence spectrophotometer with a 12-μl flow-cell operated at an excitation wavelength of 335 nm and an emission wavelength of 455 nm, a Hitachi 561 recorder (Tokyo, Japan) and a Shimadzu SCL-6A system controller. HCPI derivatives were separated at ambient temperature using methanol–water as a mobile phase with gradient elution. For the separation of authentic fatty acid derivatives with HCPI, the flow-rate used was 1.0 ml/min with isocratic elution from 0 to 15 min (70% methanol), gradient elution from 15 to 30 min (70–100% methanol) and isocratic elution from 30 to 45 min. In the case of serum sample, the flow-rate was 1.0 ml/min with isocratic elution from 0 to 5 min (90% methanol), gradient elution from 5 to 30 min (90–100% methanol) and isocratic elution from 30 to 35 min (100% methanol).

RESULTS AND DISCUSSION

Fluorescence properties of HCPI-fatty acid derivatives

Fluorescence spectra of HCPI-fatty acids (C_6, C_{12}, C_{16}, C_{18}, C_{20}) were measured in methanol, aqueous methanol, acetonitrile and *n*-heptane. Fluorescence excitation maxima (λ_{ex}), emission maxima (λ_{em}) and relative fluorescence intensities (RFI) of the derivatives are listed in Table I. Fluorescence spectra of HCPI-arachidic acid as a representative are shown in Fig. 2. The emission maxima of the derivatives showed small red-shifts compared with that of HCPI in all the solvents used. The largest RFI was obtained with HCPI-C_{16} in acetonitrile. RFIs were larger in a polar medium than in a non-polar one. The water content in the methanol slightly affected the exci-

TABLE I

FLUORESCENCE PROPERTIES OF HCPI DERIVATIVES OF C_6, C_{12}, C_{16}, C_{18}, AND C_{20}

Sample	λ_{max} (nm)		RFI[a]
	Excitation	Emission	
Acetonitrile			
HCPI	335	440	61
C_6	335	445	94
C_{12}	335	445	85
C_{16}	335	445	100
C_{18}	335	445	88
C_{20}	335	445	94
Methanol			
HCPI	335	450	44
C_6	335	455	86
C_{12}	335	455	72
C_{16}	335	455	89
C_{18}	335	455	80
C_{20}	335	455	88
80% Methanol			
HCPI	335	455	57
C_6	340	470	76
C_{12}	340	465	77
C_{16}	340	465	89
C_{18}	340	465	76
C_{20}	340	465	79
n-Heptane			
HCPI	335	430	40
C_6	335	435	68
C_{12}	340	435	63
C_{16}	335	435	80
C_{18}	335	435	73
C_{20}	340	435	70

[a] Relative fluorescence intensity: RFI of C_{16} in acetonitrile was arbitrarily taken as 100.

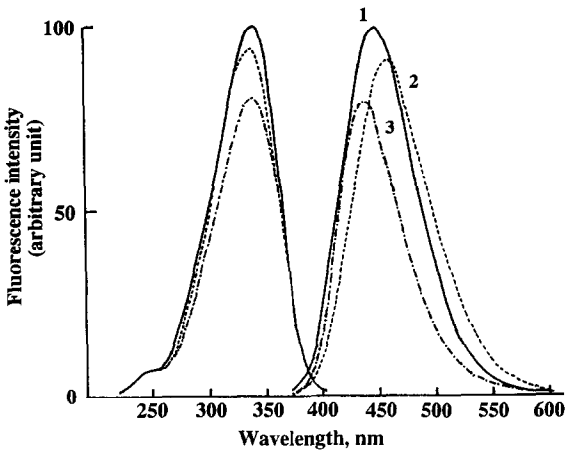

Fig. 2. Fluorescence spectra of HCPI-arachidic acid in (1) acetonitrile, (2) methanol and (3) *n*-heptane. A stock solution ($2.82 \cdot 10^{-5}$ *M*) of the derivative in methanol was diluted 100 times with each solvent.

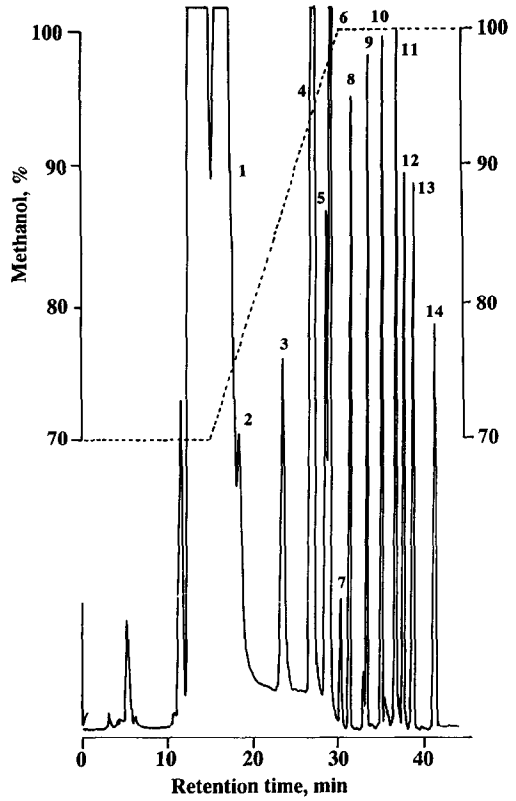

Fig. 3. Chromatogram of the HCPI-fatty acid derivatives. A 100-μl portion of a standard mixture of the fatty acids (5 nmol/ml each) was treated according to the experimental procedure. Peaks: 1, 4, 6, and 7 = reagent blank; 2 = C_5; 3 = C_6; 5 = C_8; 8 = C_{10}; 9 = C_{12}; 10 = C_{14}; 11 = C_{16}; 12 = C_{17} (I.S.); 13 = C_{18}; 14 = C_{20}.

tation and emission wavelengths, and the RFIs of the derivatives. These results suggest that aqueous methanol or acetonitrile might be useful to prepare the mobile phase for the reversed-phase separation of HCPI–fatty acids.

Separation of HCPI-fatty acid derivatives

The separation of HCPI-fatty acid derivatives was studied on a reversed-phase column, Shimpack CLC-ODS, using isocratic elution from 0 to 15 min (70%), gradient elution from 15 to 30 min (70–100%), and isocratic elution from 30 to 45 min (100%). A typical chromatogram obtained with ten saturated fatty acids is shown in Fig. 3. All peaks except for C_5 and C_8 were well separated within 45 min. The major peaks appearing just ahead of 2, between 3 and 4, following 4, and before 8 are based on the reagent blank.

Derivatization conditions

A mixture of fatty acids (C_6, C_{10}, C_{12}, C_{14}, C_{16}, C_{17}, C_{18}, C_{20}) in DMF was used as a test sample for the determination of the derivatization conditions. The effect of varying the HCPI concentration from 10 to 40 m*M* was examined. Constant and maximum peak heights were obtained over the range 20–40 m*M*; 30 m*M* was used for subsequent experiments.

The EDC concentration also affected the reaction yields of HCPI derivatives. The effect on the peak heights of varying the concentration of EDC in water from 0.1 to 2.5 *M* was studied. Constant and maximum values were obtained over 1.0 *M*. On the other hand, it was also shown that the baseline of the chromatogram gradually increased in proportion to the EDC concentration: 1.0 *M* concentration was selected.

An aqueous pyridine solution (5–20%) as a catalyst showed no significant change on the peak heights of the derivatives; 10% pyridine was chosen as a sufficient concentration.

The derivatization reaction could be carried out at room temperature. Thus the effect of the reaction time on the yield was examined (Fig. 4). The reaction seemed to be almost complete within 45 min under the conditions used. However, the peak heights of the resulting products slightly

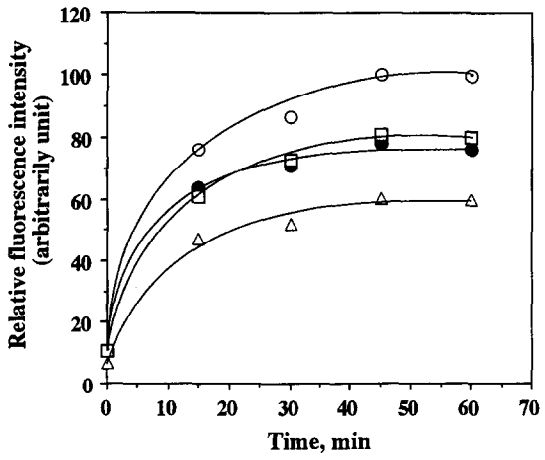

Fig. 4. Effect of reaction time on the formation of HCPI-fatty acid derivatives. A 100-μl portion of a standard mixture (5 nmol/ml each) was treated at room temperature according to the procedure: (○) C_{16}; (●) C_{17}; (□) C_{18}; (△) C_{20}.

increased after 48 h in the dark at room temperature. The reaction yields for some derivatives were determined by comparing the peak heights with those of standard authentic samples: the yields were 76% (C_{12}), 72% (C_{16}), 79% (C_{18}) and 74% (C_{20}). From these results, in this experiment, a peak-height ratio method was used for the preparation of calibration curves.

Calibration curve, detection limit and precision

The calibration curves (peak-height ratios with respect to the internal standard, C_{17}, *versus* concentration) for fatty acids (C_6, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, C_{20}) were linear over the range from 0.2 to 400 pmol per injection (r = 0.994–1.000). The lower limits of detection were 7–57 fmol at a signal-to-noise ratio of 3. The sensitivity of this method is comparable with that of the MDC method (below 100 fmol) [9,10], but lower than those of the methods using Br-MMEQ (0.2–0.8 fmol) [8], NE-OTf(4 fmol) [11], 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide (3–6 fmol) [12] and 4-(N,N-dimethylaminosulphonyl)-7-N-piperazino-2,1,3-benzoxadiazole (3.2–4.7 fmol) [14]. Relative standard deviations of the peak-height ratio for five replicate measurements of fatty acids (100 pmol per injection) were 0.7–2.6%.

Application to the determination of saturated free fatty acids in serum

Fig. 5 shows a typical chromatogram obtained with normal human serum. The free fatty acids from human serum were extracted with a chloroform–*n*-heptane (1:1, v/v) as previously reported [22,23]. Some peaks other than HCPI derivatives of saturated fatty acids appeared on the chromatogram. As a preliminary experiment, some unsaturated fatty acids ($C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$ and $C_{20:4}$) were confirmed to be effectively derivatized with HCPI by the method described. The retention times for the HCPI derivatives of unsaturated fatty acids were 17.5 min ($C_{18:3}$), 18.6

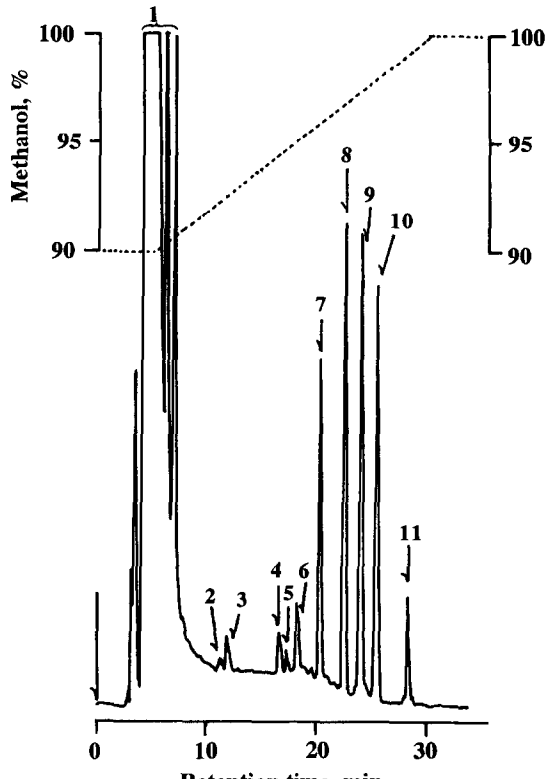

Fig. 5. Chromatogram of HCPI-fatty acid derivatives in normal human serum. A 50-μl portion of normal human serum was treated according to the procedure. Peaks: 1 and 3 = reagent blank; 2 = C_{12}; 4 = C_{14}; 5 = $C_{18:3}$; 6 = $C_{16:1}$; 7 = $C_{18:2}$; 8 = C_{16}; 9 = $C_{18:1}$; 10 = C_{17} (I.S.); 11 = C_{18}. For other conditions, see Experimental.

min ($C_{16:1}$), 20.8 min ($C_{18:2}$, $C_{20:4}$) and 24.5 min ($C_{18:1}$) by the HPLC conditions used in Fig. 5. The HCPI derivatives of $C_{18:2}$ and $C_{20:4}$ could not separated from each other under the conditions used. The peaks other than the HCPI derivatives of saturated fatty acids on the chromatogram of normal human serum were qualitatively determined by co-chromatography. However, the quantitative determination of unsaturated fatty acids has not yet been achieved. The details will be published elsewhere.

Working curves were prepared by using spiked serum with known concentrations of fatty acids (C_{12}, C_{14}, C_{16}, and C_{18}). The internal standard was 20 nmol/ml C_{17} (for C_{12}, C_{14}, and C_{18}) or 200 nmol/ml C_{17} for C_{16}. The peak-height ratios of each fatty acid to the internal standard were linear from 5 to 50 nmol/ml of serum for C_{12}, C_{14} and C_{18} (r = 0.995–0.999) and from 50 to 400 nmol/ml of serum for C_{16} (r = 0.994).

Recovery tests were carried out by adding 2 nmol of each fatty acid to 50 μl of serum. The recoveries (mean ± S.D., n = 5) were 99.6 ± 3.9% (C_{12}), 88.6 ± 1.8% (C_{14}), 108.2 ± 3.1% (C_{16}), 76.0 ± 2.8% (C_{17}) and 41.6 ± 1.4% (C_{18}). The precision was established from five replicate measurements using a normal serum. The relative standard deviations of the peak-height ratio for C_{12}, C_{14}, C_{16} and C_{18} were 3.0, 3.7, 1.6, and 1.9%, respectively.

The concentrations of saturated free fatty acids in sera from healthy volunteers were determined by the proposed method (Table II). The mean values obtained of free fatty acids were similar to those reported previously [23].

TABLE II

CONCENTRATIONS OF FREE SATURATED FATTY ACIDS IN NORMAL HUMAN SERA

Age	Sex	Concentration (nmol/ml)			
		C_{12}	C_{14}	C_{16}	C_{18}
44	M	11.3	13.4	167.7	50.6
37	M	1.2	4.7	65.2	26.6
33	M	4.2	8.1	115.5	31.1
24	M	3.0	13.9	152.2	48.6
21	M	2.4	8.6	123.6	43.9
23	F	3.6	12.0	164.5	65.8
23	F	1.9	5.3	51.3	19.5
22	F	1.5	5.8	101.4	27.3
21	F	3.9	12.4	116.3	29.2
21	F	4.0	9.6	193.3	41.1
Mean		3.7	9.4	125.1	38.4
S.D.		2.9	3.4	45.4	14.1

CONCLUSION

HCPI can be easily synthesized from commercially available materials. The derivatization reaction of fatty acids with HCPI to give intensely fluorescent derivatives proceeds readily at room temperature. The proposed method is sensitive and can be applied to the determination of free fatty acids in human serum. It may also be applied to the assay of other biologically important carboxylic acids. Furthermore, HCPI is a chemiluminogenic derivatization reagent, and thus could be used for the chemiluminescent detection of fatty acids in HPLC. A chemiluminescent study on the HCPI derivatives of fatty acids is in progress, and the details will be reported elsewhere.

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