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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE FATTY ACIDS WITH 1-NAPHTHYLAMINE

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SUMMARY

A 1% benzene solution of oxalyl chloride was added to saturated and unsaturated fatty acids and the mixture was allowed to react at 70°C for 30 min; by this procedure, each fatty acid was converted into its acid chloride in a considerably quantitative manner. By reacting this acid chloride with 1-naphthylamine at 30°C for 15 min, naphtylamine derivatives were produced, which showed strong ultraviolet absorption around 280—290 nm. Experiments were made on the recovery of the fatty acids added to 0.5 ml of human serum, and the recovery was found to fall in the range of 94—106% (coefficient of variation = 0.5—4.1%) when the following amounts of six fatty acids were added: $C_{14:0}$, 2 μg; $C_{16:0}$, 20 μg; $C_{16:1}$, 5 μg; $C_{18:0}$, 4 μg; $C_{18:1}$, 20 μg; $C_{18:2}$, 10 μg.

INTRODUCTION

In the quantitative analysis of fatty acids by high-performance liquid chromatography (HPLC), UV- and fluorescence-labeling are widely utilized to increase the detection sensitivity of fatty acids. The main derivatizing reagents reported so far are phenacyl bromide [1], 2-naphthacyl bromide [2], *p*-bromophenacyl bromide [3], 1-benzyl-2-tolyltriazene [4], and O-*p*-nitrobenzyl-N,N'-diisopropyl-isourea [5]. Many of the labeling reagents for carboxylic acids reported in the past have been developed mainly for the purpose of direct reaction with carboxylic acids. Therefore, there was a considerable limitation to the types applicable as labeling reagent.

Generally, when synthesizing the amides or esters of carboxylic acids, they are reacted with amines or alcohols after they have been derivatized to acid chorides. We attempted to apply this method to the labeling of free fatty acids (FFA). Thus, instead of producing derivatives from FFA in the free form, by changing the FFA into their acid chlorides it is easy to introduce the amines

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with UV or fluorescence properties into the FFA, thus facilitating the microdetection of FFA. In our previous report [6], we examined the reaction conditions to make the acid chlorides by reacting thionyl chloride ($SOCl_2$) with saturated fatty acids, and binding 1-naphthylamine (NA) containing a primary amine structure to the acid chlorides. As a result, it was found that saturated fatty acids were converted into their acid chlorides by $SOCl_2$, and that the acid chlorides reacted easily with NA, forming NA derivatives exhibiting strong UV absorption around 280 nm. On the basis of these results, we performed the present study in an attempt to establish a more accurate method for the quantitative analysis of FFA, which also involves a labeling method applicable to unsaturated FFA.

EXPERIMENTAL

Reagents

Myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$, PT), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), linoleic acid ($C_{18:2}$, Ll), were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Oleic acid ($C_{18:1}$) and palmitoyl chloride (PT · Cl) were purchased from Sigma (St. Louis, MO, U.S.A.). Thionyl chloride ($SOCl_2$) and margaric acid ($C_{17:0}$) were from Nakarai Chemicals (Kyoto, Japan). Linoleoyl chloride (Ll · Cl), chrysene, 1-naphthylamine (NA) and triethylamine (TEA) were from Tokyo Kasei Kogyo (Tokyo, Japan). Oxalyl chloride, $(COCl)_2$, was purchased from Wako Pure Chemical (Osaka, Japan).

Apparatus

A Hitachi high-performance liquid chromatograph Model 635A equipped with a Hitachi multiwavelength UV monitor was used. Melting point was determined with the Yanagimoto micro melting point apparatus. For measuring the infrared (IR) spectra, a Hitachi grating IR spectrometer 215 was used. ^{1}H-NMR spectra were determined on a JEOL Fx-200 NMR spectrometer with using tetramethylsilane (TMS) as an internal standard. UV spectra and mass spectra were measured with a Shimadzu UV-210A and a Hitachi RMU-7MG, respectively.

HPLC conditions

Column: μBondapak C_{18} (30 × 0.4 cm I.D., particle size 8—10 μm). Detection wavelength: 280 nm. Mobile phase: methanol—water (81:19). Flow-rate: 2.0 ml/min. Column temperature: 40° C.

Preparation of N-linoleoyl-1-naphthylamine (Ll · NA)

Ll · Cl (1 mmole), NA (1 mmole) and TEA (1 mmole) were dissolved in benzene (10 ml) in a reaction vial (15 ml), and were allowed to react with stirring for 30 min in an oil bath at 50° C. The solvent was removed at reduced pressure, and the residue was recrystallized from methanol—water to give Ll · NA: m.p. 41—42° C. IR $\nu_{max}^{CHCl_3}$ cm^{-1} : 1680 (—NHCO). NMR (C^2HCl_3) δ : 0.89 (3H, t, J = 7 Hz, $-CH_2CH_3$), 1.0—1.56 (16H, m, $-(CH_2)_5-$ and $-(CH_2)_3-$), 1.9—2.14 (4H, m, 2 × ($-CH_2-C=$)), 2.51 (2H, t, J = 7 Hz, $-CH_2-CO-$), 2.77 (2H, t, J = 5 Hz, $=C-CH_2-C=$), 5.19—5.48 (4H, m, 2 ×

(—CH=CH—)), 7.4—8.13 (7H, m, aromatic protons). Mass spectrum *m/z*: 405 (M^+).

Preparation of NA solution

NA (57.3 mg, 400 μmoles) was dissolved in benzene to give a total of 10 ml. The prepared solution was kept shielded from the light.

Preparation of TEA solution

TEA (404 mg, 4 mmoles) was dissolved in benzene to give a total of 10 ml.

Method for the quantitative analysis of PT · Cl and Ll · Cl

PT · Cl (4—200 nmoles) or Ll · Cl (14—200 nmoles) dissolved in 0.1 ml of benzene was put into reaction vials to which 0.1 ml of NA solution and 0.01 ml of TEA solution were added; the reaction was carried out at 30°C for 15 min. Then 0.1 ml of benzene solution containing chrysene (50 μg) as an internal standard was added, and the mixture was shaken sufficiently: 2 μl were injected into the chromatograph. On the chromatogram obtained, the ratio of the peak height for the internal standard to that for each acid chloride was measured, and the amount of PT · Cl or Ll · Cl was calculated using the regression equation made in advance.

Method for making the NA derivatives of FFA

FFA (2—1000 nmoles) dissolved in 0.6 ml of benzene was put into a reaction vial and shaken sufficiently after adding 0.6 ml of 2% $(COCl)_2$ solution in benzene. The reaction was carried out for 30 min in an oil bath at 70°C. After the reaction, the solvent was removed at reduced pressure. NA solution (0.1 ml) and TEA solution (0.01 ml) were added; the mixture was reacted at 30°C for 15 min to give the NA derivatives.

Methods for the extraction and the quantitative analysis of serum FFA

For the extraction of FFA from serum, we adopted the column extraction method which we had examined previously [7]. To 0.5 ml of serum was added 0.1 ml of methanol solution containing 10 μg of margaric acid as an internal standard, and then mixed with 1.4 ml of 1/15 *M* phosphate buffer (pH 7.0). After shaking sufficiently, the mixture was poured into a glass column (45 × 12 mm I.D.) packed with 1 g of Extrelut®. After adsorbing for 20 min, FFA was eluted out with 10 ml of chloroform. After removing the solvent at reduced pressure, the residue was redissolved in 0.6 ml of benzene. The reaction was carried out according to the derivatizing method and then 40 μl of the reaction mixture were injected directly into the HPLC apparatus. From the chromatogram obtained, the ratio of the peak height for each FFA to that fo the internal standard was measured, and the amount of each FFA was calculated from the calibration curves previously made for each FFA.

Experiments on the recovery of the FFA added to human serum

Into 0.5 ml of the serum separated immediately after blood sampling were added 0.1 ml of methanol solution containing the internal standard, margaric acid ($C_{17:0}$) 10 μg, and FFA of the following composition: $C_{14:0}$ 2 μg, $C_{16:0}$

20 μg, $C_{16:1}$ 5 μg, $C_{18:0}$ 4 μg, $C_{18:1}$ 20 μg, $C_{18:2}$ 10 μg. Separation and assay of FFA were performed according to the methods described above. From the values obtained, the amount of each FFA contained in the untreated fresh serum was subtracted, which was divided by the added amount to give the recovery ratio.

RESULTS AND DISCUSSION

Introduction of NA into FFA

As a result of the determination of the structure of the products of the reaction of NA with Ll · Cl, the acid chloride of Ll, it was confirmed that the structure was N-linoleoyl-1-naphthylamine (Ll · NA), which was formed by the binding of NA to the carboxyl group of Ll. In addition, UV spectra were determined using the methanol solution of Ll · NA obtained herein; as a result, as in the case of PT · NA shown in the previous report [6], strong absorption was found around 280—290 nm, which was not observed with NA (Fig. 1). These results indicate that NA which contains a primary amine can easily be introduced into saturated and unsaturated fatty acids by derivatizing them into acid chlorides. Therefore, the optimal condition when introducing NA into FFA was examined. The derivatizing method performed in the present study consisted of two reactions; derivatization of FFA into acid chlorides, and reaction of acid chlorides with NA. Thus, these two methods were examined separately.

(1) Derivatization of FFA into acid chlorides. Since the condition for the reaction to make acid chlorides of the saturated fatty acids was already examined in the previous report [6], the examination was made on the unsaturated fatty acids in the presents study. Ll was selected as the unsaturated

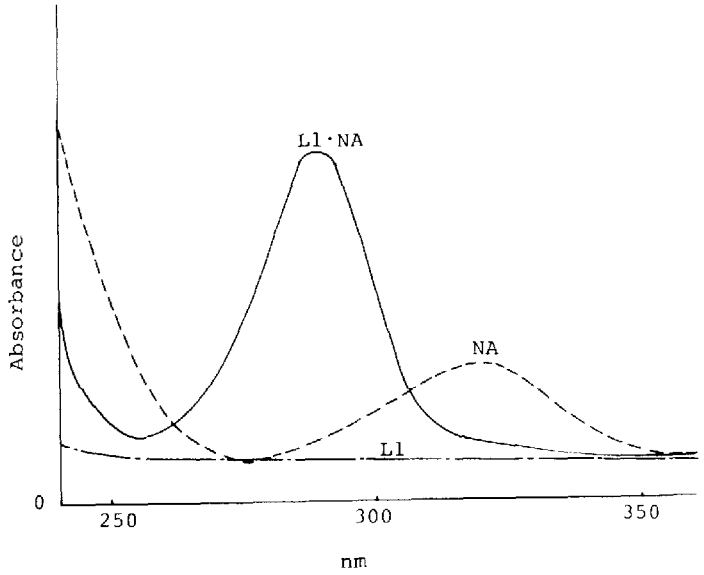

Fig. 1. Absorption spectra of Ll · NA and NA in methanol solution. Ll · NA = N-linoleoyl-1-naphthylamine, NA = naphthylamine, Ll = linoleic acid.

fatty acid, and the reaction was carried out utilizing a benzene solution of 50% $SOCl_2$ [8, 9] as the derivatizing reagent. The Ll · Cl produced was transformed to its NA derivative, and measured according to the method described in the Experimental section. As shown in Fig. 2a, the reaction ratio from Ll to Ll · Cl was low (about 17%), and several peaks other than that corresponding to Ll · Cl were observed. In the case of saturated fatty acids, their acid chlorides were produced by reaction with $SOCl_2$ at the yield of almost 100%. Therefore, in contrast to saturated fatty acids, it was found that the reaction ratio from unsaturated fatty acids to their acid chlorides was low when using $SOCl_2$. Thus, in the next step, $(COCl)_2$ was investigated as the derivatizing reagent [10]. Reaction was carried out by adding a 50% benzene solution of $(COCl)_2$ to Ll. The yield of Ll · Cl was high (about 88%) and side-products were not formed (Fig. 2b). From these results, it was decided to use $(COCl)_2$ for derivatizing FFA, including unsaturated fatty acids, into their acid chlorides, and the optimal concentration of $(COCl)_2$ was therefore examined. $(COCl)_2$ was adjusted at six different concentrations with benzene: 50, 10, 5, 1, 0.5 and 0.1%, and each solution was reacted with PT or Ll. The acid chlorides produced were changed into NA derivatives and the measurements made; the amounts of acid chlorides were calculated on the basis of NA derivatives. The yield of the acid chlorides increased with increasing concentration of $(COCl)_2$ in the case of both PT and Ll. The maximum yields were obtained using 1% $(COCl)_2$ (PT, 100%; Ll, 92%); there was little change in the yield of acid chloride if the concentration of $(COCl)_2$ was further increased (Fig. 3). Therefore, the concentration of $(COCl)_2$ for derivatizing FFA into their acid chlorides was set at 1%.

In the next step, we examined the optimal reaction temperature and reaction time when leading FFA into their acid chlorides with using 1% $(COCl)_2$. PT and

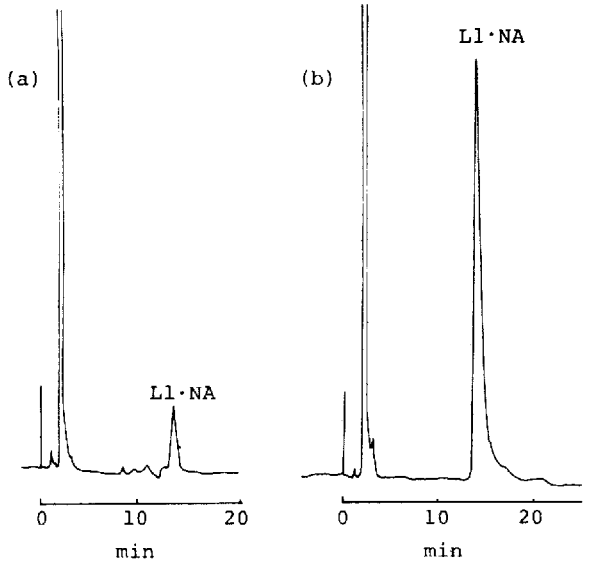

Fig. 2. Comparison of reagents to prepare Ll · Cl from Ll. (a) Ll (0.78 μmole) was reacted with 50% $SOCl_2$ in benzene; (b) Ll (0.78 μmole) was reacted with 50% $(COCl)_2$ in benzene. Ll · Cl produced was changed into the NA derivative for determination by HPLC.

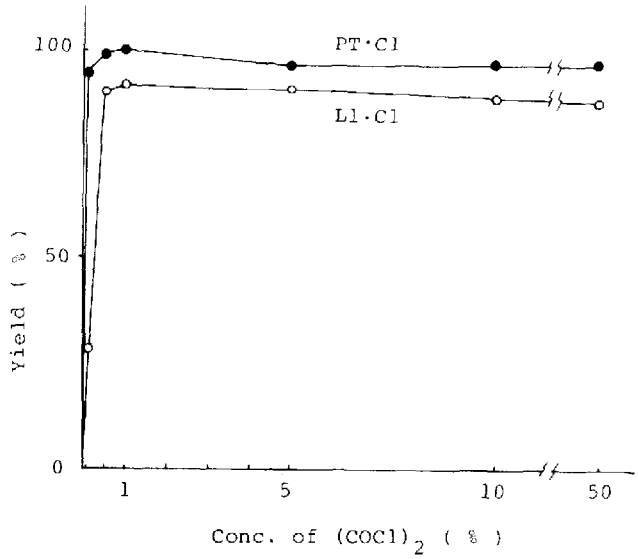

Fig. 3. Effect of concentration of $(COCl)_2$ on production of PT · Cl and Ll · Cl. Each 0.78 μmole of PT and Ll was dissolved in 0.2 ml of six different concentrations of $(COCl)_2$ solution. The mixture was heated at 70°C for 30 min. Each PT · Cl and Ll · Cl thus produced was changed into NA derivatives.

Ll were selected as the saturated and unsaturated fatty acids, respectively, and they were reacted in a 1% benzene solution of $(COCl)_2$ with varying temperatures and reaction times. The production of PT · Cl from PT increased with increasing reaction temperature: PT · Cl was produced at a yield of about 100% after the reaction at 70°C for 30 min. Further increase in temperature up to 90°C resulted in a decrease in the production of acid chlorides compared to the value at 70°C. Moreover, when the reaction was carried out at 90°C, the amount of acid chloride produced varied considerably depending on the reaction time. With respect to Ll, the production of Ll · Cl increased with increasing reaction temperature, and a maximum yield of 92% was obtained at 70°C. Thus, the yield of the reaction from Ll to its acid chloride was found to be low compared to PT. From these results, the temperature and time of reaction for converting FFA into acid chlorides were fixed at 70°C and 30 min, respectively.

(2) Reaction of acid chlorides with NA. With respect to the condition for the reaction of PT · Cl with NA, as described in the previous report [6], PT · NA was confirmed to be produced at a yield close to 100% after reaction at 30°C for 15 min. In the present study, the reaction with NA and Ll · Cl was examined. To the benzene solution of Ll · Cl was added the NA solution, and TEA solution to neutralize a side-product, HCl. The amount of Ll · NA produced was compared by varying the reaction time and temperature. The amounts of Ll · NA were calculated from the calibration curve which was constructed using the derivative prepared according to the method described in the experimental section as a standard and using chrysene as an internal standard. It was found that changes in reaction temperature and time hardly affected the amount of NA derivative produced, and that Ll · NA was obtained at a yield of almost 100% even at a temperature as low as 30°C and a short

reaction time (15 min). These results coincide with those for the reaction conditions for PT · Cl with NA; thus, the conditions for the reaction of acid chlorides with NA were decided to be 30°C and 15 min.

HPLC of NA derivatives of FFA

Seven NA derivatives were produced of the main FFA contained in human serum — $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ — and an internal standard, $C_{17:0}$. Examinations on the separation of these derivatives by HPLC indicated that good separation was obtained when using methanol—water (81:19) for the mobile phase and operating at the flow-rate of 2.0 ml/min (Fig. 4). Before applying the present derivatizing method to the quantitative analysis of human serum FFA with HPLC, calibration curves were made from the chromatogram obtained by injecting the following amounts of FFA for HPLC: $C_{14:0}$, 0.73—2.91 μg; $C_{16:0}$, 3.64—14.56 μg; $C_{16:1}$, 0.73—2.91 μg; $C_{18:0}$, 0.73—2.91 μg; $C_{18:1}$, 3.64—14.56 μg; $C_{18:2}$, 1.46—5.28 μg. The regression lines between the amount injected into the column (X) and the peak height ratio (Y) to the internal standard ($C_{17:0}$, 3.64 μg) were: $C_{14:0}$, $Y = 0.788X - 0.080$ ($R = 0.998$); $C_{16:0}$, $Y = 0.382X + 0.125$ ($R = 0.999$); $C_{16:1}$, $Y = 0.589X + 0.05$ ($R = 0.999$); $C_{18:0}$, $Y = 0.192X + 0.04$ ($R = 0.998$); $C_{18:1}$, $Y = 0.320X + 0.115$ ($R = 0.999$): $C_{18:2}$, $Y = 0.421X + 0.130$ ($R = 0.999$). The detection limit for $C_{16:0}$ was 4 ng, assuming a signal-to-noise ratio of 3.

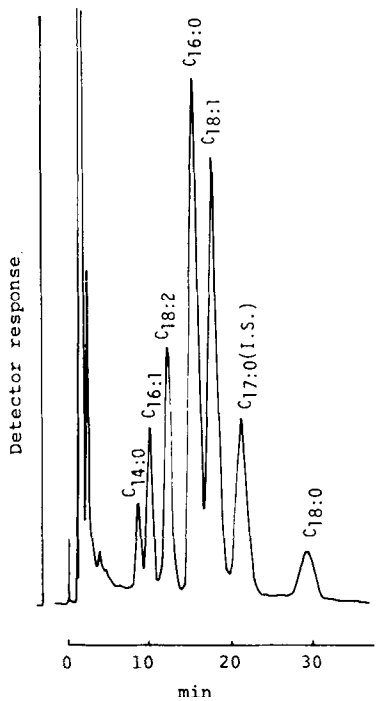

Fig. 4. HPLC of some NA-derivatized FFA. Column: μBondapak C_{18}. Mobile phase: methanol—water (81:19). Flow-rate: 2.0 ml/min. Detector: 280 nm. Temperature: 40°C.

Amount of human serum FFA determined by the present method

Before determining the amount of human serum FFA with the present derivatizing method, we performed the recovery experiments of FFA added to human serum. Six FFA were selected, which are the main FFA contained in human serum. The amount of each FFA added was close to that naturally contained in 0.5 ml of human serum. The percentage recovery of each FFA was calculated, and was found to fall in the range 94—106% for all six FFA (coefficient of variation = 0.5—4.1%, n = 4). Thus, it was confirmed that the present derivatizing method can be applied to the quantitative analysis of serum FFA (Table I).

TABLE I

ANALYTICAL RECOVERY OF FATTY ACIDS ADDED TO HEALTHY HUMAN SERUM

Fatty acid	Added* (μg)	Found** (μg)	Recovery** (%)	C.V. (%)
$C_{14:0}$	2.0	2.0 ± 0.1	100.0 ± 4.1	4.1
$C_{16:0}$	20.0	21.0 ± 0.3	105.0 ± 1.4	1.3
$C_{16:1}$	5.0	4.9 ± 0.1	98.0 ± 1.6	1.6
$C_{18:0}$	4.0	3.8 ± 0.1	94.0 ± 3.4	3.6
$C_{18:1}$	20.0	21.2 ± 0.3	105.8 ± 1.5	1.4
$C_{18:2}$	10.0	10.3 ± 0.1	103.3 ± 0.5	0.5

*Each FFA was added to 0.5 ml of serum.
**Mean ± S.D., n = 4.

In the next step, blood was collected from five volunteers (adult, either sex), and the serum was immediately separated. The amount of FFA in 0.5 ml of each serum was determined with the present method (Table II). Values obtained were in good agreement with the amount of each FFA in normal human serum reported previously [11, 12]. Moreover, the amount of fatty acid was repeatedly determined in a particular person, and the amount of each fatty acid can be determined accurately with a coefficient of variation of 1.4—5.9%, as shown in Table III. Especially, when compared with the results of repeated determinations of serum FFA with gas chromatography as reported by Haan et al. [13], $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ were found to be determined more accurately with the present method.

TABLE II

FFA CONCENTRATION IN HEALTHY HUMAN SERUM DETERMINED BY HPLC

Volunteer	FFA (μg/ml)					
	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
M.N.	3.4	18.8	2.0	3.0	21.0	18.4
Y.I.	3.2	16.4	1.4	2.8	12.4	7.4
K.S.	4.0	21.0	2.2	2.2	25.4	22.2
M.S.	1.8	7.8	1.2	0.8	8.2	5.2
M.I.	4.0	19.6	2.4	6.4	27.6	24.6

TABLE III

REPRODUCIBILITY OF FOUR ANALYSES OF THE SAME HEALTHY HUMAN SERUM

	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Mean (μg per 0.5 ml)	1.7	8.5	1.4	1.8	9.2	7.4
S.D.	0.1	0.4	0.1	0.1	0.3	0.1
C.V. (%)	5.9	4.7	7.1	5.6	3.3	1.4

From the experimental results described, the following conclusion can be drawn. In the present derivatizing method, simply by converting fatty acids into their acid chlorides, the primary amine which has rarely been utilized as the UV- or fluorescence-labeling reagent in the past was found to be applicable to the determination of fatty acids. Thus, the present method is considered to be widely applicable as a labeling method for fatty acids because there are many reagents containing primary amines which exhibit UV absorption or fluorescence. Moreover, quantitative analysis of FFA in healthy human serum was performed with the present method, and it was confirmed that the method can be applied to the clinical fields.

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