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Liquid chromatography–mass spectrometry of hydroxy and non-hydroxy fatty acids as amide derivatives

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ABSTRACT

A useful method for analyzing fatty acids by liquid chromatography–mass spectrometry with an atmospheric-pressure chemical-ionization interface system has been developed. The sensitivity of six kinds of palmitamide derivatives monitored by a single ion of $[M+H]^+$ was, in decreasing order: N-*n*-propylamide > anilide > N,N-diethylamide, amide > N,N-diphenylamide > N-1-naphthylamide. Individual fatty acids were identified from a mixture of amide derivatives of authentic fatty acids from $C_{16:0}$ to $C_{30:0}$ on a mass chromatogram. This method was used to detect both hydroxy and non-hydroxy fatty acids. Many kinds of fatty acid, including hydroxy fatty acids of the rat brain, were detected in a single run.

INTRODUCTION

Although the most commonly used method for fatty acid analysis at present is gas chromatography–mass spectrometry (GC–MS), some problems are still encountered. For example, shortening of the GC-column is inevitable to vaporize and detect extremely long-chain fatty acids [1,2], and repetition of GC–MS with columns of different polarities is sometimes necessary to obtain the best separation of fatty acids in samples. Hydroxy fatty acids are found widely not only in animals, but also in plants, bacteria and fungi. To detect hydroxy fatty acids by GC, it is usually necessary to separate hydroxy fatty acid groups from non-hydroxy fatty acid groups and analyse them separately. Polyunsaturated hydroxy fatty acids, prostaglandins and some other hydroxy fatty acids are not vaporized as easily as non-hydroxy fatty acids.

As reported previously [3], anilide derivatives of fatty acids have been found to be suitable for liquid chromatography (LC)–MS with an atmospheric-pressure chemical-ionization (APCI) interface system. In this study, we examined other amide derivatives to find a more suitable derivative for analysing fatty acids by LC–MS. An N-*n*-propylamide derivative was found to be excellent. A method for preparing hydroxy fatty acid amides was developed, and hydroxy and non-hydroxy fatty acids in the rat brain were simultaneously detected in a single LC–MS run.

EXPERIMENTAL

Reagents

Icosanoic acid ($C_{20:0}$), docosanoic acid ($C_{22:0}$) and ricinoleic acid (12-OH-$C_{18:1}$) were purchased from Nu-Chek-Prep through Funakoshi Pharmaceutical (Tokyo, Japan), tetracosanoic acid ($C_{24:0}$) from P-L Biochemicals (Milwaukee, WI, USA), hexacosanoic acid ($C_{26:0}$) from Sigma (St. Louis, MO, USA), 12-hydroxystearic acid (12-OH-$C_{18:0}$) from Serdary Research Labs. (London, Ontario, Canada) and octacosanoic acid ($C_{28:0}$) from Aldrich (Milwaukee, WI,

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USA). Tricontanoic acid ($C_{30:0}$), 2-hydroxyoctadecanoic acid (2-OH-$C_{18:0}$), 2-hydroxyicosanoic acid (2-OH-$C_{20:0}$) and 2-hydroxydocosanoic acid (2-OH-$C_{22:0}$) were obtained from Larodan Fine Chemicals (Malmö, Sweden), and aniline, diphenylamine and *n*-propylamine were purchased from Nakarai Chemicals (Kyoto, Japan). Prostaglandins E_2 (PGE_2) and B_2 (PGB_2) were obtained from Funakoshi Pharmaceutical. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Nakarai Chemicals. Redistilled water was used for HPLC. Thin-layer chromatography (TLC) was carried out with precoated plates of silica gel LK5 (thickness 0.25 mm) (Whatman, Clifton, NJ, USA).

Equipment

A Hitachi (Tokyo, Japan) M-2000-type double-focusing mass spectrometer–computer system, equipped with a Hitachi L-6200-type HPLC instrument through a Hitachi APCI interface system, was used. HPLC was performed using a reversed-phase Cosmosil $5C_{18}$-packed column with 5-μm particles (250 mm × 4.6 mm I.D., Nakarai Chemicals). The starting mobile phase was 100% methanol, and dichloromethane or tetrahydrofuran was added in a linear gradient if necessary. The drift voltage of the APCI system was 100 V, and the temperatures of the vaporizer and desolvator were 250 and 385°C, respectively. The multiplier voltage during mass spectrometry and mass chromatography was 1500 V, but this was raised to 2000 V for monitoring a single ion. Electron impact (EI) mass spectra were determined with the direct inlet system of a Hitachi M-80 mass spectrometer at 70 eV. ^{1}H NMR spectra were determined with a Varian XL-200 NMR spectrometer (200 MHz), using tetramethylsilane (TMS) as an internal standard. Patterns of NMR spectra are reported as follows: t, triplet; m, multiplet; br, broad. All melting temperatures were determined with a Yanagimoto MP-type melting-temperature apparatus and are uncorrected.

Preparation of authentic fatty amide derivatives

Amide derivatives of palmitic acid or saturated fatty acid mixture ($C_{16:0}$–$C_{30:0}$) of the same molar content as each acid were prepared by a slight modification of the method reported previously [3]. A palmitoyl chloride (1 mmol) or fatty acid chloride mixture ($C_{16:0}$–$C_{30:0}$, total 1 mmol) was dissolved in 10 ml of dry benzene, and each of the following amines was added separately: aniline (1.5 mmol), *n*-propylamine (2 mmol), 20% aqueous ammonia (2 mmol), 1-naphthylamine (1 mmol), diethylamine (2 mmol) or diphenylamine (1 mmol). The reaction mixtures with 1-naphthylamine and diphenylamine were heated at 30°C for 30 min. The mixtures with the other four amines were left at room temperature for 1.5 h. After separating the organic solvent from the aqueous phase, these mixtures were washed thoroughly with 2 *M* hydrochloric acid. Then the organic solvent was evaporated under reduced pressure. The residues of palmitamides thus obtained were observed as a single spot on silica gel TLC plates (*n*-hexane–diethyl ether–acetic acid, 90:10:2, v/v/v as the developing system). The spots were revealed in iodine vapour throughout the experiments. Some of the physical characteristics of the amide derivatives were as follows.

Palmitanilide. Colourless needles after recrystallization from ethanol–water; melting point, 90.5–91°C; R_F = 0.11; EI MS ions at *m/z* 331 (M^+), 239, 93, 77; NMR (C^2HCl_3) δ: 0.89 (3H, t, CH_3), 1.2–1.8 (26H, m, $(CH_2)_{13}$), 2.40 (2H, t, CH_2CO), 7.10–7.60 (5H, m, aromatic *H*).

N-n-Propylpalmitamide. Colourless needles after recrystallization from ethanol–water; melting point, 74–74.5°C; R_F = 0.09; EI MS ions at *m/z* 297 (M^+), 254, 239, 101; NMR (C^2HCl_3) δ: 0.9–1.0 (6H, m, CH_3), 1.2–1.8 (28H, m, $CH_3(CH_2)_{13}$-$CONHCH_2CH_2CH_3$), 2.1–2.3 (4H, m, CH_2-$CONHCH_2$), 5.78 (1H, br, $CONH_2$).

Palmitamide. Pale yellow needles after recrystallization from ethanol–water; melting point, 104–104.5°C; R_F = 0.05; EI MS ions at *m/z* 255 (M^+), 59, 44; NMR(C^2HCl_3) δ: 0.90 (3H, t, CH_3), 1.2–1.9 (26H, m, $(CH_2)_{13}CH_2CO$), 2.26 (2H, t, CH_2CO), 5.78 (1H, br, NH).

N-1-Naphthylpalmitamide. Colourless needles after recrystallization from tetrahydrofuran–ethanol–water; melting point, 112.5–113°C; R_F =

0.11; EI MS ions at m/z 381 (M^+), 143, 127; NMR (C^2HCl_3) δ: 1.02 (3H, t, CH_3), 1.1–1.7 (26H, m, $CH_3(CH_2)_{13}CH_2CO$), 2.48 (2H, t, CH_2CO), 7.4–8.0 (7H, m, aromatic *H*).

N,N-Diethylpalmitamide. Pale yellow oil; R_F = 0.25; EI MS ions at m/z 311 (M^+), 282, 115, 100; NMR(C^2HCl_3) δ: 0.09 (3H, t, $CH_3(CH_2)_{14}$-CO), 1.1–1.7 (32H, m, $CH_3(CH_2)_{13}$-$CH_2CONH(CH_2CH_3)_2$), 2.34 (2H, t, CH_2CO).

N,N-Diphenylpalmitamide. The residue had to be purified once by preparative silica gel TLC before recrystallization; colourless needles after recrystallization from ethanol; melting point, 70–70.5°C; EI MS ions at m/z 407 (M^+), 169; NMR (C^2HCl_3) δ: 0.91 (3H, t, CH_3), 1.2–1.7 (26H, m, $CH_3(CH_2)_{13}CH_2CO$), 2.28 (2H, t, CH_2CO), 7.1–7.5 (10H, m, aromatic *H*).

N-n-Propylamidation of fatty acids under mild conditions

N-*n*-Propylamidation of a fatty acid was performed according to a slight modification of the peptide synthesis developed by Shioiri and Yamada [4]. To a solution of a fatty acid (10 mmol) and propylamine · HCl (12 mmol) in dry dimethylformamide (DMF) (1 ml), diphenylphosphoramide (12 mmol) in DMF (1 ml) was added at 0°C or room temperature, followed by addition of triethylamine (12 mmol) in DMF. The mixture was stirred at room temperature for the time indicated later, then the reaction mixture was applied to LC–MS.

The reaction mixture of ricinoleic acid was added to water, extracted with *n*-hexane–diethyl ether (1:1, v/v) and concentrated. The residue was purified twice by silica gel TLC (*n*-hexane–diethyl ether–acetic acid, 90:30:20, v/v/v). The purified compound was observed as a single spot (R_F = 3.1) on a silica gel TLC plate and as a single peak on an LC mass chromatogram [total ion current (TIC) above m/z 200, with methanol as mobile phase]. It was a pale yellow oil. EI MS ions at m/z 321 ($[M - H_2O]^+$), 292, 254 and 225 and LC–MS ions at m/z 340 ($[M + H]^+$), 322 ($[M - H_2O + H]^+$) were observed.

Preparation of total fatty acid in rat brain

Male Wistar rats (aged three weeks) were killed by decapitation and the brains were rapidly excised. Lipids were extracted from the whole brain as mentioned by Folch *et al.* [5]. The lipid fraction was hydrolysed with 0.5 *M* hydrochloric acid at 100°C for 45 min according to Aveldano and Sprecher [2].

RESULTS

LC–MS of various kinds of fatty acid amide derivatives

LC–MS of six kinds of authentic palmitamide derivatives was performed with methanol as the mobile phase. Each spectrum of anilide [3] and N-*n*-propylamide derivatives exhibited a single and intense $[M + H]^+$ ion. In the spectra of N,N-diethylamide and amide derivatives, $[M + H]^+$ ions were observed as the base peaks. In the case of N-1-naphthylamide and N,N-diphenylamide derivatives, in addition to $[M + H]^+$ ions, ions were observed at m/z 144 ($[C_{10}H_7NH_2 + H]^+$) and m/z 170 ($[(C_6H_5)_2NH + H]^+$), respectively, due to cleavage of the amide group.

Sensitivity of palmitamide derivatives for detection with LC–MS

The sensitivity of the palmitamide derivatives was evaluated by monitoring a single ion of $[M + H]^+$ from the derivatives. The peaks from the N-*n*-propylamide and anilide derivatives are shown in Fig. 1. The peak heights were plotted against the amount of the amides (Fig. 2). The points of each amide derivative were plotted as a linear line. The relative sensitivities of the derivatives were roughly estimated from the slope of each line as follows (the ratios to anilide are in parentheses): N-*n*-propyl amide (2.1) > anilide (1.0) > N,N-diethylamide (0.66), amide (0.62) > N,N-diphenylamide (0.44) > N-1-naphthylamide (0.06).

Mass chromatograms of amide derivatives of the fatty acid mixtures

Using LC–MS with various amide derivatives of the authentic fatty acid mixtures ($C_{16:0}$–

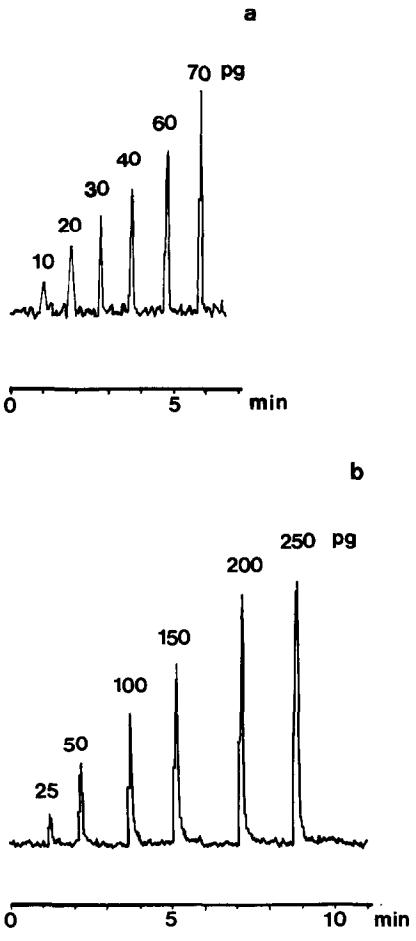

Fig. 1. Sensitivity of LC–MS for various palmitamides. The mass spectrometer–computer system was set to detect a single ion ([M + H]$^+$) with the maximum sensitivity for (a) N-*n*-propylpalmitamide and (b) palmitanilide.

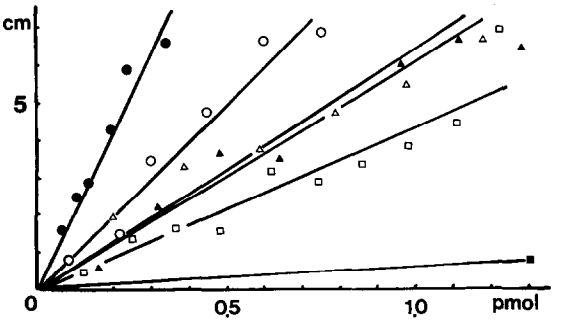

Fig. 2. Peak heights plotted against the amount of palmitamides: (●) N-*n*-propylamide; (○) anilide; (▲) N,N-diethylamide; (△) amide; (□) N,N-diphenylamide; and (■) N-1-naphthylamide derivatives.

$C_{30:0}$), it was found that mass chromatography is very convenient for identifying each fatty acid, as shown for the N-*n*-propylamide derivatives in Fig. 3.

LC–MS analysis of hydroxy fatty acids

N-*n*-Propylamidation of fatty acids under the mild conditions mentioned in Experimental was applied to hydroxy fatty acid amide synthesis. The retention time on LC–mass chromatography and the mass spectrum of the product from palmitic acid were found to be identical with those of authentic N-*n*-propylpalmitamide (mobile phase, methanol). Fig. 4A shows the calibration curve of TIC peak areas on a mass chromatogram in relation to the amount of authentic N-*n*-propylpalmitamide. The time course of the reaction was determined from this calibration curve (Fig. 4B). After 3 h of reaction time, the yield was made quantitative. The derivative was stable for more than a week in the reaction mixture at room temperature.

The N-*n*-propylamide of ricinoleic acid was prepared under these mild conditions. To determine the yield of the amide preparation, the reaction mixture was applied to LC–MS to estimate the TIC peak area on the mass chromatogram for the amide. Purified N-*n*-propylricinolamide was used to obtain the calibration curve. The yield of the amide could be made quantitative.

A mixture of various kinds of hydroxy fatty acid (12-OH-$C_{18:0}$, 12-OH-$C_{18:1}$, 2-OH-$C_{18:0}$, 2-OH-$C_{20:0}$, 2-OH-$C_{22:0}$) was derivatized to N-*n*-propylamide under the same mild conditions and applied to LC–MS. Figs. 5 and 6 show the mass chromatograms of this mixture and the mass spectra, respectively. The spectra of 2-hydroxy fatty acids showed intense [M + H]$^+$ ions as base peaks, whereas those of 12-hydroxy fatty acids showed [M + H]$^+$ ions and [M − H_2O + H]$^+$ ions as base peaks.

We tested prostaglandins as an example of more heat-labile hydroxy fatty acid derivatives. The presence of a hydroxy group and a double bond in the fatty acids did not affect the yield and the reaction time of the preparation of amide derivatives under mild conditions. Therefore, the

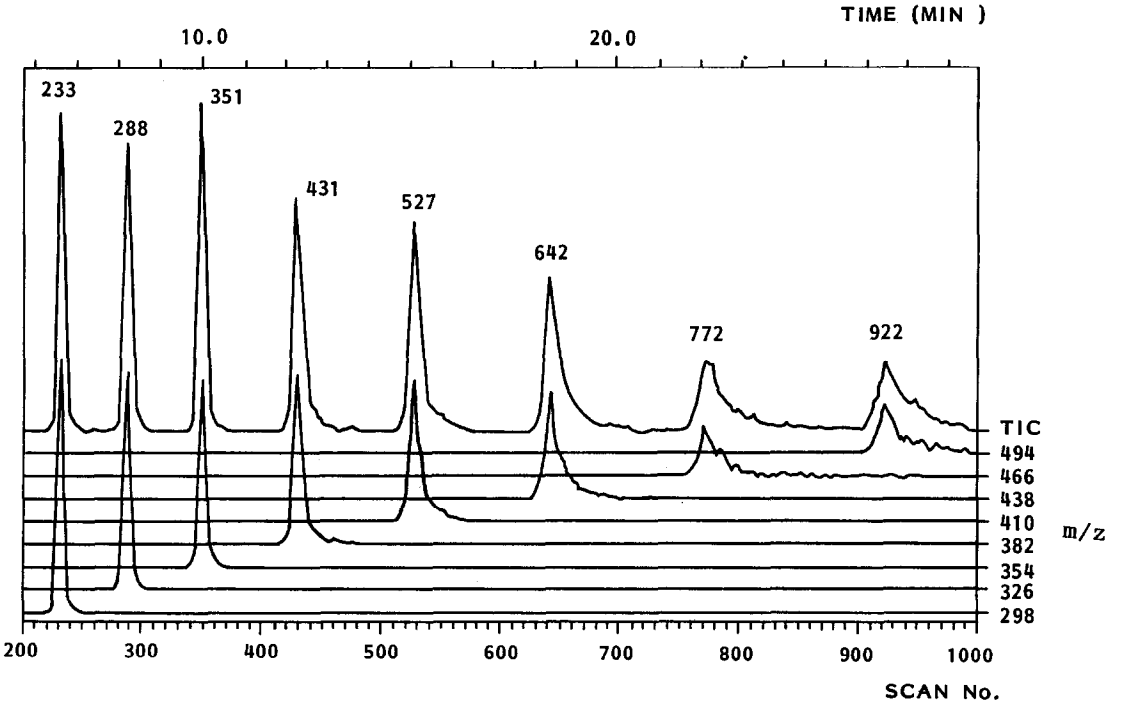

Fig. 3. Mass chromatogram of N-*n*-propylamide derivatives of a fatty acid mixture ($C_{16:0}$–$C_{30:0}$): 3 μmol of each fatty acid were used. Mobile phase, 100% methanol, with dichloromethane added at 0.5%/min.

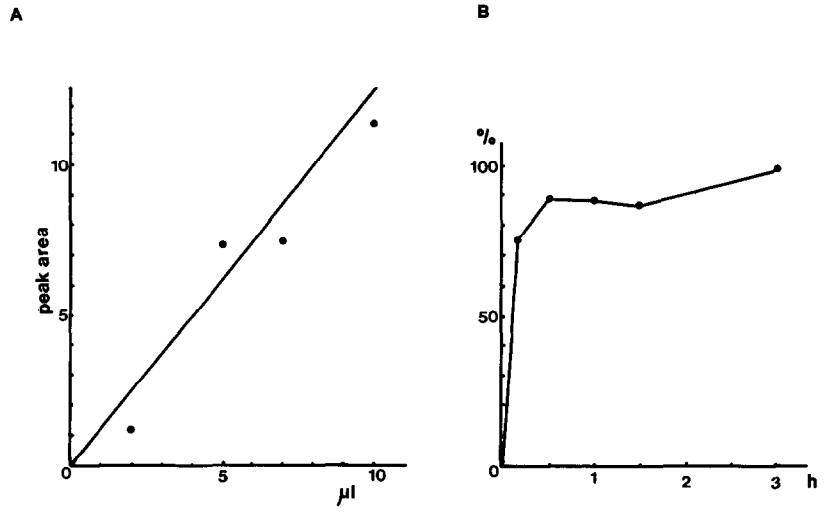

Fig. 4. Time course for synthesis of N-*n*-propylpalmitamide. Authentic N-*n*-propylpalmitamide (8.2 mg/ml) was applied to LC–MS (mobile phase, methanol). (A) Peak areas of total ion current above *m/z* 200 are plotted against the amount (input volume). (B) The reaction mixture for synthesizing N-*n*-propylpalmitamide under the mild conditions (described in Experimental) was analysed by LC–MS at the indicated time (mobile phase, methanol).

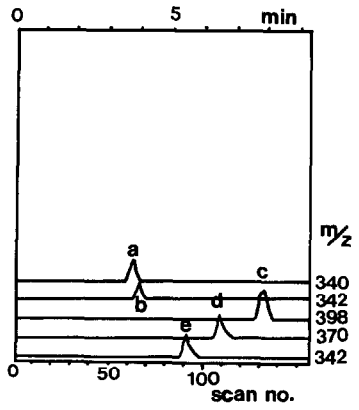

Fig. 5. Mass chromatograms of N-*n*-propylamide derivatives of hydroxy fatty acids. A mixture of hydroxy fatty acids was derivatized according to the mild conditions (described in Experimental) and analysed by LC–MS (mobile phase, methanol): (a) 12-OH-$C_{18:1}$; (b) 12-OH-$C_{18:0}$; (c) 2-OH-$C_{22:0}$; (d) 2-OH-$C_{20:0}$; (e) 2-OH-$C_{18:0}$. The corresponding mass spectra are shown in Fig. 6.

mass spectra and the ion chromatograms were examined immediately after amidation. After mild amidation of the mixture of PGE_2 and PGB_2, the reaction mixture was applied to LC–MS. Fig. 7 shows the total ion chromatogram, and the mass spectra of peaks a and b are shown in Fig. 8. Here, we could detect intense $[M + H]^+$ ions as base peaks in the mass spectra of peaks a (PGE_2) and b (PGB_2). In addition, $[M - H_2O + H]^+$ and $[M - 2H_2O + H]^+$ (weak) ions were observed in peak a (PGE_2). A weak $[M - H_2O + H]^+$ ions was detected in peak b (PGB_2). In the mass spectrum of N-*n*-propylamide of PGE_2 with methanol as mobile phase, a very weak $[M + H]^+$ ion, and intense $[M - H_2O + H]^+$ and $[M - 2H_2O + H]^+$ ions were observed (data not shown).

Analysis of fatty acids in the rat brain

As an example of the LC–MS analysis of hydroxy fatty acids of natural origin, an experiment with the rat brain is described. N-*n*-Propylamide derivatives of fatty acids from the rat brain were prepared under the mild conditions mentioned previously, and LC–MS was performed under the same conditions as those in Fig. 3. As shown

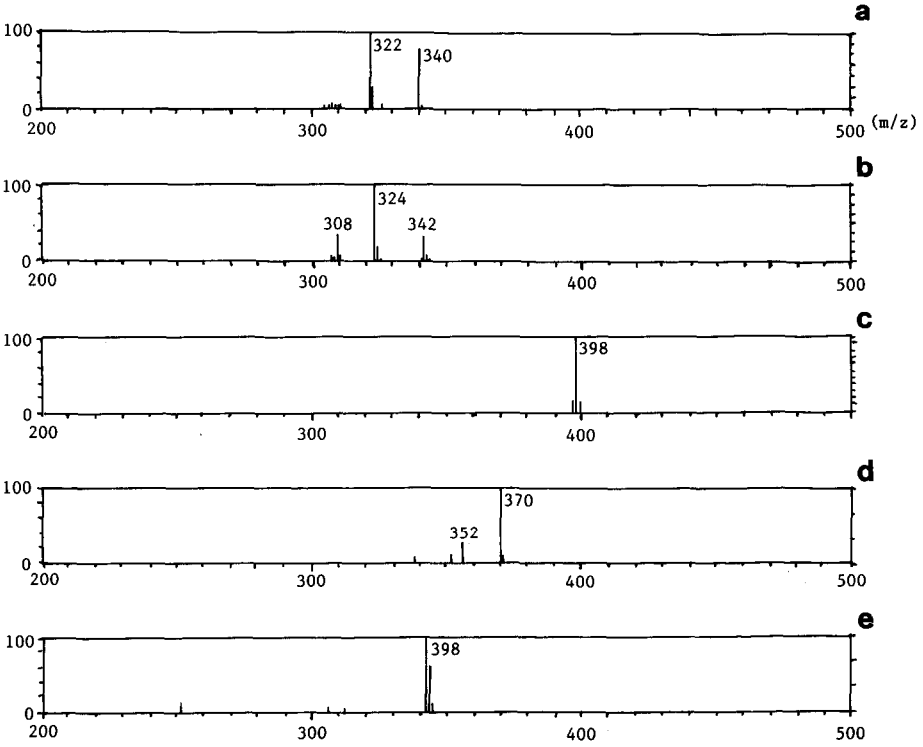

Fig. 6. Mass spectra of the N-*n*-propylamide derivatives of fatty acids described in Fig. 5.

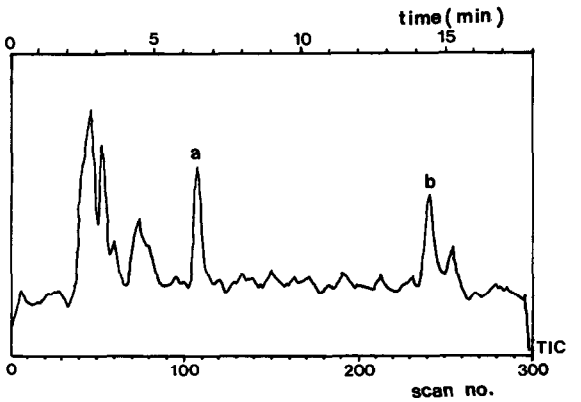

Fig. 7. Total ion chromatogram of N-*n*-propylamide derivatives of prostaglandins E_2 (a) and B_2 (b). The prostaglandins were derivatized under the mild conditions described in Experimental and analysed by LC–MS (mobile phase, acetonitrile–water, 1:1).

in Fig. 9, the even-numbered saturated acid series from C_{14} to C_{26}, the odd-numbered saturated acid series from C_{15} to C_{25} (observed in the other LC–MS manipulation), the monoenoic acid series from C_{16} to C_{26} and the dienoic acid series from C_{18} to C_{24}, the trienoic acid series from C_{20} to C_{22} and hydroxy acids (2-OH-$C_{18:0}$, 2-OH-$C_{22:0}$, 2-OH-$C_{23:0}$, 2-OH-$C_{24:0}$, 2-OH-$C_{24:1}$) could be detected successively from mass chromatograms of the N-*n*-propylamide derivatives in a single LC–MS run. The mass chromatograms of the anilide derivatives were almost the same as those of the N-*n*-propylamide derivatives (data not shown).

DISCUSSION

Among six kinds of amide derivative of fatty acids, the N-*n*-propylamides were found to be the most sensitive to LC–MS with APCI, as shown in Fig. 2. Moreover, these amides can be prepared more easily than others, since excess unreacted *n*-propylamine can be evaporated together with the solvent. The anilide derivatives were the second most sensitive. It is also advantageous that the anilides have an absorption at 244 nm. Therefore, they could be useful when fractionation or quantification of fatty acids is necessary. Another excellent method for preparing the amide derivatives of the hydroxy fatty acids was developed because oxalyl chloride, which is used for fatty acid amide preparation [3], reacts not only with the carboxy group, but also with the hydroxy group.

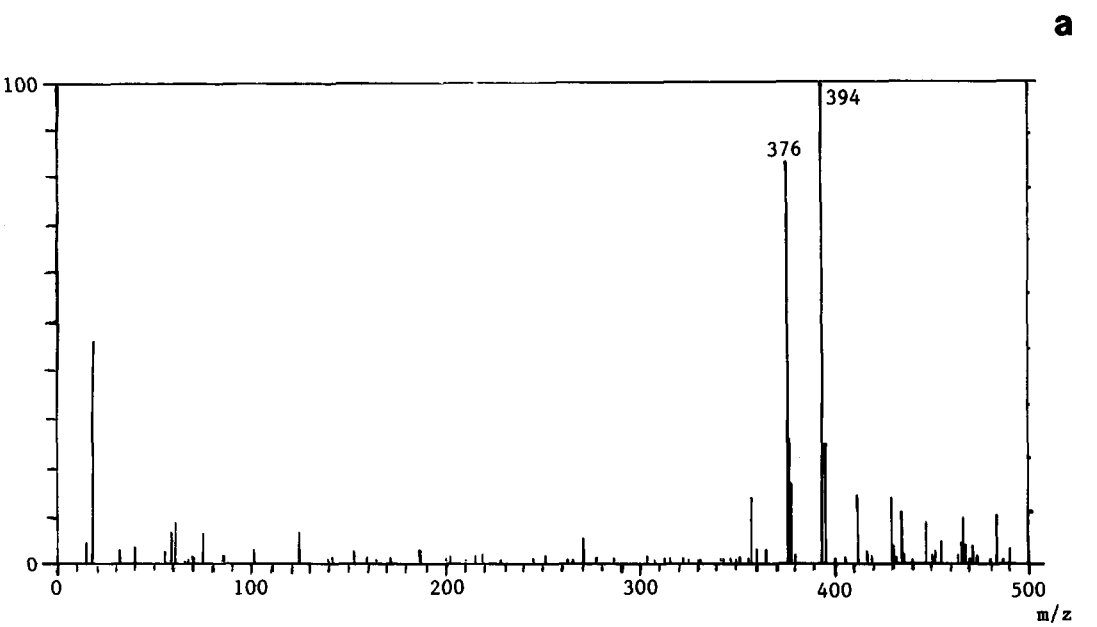

Fig. 8.

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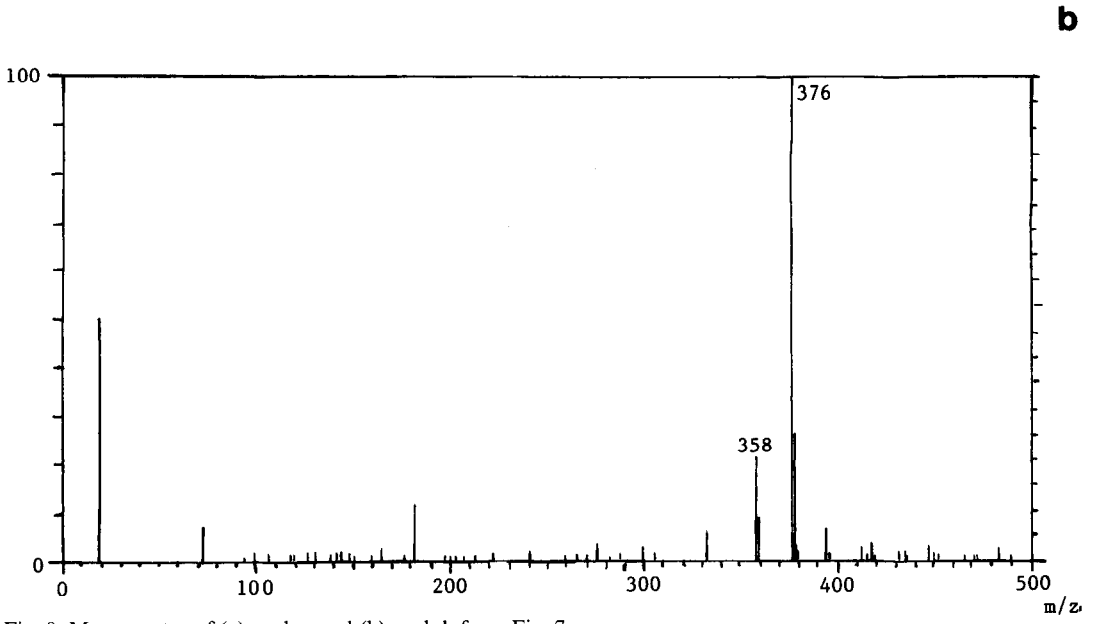

Fig. 8. Mass spectra of (a) peak a and (b) peak b from Fig. 7.

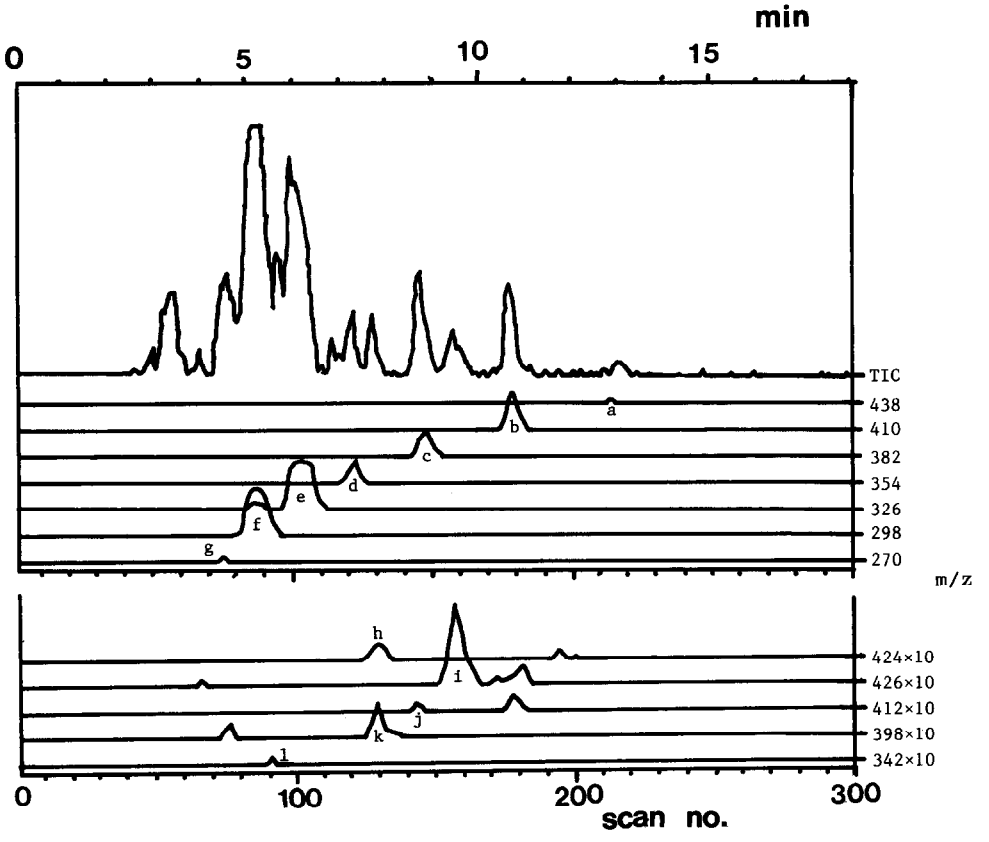

Fig. 9. Mass chromatograms of N-*n*-propylamide derivatives of fatty acids in the rat brain. Derivatives were prepared under the mild conditions described in Experimental and analysed by LC–MS (mobile phase, 100% methanol, with tetrahydrofuran added gradually 1%/min). (a) $C_{26:0}$; (b) $C_{24:0}$; (c) $C_{22:0}$ (d) $C_{20:0}$; (e) $C_{18:0}$; (f) $C_{16:0}$; (g) $C_{14:0}$; (h) 2-OH-$C_{24:1}$; (i) 2-OH-$C_{24:0}$; (j) 2-OH-$C_{23:0}$; (k) 2-OH-$C_{22:0}$; (l) 2-OH-C_{18-0}.

The amide derivatives of fatty acids are highly sensitive for detecting fatty acids by LC–MS [3] (Fig. 1). Detection by LC–MS also has the following advantages. First, many series of fatty acids can be detected simultaneously, as shown in Fig. 9, even when they have been poorly separated by the LC column. This is because the peak of the $[M + H]^+$ ion is usually the single and base peak for each fatty acid derivative. In the case of GC–MS, separation of each fatty acid is often critical for a precise analysis, but it is practically impossible to use only one column to separate all fatty acids owing to the wide differences in carbon chain-length, double bond number, side-chains, etc. Second, LC–MS seems to be more suitable than GC–MS for analysing extremely long-chain fatty acids (especially longer than C_{30}). We could clearly detect N-*n*-propylamide derivatives of the fatty acids of *Mycobacterium lepraemurium* and *Mycobacterium smegmatis* with a wide range of carbon chain lengths (C_{30}–C_{54}) in a single LC–MS run [6]. The analysis of material containing short-chain fatty acids would be possible because of the low volatility of the amide derivatives. The retention times of the peaks in the mass chromatogram were useful for identification of the fatty acids. A series of fatty acids line up successively, as shown partly in Fig. 9. The fragmentation by hydroxy group elimination may be helpful for the structural determination of hydroxy fatty acids. In addition, it is evidently advantageous to use LC–MS with APCI in combination with GC–MS to analyse some special kinds of fatty acid of natural origin, such as those from the brain and mycobacteria.

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