CHROM. 17,357

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SHORT- AND LONG-CHAIN FATTY ACIDS AS 2-NITROPHENYLHYDRAZIDES

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(Received October 22nd, 1984)

SUMMARY

Both short- and long-chain fatty acids, including mono- and polyunsaturated fatty acids, were coupled with 2-nitrophenylhydrazine hydrochloride in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and analysed by high-performance liquid chromatography. Elutions of mixtures of both short- (C_2–C_8) and long-chain (C_{10}–C_{18}) fatty acid hydrazides were achieved within 15 min. By use of a visible-range detector (400 nm) the detection limits of both short- and long-chain fatty acids were found to be in the range 10–15 pmol per injection, and linear relationships were obtained over the concentration range 20 pmol–1 nmol per injection. Ultraviolet detection (230 nm) for the long-chain fatty acids demonstrated that the detection limits were 2.5–5 pmol per injection with linearity over the range 5 pmol–1 nmol per injection.

INTRODUCTION

Most fatty acids show no useful absorption in the visible and ultraviolet (UV) regions or no fluorescence for detection in high-performance liquid chromatography (HPLC), and various labelling techniques have been developed in order to perform the sensitive separation and analysis of the acids. These include UV labelling techniques to give phenacyl[1-3], *p*-bromophenacyl[4-6], methoxyphenacyl[7], and naphthacyl[8] esters, *p*-methoxyanilide[9], naphthyldiazoalkane[10] and 1-chlormethylisation derivatives[11], and fluorescence labelling techniques to give 4-bromomethyl-7-methoxycoumarin[12,13], 4-bromomethyl-7-acetoxycoumarin[14], 9,10-diaminophenanthrene[15], 9-anthryldiazomethane[16,17] and 9-aminophenanthrene[18] derivatives. These methods, however, have not always been satisfactory with regard to sensitivity, separation and analysis time.

In a previous paper it was demonstrated that both aliphatic and aromatic acids reacted sensitively with 2-nitrophenylhydrazine hydrochloride (2-NPH · HCl) using dicyclohexylcarbodiimide (DCC) as a coupling agent to give acid hydrazides, which were useful for the sensitive colorimetric determination of the acids[19]. This paper describes the labelling of both short- and long-chain fatty acids with 2-NPH · HCl and the separation and analysis of the hydrazides by reversed-phase HPLC. In this

study, the coupling reagent DCC was replaced with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC · HCl), which is water soluble and suitable for reversed-phase HPLC.

EXPERIMENTAL

Reagents and chemicals
Oleic, linoleic, linolenic acids and 1-EDC · HCl were purchased from Sigma (St. Louis, MO, U.S.A.). Lauric, myristic, palmitic, palmitoleic and stearic acids were obtained from Japan Chromato Kogyo (Tokyo, Japan). Other fatty acids, pyridine and 2-NPH · HCl were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Analytical-reagent grade methanol was purchased from Wako (Osaka, Japan). Methanol and water were distilled before use and the other reagents and chemicals were used without further purification.

Reagent solutions
2-NPH · HCl solution (0.02 M) was prepared by dissolving the reagent in water. Pyridine solution (3%, v/v) in ethanol and 1-EDC · HCl solution (0.25 M) in ethanol were prepared, then a working 1-EDC · HCl solution was prepared by mixing equal volumes of the 1-EDC · HCl and pyridine solutions. Potassium hydroxide solutions, 15% w/v) in methanol–water (80:20), was prepared.

Derivatization procedure
Fatty acids were dissolved in water, aqueous ethanol or ethanol in the concentration range 20–4000 nmol/ml. To 0.1 ml of a sample solution, 0.4 ml of 1-EDC · HCl working solution and 0.2 ml of 2-NPH · HCl solution were added and the mixture was heated at 60°C for 20 min. After the addition of 0.1 ml of potassium hydroxide solution, the resulting mixture was further heated at 60°C for 15 min, then cooled in running water. An aliquot (1–2 μl) of the mixture was injected directly into the liquid chromatograph.

HPLC analysis
All analyses were performed with a Shimadzu Model LC-5A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with both a variable-wavelength UV–visible detector (Shimadzu Model SPD-1) and a variable-wavelength UV detector (Shimadzu Model SPD-2A). The former, with a flow cell volume of 6.4 μl, was normally set to monitor the absorbance at 400 nm, and the latter, with a flow cell volume of 8 μl, for continuous monitoring at 230 nm. The detector signals were recorded on a multi-pen recorder (Rikadenki Kogyo, Tokyo, Japan).
The separation was performed with a C$_8$ reversed-phase column (250 × 4.6 mm I.D.) packed with YMC-C8 (particle size 5 μm), which was obtained from Yamamuta Chemical Research Institute (Kyoto, Japan). The column temperature was maintained at 50°C.
All analyses were carried out isocratically using methanol–water as the solvent system at a flow-rate of 1.2 ml/min. The pH of the solvents was maintained at 4.5 by adding 0.1 N HCl. The solvents were filtered through Fluoropore filters (pore size 0.45 μm) (Sumitomo Electric Ind., Osaka, Japan) and degassed with a Sonifer B-12 (Branson Sonic, CT, U.S.A.) before use.

Absorption spectra

The absorption curves of the fatty acid hydrazides were measured with a Hitachi Model 340 recording spectrophotometer (Hitachi, Tokyo, Japan).

RESULTS AND DISCUSSION

The reaction conditions were investigated with *n*-valeric and myristic acids, chosen to represent short- and long-chain fatty acids, respectively.

In order to ensure the maximum derivatization of the fatty acids, the time-dependent increase in the peak heights of the fatty acid hydrazides was monitored by HPLC. The results are shown in Fig. 1. The peak heights became constant after *ca.* 10 min at 60°C, which suggests that the derivatization was maximal after this period. Using an optimum reaction time of 20 min, the other fatty acids were also converted into hydrazides.

Fig. 2 shows the relationship between the peak heights of the acid hydrazides and the concentration of 2-NPH · HCl. The peak height increased with increase in concentration of 2-NPH · HCl. A 0.02 *M* solution was preferred for the HPLC analyses because a more concentrated solution of the reagent gave impurity peaks on the chromatogram.

The effect of the 1-EDC · HCl concentration on the peak height is shown in Fig. 3. Relatively higher peak heights were obtained in the concentration range 0.2–0.3 *M* without affecting the impurity peaks and 0.25 *M* was selected in subsequent studies.

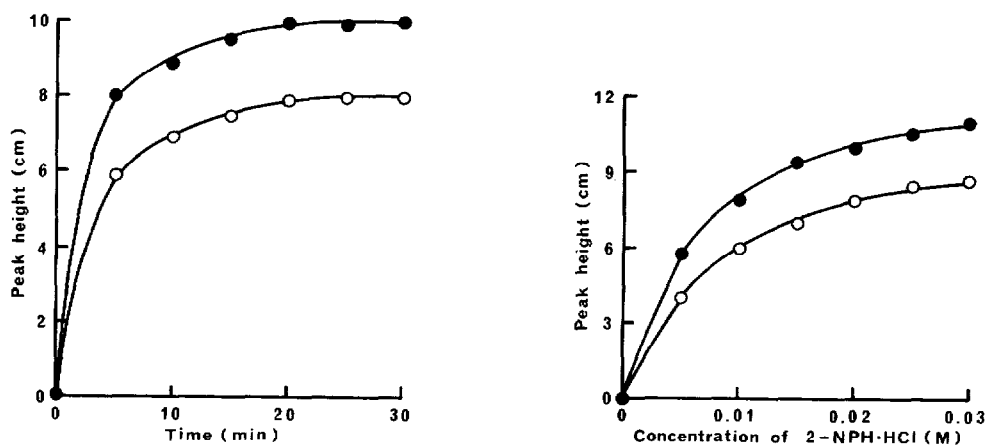The influence of the concentration of pyridine on the peak heights of the *n*-

Fig. 1. Effect of reaction time on colour formation at 60°C. A 0.8-μmol amount of each acid was treated by the derivatization procedure with various reaction times. An aliquot of 2 μl of the reaction mixture was injected on to the column and was detected at 400 nm with 1×10^{-2} units absorbance range. ●, *n*-Valeric acid; ○, myristic acid.

Fig. 2. Effect of concentration of 2-NPH · HCl on colour formation. A 0.8-μmol amount of each acid was treated by the derivatization procedure using various concentrations of 2-NPH · HCl. An aliquot of 2 μl of the reaction mixture was injected on to the column and was detected at 400 nm with 1×10^{-2} units absorbance range. ●, *n*-Valeric acid; ○, myristic acid.
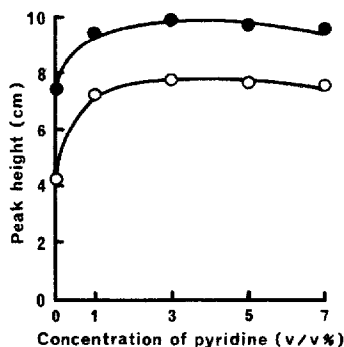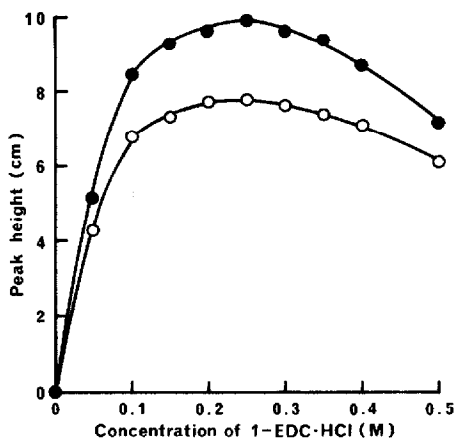
Fig. 3. Effect of concentration of 1-EDC · HCl on colour formation. A 0.8-μmol amount of each acid was treated by the derivatization procedure using various concentrations of 1-EDC · HCl. An aliquot 2 μl of the reaction mixture was injected on to the column and was detected at 400 nm with 1×10^{-2} units absorbance range. ●, n-Valeric acid; ○, myristic acid.

Fig. 4. Effect of concentration of pyridine on colour formation. A 0.8-μmol amount of each acid was treated by the derivatization procedure using various concentrations of pyridine. An aliquot of 2 μl of the reaction mixture was injected on to the column and was detected at 400 nm with 1×10^{-2} units absorbance range. ●, n-Valeric acid; ○, myristic acid.

valeric and myristic derivatives is shown in Fig. 4. The peak heights were almost constant over the range of pyridine concentrations investigated. These experiments indicated that the optimum concentration of pyridine was 3% (v/v).

Potassium hydroxide removed the blank brown colour[19], and a concentration of 15% (w/v) was needed in order to eliminate the interference in the chromatogram due to the excess of the reagents and the reaction by-products that might be formed during the coupling reaction.

Table I gives the absorbance peak maxima and the molar absorptivities of 18 fatty acid hydrazides in a basic medium. All of the fatty acids, including unsaturated compounds, were converted into acid hydrazides and the latter were stable for at least 10 days when kept in the reaction mixture at room temperature.

The absorption curves of acetic acid hydrazide in solutions of various pH are shown in Fig. 5. The hydrazide ionizes at high pH (<12) to give an intense violet colour. At low pH (<8.5), however, the absorption maximum of the hydrazide shifts considerably towards the blue region. On the other hand, with the reversed-phase HPLC column the pH of the eluent was restricted to the range 2–8, and the eluting system of mixtures of methanol and water was chosen to maintain the pH at 4.5. All of the fatty acid hydrazides gave absorption maxima at 400 nm in the acidic medium, and were detectable photometrically by monitoring at this wavelength. These derivatives also showed strong absorption in the UV region, with maximal absorption at around 230 nm, and were monitored with a UV detector.

The HPLC of fatty acid hydrazides using a YMC-C8 column and methanol–water as the eluent was also studied. An excess of the reagents and the reaction by-products did not interfere with the HPLC analyses in the visible range, because they

TABLE I

ABSORPTION MAXIMA AND MOLAR ABSORPTIVITIES OF FATTY ACID HYDRAZIDES IN BASIC MEDIUM

Molar absorptivities $\pm$ standard deviation ($n = 3$).

| Fatty acid	λ_{max} (nm)	Molar absorptivity ($l\ mol^{-1}\ cm^{-1}$)
Acetic acid	545	5151 ± 20
Propionic acid	550	5285 ± 17
Isobutyric acid	555	5149 ± 17
Butyric acid	550	6003 ± 28
Isovaleric acid	555	6328 ± 20
Valeric acid	550	5379 ± 27
Caproic acid	550	5093 ± 25
Heptanoic acid	550	5248 ± 19
Caprylic acid	550	5843 ± 31
Capric acid	550	5320 ± 28
Lauric acid	550	5155 ± 24
Myristic acid	550	5864 ± 28
Linolenic acid	550	5477 ± 15
Palmitoleic acid	550	5728 ± 26
Linoleic acid	550	5525 ± 20
Palmitic acid	550	5787 ± 19
Oleic acid	550	5808 ± 26
Stearic acid	550	5485 ± 22
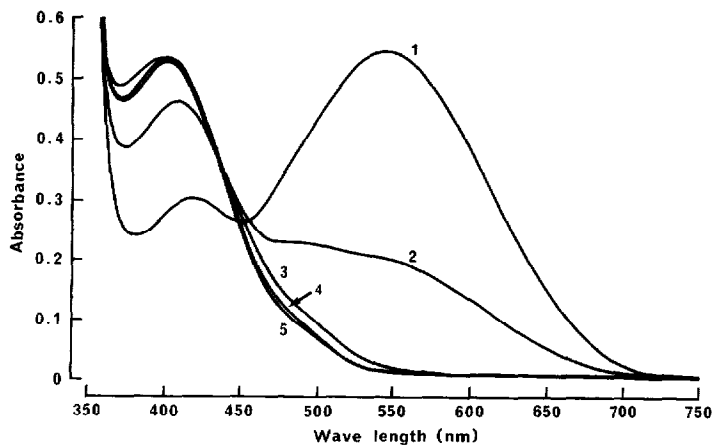
Fig. 5. Absorption spectra of solutions of acetic acid 2-nitrophenylhydrazine of various pH. A 0.5-μmol amount of acetic acid was treated by the derivatization procedure. The reaction mixture was adjusted with 3 N HCl to the desired pH and was measured from 350 to 750 nm. pH: 1, 13.85; 2, 10.5; 3, 8.5; 4, 6.5; 5, 4.5.

did not absorb visible radiation at 400 nm and were eluted before any of the fatty acid hydrazides. However, the impurity peaks in the UV region could interfere in the determination of some short-chain fatty acid hydrazides.

Fig. 6 shows a typical separation of straight- and short-chain fatty acid hydrazides by HPLC analysis with methanol–water (62:38) as the eluent and detection in the visible range. Complete separation of these compounds was obtained with a short retention time (15 min) and symmetrical peaks.

Fig. 7 shows that when the mobile phase was changed to methanol–water
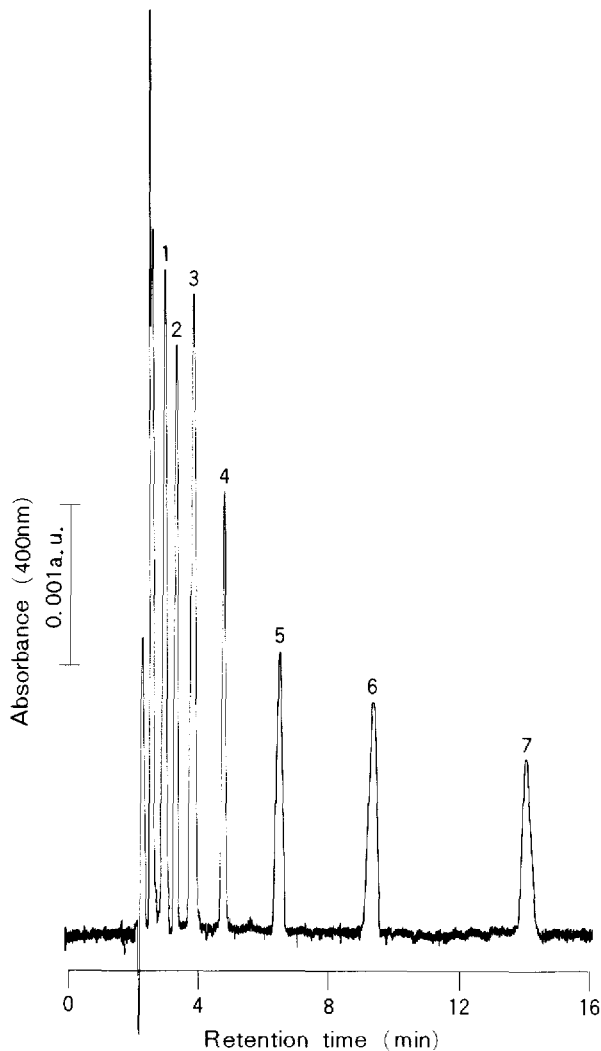

Fig. 6. Chromatogram of the 2-nitrophenylhydrazides of straight- and short-chain fatty acids obtained with visible-range detection. Mobile phase, methanol–water (62:38); flow-rate, 1.2 ml/min. Peaks: 1 = acetic; 2 = propionic; 3 = butyric; 4 = valeric; 5 = caproic; 6 = heptanoic; 7 = caprylic acid. Each peak corresponds to 150 pmol.
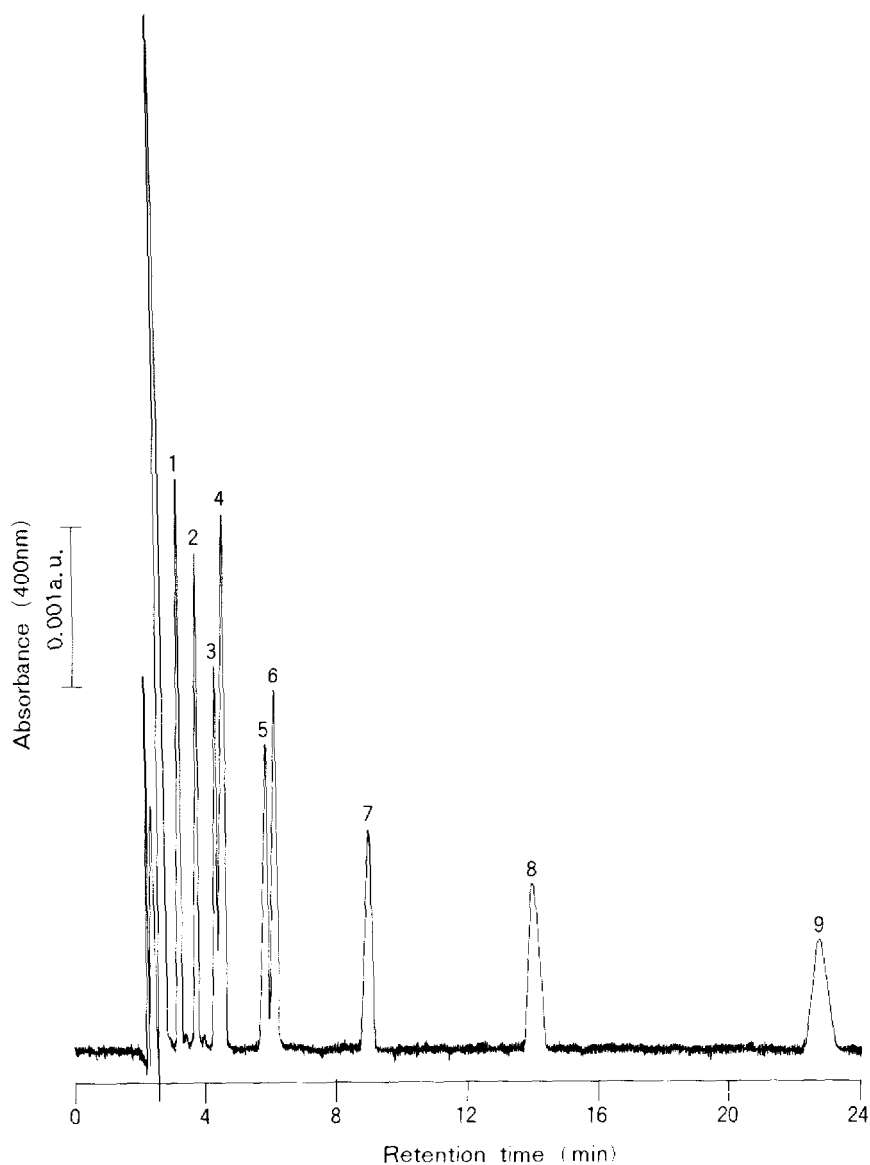
Fig. 7. Chromatogram of the 2-nitrophenylhydrazides of short-chain fatty acids, including isobutyric and isovaleric acids, obtained with visible-range detection. Mobile phase, methanol–water (58:42); flow-rate, 1.2 ml/min. Peaks: 1 = acetic; 2 = propionic; 3 = isobutyric; 4 = butyric; 5 = isovaleric; 6 = valeric; 7 = caproic; 8 = heptanoic; 9 = caprylic acid. Each peak corresponds to 150 pmol.

(58:42), short-chain fatty acids, including isobutyric and isovaleric acids, were eluted within 23 min, and iso-isomers were eluted faster than the normal isomers.

Chromatograms of a mixture of saturated and unsaturated long-chain fatty acid hydrazides, obtained using methanol–water (86:14) as the eluent and with both visible-range and UV detection, are shown in Fig. 8. No interfering peaks were ob-

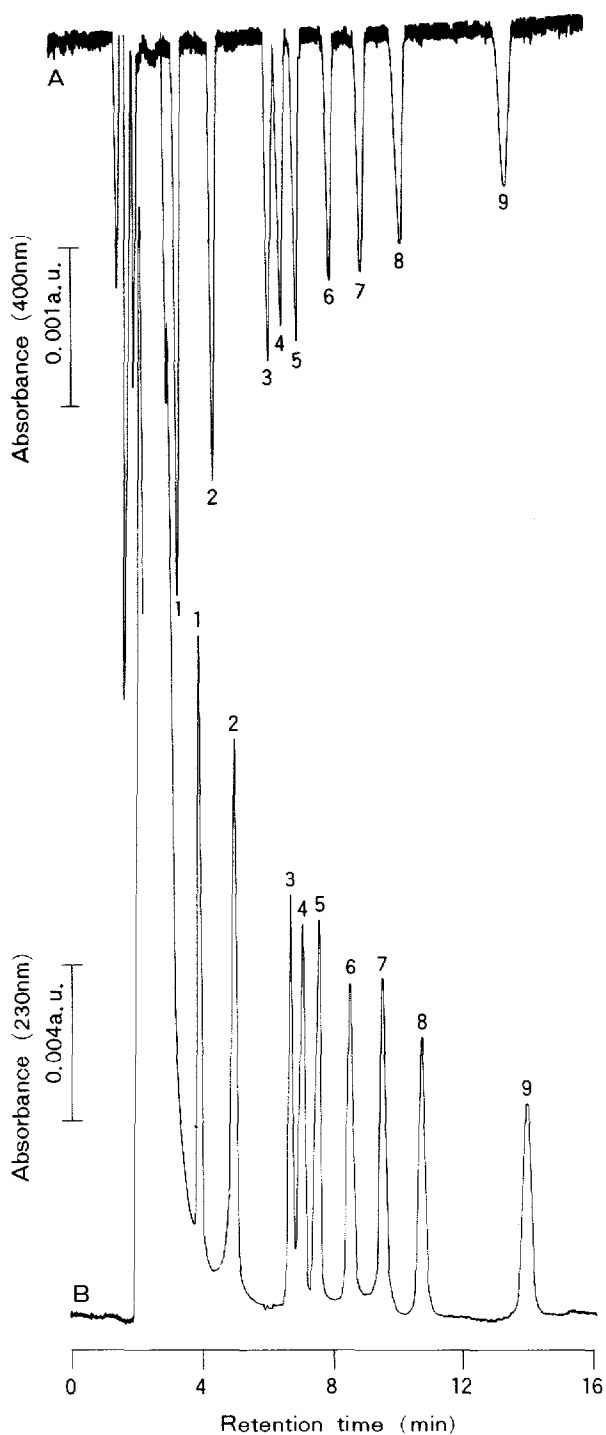Fig. 8. Chromatograms of the 2-nitrophenyl-hydrazides of a mixture of saturated and unsaturated long-chain fatty acids obtained with visible-range (A) and UV (B) detection. Mobile phase, methanol–water (86:14); flow-rate, 1.2 ml/min. Peaks: 1 = capric; 2 = lauric; 3 = myristic; 4 = linolenic; 5 = palmitoleic; 6 = linoleic; 7 = palmitic; 8 = oleic; 9 = stearic acid. Each peak corresponds to 150 pmol.

TABLE II

EQUATIONS FOR CALIBRATION GRAPHS AND CORRELATION COEFFICIENTS (r) FOR VISIBLE-RANGE DETECTION

The equation is defined as $h = ax + b$, where h is the peak height ($\times 10^{-2}$ absorbance units at 400 nm) and x is the amount of sample (pmol).

Fatty acid	Equation	r	Linear range (pmol)
Acetic acid	$h = 0.0626x + 0.4242$	0.9999	20–1000
Propionic acid	$h = 0.0578x + 0.4785$	0.9997	20–1000
Butyric acid	$h = 0.0538x + 1.1575$	0.9999	20–1000
Valeric acid	$h = 0.0402x + 0.7065$	0.9992	20–1000
Caproic acid	$h = 0.0287x + 0.0058$	0.9986	20–1000
Heptanoic acid	$h = 0.0232x - 0.0530$	0.9985	20–1000
Caprylic acid	$h = 0.0173x + 0.0285$	0.9985	20–1000
Capric acid	$h = 0.0512x + 0.7180$	0.9992	20–1000
Lauric acid	$h = 0.0422x + 0.4430$	0.9997	20–1000
Myristic acid	$h = 0.0311x + 0.5310$	0.9992	20–1000
Linolenic acid	$h = 0.0278x + 0.4821$	0.9994	20–1000
Palmitoleic acid	$h = 0.0287x + 0.5396$	0.9991	20–1000
Linoleic acid	$h = 0.0231x + 0.5020$	0.9989	20–1000
Palmitic acid	$h = 0.0224x + 0.4884$	0.9986	20–1000
Oleic acid	$h = 0.0191x + 0.4365$	0.9990	20–1000
Stearic acid	$h = 0.0143x + 0.4028$	0.9987	20–1000

served with the derivatization conditions used. The only difference between chromatograms of the same sample obtained at 400 and 230 nm was a several-fold increase in the size of the peaks at the latter wavelength. Baseline separation of these derivatives was obtained in less than 15 min. Retention times increased with increasing chain length for the saturated fatty acid hydrazides and inversely with the degree of unsaturation for the unsaturated fatty acid hydrazides.

To construct calibration graphs for quantitation, increasing amounts of mixtures of short- or long-chain fatty acids were derivatized and analysed as described above. From the chromatograms obtained, with visible-range and UV detection, the relationships between peak height and concentration of the acids were calculated by the least-squares method.

Table II gives the equations and correlation coefficients obtained with visible-range detection. For the short-chain fatty acids, linear relationships were obtained in the range 20 pmol–1 nmol per injection. Linearity for the long-chain fatty acids was also found to hold in the same concentration range. The coefficients of variation were less than 1.7% ($n = 7$) for 100 pmol per injection and the limits of detection, based on a signal-to-noise ratio of 3:1, were 10–15 pmol per injection for both short- and long-chain fatty acids.

Table III gives the equations and correlation coefficients obtained with UV detection. The calibration graphs for the long-chain fatty acids were linear over the range 5 pmol–1 nmol per injection and the coefficients of variation were less than 2.1% ($n = 7$) for 100 pmol per injection. With UV detection the limits of detection were 2.5–5 pmol per injection for long-chain fatty acids with a signal-to-noise ratio of 5:1. The UV detection of long-chain fatty acid derivatives was approximately four

TABLE III

EQUATIONS OF CALIBRATION GRAPHS AND CORRELATION COEFFICIENTS (r) FOR UV DETECTION

The equation is defined as $h = ax + b$, where h is the peak height (5×10^{-3} absorbance units at 230 nm) and x is the amount of sample (pmol).

Fatty acid	Equation	r	Linear range (pmol)
Capric acid	$h = 0.4569x + 3.1820$	0.9994	5–1000
Lauric acid	$h = 0.3835x + 5.6061$	0.9992	5–1000
Myristic acid	$h = 0.3095x + 2.9100$	0.9991	5–1000
Linolenic acid	$h = 0.2806x + 2.8512$	0.9990	5–1000
Palmitoleic acid	$h = 0.2934x + 3.0311$	0.9990	5–1000
Linoleic acid	$h = 0.2390x + 2.1003$	0.9990	5–1000
Palmitic acid	$h = 0.2357x + 2.2027$	0.9990	5–1000
Oleic acid	$h = 0.2005x + 2.1956$	0.9986	5–1000
Stearic acid	$h = 0.1558x + 1.9564$	0.9979	5–1000

times more sensitive than visible-range detection. This sensitivity was comparable to that of fluorescence labelling methods[13,16].

In conclusion, the method presented is simple, rapid and reliable for labelling fatty acids and has several advantages with regard to resolution, analysis time and sensitivity over previously published methods.

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