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THE USE OF FORMIC ACID IN CARRIER GAS

RAPID METHOD FOR IDENTIFICATION AND DETERMINATION OF PHYTANIC ACID BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY—CHEMICAL IONIZATION

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SUMMARY

A rapid gas chromatographic method to determine phytanic acid in plasma from Refsum's disease is described. After a brief alkaline hydrolysis of lipids, the biological sample is directly injected into a glass pre-column; an acid carrier gas (formic acid in nitrogen) is used to displace the long-chain fatty acids from their sodium salts and from their binding to proteins. Formic acid introduced through the column may also be used as a reagent gas for chemical ionization in combined gas chromatography—mass spectrometry; fatty acids (C_{14} to $C_{18:2}$ and phytanic acid) are easily identified by their M + 1 (base peak) and M — 17 peaks. The described procedure is also suitable for studying normal fatty acids from plasma lipids.

INTRODUCTION

The use of an acid carrier gas in gas chromatography (GC) has been shown to

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be useful in the screening of some metabolic diseases by allowing direct injection of plasma volatile fatty acids (VFA) and limiting artefacts due to the adsorption of free VFA on the column [1—3]. Moreover, formic acid in carrier gas may be used as the reagent gas of chemical ionization in combined gas chromatography—mass spectrometry (GC—MS) [4] which allows an easy identification of the acids by their (M + 1) ions. When used with Carbowax 20M as stationary phase, this technique is suitable for the determination of C_2 to C_5 plasma VFA, saturated or not, branched or not, but this column packing is not polar enough to allow a rapid separation of long-chain fatty acids.

As previously described [5], the use of 10% SP 216 PS as stationary phase allows a rapid elution of C_{10} to C_{18} free fatty acids. So it seems interesting to adapt the technique of on-column elution to the analysis of long-chain fatty acids by using SP 216 PS as stationary phase. This procedure, which bypasses the tedious extraction and derivatization steps, should be much more rapid and simple than previously described methods for the identification and estimation of phytanic acid in the diagnosis and care of Refsum's disease [6-8].

Refsum's disease is an inherited lipid storage disease in which phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) accumulates in various organs and fluids; this accumulation is due to the failure of the α-oxidation system which allows the conversion of phytanic acid to the α-hydroxy derivative. Phytanic acid, virtually undetectable in normal plasma, has been found, up to 1.7 mg/ml in the blood plasma of patients with Refsum's disease [9,10].

MATERIALS AND METHODS

Principle

After a rapid alkaline hydrolysis, the biological sample is directly injected into a glass pre-column. Fatty acids are displaced from their salts and protein binding by a formic acid flow saturating the carrier gas.

Reagents

Formic acid (98%) was purchased from Baker (Deventer, The Netherlands), stationary phase SP 216 PS from Sulpelco (Bellefonte, PA, U.S.A.), other chromatographic reagents from Varian Aerograph (Walnut Creek, CA, U.S.A.), phytanic acid from Applied Science Europe (Oud-Beijerland, The Netherlands) and other chemicals from Merck (Darmstadt, G.F.R.).

All volumes were measured with Hamilton syringes.

Gas chromatography

An Intersmat IGC 120 DFL dual-column gas chromatograph equipped with a flame ionization detector (FID) was used without modification. The injector was equipped with a glass pre-column (Insert tube Intersmat) carefully acid-washed, rinsed out, then heated overnight at 270°C in the injector port. This injector was maintained at 270°C and the FID at 200°C.

The stainless-steel column (2 m × 2 mm I.D.) was packed with 10% SP 216 PS on Supelcoport (100—120 mesh) and was operated isothermally at 170°C. Just before gas entry, the nitrogen flow (40 ml/min) was saturated with acid by flowing without bubbling over 1 ml of pure formic acid contained in a 5-ml

screw-cap vial as previously described [3]. The use of pure anhydrous formic acid was necessary to avoid a premature deterioration of the column, especially with a polar phase such as SP 216 PS.

Combined gas chromatography—mass spectrometry

Combined GC—MS was performed on a Ribermag 10-10 B equipped with a Girdel 30 gas chromatograph and a Ribermag 400 data system computer. GC conditions were identical, except for the carrier gas (helium instead of nitrogen) and column temperature (200°C instead of 170°C). GC—MS interface temperature was 250°C, electron energy 70 eV, formic acid in the carrier gas was used as chemical ionization reagent gas, each mass from *m/e* 100 to *m/e* 400 was integrated during 5 msec.

Procedure

Internal standard, pentadecanoic acid in methanol (5 μl) and 20 μl of 10 *M* sodium hydroxide were added to 100 μl of heparinized plasma obtained from blood drawn at the finger tip. After 2 min at 20°C, the solution was rapidly centrifuged and 1 μl injected into the gas chromatograph. Between injections of biological samples 10 μl of 50% (v/v) formic acid were injected in order to clean the pre-column from residual compounds possibly present. In such conditions, the pre-column was used for up to about 20 injections with protein-containing samples.

RESULTS AND DISCUSSION

As noticed by Skrbic and Cumings [11] phytanic acid in plasma from Refsum's disease is esterified and represents up to 35% of total fatty acids. So it is necessary to hydrolyze phytanic acid from its plasmatic esters, which is rapidly performed under strong alkaline conditions (1.7 *M* sodium hydroxide, final concentration) (Fig. 1). The injection of more than 1 μl of such a solution into the gas chromatograph raises some difficulties with regard to the quantity of formic acid found in the injection port during the injection time and it is necessary to displace the sodium salts of fatty acids. One microlitre gives sufficient sensitivity and creates no problem, but the injection of a larger volume (e.g. 5 μl) of alkaline solution leads to a non-quantitative recuperation of injected material and appearance of ghost peaks due to the displacement of the residual salts during subsequent aqueous injections. The linearity of the detector response has been tested on six concentrations from 0.5 m*M* to 10 m*M* for C_{14}, C_{16} and C_{18} in chloroformic solutions. This study has shown that detector response was quite linear for each acid at the quoted concentrations and the response coefficient was very close from one acid to another (1.33 per mmol for C_{18}, 1.31 per mmol for C_{14}).

Thus, results of phytanic acid determinations are expressed in reference to C_{15}, assuming a similar response coefficient; C_{15} was chosen as an internal standard, because it is chemically related to phytanic acid, practically absent from biological fluids, and is well separated from C_{14} and C_{16}. It was added in the concentration range of phytanic acid in plasma from Refsum's disease. The small systematic error possibly arising from reference to C_{15} is not a real problem in clinical applications.

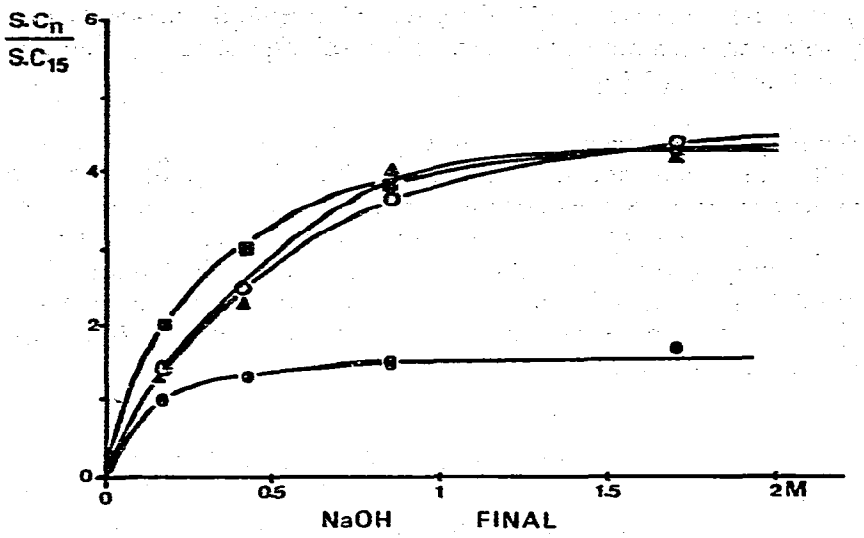

Fig. 1. Influence of final sodium hydroxide concentration on the liberation of fatty acids from normal plasma lipids. Results are expressed as area ratios to internal standard (C_{15}). ■, C_{16}; ●; C_{18}, ○, $C_{18:1}$ and ▲, $C_{18:2}$.

An error by excess in quantification could be made because of the poor separation of C_{17} and phytanic acid on SP 216 PS, but C_{17} is present in a small amount relative to phytanic acid in Refsum's disease.

Standard deviation, studied by ten repetitive determinations on plasma from

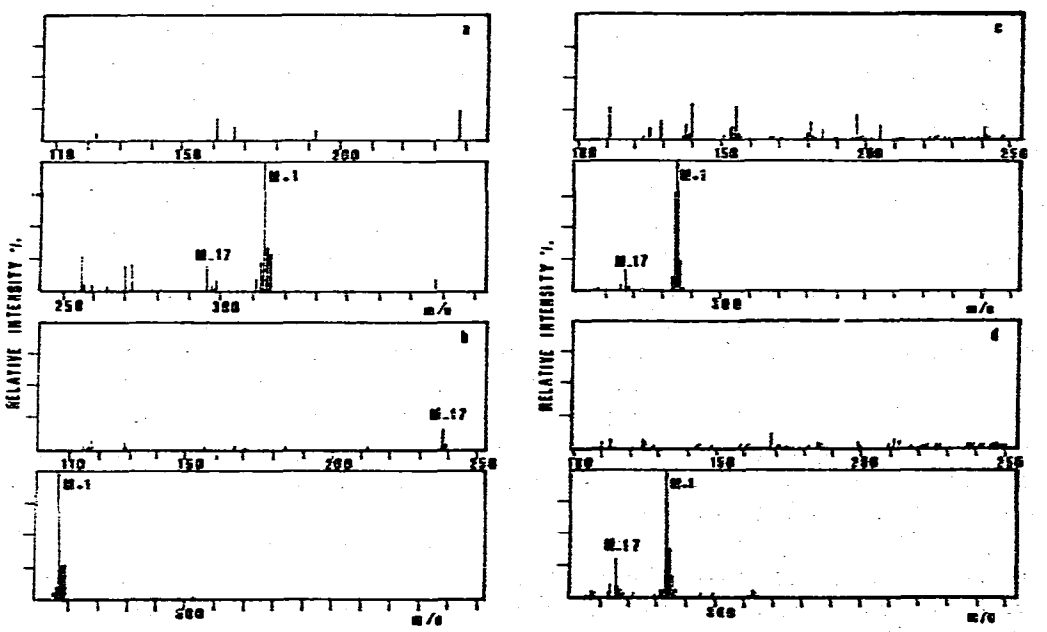

Fig. 2. Chemical ionization (formic acid) mass spectra of four fatty acids. (a) Phytanic acid, M + 1 = 313; (b) palmitic acid C_{16}, M + 1 = 257; (c) stearic acid C_{18}, M + 1 = 285; (d) oleic acid $C_{18:1}$, M + 1 = 283. Spectra were identical with those of authentic acids.

Refsum's disease, was 0.013 at a concentration of 0.265 mg/ml (5%).

As shown by Figs. 2 and 3 the identification of C_{16}, C_{18}, $C_{18:1}$ and phytanic acid may be achieved by formic acid—chemical ionization—mass spectrometry combined to the above-described GC procedure. Characteristic spectra with M + 1 (base peak) and M — 17 peaks were obtained (Fig. 2). They were identical with those of authentic acids. Thus, mass chromatograms of the M + 1 base peak allow unambiguous identification of phytanic acid (Fig. 3B) in plasma from Refsum's disease and the absence of *m/e* 313 (M + 1) base peak in normal plasma (Fig. 3A).

Results in Refsum's disease; comparison with other procedures

The described technique was first tested to quantify the plasmatic non-

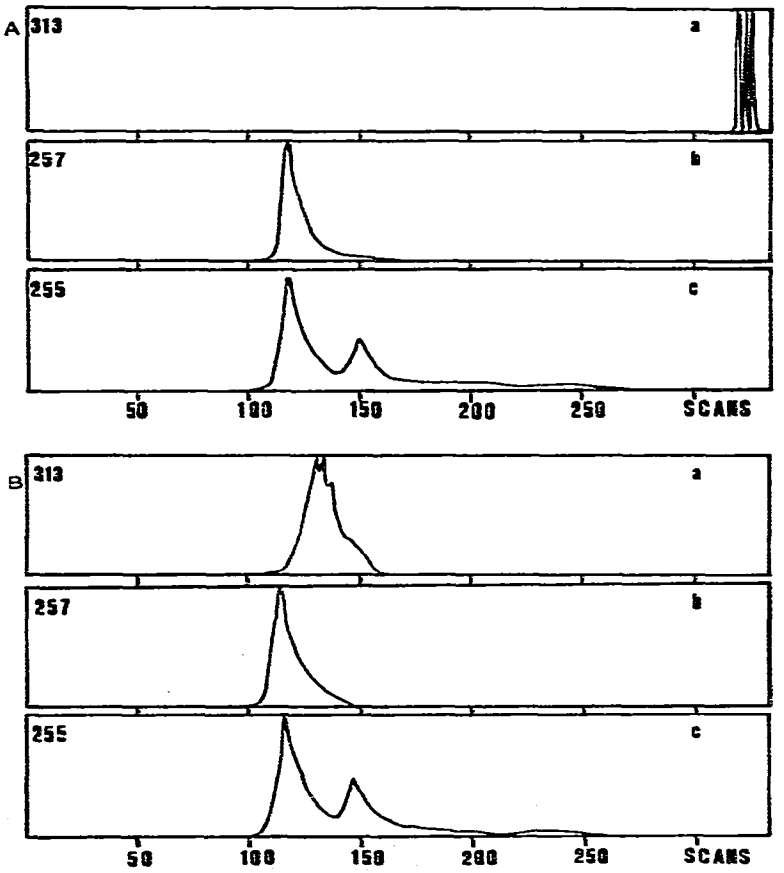

Fig. 3. Mass chromatograms of the fatty acids hydrolysed by 1.7 *N* sodium hydroxide. For GC—MS conditions see text. (A) Normal plasma and (B) Refsum's disease plasma. To achieve a more rapid separation, mass chromatograms in the GC—MS system were performed at 200°C whereas simple chromatographic separations had been realized at 170°C; the elution time of phytanic acid relative C_{16} and $C_{16:1}$ was modified by this temperature difference. Formic acid was used as carrier gas and as a reagent gas in chemical ionization. Each mass corresponds to the M + 1 base peak of a fatty acid. Each chromatogram is normalized on the largest peak so that 313 peak of Fig. 3A is practically non-existent and does not correspond to phytanic acid.

esterified fatty acid (NEFA) but it is not sensitive enough under the described conditions to allow an estimation of each class of NEFA. In Refsum's disease, Fig. 4 shows that phytanic acid is undetectable in the NEFA plasma fraction.

The phytanic acid content of hydrolyzed plasma lipids of Refsum's disease (three patients) (Fig. 5) was compared by two procedures (Table I), the above described on-column injection method and the classical extraction and methylation procedure. Samples were treated by chloroform—methanol (2:1, v/v), and the extract evaporated and methylated [8]. After concentration, the resulting solution was injected onto 10% polyethylene glycol adipate on diatomite (100—120 mesh). All results were expressed with reference to C_{15}.

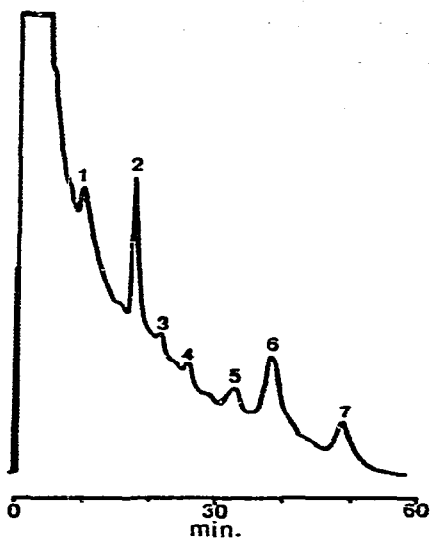

Fig. 4. Non-esterified fatty acids profile in plasma from Refsum's disease. Peaks: 1 = C_{14}; 2 = C_{16}; 3 = $C_{16:1}$; 4 = C_{17} and phytanic acid; 5 = C_{18}; 6 = $C_{18:1}$; 7 = $C_{18:2}$. For chromatographic procedure, see text. Column temperature, 170°C.

TABLE I

COMPARISON OF TWO METHODS, EXTRACTION—METHYLATION AND ON-COLUMN ELUTION DIRECT PROCEDURE, FOR THE ESTIMATION OF PHYTANIC ACID

All results were referred to C_{15} (internal standard).

Sample		Phytanic acid (μg/ml)	
		Extraction—methylation, fresh plasma	Direct procedure, deep frozen plasma
M.G.	17/4	65	90
	31/10	51	65
M.R.	10/10	205	265*
	31/10	50	60
M.S.	untreated	920	870**

*n = 10; S.D. = ± 0.013.
**Fresh plasma.

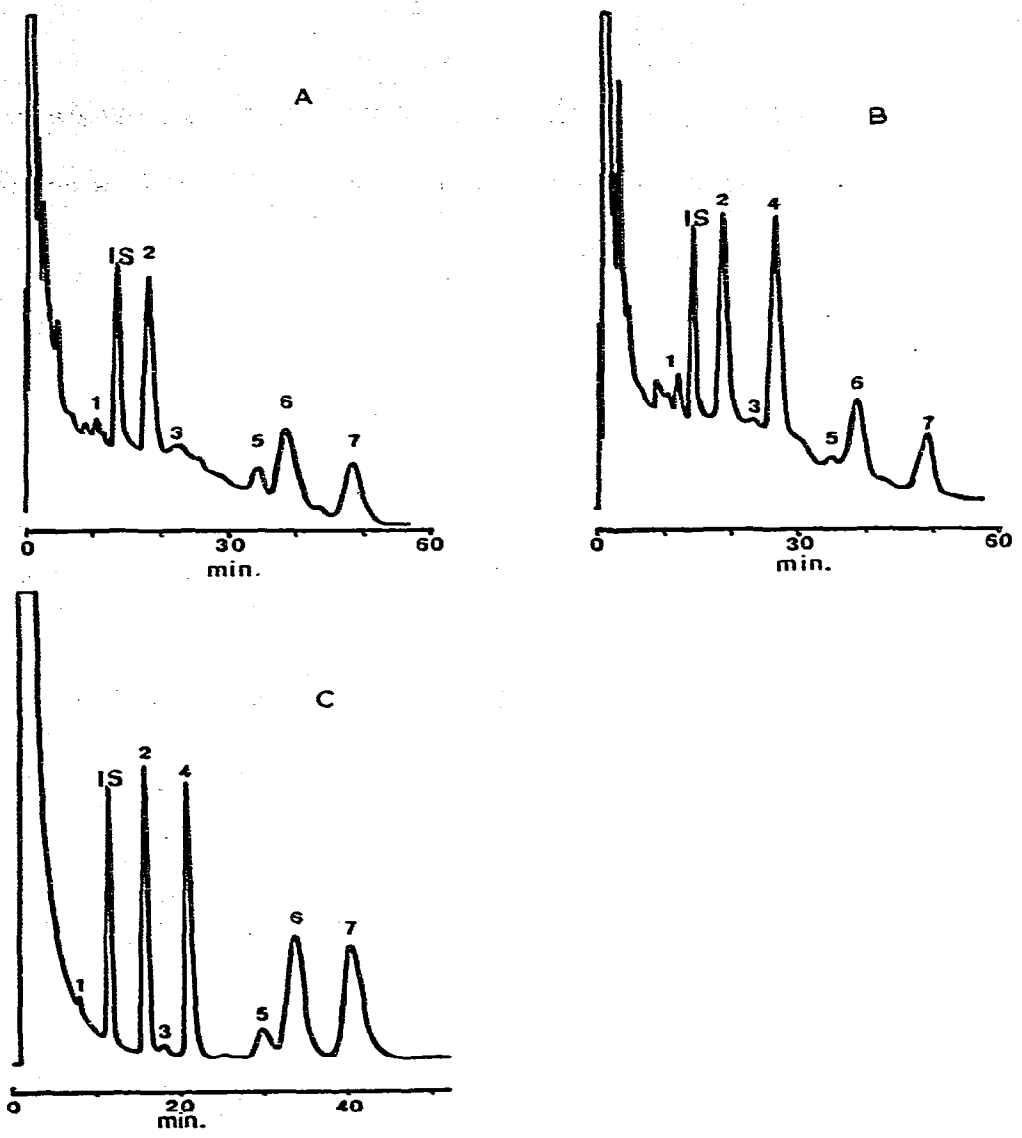

Fig. 5. Plasma fatty acids profile after alkaline hydrolysis. (A) Normal plasma after alkaline hydrolysis direct injection. (B) Plasma from Refsum's disease after alkaline hydrolysis. Direct injection (phytanic acid, 870 μg/ml). (C) Plasma from Refsum's disease (same sample as B) after extraction and methylation (phytanic acid, 920 μg/ml). For chromatographic details see text. Column temperature, 170°C. Peaks: 1 = C_{14}; 2 = C_{16}; 3 = $C_{16:1}$; 4 = C_{17} and phytanic acid; 5 = C_{18}; 6 = $C_{18:1}$; 7 = $C_{18:2}$; IS = internal standard, pentadecanoic acid in methanol.

The fatty acid composition of plasma lipids of one patient (untreated Refsum's disease) was also compared by the two procedures (Table II) which gave similar results and confirmed the high percentage of phytanic acid already noticed by Skrbic and Cumings [11].

CONCLUSION

The described method presents several advantages. First, it allows a very

TABLE II

FATTY ACID COMPOSITION OF PLASMA LIPIDS FROM UNTREATED REFSUM'S DISEASE

Fresh plasma of patient M.S. Total fatty acids: extraction procedure, 920 μg/ml and direct procedure, 870 μg/ml. All results were referred to C_{15} internal standard.

	Total fatty acids (%)	
	Extraction—methylation	Direct
C_{14}	1	2
C_{15}	22	23
Phytanic acid + C_{17}	30	31
C_{18}	5	5
$C_{18:1}$	20	22
$C_{18:2}$	22	17

simple and rapid estimation of phytanic acid in Refsum's disease; no prior manipulations, neither extraction nor derivatization are necessary; a very small sample (1 μl) of alkalinized plasma is directly injected into the pre-column and the whole experiment does not last more than 60 min. Second, it appears to be also suitable for the determination of the total fatty acid composition of plasma lipids. Third, though not very sensitive, it may eventually be used without hydrolysis for a rapid estimation of the main plasmatic non-esterified fatty acid (formic acid does not break the ester bonds of lipids). Finally, formic acid in the carrier gas may also be used to achieve chemical ionization in combined mass spectrometry. Thus, if such an equipment is available, the identification of fatty acids may be easily confirmed, and the sensitivity largely increased by using mass chromatography or fragmentography of the main characteristic peaks (M + 1, M − 17).

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