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Note

Simple and rapid method for the quantitation of plasma phytanic acid by gas chromatography

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Phytanic acid accumulates in variable quantities (20—250 mg/l), in plasma, tissues and lipids [1, 2] of humans suffering from a rare neurological disorder, Refsum's disease, while it is absent in normal subjects. Preformed phytanic acid in food seems to be the principal source of diterpenoid acid found in these patients [3]. It has been observed that in Refsum's disease accumulation of phytanic acid in lipids of plasma and tissues might be due to a failure in the oxidation pathway [4, 5]. Phytanic acid accumulation seems at present to be the most characteristic single sign of Refsum's disease.

In this report we describe a simple gas—liquid chromatographic method for the determination of phytanic acid. The usual esterification techniques adopted for detecting plasma lipids [6—8] require a number of manipulative steps which are time-consuming and which may also result in loss or contamination of the sample. A simple esterification technique is desirable. Some authors have developed transesterification methods for methylating triglycerides in fats and in oils, using an organic base catalyst [9, 10]. We decided to apply a similar technique to a small amount of plasma to determine rapidly and conveniently the total phytanic acid in subjects with Refsum's disease.

The free acid, with the other free fatty acids which may be present in the

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sample, is converted to trimethylphenylammonium salts which thermally degrade to methyl esters by the on-column pyrolysis. The transesterification of triglycerides and other lipids containing ester bonds with phytanic acid cannot be demonstrated as at present we do not have any patient suffering from Refsum's disease.

We have compared our final product to the product obtained with a traditional method [11] by mass spectrometry to check methylation. The result was excellent.

MATERIALS AND METHODS

Instruments

A Perkin-Elmer 3920B gas chromatograph equipped with a flame ionization detector and a 2 m × 2 mm I.D. spiral glass column packed with 15% STAB DEGS WAV 80—100 mesh was used for the gas chromatographic analysis. The column was conditioned overnight at 210°C before use. Column, injector and detector temperatures were fixed at 200°C, 250°C, and 250°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min.

The chemical structure of methyl phytanate obtained by the organic base catalyst was confirmed by using a MAT 44 Varian mass spectrometer coupled to a gas chromatograph.

Reagents

Phytanic acid was obtained from Analabs (North Haven, CT, U.S.A.). Methyl pentadecanoate (internal standard) was obtained from Carlo Erba (Milan, Italy). Trimethylphenylammoniumhydroxide (TMPAH) was obtained from Pierce Eurochemie (Rotterdam, The Netherlands). All organic solvents of analytical grade and methyl pentadecanoate were obtained from Carlo Erba.

A stock solution of the tested substance was prepared in pure chloroform at a concentration of 2.5 mg/ml; the internal standard was made separately in solution at a concentration of 0.4 mg/ml. The two solutions were stored at 4°C. Prior to the assay, aliquots of the stock solutions were diluted to the desired concentrations in blank samples.

Extraction procedure

Aliquots of 0.1 ml of the internal standard (0.4 mg/ml), and 0.1, 0.3, 0.5, 0.8, 1.0, 1.5 ml of phytanic acid solution (0.1 mg/ml) were added to a series of 13-ml glass centrifuge tubes. After evaporating to dryness in a water bath at 60°C, 1.0 ml of human serum or plasma was added to each tube. After mixing for 10 min on a mechanical shaker, 0.1 ml of 12 *M* hydrochloric acid solution was added. The samples were left for 20 min in a water bath at 30°C. After the addition of 8 ml of diethyl ether and mechanical agitation for 30 min, the tubes were centrifuged and 7 ml of the upper layer were transferred to a second series of test tubes; 0.2 ml of 0.2 *M* TMPAH was added to each tube. The tubes were then allowed to stand at room temperature for 30 min. The samples were then evaporated to dryness under nitrogen; the residue was re-dissolved in 0.1 ml of TMPAH; 2 μl of the solution were injected into the gas chromatograph.

RESULTS

Table I shows the within-run precision at two different concentrations (n = 14) for the tested substance. The coefficient of variation did not exceed 8.0%.

Standard curves were constructed by plotting peak area ratios (substance/internal standard) versus substance concentrations (μg/ml). The instrument responses and the concentrations were linearly related over the range 10—150 μg/ml: slope = 19.226, intercept = 1.336, r = 0.999.

The extraction efficiency from serum samples containing known amounts of phytanic acid was 90%.

Fig. 1 depicts a chromatogram of phytanic acid extracted from serum. The retention times for phytanic acid and internal standard were 3 min 59 sec

TABLE I

WITHIN-RUN PRECISION OF THE ANALYSIS OF PHYTANIC ACID

Results are based on fourteen analyses for each of two concentrations of phytanic acid.

Concentration (μg/ml)	Peak area ratio phytanic acid/ internal standard (mean ± S.D.)	Coefficient of variation (%)
50	849.0 ± 68.1	8.0
100	1495.4 ± 81.0	5.4

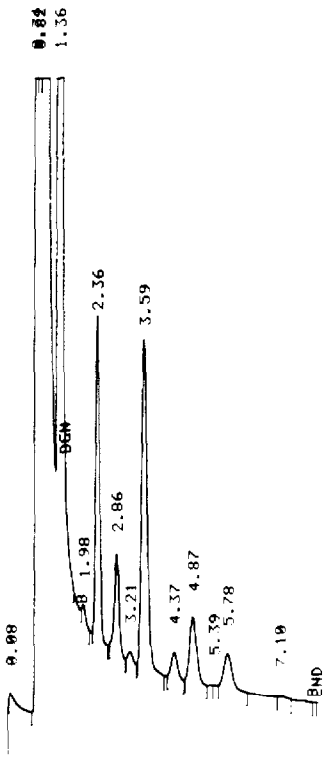

Fig. 1. Gas chromatogram of an extract of 1 ml of plasma pretreated with phytanic acid (retention time 3 min 59 sec) and internal standard (retention time 2 min 36 sec).

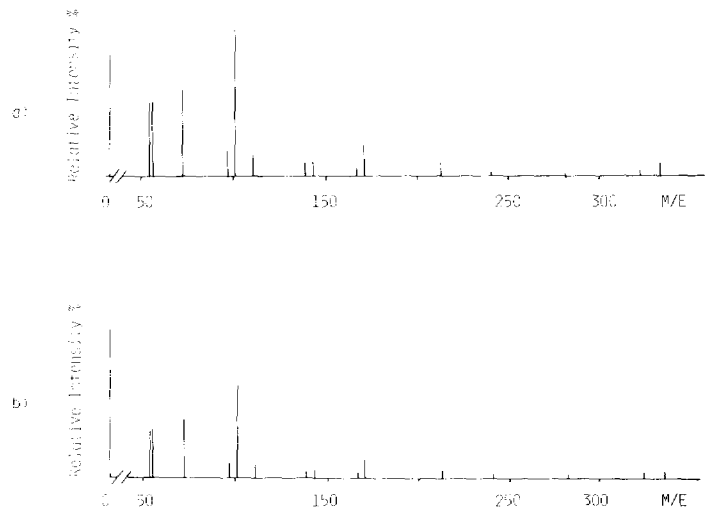

Fig. 2. Mass spectra of phytanic acid obtained by our method (a) and a traditional method (b).

a)

#	MASS	TIME	INTENSITY	
1	[illegible]	3.5	83.2	[illegible]
2	213.0	3.5	7.6	9.1
3	171.0	3.5	20.7	24.8
4	326.0	3.5	13.3	15.9

b)

#	MASS	TIME	INTENSITY	
1	101.0	3.5	163.0	100.0
2	213.0	3.5	13.5	8.2
3	171.0	3.5	39.6	24.2
4	326.0	3.5	26.7	16.3.

Fig. 3. Percentage values for the intensity of four peaks of mass spectra obtained by our method (a) and a traditional method (b).

and 2 min 36 sec, respectively. No interference from serum components was observed and 300 μl of 0.2 *M* TMPAH was sufficient for methylation.

In Fig. 2 the mass spectra of the substances show the formation of methyl phytanate. Fig. 3 shows the ratios of the values of the more significant ions: *m/e* 101, *m/e* 171, *m/e* 213, *m/e* 326.

CONCLUSIONS

The proposed method possesses the important characteristics of precision, sensitivity and selectivity over a wide range of phytanic acid concentrations, in addition to extreme rapidity and simplicity. The retention times for phytanic acid and internal standard are very satisfactory and the two peaks are well separated from the other fatty methyl esters occurring on the chromatogram.

The greatest advantages of this technique are that the number of manipulative steps is kept to a minimum, and loss or contamination of the sample is reduced.

The excess of TMPAH and the high temperature in the pyrolytic methylation often cause isomerization and degradation of unsaturated fatty acids [12], chiefly linoleic acid, but this does not involve phytanic acid, which is a saturated fatty acid.

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