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Note

Simple capillary gas chromatographic method for the quantitation of phytanic acid in serum

K. JACOB*, S. MEHLIN, M. KNEDEL and W. VOGT

Institut für Klinische Chemie am Klinikum Grosshadern der Universität München, Postfach 701260, D-8000 Munich 70 (F.R.G.)

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Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a trace component of human lipids originating from the diet [1]. Large amounts of this exogenous branched-chain fatty acid are accumulated in patients suffering from Refsum's disease, an inherited disorder of phytanic acid metabolism [2]. Diagnosis and therapeutic monitoring of this rare neurological disease are performed by quantitating phytanic acid in serum [3].

Conventional methods for the determination of phytanic acid in serum are laborious and time-consuming. Usually they consist of a series of procedural steps, e.g. extraction, saponification, back-extraction, methylation and, finally, gas chromatographic (GC) analysis of the methyl ester formed. A decisive improvement in the estimation of fatty acids from edible oils and fats was achieved by McCreary et al. [4] using a direct transesterification method with *m*-trifluoromethylphenyltrimethylammonium hydroxide as the reagent. Thus, saponification, back-extraction and re-esterification could be replaced by a simple pyrolysis methylation step. To our knowledge, the first application of this transesterification technique to the direct analysis of total phytanic acid from crude extracts of human serum lipids was reported by our group in 1981 [5]. Recently, two papers were published dealing with a similar technique for the direct analysis of serum phytanic acid [6, 7]. However, these authors were using trimethylphenylammonium hydroxide for the transmethylation step and packed columns for GC separation. In our opinion, packed-column GC cannot be recommended for sufficient separation of analytes in such a complex matrix as serum.

The aim of this contribution is to demonstrate the inherent superior

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sensitivity and separation performance of our capillary GC, direct-methylation method for the quantitation of phytanic acid in serum.

EXPERIMENTAL

Reagents and materials

Methanol and chloroform were for residue analysis and purchased from Merck (Darmstadt, F.R.G.). Dichloromethane, toluene, isopropanol and pentadecanoic acid were of analytical grade (Merck). Methyl propionate, with a purity of 97—99%, was delivered from EGA-CHEMIE (Steinheim, F.R.G.). Phytanic acid, with a purity of 99%, was from Antechnika (Karlsruhe, F.R.G.). *m*-Trifluoromethylphenyltrimethylammonium hydroxide was used as a 0.2 mol/l methanolic solution (METH-PREP II), which was obtained from Applied Science (State College, PA, U.S.A.).

Apparatus

GC analyses were performed with a Model 2800 gas chromatograph (Varian, Darmstadt, F.R.G.) equipped with either a 25-m SP-1000 glass capillary column (LKB, Bromma, Sweden) or a 30-m DB-225 fused-silica capillary column (J&W Scientific, Rancho Cordova, CA, U.S.A.). Carrier gas was nitrogen at a flow-rate of ca. 2 ml/min. Other conditions: injector temperature, 220°C; column temperature, 100—235°C; heating rate, 6°C/min; flame-ionization detector temperature, 235°C; splitting ratio, 1:10.

Method

Serum (0.2 ml), distilled water (4 ml) and 1 *M* hydrochloric acid (10 µl) were placed in a separatory funnel and extracted with 40 ml of chloroform—methanol (2:1) by vigorous shaking for 10 min, according to Lin and Horning [8]. The mixture was held for 6—12 h at 6°C for phase separation. The lower phase was concentrated in a rotary evaporator at 25°C. A solution of 100 µg of pentadecanoic acid in 100 µl of isopropanol was added, the sample was transferred with three portions of 500 µl of dichloromethane into a 3-ml conical glass tube and evaporated to dryness in a stream of nitrogen. The residues were dissolved in 150 µl of toluene and allowed to react with 60 µl of METH-PREP II for 30 min at ambient temperature. A 1-µl aliquot of this mixture was injected, together with 1 µl of methyl propionate, into the gas chromatograph.

RESULTS AND DISCUSSION

Extraction

Extraction of serum samples was performed according to the method of Folch et al. [9]; however, the modified procedure of Lin and Horning [8] was used. Solid-phase extraction with Extrelut as described by Klump et al. [10] resulted in greater variations of recovery compared with liquid—liquid extraction. Pentadecanoic acid served as internal standard, which was added after the extraction step in order to compensate for losses during sample transfer and to enable GC quantitation.

Methyl ester formation

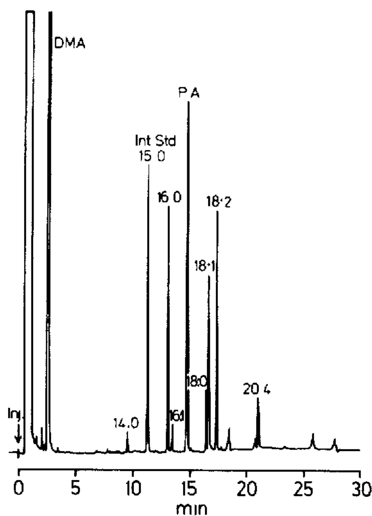
The methanolic solution of *m*-trifluoromethylphenyltrimethylammonium hydroxide works not only as a transesterification reagent but also as a methylation reagent for free fatty acids [4]. We were able to lower the pyrolysis temperature appreciably in the GC injection step from 250°C as described by McCreary et al. [4] down to 220°C by co-injection of methyl propionate. This compound, in addition, protects unsaturated fatty acids from degradation at elevated temperatures [11].

Gas chromatography

Complete chromatographic separation of phytanic acid methyl ester from the principal fatty acid methyl esters from human serum was achieved either on a 25-m SP-1000 glass capillary column (Fig. 1) or on a 30-m DB-225 fused-silica capillary column. However, heptadecanoic acid, a trace lipid component, exhibited nearly the same retention time as phytanic acid. Therefore, quantitation of normal levels of phytanic acid in human serum (0.5—3.5 μg/ml) requires a more specific detection mode such as mass fragmentography [12].

The chromatographic performance of the capillary columns is not influenced by the methylation reagent, which is decomposed in the hot zone of the injector to give N,N-dimethyl-(*m*-trifluoromethyl)aniline [4]. No deterioration of the columns was observed after several hundred injections.

Additional peaks are eluted at the end of the temperature programme after a series of ten to fifteen injections. These peaks were identified as free cholesterol, which is formed during the transesterification process. In this case,



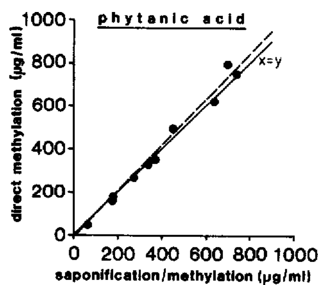


Fig. 1. Fatty acid profile of an untreated patient suffering from Refsum's disease, obtained by direct methylation. DMA = N,N-Dimethyl-(*m*-trifluoromethyl)aniline; Int. Std. = internal standard; P.A. = phytanic acid methyl ester. Conditions: 25-m SP-1000 glass capillary column, 100—235°C (6°C/min).

Fig. 2. Comparison of two methods for the quantitation of phytanic acid in serum of patients with Refsum's disease (n = 10, r = 0.9921): (—) 45°-line (x = y); (- - - -) regression line.

the column has to be kept at 235°C in order to remove these trapped compounds. Free glycerol could not be detected under the chromatographic conditions applied.

Criteria of the method

The calibration curve is quite linear in the range 20—800 μg/ml (r = 0.9998), using phytanic acid for establishing the standard curve. A coefficient of variation of 4% was obtained at a concentration of 450 μg/ml (n = 8), calculating the results from the peak-height ratios. The detection limit was found at 5—10 μg/ml, with a sample volume of 200 μl.

The accuracy was checked by analysing ten different serum samples of Refsum patients with a conventional method [3] and the method presented here. The results were quite similar, offering a correlation coefficient of 0.9921 (Fig. 2). Thus it can be assumed that the direct-methylation technique leads to results comparable with those obtained with conventional saponification/re-esterification techniques for the quantitation of phytanic acid in serum.

CONCLUSION

The direct-methylation method for the determination of phytanic acid in serum, combined with capillary GC, is a highly sensitive, reliable and accurate technique. A special advantage of this method is its great simplicity, saving much time and labour.

Up to now, this method enabled us to detect five patients suffering from Refsum's disease. Furthermore, it was successfully applied to the therapeutic surveillance of Refsum patients by dietary treatment and long-term plasma exchange over a period of several years [13].

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