Capillary gas-liquid chromatographic-mass spectrometric measurement of very long chain (C_{22} to C_{26}) fatty acids in microliter samples of plasma

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Summary In order to quantify accurately the plasma content of very long chain fatty acids, we have developed a selected ion monitoring gas-liquid chromatographic-mass spectrometric micromethod which allows all of these acids (22:0, 24:1, 24:0, 26:1, and 26:0) to be determined simultaneously in the same 0.5-ml plasma sample; 17:0 and 27:0 fatty acids are used as assay internal standards. For plasma samples in the range equivalent to the various very long chain fatty acid physiological concentrations, assay precision was ±2%. The present method has been successfully applied to the biological recognition of patients with adrenoleukodystrophy, their heterozygote relatives, and of cerebro-hepato-renal syndrome and neonatal adrenoleukodystrophy. —**Aubourg, P., P. F. Bougnères, and F. Rocchiccioli.** Capillary gas-liquid chromatographic-mass spectrometric measurement of very long chain (C_{22} to C_{26}) fatty acids in microliter samples of plasma. *J. Lipid Res.* 1985. **26:** 263-267.

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Adrenoleukodystrophy (ALD) is a genetically determined progressive disorder which affects mainly the adrenal cortex and the white matter of the nervous system. It is associated with the accumulation of very long chain saturated fatty acids (VLCFA), particularly hexacosanoic acid (26:0), in nervous tissue (1), fibroblasts (2), plasma lipids (3), and leukocytes (4). This provided the basis for the biological recognition of this disease, using conventional plasma lipid extraction procedures and quantitation of this fatty acid by capillary gas-liquid chromatography (3).

However, since hexacosanoic acid accounts for only a minute fraction of plasma lipids, even in ALD, these techniques had to involve elaborate and tedious procedures for fatty acid purification from neutral lipids, which largely limited their clinical use.

Moreover, identification of ALD heterozygotes as well as antenatal recognition of affected fetus may be unsatisfactory with these techniques when small increments of hexacosanoic acid have to be accurately quantified.

Therefore, we designed a method suitable for these purposes, using simple procedures for extraction of plasma lipids and derivatization, and then simultaneous quantitation of 22:0, 24:1, 24:0, 26:1, and 26:0 fatty acids by gas-liquid chromatography-mass spectrometry (GLC-MS) using 17:0 and 27:0 fatty acids as internal standards.

MATERIALS AND METHODS

Patients

One-ml samples of venous blood were obtained from three patients with classical ALD, eight patients with the so-called "neonatal ALD," and from two patients with cerebro-hepato-renal syndrome (CHRS). Six subjects known to be heterozygotes for ALD were also studied and the results were compared with those from eleven age-matched normal controls.

Materials

Heptadecanoic (17:0), docosanoic (22:0), tetracosanoic (24:0), hexacosanoic (26:0), and heptacosanoic (27:0) acids were purchased from Analabs (North Haven, CT). Chemical purity of these reagents was confirmed by conventional GLC-MS analyses of their respective trimethylsilyl derivatives. N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Pierce (Rockford, IL); chloroform, methanol, hexane, and acetonitrile were purchased from Fluka (Buchs, Switzerland).

Stock solutions of 17:0, 22:0, and 24:0 acids were prepared by dissolving 5 mg of accurately weighed fatty acid in 100 ml of chloroform. Stock solutions of 26:0 and 27:0 acids were similarly prepared by dissolving 1 and 3 mg of each fatty acid in 1000 ml of chloroform. Working calibration standard solutions were prepared by serially diluting various combinations of stock solutions of each fatty acid (see Results) which were kept stored at −20°C. For internal standards, the desired amounts of 17:0 and 27:0 fatty acids in chloroform were added to the initial chloroform-methanol extract containing plasma lipids as described.

Analytical methods

In order to isolate total lipids from plasma, the conventional procedure of Folch, Lees, and Sloane Stanley (4) was modified as follows: 0.5 ml of plasma was added to 4.5 ml of a solution of chloroform-methanol 1:1 and the lipids were extracted by vigorous shaking. After centrifugation, the supernatant was added to 1.5 ml of double-distilled water and 3.5 ml of chloroform containing 99 nmol of 17:0

Abbreviations: ALD, adrenoleukodystrophy; VLCFA, very long chain fatty acids; GLC-MS, gas-liquid chromatography-mass spectrometry; CHRS, cerebro-hepato-renal syndrome.

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fatty acid and 7.5 nmol of 27:0 fatty acid. The samples were reshaken and centrifuged, and the upper phase was discarded. The lower phase containing the lipids was washed twice with a volume of Folch solution (methanol–saline–chloroform 48:47:3) (5) equal to that of the discarded phase; it was then evaporated to dryness under N_2 at room temperature. The residues were resuspended in 2.5 ml of a 0.5 M solution of HCl in acetonitrile–water 9:1, flushed with N_2, and heated at 100°C for 45 min in order to release fatty acids from lipids, as recently described by Aveldaño and Horrocks (6). After cooling, 5 ml of hexane was added to each sample, and the fatty acids were extracted into the hexane. The hexane phase was transferred to another tube containing 2 ml of 6 M KOH. The mixture was again shaken vigorously, the upper hexane phase was discarded, and the lower phase was washed with 5 ml of hexane. The alkaline phase containing the fatty acids was then acidified with 2 ml of 10 M HCl, and the free fatty acids were extracted twice into 5 ml of hexane. The combined hexane extracts were pooled and evaporated to dryness under N_2 at room temperature in a conical reaction vial. The trimethylsilyl esters of the fatty acids were obtained by dissolving the residues in 50 μl of (BSTFA/TMCS)–pyridine 2:1 mixture and heating at 60°C for 10 min. Samples were stored at −20°C until analysis.

Gas–liquid chromatographic–mass spectrometric analyses

Plasma fatty acid trimethylsilyl esters were separated on a gas chromatograph (Carlo Erba, Italy) coupled with a quadrupole mass spectrometer (Nermag, Rueil-Malmaison, France) equipped with an INCOS 2000 Series Data System (Finnigan MAT, Sunnyvale, CA). Chromatographic conditions were as follows: temperature was programmed from 70 to 295°C (30°C per min) on a CPtm Sil CB capillary column (25 m × 0.32 mm, Chrompack, The Netherlands) using helium carrier gas (2 ml per min) in an oven equipped with an "on column" injector (Carlo Erba, Italy). Mass spectrometer ion source temperature was maintained at 300°C, multiplier voltage was 2.2 kV, emission current was 0.2 mA, and electron energy was 70 eV. Ions at m/z 327, m/z 397, m/z 423, m/z 425, m/z 451, m/ 453, and m/z 467, corresponding to the respective [M − 15] ions of 17:0, 22:0, 24:1, 24:0, 26:1, 26:0, and 27:0 fatty acid trimethylsilyl esters, were selectively monitored (**Fig. 1**). The corresponding peaks were integrated and the content of each fatty acid was calculated as described below.

Since each analytical run requires injection of only approximately 2 μl of the final (BSTFA/TMCS)–pyridine solution, several replicate measurements can be made on each 0.5-ml plasma sample.

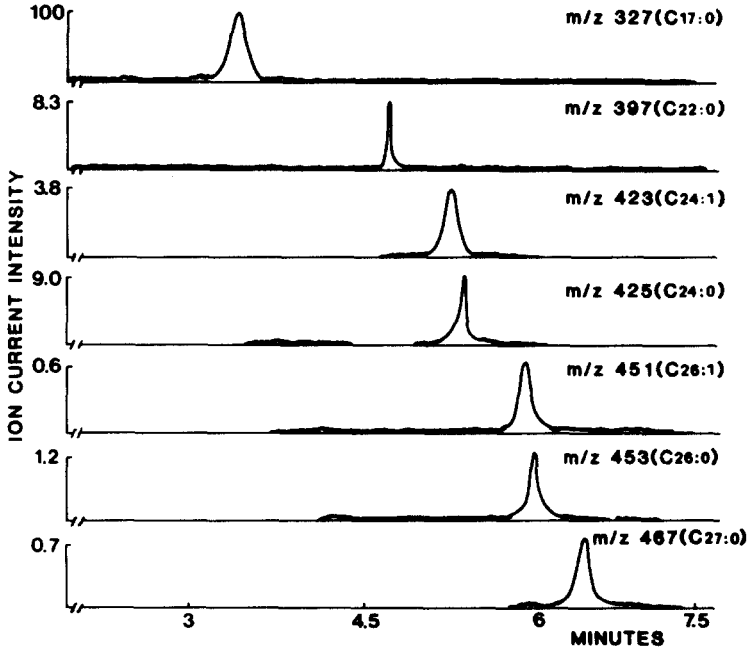

Fig. 1. Mass chromatograms of the [M − 15] ions of 22:0, 24:1, 24:0, 26:1, and 26:0 fatty acids and the internal standard 17:0 and 27:0 fatty acids selected for their quantitation in a 0.5-ml plasma sample. Fatty acids 17:0 and 27:0 were selected because they had both chromatographic retention times and m/z of [M − 15] ions located, respectively, at each edge of the desired range of retention times and ion masses. Ion current intensities are expressed as percentage of the peak at m/z 327.

Calculations

Prior to plasma analysis, calibration standards containing the same amounts of 17:0 and 27:0 fatty acids as internal standards, but different amounts of 22:0, 24:0, and 26:0 fatty acids were analyzed by GLC–MS to establish a set of standard curves defined by the following equations:

$$RC1\ (397/327) = k1\ (N\ 22:0/N\ 17:0) \quad Eq.\ 1$$

$$RC2\ (425/327) = k2\ (N\ 24:0/N\ 17:0) \quad Eq.\ 2$$

$$RC3\ (453/467) = k3\ (N\ 26:0/N\ 27:0) \quad Eq.\ 3$$

where RCi is the observed ion current ratio corresponding to the [M − 15] ion of each fatty acid and of the internal standard selected for its quantitation; k1, k2, and k3 are the slope of each calibration line; N 17:0, N 22:0, N 24:0, N 26:0, and N 27:0 were the absolute amounts (in moles) of the corresponding fatty acids, respectively, in the calibration standards. Since these amounts are known and RCi values are measured, equations 1, 2, and 3 can be solved for k1, k2, and k3.

The absolute amounts of endogenous 22:0, 24:0, and 26:0 fatty acids in the plasma samples (N 22:0, N 24:0, and N 26:0) were then calculated from the measured 397/327, 425/327, and 453/467 ion current ratios (RS1, RS2, and RS3, respectively), according to the following rearrangements of equations 1, 2, and 3:

$$N\ 22:0 = (N\ 17:0/k1) \times RS1\ (397/327) \quad Eq.\ 4$$

$$N\ 24:0 = (N\ 17:0/k2) \times RS2\ (425/327) \quad Eq.\ 5$$

$$N\ 26:0 = (N\ 27:0/k3) \times RS3\ (453/467) \quad Eq.\ 6$$

The amounts of 24:1 and 26:1 fatty acids in plasma samples (N 24:1 and N 26:1) were calculated from the observed 423/425 and 451/453 ion current ratios, respectively, m/z 423 and m/z 451 being the masses of the [M − 15] ions of the respective trimethylsilyl esters of 24:1 and 26:1 fatty acids, and from the measured amounts of plasma 24:0 and 26:0 fatty acids:

$$N\ 24:1 = N\ 24:0 \times RS4\ (423/425) \quad Eq.\ 7$$

$$N\ 26:1 = N\ 26:0 \times RS5\ (451/453) \quad Eq.\ 8$$

The results were corrected for sample volume and expressed as nanomoles of 22:0, 24:1, 24:0, 26:1, and 26:0 fatty acids per liter of plasma.

RESULTS

To test the linearity of the assay (**Fig. 2**), a series of standards containing 14.7–117.6 nmol of 22:0, 9.64–135.05 nmol of 24:0, and 1.11–5.0 nmol of 26:0 fatty acids in 500 μl of chloroform was prepared in triplicate. Internal standards, 99 nmol of 17:0 and 7.31 nmol of 27:0 fatty acids, were added to each sample and the sample was analyzed as described. Fig. 2 shows that the assay was linear

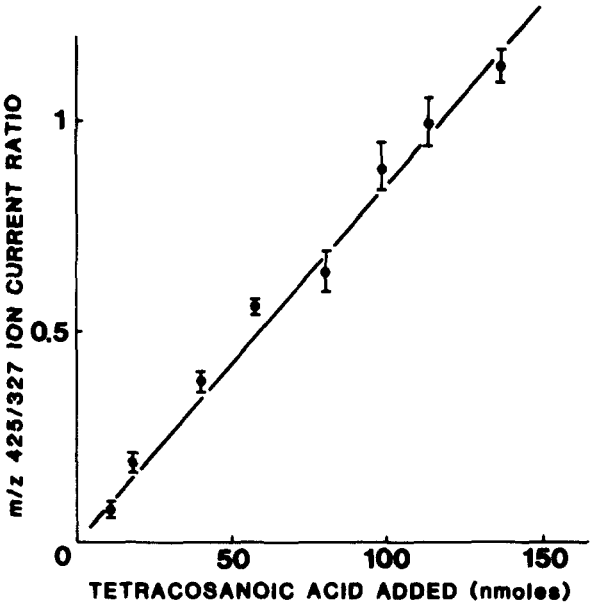

Fig. 2. Calibration curve for determinations of tetracosanoic (24:0) acid using heptadecanoic (17:0) acid internal standard as described. The equation describing the experimental line is $y = 0.0084\ x$; $r = 0.998$.

throughout the measured range and the average precision (standard deviation/mean) of individual estimate was 5%. Similar results were obtained for docosanoic ($y = 0.0086\ x$; $r = 0.998$) and hexacosanoic ($y = 0.58\ x + 0.0513$; $r = 0.998$) acids, with an average relative precision of 3.1 and 4.2%, respectively. Except for amounts of 22:0, 24:0, and 26:0 fatty acid corresponding to less than 100 pg injected into the GLC–MS system, there was no concentration-dependent effect on measurement precision. In the physiological range of plasma 26:0 fatty acid concentrations, the average relative precision was 2.1%, while it was 2.5% for 22:0 and 1.5% for 24:0 fatty acids, respectively.

To examine the effects of the extraction and hydrolysis procedures on the results, triplicate 0.5-ml aliquots of the above standard solutions were evaporated to dryness, redissolved in 0.5 ml of plasma, and analyzed by the present method using 17:0 and 27:0 fatty acids as internal standards. The content of 22:0, 24:0, and 26:0 fatty acids was thus determined in unspiked and various spiked plasma samples, showing no detectable significant differences with the calculated expected values. **Fig. 3** shows the results obtained in eleven normal children, three children with classical ALD, and relatives known to be heterozygotes for classical ALD, neonatal ALD and CHRS, for each of the measured fatty acids. Clear-cut differences were observed between these groups for 26:0 fatty acid concentration in plasma lipids, allowing, in particular, for unambiguous recognition of heterozygotes for ALD disease ($P < 0.0001$ for the whole group vs. controls). Likewise, average 22:0, 24:1, 24:0, and 26:1 fatty acid concentrations were increased in patients with ALD,

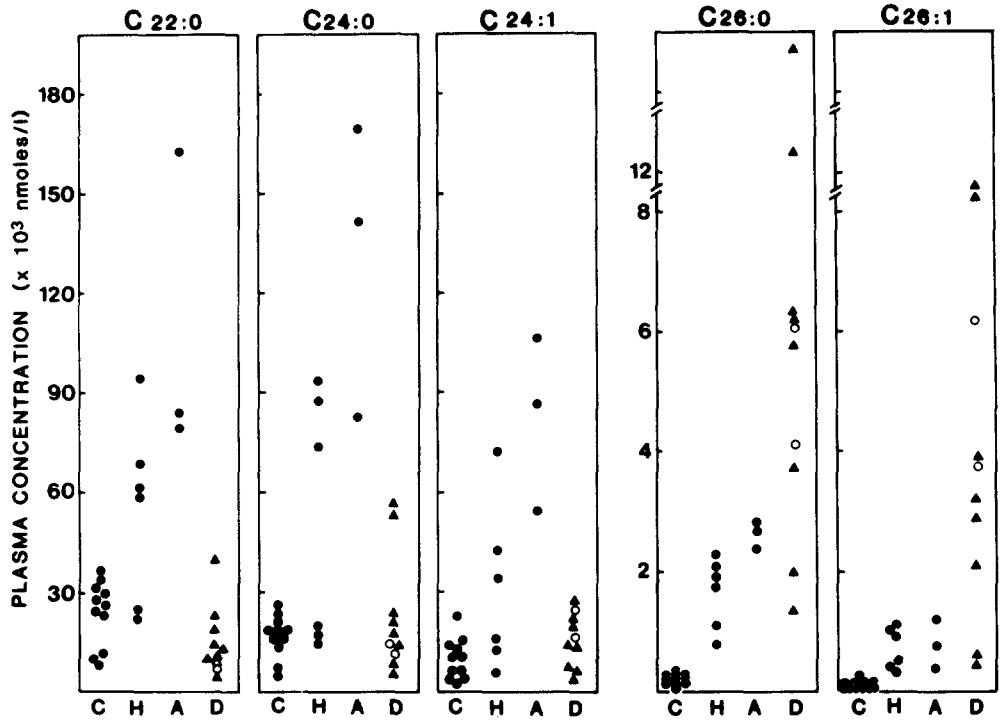

Fig. 3. Plasma concentration of VLCFA in controls (C), heterozygotes for ALD (H), patients with ALD (A), or related disorders (D), neonatal ALD (▲), and cerebro-hepato-renal syndrome (○).

and intermediary in heterozygotes, although overlapping zones of values existed between the groups. Interestingly, neonatal ALD and CHRS plasma had distinctively elevated 26:0 and 26:1 fatty acid concentrations, while 22:0 and 24:0 fatty acid concentrations were in the low normal range.

The present method and a conventional GLC assay of plasma 26:0 fatty acid (3, 7) gave almost identical results in the same patients: 1828 ± 490 nmol/l (mean ± standard deviation) versus 1785 ± 722 nmol/l in ALD heterozygotes, and 2899 ± 88 nmol/l versus 2616 ± 214 nmol/l in ALD patients, respectively. The relative precision of plasma content of hexacosanoic acid averaged 2% in the present method.

DISCUSSION

Increased plasma content of 26:0 fatty acid is the only distinctive biochemical marker available today for the recognition of ALD patients (3). The selected ion monitoring GLC-MS method presented fulfills the requirements for extending measurement of 26:0 fatty acid to situations where the amount of available material is limited by sample size: capillary blood samples in neonates, amniotic fluid and trophoblastic tissue samples during pregnancy in a heterozygote mother, and culture media in in vitro studies. The measurement of 26:0 fatty acid in these situations is a necessary step for a better understanding and management of ALD in the future.

Moreover, the present method provides simultaneous measurement of 22:0, 24:1, 24:0, and 26:1 fatty acid concentrations, for which no other technical alternative exists so far. The practical nature of the method is illustrated by analysis of these fatty acids in normal subjects, ALD hetero- and homozygotes, and other disorders; to our knowledge, these are the first quantitative estimates of the plasma content of 22:0, 24:1, and 24:0 in human subjects. Finally, while specifically designed for recognition of ALD patients, the sensitivity and accuracy of the current method may find additional application in in vivo studies of VLCFA physiology and pathophysiology, as well as in vitro investigations of their metabolism.

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