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Long-chain fatty alcohol quantitation in subfemtomole amounts by gas chromatography–negative ion chemical ionization mass spectrometry

Application to long-chain acyl coenzyme A measurement

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ABSTRACT

We describe a simple and sensitive method to identify and quantitate long-chain fatty alcohols. Long-chain fatty alcohols were converted to their pentafluorobenzoyl derivative and analyzed by gas chromatography (GC)–mass spectrometry in the negative ion chemical ionization (NICI) mode with selected ion monitoring. GC resolution was obtained for myristyl, palmityl, heptadecyl, stearyl, oleyl, linoleyl and arachidonyl alcohols. As little as 0.4 fmol of fatty alcohol can be detected, which represents a six order-of-magnitude increase in sensitivity over previously described methods. This assay can be used to measure femtomolar amounts of long-chain acyl coenzyme A thioesters after reduction to the corresponding fatty alcohols with sodium borohydride. Other potential applications of this assay include identification and quantitation of long-chain fatty alcohol production by microorganisms.

INTRODUCTION

Measurement of endogenous cellular levels of long-chain acyl CoA thioesters and the identification of the long-chain acyl composition of these compounds is attracting interest in a variety of settings. Long chain acyl coenzyme A (CoA) thioesters have been implicated as substrates in a number of metabolic reactions at the cellular level[1,2]. Increased amounts of these compounds have been described in certain pathological conditions, including myocardial ischemia, and may contribute to cellular injury in such settings[3,4]. More recently, *de novo* synthesis of diacylglycerol from glucose has been implicated in the pathogenesis of the vascular complications of diabetes mellitus[5]. There is evidence in various tissues, including pancreatic islets and retinal capillary endothelial cells, that this pathway involves metabolism of glucose to triose phosphates, which are then acylated using long-chain acyl CoA as the fatty acyl

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donor. The resultant acyl-dihydroxyacetone-phosphate is then reduced to lysophosphatidic acid, which is hydrolyzed[5–10].

A number of assays have been decribed for the measurement of long-chain acyl CoA thioesters[11–19]. High-performance liquid chromatographic (HPLC) methods achieve sensitivity in the nanomolar range[16]. Recently, Prasad *et al.*[17] have described a technique which takes advantage of the ability of sodium borohydride to reduce the thioester linkage of CoA esters[18] to obtain fatty alcohols, which were then identified and quantitated in the subnanomolar range by gas chromatography (GC) with flame ionization detection. These authors suggested that the sensitivity of this technique might be increased with the use of mass spectrometric (MS) detection. We describe here a GC–negative ion chemical ionization (NICI)-MS method for the measurement of fatty alcohols derived from long-chain acyl CoA esters in the subfemtomolar range.

MATERIALS AND METHODS

Materials

Fatty alcohols were purchased from Avanti (Pelham, AL, U.S.A.). Long-chain acyl CoA esters were obtained from Sigma (St. Louis, MO, U.S.A.). [1-^{14}C]Myristoyl CoA was from Amersham (Arlington Heights, IL, U.S.A.). All other chemicals including pentafluorobenzoyl chloride were obtained from Sigma. All solvents (HPLC grade) were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). Screw-cap septum vials (1.5 ml, No. 13208) were obtained from Pierce (Rockford, IL, U.S.A.).

Derivatization of fatty alcohols

Long-chain fatty alcohols were concentrated to dryness under nitrogen in silanized, acid-washed 1.5-ml glass screw-cap vials. Derivatization was performed by adding 0.1 ml of pentafluorobenzoyl chloride and incubating for 45 min at 120°C. The contents were then concentrated to dryness (nitrogen) and water (0.1 ml) was added. Extraction was performed with methylene chloride (0.2 ml), followed by one water (0.1 ml) wash. The organic extract was evaporated under nitrogen and the sample was reconstituted in heptane (0.1 ml) prior to analysis.

Gas chromatographic–mass spectrometric analysis

GC was performed on a Hewlett-Packard 5890 gas chromatograph interfaced with a Hewlett-Packard 5988 mass spectrometer. A capillary column (Hewlett-Packard Ultraperformance, 8 m × 0.31 mm I.D., cross-linked methylsilicone, film thickness 0.17 μm) was operated with a Grob-type injector in the splitless mode with helium as carrier gas (inlet pressure 4 p.s.i., injector temperature 280°C). The distal end of the column was inserted directly into the ion source (interface temperature 280°C). The GC oven temperature was programmed from 85 to 200°C at a rate of 30°C/min starting 0.8 min after the injection, and maintained at 200°C for 6 min. The mass spectrometer was operated in the NICI mode with methane as reagent gas (source pressure 0.6 Torr). The ionizing potential was 240 eV. Selected ions were monitored which corresponded to the molecular ion of the fatty alcohol pentafluorobenzoyl ester derivative.

Long-chain acyl CoA analysis

Two extraction techniques of long-chain acyl CoA were investigated. The first scheme was described by Prasad *et al.*[17] and involves chloroform–methanol extraction, acetonitrile precipitation, adsorbtion on Al_2O_3, and reduction of the adsorbed fatty acyl CoA to alcohols with sodium borohydride. Fatty alcohols were extracted with pentane, converted to the pentafluorobenzoyl ester, and then analyzed by GC–NICI-MS as described above.

In the second scheme, a chloroform–methanol (1:2, v/v) extract containing long-chain fatty acyl CoA thioesters with heptadecanoyl CoA as an internal standard (3 nmol) was applied to a channeled thin-layer chromatography (TLC) plate (Whatman LK6) which was developed in butanol–acetic acid–water (100:40:60, v/v/v) for *ca.* 4–5 h[20]. Long-chain acyl CoA spots were localized by light iodine staining of the standards run on the same plate and the corresponding silica was scraped into silanized conical 5-ml borosilicate tubes (with PTFE screw caps). Long-chain acyl CoA thioesters were extracted from the silica by adding 0.5 ml of the TLC developing solvent, vortexing 30 min and centrifuging 5 min at 800 *g*. The supernatant was then transferred to a clean 5-ml tube. (The extraction was repeated two more times). Following concentration under nitrogen, the following solution was added to each tube: 1 ml of diethyl ether, 0.05 ml of 0.02 *M* borate buffer pH 8.0, 10 m*M* $CaCl_2$ and 20 units of phospholipase C from *B. cereus*. Tubes were incubated 2 h at 37°C. The contents of each tube were concentrated to dryness (nitrogen); reconstituted in 0.05 ml of ethanol–acetic acid (50:50, v/v); and applied to a channeled Whatman LK6 plate, which was developed in the same solvent system as described above. Long-chain acyl CoA thioester spots were scraped and reduced to their corresponding long-chain fatty alcohols by the addition of 1.25 ml of methanol–water (50:50, v/v) and 16 mg of $NaBH_4$ and were then incubated 60 min at 37°C in a shaking water bath. The reaction was terminated with 0.6 ml of 1 *M* hydrochloric acid. Fatty alcohols were extracted with 2 ml of pentane twice. The pentane fraction containing the fatty alcohols was washed with water (1 ml) and concentrated to dryness (nitrogen). Derivatization and analysis of the pentafluorobenzoyl derivatives of fatty alcohols were performed as described above.

RESULTS AND DISCUSSION

Fatty alcohol analysis

Long-chain fatty alcohols were converted to their pentafluorobenzoyl derivatives and analyzed by GC–NICI-MS. Fig. 1 shows the total ion current tracing and corresponding NICI-mass spectra for the pentafluorobenzoyl derivative of stearyl alcohol. A strong molecular ion (*m/z* 464) was observed. The mass spectra of the polyunsaturated fatty alcohol arachidonyl alcohol is shown in Fig. 2. A strong molecular ion (*m/z* 484) was noted. Pentafluorobenzoyl derivatives of other fatty alcohols can also be monitored by their molecular ion in the NICI mode: *m/z* 408 (myristyl alcohol), 436 (palmityl alcohol), 450 (heptadecyl alcohol), 460 (linoleyl alcohol), 462 (oleyl alcohol).

As illustrated in Fig. 3, when increasing amounts of the pentafluorobenzoyl derivative of stearyl alcohol were analyzed by GC–NICI-MS, the peak area was a linear function of the amount of stearyl alcohol added (between 0.4 and 400 fmol). As

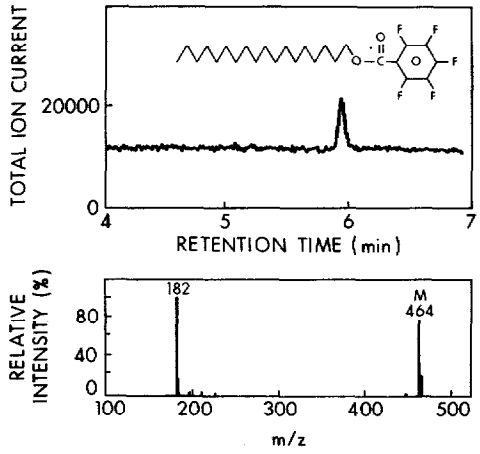

Fig. 1. NICI-MS of the pentafluorobenzoyl derivative of stearyl alcohol. A 3-pmol amount of a pentafluorobenzoyl derivative of stearyl alcohol was injected into the gas chromatograph as described in Materials and Methods. The mass spectrometer was operated in the NICI mode with methane as the reagent gas (source pressure: 0.6 Torr). Top: total ion current chromatogram of the pentafluorobenzoyl derivative of stearyl alcohol. Bottom: mass spectra of the peak eluting at 5.97 min (electron multiplier voltage setting of 1400).

little as 0.37 fmol of stearyl alohol can be detected with a satisfactory signal-to-noise ratio (Fig. 3, lower right panel).

Fig. 4 illustrates a standard curve of a mix of pentafluorobenzoyl derivatives of fatty alcohols (14:0, 16:0, 18:0, 18:1, 18:2, and 20:4) using 4 pmol of the pentafluorobenzoyl derivative of heptadecyl alcohol as the internal standard with analysis by GC–NICI-MS. Fatty alcohols were identified based on their retention time and

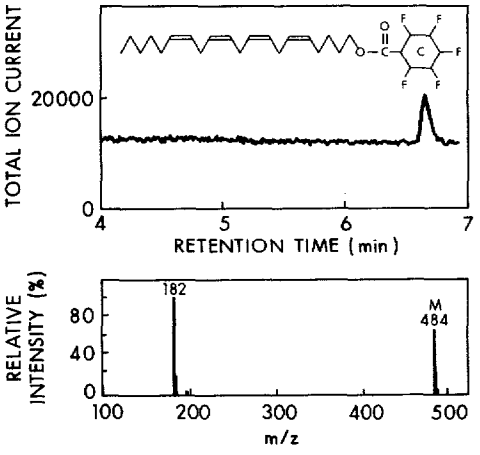

Fig. 2. NICI-MS of the pentafluorobenzoyl derivative of arachidonyl alcohol. A 3-pmol amount of a pentafluorobenzoyl derivative of arachidonyl alcohol was injected into the gas chromatograph as described in Fig. 1. Top: total ion current chromatogram. Bottom: mass spectra of the peak eluting at 6.73 min (electron multiplier voltage setting of 1400).

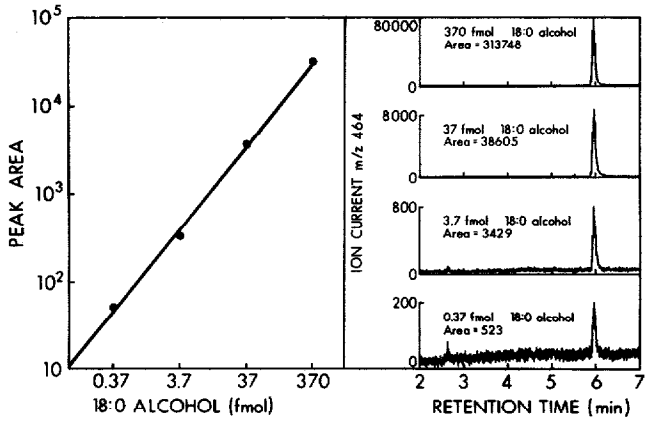

Fig. 3. Response curve of the pentafluorobenzoyl derivative of stearyl alcohol by GC–NICI-MS. Varying amounts of the pentafluorobenzoyl derivatives of stearyl alcohol were injected into the gas chromatograph and eluted as described in Fig. 1. The eluent was monitored by NICI-MS at a *m/z* ion of 464 (mass defect = 0.3). The chromatograms obtained are shown in the right panel. The area under the peak was integrated by the Hewlett-Packard computer and the dilution curve obtained is shown in the left panel as a function of the amount of fatty acid stearyl alcohol (0.4–400 fmol).

molecular ion (Fig. 4, right panel) and quantitated by selected ion monitoring: the area under the peak was expressed as a ratio to that of the internal standard (heptadecyl alcohol) and a standard curve constructed (Fig. 4, left panel). The amount of each fatty alcohol recovered is a linear function of the amount added with a slope (determined by linear regression) approaching 1 (myristyl alcohol: slope = 1.00, intercept = 0, $r = 1.000$; palmityl alcohol: slope = 1.00, intercept = 0.02, $r = 0.997$; linoleyl alcohol: slope = 0.99, intercept = 8.48, $r = 0.998$; oleyl alcohol: slope = 0.96, intercept = 37.40, $r = 0.998$; stearyl alcohol: slope = 1.00, intercept = 1.29, $r = 0.995$; arachidonyl alcohol: slope = 0.99, intercept = 4.48, $r = 0.996$).

These observations demonstrate the feasibility of measuring fatty alcohols by GC–MS with quantitation achievable in the subfemtomolar range. Prasad *et al.*[17] have recently described a GC method for the analysis of fatty alcohols which achieved subnanomolar sensitivity using *tert.*-butyldimethylsilyl derivatives of the fatty alcohols. Problems associated with that technique are the presence of contaminants after silylation which can interfere with the analysis. The use of MS to analyze pentafluorobenzoyl derivatives of fatty alcohols bypasses the contaminant problem associated with GC analysis by flame ionization detection. The GC–MS method also identifies the fatty alcohol derivative based on its molecular ion and achieves a six order-of-magnitude increase in sensitivity. A potential application of this sensitive technique is the identification and quantitation of long-chain fatty alcohol production by yeasts and bacteria[21,22].

Long-chain acyl CoA analysis

Several approaches have been used to assay long-chain acyl CoA thioesters. Enzymatic assays measure coenzyme A released from long-chain acyl CoA with a sensitivity in the subnanomolar range[19]. This method, however, provides no information on the fatty acyl composition of the CoA esters and is subject to a number

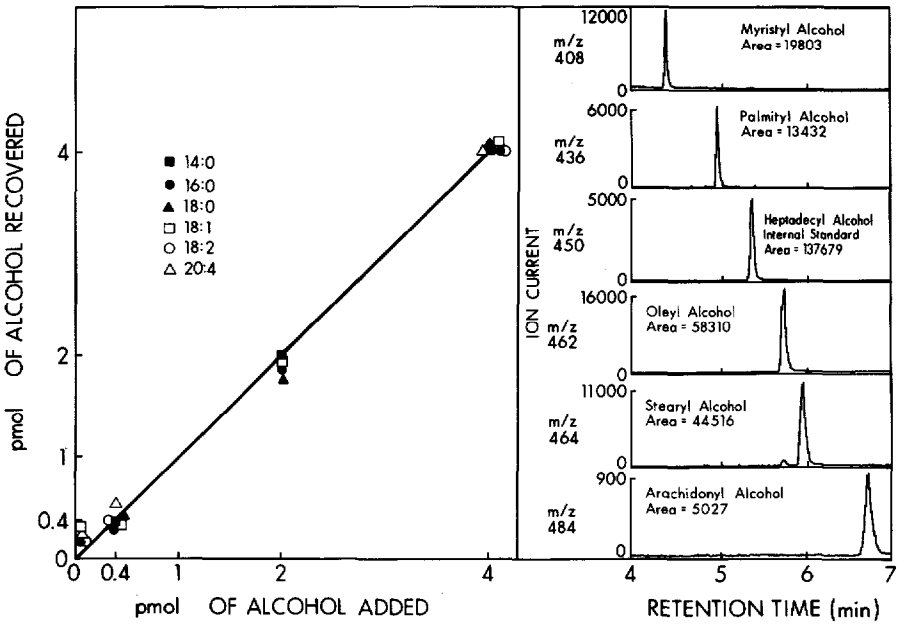

Fig. 4. Standard curve of a mix of pentafluorobenzoyl derivatives of long-chain fatty alcohols. Increasing amounts of pentafluorobenzoyl derivative of long-chain fatty alcohols were injected into the gas chromatograph and eluted as described in Fig. 1. The eluent was monitored by NICI-MS at the following *m/z* (dwell-time 50 ms): 408 (■, myristyl alohol), 436 (●, palmityl alcohol), 450 (heptadecyl alcohol as internal standard), 460 (○, linoleyl alcohol), 462 (□, oleyl alcohol), 464 (▲, stearyl alcohol), 484 (△, arachidonyl alcohol). Left: Standard curve of mixes of fatty alcohols. Linear regression analysis was performed for each long-chain fatty alcohol (see Results and Discussion section for the slope and intercept of the calculated regression line for each long-chain fatty alcohol) and used to calculate the amount of fatty alcohol recovered. The solid line indicates the line of identity. Right: Chromatograms of a mix of pentafluorobenzoyl derivative of fatty alcohols. This tube contained 400 fmol of myristyl alcohol, 400 fmol of palmityl alcohol, 4000 fmol of heptadecyl alcohol, 4000 fmol of oleyl alcohol, 2000 fmol of steaoyl alcohol and 40 fmol of arachidonyl alcohol. Each section of the panel represents the individual chromatogram obtained at the *m/z* ion specific for each long-chain fatty alcohol monitored by NICI-MS as indicated above.

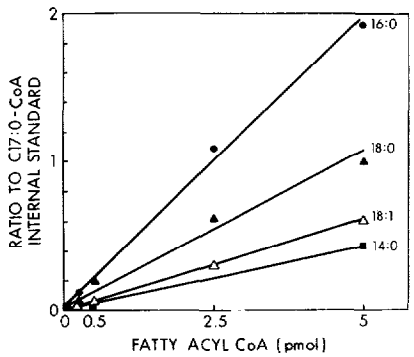

Fig. 5. Standard curve of long-chain acyl CoA thioesters measured as pentafluorobenzoyl derivative of corresponding fatty alcohols. Increasing amounts of long-chain acyl CoA thioesters were purified according to Materials and Methods, second scheme, reduced to their corresponding fatty alcohols, derivatized to pentafluorobenzoyl esters and analyzed as described in Fig. 4. Results are expressed as a ratio of the area under the peak of the corresponding fatty alcohol divided by that of heptadecyl alcohol, the internal standard. ■ = Myristoyl CoA; ● = palmitoyl CoA; ▲ = stearoyl CoA; △ = oleoyl CoA.

of interferences[14]. Recently, a reversed-phase HPLC technique has been described which separates the individual long-chain acyl CoA thioesters. UV detection used with this technique achieves a sensitivity in the nanomolar range[16]. Finally, Prasad *et al.*[17] have used sodium borohydride to reduce long-chain acyl CoA thioesters to their corresponding long-chain alcohols and have quantitated these compounds by GC–flame ionization detection with a reported sensitivity in the subnanomolar range. Using their technique for extracting and reducing long-chain acyl CoA (see Materials and Methods) and measuring the resultant fatty alcohols by GC–NICI-MS as described above, we found that the addition of 21, 19 and 19 pmol of palmitoyl, stearoyl and oleoyl CoA resulted in the recovery of 24, 22 and 11 pmol, respectively, of the corresponding fatty alcohol using heptadecanoyl CoA (3 nmol) as an internal standard. Recovery (as assessed with [^{14}C]myristoyl CoA) was 22%.

We have also developed an alternate long-chain acyl CoA purification scheme aimed at separating long-chain acyl CoA from other lipids, most notably phosphatidylcholine, which is difficult to separate from long-chain acyl CoA species on TLC. Fatty acyl oxoesters in phospholipids are also reduced by sodium borohydride to the corresponding fatty alcohols. This alternate procedure involves an initial TLC separation, which achieves good resolution of fatty acyl CoA (R_F = 0.47–0.51 for C14:0 to C20:4 CoA) from unesterified fatty acids (R_F = 0.86), diacylglycerols (R_F = 0.91), triacylglycerols (R_F = 0.94), phosphatidic acid (R_F = 0.70), cardiolipin (R_F = 0.69), phosphatidylethanolamine (R_F = 0.67) and phosphatidylinositol/phosphatidylserine (R_F = 0.62). Phosphatidylcholine, however, co-migrates (R_F = 0.53) with the long-chain acyl CoA esters. The spot corresponding to long-chain acyl CoA thioesters is scraped, extracted, and digested with phospholipase C (from *B. cereus*) to hydrolyze (>99.8 complete) phosphatidylcholine to diacylglycerol and phosphocholine. Digestion is then followed by another TLC purification. The purified long-chain acyl CoA tioesters are then directly reduced on silica to their corresponding fatty alcohols with sodium borohydride (70% recovery as compared to 55% on Al_2O_3). Fig. 5 shows a standard curve of a mix of long-chain fatty CoA processed in the presence of nmole amounts of lipids. A linear response (ratio of the peak area of the pentafluorobenzoyl derivative of the corresponding fatty alcohol to that of the heptadecyl alcohol internal standard) was obtained in the femtomolar and picomolar range for saturated and unsaturated long-chain acyl CoA esters.

CONCLUSIONS

We have described a GC–MS method to identify and quantitate long-chain fatty alcohols in the subfemtomolar range. Advantages of this simple technique are that it provides identification of each species of long-chain fatty alcohol, is not subject to interferences present with GC flame ionization detection techniques and is extremely sensitive. Potential applications of this sensitive technique include measurement of the long-chain acyl CoA content of cells and tissues.

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