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Gas chromatographic measurement of 3- and 4-thia fatty acids incorporated into various classes of rat liver lipids during feeding experiments

Hans J. Grav[a,*], Daniel K. Asiedu[b], Rolf K. Berge[b]

[a]*Institute for Nutrition Research, University of Oslo, P.O. Box 1046, Blindern, N-0316 Oslo, Norway*
[b]*Laboratory of Clinical Biochemistry, University of Bergen, Haukeland Hospital, Bergen, Norway*

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Abstract

A practical procedure is described for the quantitative measurement of the amount of acyl units derived from tetradecylthioacetic acid (effecting hypolipemia in rats) and tetradecylthiopropionic acid (effecting hyperlipemia). The procedure involves three main successive steps: (1) extraction; (2) solid-phase lipid class separation yielding free fatty acids, phospholipids, triacylglycerides, cholesterol esters, and diacylglycerides without crosscontamination; and (3) gas chromatography of hydrolyzed lipids derivatized to picolinyl esters, combined with unambiguous identification by gas chromatography–mass spectrometry. The overall recoveries of heptadecanoyl lipids added as internal standards during extraction were 94–96%, except for cholesteryl heptadecanoate where the recovery was 60% owing to incomplete hydrolysis. Recoveries of thia fatty acids from samples spiked with these compounds were 95%. Flame-ionization response factors were found to be 0.92 and 0.81 for the tetradecylthioacetic acid and tetradecylthiopropionic acid picolinyl esters, respectively, compared to that of heptadecanoic acid. The lower limit of quantitation was 25 pmol as injected. Measurement of the amount of thia fatty acyl units in rat plasma and in liver lipids 4 h after administration of single doses by gastric intubation indicated efficient absorbtion and rapid incorporation into liver lipids, particularly in the phospholipid fraction. Both plasma clearance and channelling into lipids was slower for the 4-thia fatty acid.

1. Introduction

Long-chain fatty acids in which sulphur atoms substitute for one or more methylene groups in the alkyl chain, have been shown to perturb normal lipid metabolism. Thus 3-thia fatty acids (non-β-oxidizable) [1] have been shown to induce hypolipemia and liver peroxisome proliferation in rats [2–4] and to induce peroxisomal β-oxidation in Morris 7800 C1 hepatoma cells [5]. In contrast, fatty acids with the sulphur atom in the 4-position (β-oxidizable) caused only weak induction of peroxisomal β-oxidation when fed to rats and, as the feeding regime continued, the rats developed fatty livers and hypertriglyceridemia [2–4]. In part these latter effects may be explained as a result of 4-thia fatty acids serving as irreversible inhibitors of mitochondrial and peroxisomal β-oxidation [6] and channelling activated fatty acids into phospholipids and tri-

* Corresponding author.

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acylglycerides as a consequence [7]. In isolated hepatocytes, the oxidative fate of 3- [8] and 4-thia fatty acids [9] has been shown to involve ω-oxidation followed by peroxisomal β-oxidation, and mitochondrial/peroxisomal β-oxidation to acetyl-CoA and a long-chain alkyl thiol, respectively.

Much less is known of the possible incorporation of thia fatty acids into lipids and the time course of their occurrence in target tissues, information essential for understanding in vivo thia acid metabolism, and for pinpointing their locus of action. To obtain this type of information a sensitive, reliable method is required for quantitation of thia acyl moieties and their metabolites. Available so far are procedures based upon measurement of radioactivity from ^{14}C-labelled thia acids [8,10] involving TLC lipid class separation followed by subfractionation of phospholipids with HPLC. While useful for the qualitative assessment of metabolic pathways, such methods are not easily standardized to yield quantitative data. Spectrophotometric methods have been applied in in vitro experiments with mitochondria where thia acyl-CoA esters were used to probe the mechanism of medium-chain acyl-CoA dehydrogenase [1,11], but such procedures are not easily adapted for the measurement of acyl chain incorporation into lipids.

The procedure presented here provides a sensitive, accurate method for measuring the mass of thia fatty acids, administered per os or in vitro, and incorporation into tissue or cellular lipids. The method involves three basic steps: extraction, solid-phase fractionation of lipid classes, and GC of derivatized fatty acyl groups.

2. Experimental

2.1. Chemicals and reagents

Tetradecylthioacetic- and tetradecylthiopropionic acids were prepared as described previously [5,10]. Heptadecanoic acid (>99% pure) was obtained from Nu-Chek-Prep (Elysian, MN, USA), while metyl heptadecanoate (>99% pure) as well as other lipid compounds were products of Sigma Chemical Co. (St. Louis, MO, USA). Solvents of pro analysi grade were obtained from E. Merck (Darmstadt, Germany). CPL Fish oil 30 triglyceride standard was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Radioactive lipids were from either New England Nuclear (Boston, MA, USA) or Amersham International plc (Aylesbury, UK).

2.2. Treatment of animals

Groups of male Wistar rats were housed, fed and treated as described elsewhere [12]. Each animal received a single dose (150 mg/kg body weight) of either tetradecylthioacetic- or tetradecylthiopropionic acid by gastric tube delivery. After 4 h animals were killed and plasma and tissue samples were kept at −80°C until analyzed.

2.3. Lipid extraction and sample preparation

Frozen lipid samples were crushed in liquid nitrogen and weighed aliquots (0.2–0.5 g) of powdered tissue were subsequently extracted according to a modified [13] Folch et al. [14] procedure. During homogenization heptadecanoic acid (444 nmol), triheptadecanoylglycerol (71 nmol), L-α-diheptadecanoyl phosphatidylcholine (131 nmol), and cholesteryl heptadecanoate (156 nmol) were added as internal standards. Plasma samples (0.4 ml) were thawed and likewise extracted in the presence of 89 nmol heptadecanoic acid and 0.1% ethanolic butylated hydroxytoluene. Each liver extract was subjected to liquid anion-exchange chromatography on 1-ml (100 mg) Supelclean LC-NH_2 SPE columns, using aliquots per column of <2 mg total lipid (by weight, as measured by evaporation). Before use each column was washed with hexane (4 × 2 ml) to remove possible contamination from cartridge-associated nonesterified fatty acids. Chromatography was performed at room temperature using a Visiprep solid-phase extraction vacuum manifold (Supelco, Bellefonte, PA, USA) to separate the lipid classes using eluents as described by Kaluzny et al. [15]. The fractions

containing triacylglycerides, diacylglycerides, cholesterol esters, and phospholipids, as well as aliquots from the plasma extracts, were separately hydrolyzed in 15% methanolic KOH for 45 min at 65°C, followed by acidification with HCl and repeated extractions with hexane. The free fatty acid fractions obtained by chromatography from liver extracts were evaporated under a stream of nitrogen and dissolved in hexane.

The fatty acid containing hexane solutions were then evaporated under a stream of nitrogen and each converted to picolinyl esters using the method of Christie and Stefanov [16] as described [17]. The picolinyl esters were purified as recommended by Christie [17] using Analytichem Bondesil aminopropyl silica gel (Varian Sample Preparation Products, Harbor City, CA, USA) as a vehicle for removal of any free fatty acid.

For determination of the recovery of the different lipid classes, separate Supelclean columns were charged with 1.0 ml of chloroform solutions containing 2.0 mg of either [1-^{14}C]palmitic acid, tri-[1-^{14}C]palmitoyl glycerol, cholesteryl-[1-^{14}C]oleate, or L-α-[1-^{14}C]dipalmitoyl phosphatidylcholine, each with specific radioactivity of 10 μCi/mg, and eluted as above. Samples of each of 5 sequential eluates were mixed with 5 ml of Insta-gel II (Pachard Instrument Co., Downers Grove, IL, USA) and radioactivity was measured with a scintillation spectrometer.

2.4. Gas chromatography

Derivatized fatty acids were injected into a Shimadzu GC-14A gas chromatograph connected to a Shimadzu C-R4AX Chromatopac data unit (Shimadzu Europe, Duisburg, Germany). The capillary columns from S.G.E. International (Ringwood, Vic., Australia) contained either a BP1 (50 m × 0.22 mm I.D., film thickness 0.25 μm) or a polar BPX70 (30 m × 0.32 mm I.D., film thickness 0.25 μm) phase, for separation of the 3- or 4-thia fatty acids, respectively. Helium was used as carrier gas (average velocity 29 cm/s) as well as make-up gas for flame-ionization detection at 290°C. Injections (1 μl) were made in the splitless mode (closed split 40 s; injector temperature 290°C). For samples containing tetradecylthioacetic acid the column was initially held at 40°C for 3 min, then programmed at 40°C/min to 140°C, held there for 3 min, and finally programmed at 40°C/min to 290°C.

For samples containing tetradecylthiopropionic acid the column was initially held at 40°C for 3 min, then programmed at 40°C/min to 160°C, held there for 10 min, programmed at 40°C/min to 200°C, held there for 3 min, and finally allowed to rise to 250°C at a rate of 3°C/min. Amounts of 3- and 4-thia fatty acids were calculated using the internal standards method (see e.g. Refs. [18,19]) relative to the heptadecanoyl moiety, making appropriate corrections for the different amounts of this acid in the added internal standard lipids, as well as for the differing "response factors" of the thia fatty acids relative to heptadecanoic acid (see Results and Discussion).

To serve as chromatographic standard 1 ml of Larodan Fish Oil 30 was hydrolysed, the fatty acids liberated and extracted, and finally converted to picolinyl esters as described above.

2.5. Gas chromatography–mass spectrometry

The identities of the tetradecylthioacetate and tetradecylthiapropionate derivative gas chromatographic peaks were verified by injecting parallel samples on a Shimadzu GCMS QP2000 and post-chromatographic analysis by the Shimadzu MSPAC 200 data programme. Cold on-column injections of 1-μl samples were made on a DB1 (J and W Scientific, Folsom, CA, USA) nonpolar capillary column (40 m × 0.18 mm I.D., film thickness 0.25 mm). Helium (average velocity 20 cm/s) was used as carrier gas. The oven temperature was held at 40°C for 3 min, then programmed at 40°C/min to 140°C, held there for 3 min, then allowed to rise to 275°C at a rate of 3°C/min. The mass spectrometer was operated in the electron-impact mode at 70 eV; the temperature of the ion source was 250°C.

3. Results and discussion

During studies of thia fatty acid turnover in vivo we needed an accurate and sensitive method for quantitation of thia acyl moieties. We also needed to determine the lipid class from which such groups originated. In order to achieve this we opted for a procedure consisting of thee main consecutive steps: extraction, lipid class separation, and derivatization followed by gas chromatographic and GC–MS analysis.

3.1. Lipid class separation

In order to avoid possible sulphoxidation [7,8], extraction and lipid class fractionation were, whenever practicable, performed at low temperature (+ 5°C) and under nitrogen. Although employing eluents as suggested by Kaluzny et al. [15], the small sample sizes available in our experiments necessitated the use of smaller columns for the aminopropyl-bonded, anion-exchange chromatography. The classes of interest were: free fatty acids, phospholipids, triacylglycerides, cholesterol esters, and diacylglycerides. In order to test the column capacities and the fractionation efficiency proper analysis was mimicked by charging columns with different ^{14}C-labelled lipids and measuring their recoveries in the eluted fractions. Amounts of < 2 mg total lipid yielded fractions almost quantitatively separated and recovered (Table 1), while addition of 2.5 mg lipid (not shown) led to significant overlap between adjacent fractions. The completeness of lipid class separation at the 2 mg level was confirmed by chromatography of pairs (1 mg each) of ^{14}C- and ^{3}H-labelled model lipids. Under the conditions used we did not find the incomplete separation nor the FFA contamination from the sample preparation cartridges noted by others [20].

3.2. Gas chromatography

As small amounts of thia acids would be expected to occur in tissue lipids after gastric intubation delivery, the gas chromatographic system should be capable of base-line resolution of thia acyl units from endogenously occurring fatty acids, derivatization should be quantitative, resulting derivatives should exhibit thermal stability and possess sufficiently low polarity to allow elution below the temperature limits of the columns. For identification purposes, derivatization should ensure charge stabilization to yield informative mass spectra. Conversion of 3- and 4-thia fatty acids to picolinyl esters [21] fulfilled these requirements. Figs. 1A and 1B present typical picolinate chromatograms obtained from spiked, hydrolysed fish oil triglyceride. Each thia

Table 1
Separation and recovery of lipid classes using solid-phase extraction columns

Eluent	Eluent volume (ml)	Recovery (mean ± S.E.M., $n = 5$) (%)			
		Palmitic acid	Glycerol tripalmitate	Cholesteryl oleate	Dipalmitoyl phosphatidyl choline
Chloroform–isopropanol (2:1)	2 + 2	0.2	98.5 ± 0.5	99.1 ± 0.3	0.1
2% Acetic acid in diethyl ether	2 + 2	98.2 ± 1.1	n.d.	n.d.	n.d.
Methanol	2 + 2	n.d.	n.d.	n.d.	97.1 ± 0.6
Hexane	2 + 2	n.d.	0.5	96.5 ± 1.1	n.d.
1% Diethyl ether + 10% methylene chloride in hexane	4 + 4	n.d.	97.1 ± 0.6	0.6	n.d.

n.d. = not detected.
Portions (2 mg) of ^{14}C-labelled lipids were separated on 1-ml Supelclean LC-NH_2 SPE columns as described in Experimental.

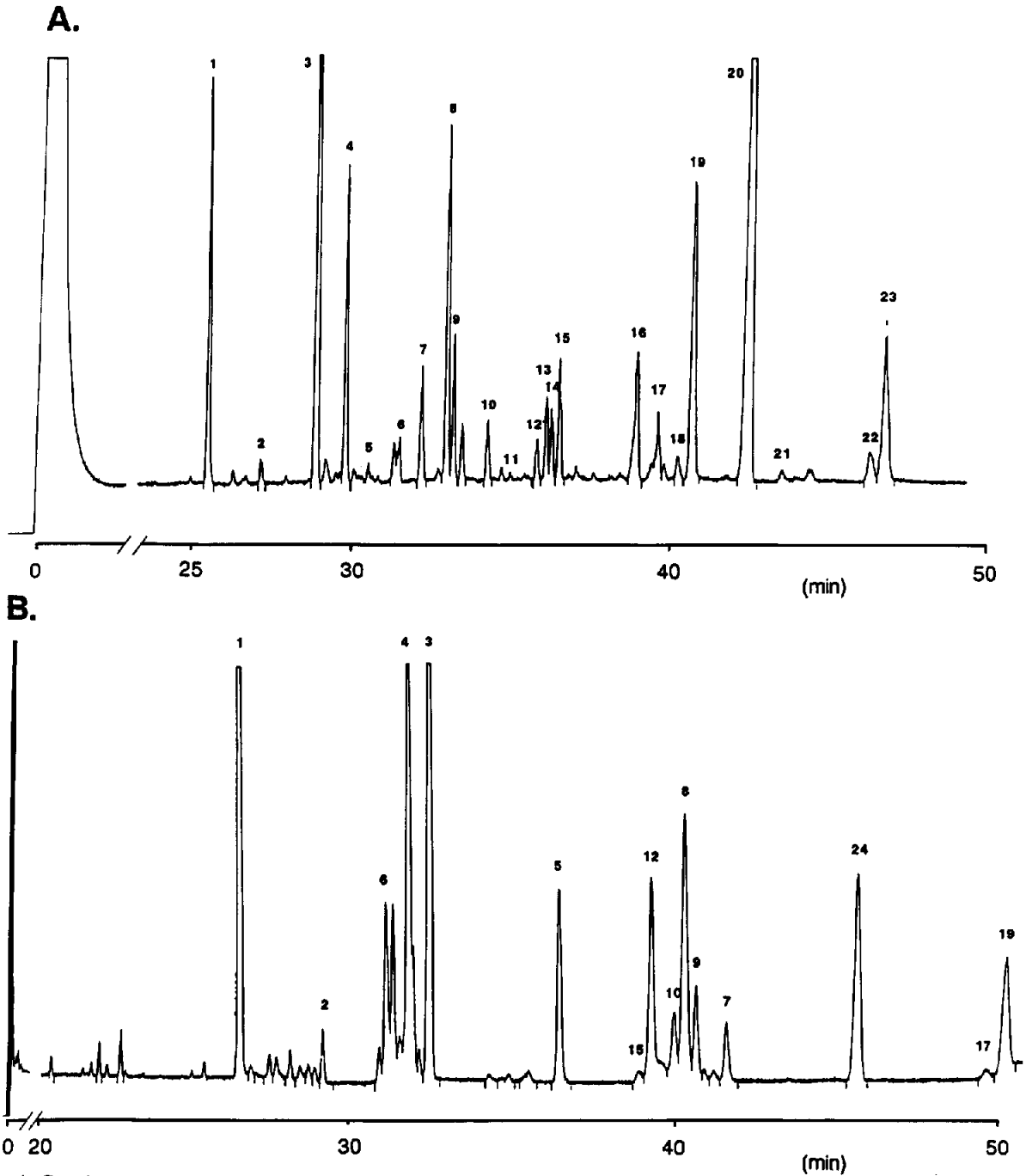

Fig. 1. Gas chromatography of fatty acid picolinyl esters derived from hydrolysed Larodan Fish Oil triglyceride with added 4- or 3-thia fatty acids. (A) Added tetradecylthiopropionic acid run on a polar BP × 70 quartz capillary column, and (B) added tetradecylthioacetic acid run on a nonpolar BP1 quartz capillary column using the conditions specified in Experimental. Peak designations were as follows: 1 = 14:0; 2 = 15:0; 3 = 16:0; 4 = 16:1 (n–9); 5 = 17:0; 6 = 16:4 (n–3); 7 = 18:0; 8 = 18:1 (n–9); 9 = 18:1 (n–7); 10 = 18:2 (n–6); 11 = 18:3 (n–6); 12 = 18:3 (n–3); 13 = 20:1 (n–11); 14 = 20:1 (n–9); 15 = 18:4 (n–3); 16 = 20:3 (n–6); 17 = 20:4 (n–6); 18 = 20:4 (n–3); 19 = 20:5 (n–3); 20 = Tetradecylthiopropionic acid; 21 = 22:1 (n–11); 22 = 22:5 (n–3); 23 = 22:6 (n–3); 24 = Tetradecylthioacetic acid.

acid appeared as a baseline resolved peak, resolution being dependent upon choice of capillary column. A nonpolar column (Fig. 1B) completely separated tetradecylthioacetate while tetradecylthiopropionate was found to co-chromatograph with docosamonoenoate. Conversely, a polar column resolved the 4-thia acyl derivative from common fatty acyl units (Fig. 1A), but the tetradecylthioacetate derivative separated poorly from arachidonate.

3.3. Gas chromatography–mass spectrometry

The mass spectra of the 3- and 4-thia fatty acyl picolinates conformed to the general pattern given by Christie et al. [22] for a series of dodecyl- and octadecyl thia fatty acids. Typical mass spectra of picolinyl esters of tetradecylthiopropionic (Fig. 2A) and -acetic (Fig. 2B) acids, taken from appropriate mass chromatograms, exhibited easily recognizable ions derived from the picolinyl moiety (m/z 92 and 108). The ion at m/z 151 (Fig. 2B) resulting from McLafferty rearrangement [21] is the base ion for the tetradecylthioacetic acid derivative, and also represents cleavage on the proximal side of the sulphur atom, but does not occur with the 4-thia acid derivative (Fig. 2A) since no extractable hydrogen is available at position 4 of the alkyl chain. The ion at m/z 165, prominent in the 4-thia acid spectrum, presumably is the protonated form of a fragment formed by cleavage between carbon atom 3 and the sulphur atom (and containing the picolinyl moiety). The major diagnostic ions are those derived from α-cleavage on either side of the sulphur atom (m/z 197 and 243, respectively, in Fig. 2A; m/z 183 and 243, respectively, in Fig. 2B), both pairs of ions being protonated. The ions seen at m/z 301 (Fig. 2A) and at m/z 287 (Fig. 2B) may tentatively result from a loss of the 3-methylpyridino moiety. Distinctive mass ions are seen in each case, but ions resulting from stepwise, radical-induced cleavage between methylene groups of the alkyl chain are less clearly discernable than those from picolinyl esters of normal saturated fatty acids [21]. We would judge the ions resulting from cleavage around the sulphur atom and those at m/z 151 and 165 for the 3- and 4-thia acids, respectively, to be sufficiently prominent and distinctive to serve as a basis for alternative quantitative measurement of these acids by selected-ion monitoring.

3.4. Sensitivity and precision

As pure fatty acyl picolinyl esters are not yet commercially available we tested the linearity of flame-ionization responses of selected picolinyl esters prepared by derivatization against pure methyl heptadecanoate. Using a capillary column with a 0.25-μm thin film, nonpolar stationary phase, all esters exhibited linear responses within the range 25–2500 pmol, as injected (Fig. 3). Applying a Student's t-test procedure [23] to coefficients obtained from the regression lines of Fig. 3, we conclude that formation of picolinyl esters of heptadecanoate and linolenate was quantitative since the slopes for these derivatives did not differ from that of methyl heptadecanoate at the 5% level. At this level slopes of the regression lines for tetradecylthioacetyl- and propionyl picolinates differed significantly from the former group of derivatives, as well as from each other, yielding "response factors" of 0.92 and 0.81, respectively, relative to methyl heptadecanoate. As used here the term "response factor" refers to a combination of derivatization yield and FID response.

The efficiency of the hydrolytic procedure towards the various lipid classes was investigated by subjecting known amounts of triheptadecanoyl glycerol, L-α-diheptadecanoyl phosphatidylcholine, cholesteryl heptadecanoate, and 1,3-dihexadecanoyl glycerol to hydrolysis as described above, liberating and extracting the resulting fatty acids, and subjecting the formed picolinyl esters to gas chromatography. The recoveries of acyl moieties from the triacylglyceride, diacylglyceride, and phospholipid classes were $98.5 \pm 0.6\%$ ($n = 5$), $99.2 \pm 0.5\%$, and $97.2 \pm 0.8\%$, respectively, while hydrolysis of cholesterol esters was incomplete, yielding $62.3 \pm 3.1\%$ of the added heptadecanoyl moieties, in agreement with the experience of others [24]. As transesterification to picolinyl esters is

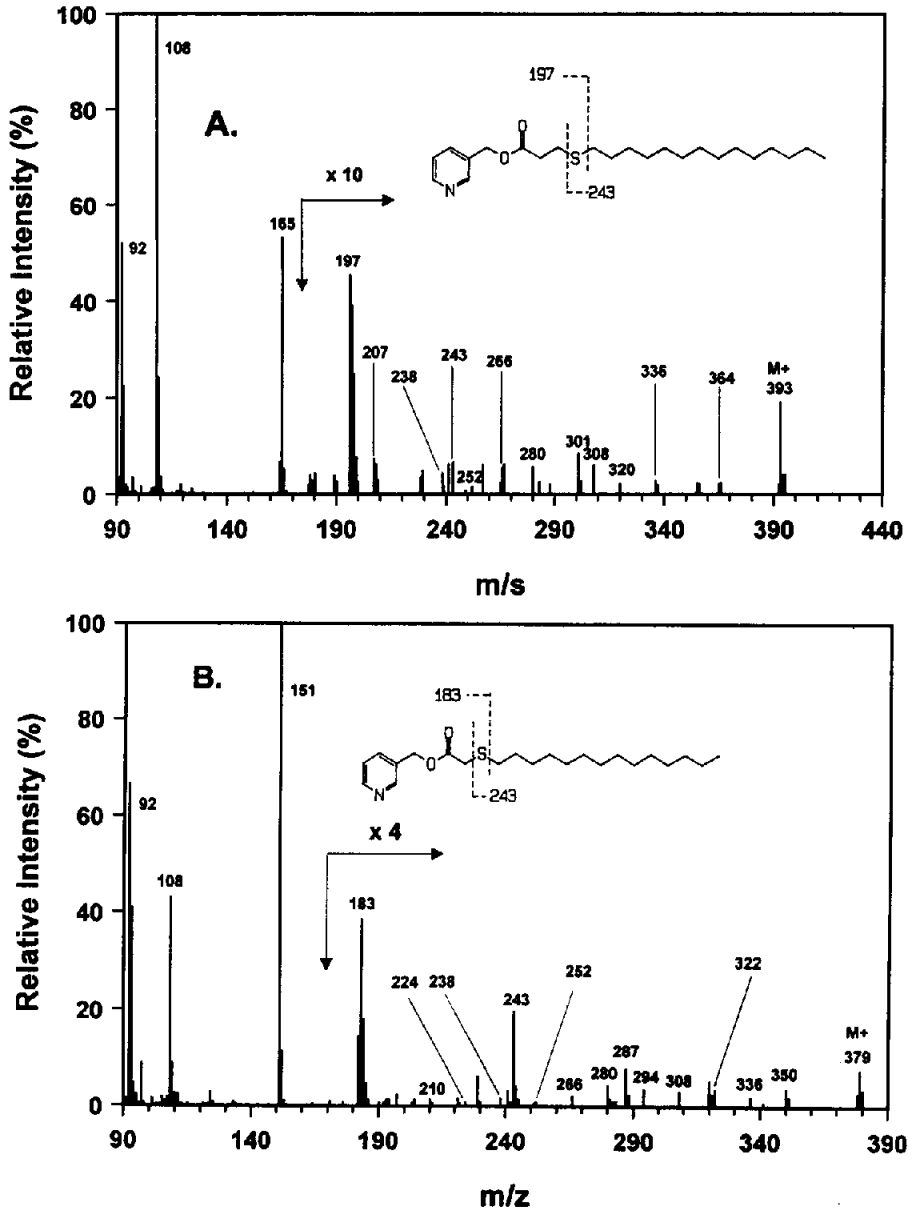

Fig. 2. Mass spectra of picolinyl esters of 4- and 3- thia fatty acids. Data were taken from mass chromatograms run parallel to those in Fig. 1 using the conditions given in Experimental. Broken lines on insert indicate presumed origin of major diagnostic fragments. (A) Tetradecylthiopropionic acid, and (B) tetradecylthioacetic acid.

not provided by present derivatization methods [21] which require free carboxyl group(s), any approach for the quantitative hydrolysis of cholesterol esters should include specialized hydrolysis [25] or enzymatic conversion by e.g. cholesterol ester hydrolase (EC 3.1.1.13). Thia fatty acids have been reported to be stable in mild alkali (Jon Songstad, personal communication), and methanolic KOH as employed here did not cause loss of 3- or 4-thia fatty acids since

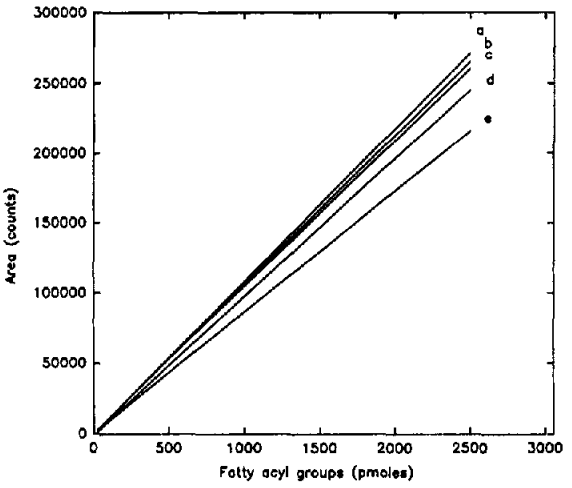

Fig. 3. Sensitivity and linearity of flame-ionization response to thia fatty acyl picolinates. Fatty acids were derivatized to picolinyl esters prior to injection whereas heptadecanoic acid methyl ester was dissolved in hexane and injected as such. Regression lines were each constructed from the means of 11 data points, each in triplicate, in the range 25–2500 pmol of fatty acid as injected. Conditions were as for chromatogram (B) in Fig. 1. The regression lines were: a. picolinyl ester of heptadecanoic acid ($y = -109 + 108x$; $r^2 = 0.98$); b. methyl ester of heptadecanoic acid ($y = 673 + 105.9x$; $r^2 = 0.97$); c. picolinyl ester of linolenic acid ($y = 955 + 103.8x$; $r^2 = 0.95$); d. picolinyl ester of tetradecylthioacetic acid ($y = -718 + 98.3x$; $r^2 = 0.98$); e. picolinyl ester of tetradecylthiopropionic acid ($y = 462 + 86.1x$; $r^2 = 0.97$).

recoveries of 98.7 ± 0.5% ($n = 4$) and 97.9 ± 0.6% of the added acids, respectively, were measured by gas chromatography as compared to tetradecylthioacetic and -propionic acids derivatized without the prior hydrolysis step.

Overall recoveries of acyl units measured by dissolving known amounts of heptadecanoic acid, triheptadecanoyl glycerol, 1,3-dihexadecanoyl glycerol, L-α-diheptadecanoyl phosphatidylcholine, or cholesteryl heptadecanoate in chloroform and processing each sample as described in the Experimental section (from the "Folch" extraction stage onward) were 95.8 ± 1.1% ($n = 5$), 95.1 ± 1.4%, 94.1 ± 1.2%, 94.2 ± 1.9%, or 59.2 ± 2.3%, respectively. In agreement with this, tetradecylthioacetic or -propionic acid added to control rat plasma and processed as described in the Experimental section gave recoveries of 95.2 ± 0.9% ($n = 5$) or 94.8 ± 1.1%, respectively. Because of uncertainties as to the efficiency of the initial lipid extraction, as well as the low yield of fatty acyl units from cholesterol esters reported here, we have found it prudent to conduct analysis in the presence of appropriate internal lipid standards, and calculating the amount of thia fatty acyl units with reference to these.

3.5. Incorporation of thia fatty acids into liver lipids

The procedures described above have proved capable of measuring the amounts of circulating 3- and 4-thia fatty acids as well as the thia acyl moieties incorporated into tissue lipids, following dietary treatment of rats in vivo. Table 2 indicates that both acids were rapidly absorbed when administered by intubation, and that the 4-thia acid exhibited a slower rate of plasma clearance. Whether the measured thia fatty acids were preferentially carried in chylomicrons or in VLDL excreted from the liver was not addressed in this experiment, which depended on total hydrolysis of plasma lipids. The predominant incorporation in liver phospholipids was amply demonstrated, while the flux of thia acyl units into triacylglycerols and/or into cholesterol esters appeared less efficient. Again, metabolic activities towards tetradecylthiopropionic acid were less active, in keeping with its slower plasma clearance. Thus both acids presumably were substrates for the long-chain fatty acyl-CoA synthetase, as found by Aarsland et al. [26], while their qualitatively similar anabolism implies that the reason for their opposing biological effects [2–4] has to be sought elsewhere.

3.6. Determination of other alkyl chain substituted fatty acids

Apart from measurement of the two thia fatty acids employed here, the procedure has proved successful for several additional thia- as well as oxa-monocarboxylic (medium- and long-chain) fatty acids. Dithiadicarboxylic fatty acids which also cause lowering of plasma triacylglycerides

Table 2
Contents of tetradecylthioacetate and tetradecylthiopropionate in rat plasma and liver 4 h after dietary treatment with these acids

Thia acid	Contents					
	Plasma	Liver				
		FFA	Phospholipid	Triacyl glyceride	Diacyl glyceride	Cholesterol ester
Tetradecylthio-acetic acid	80.9 ± 5.9	32.2 ± 1.2	634 ± 31	89.5 ± 5.5	+ [a]	+ [a]
Tetradecylthio-propionic acid	179 ± 6.4	n.d.	250 ± 13	n.d.	n.d.	n.d.

[a] = Detected, not measured. n.d = Not detected.
Plasma values are given as nmol/ml plasma ± S.E.M., while values for liver lipid classes are given as nmol/g liver wet weight ± S.E.M. ($n = 4$).

when given to rats [12,27] have been determined by us using a similar procedure with derivatization to 4,4′-dimethyloxazoline derivatives [28]. Long-chain fatty acid sulphoxides–important metabolites of thia fatty acids [7–9]–may also be measured as picolinates provided that samples are introduced to the gas chromatograph by cold, on-column injection, as also discussed by others [29,30].

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References

[1] S.-M. Lau, R.K. Brantley and C. Thorpe, *Biochemistry*, 27 (1988) 5089.
[2] A. Aarsland, N. Aarsaether, J. Bremer and R.K. Berge, *J. Lipid Res.*, 30 (1989) 1711.
[3] R.K. Berge, A. Aarsland, H. Kryvi, J. Bremer and N. Aarsaether, *Biochem. Pharmacol.*, 38 (1989) 3869.
[4] R.K. Berge, A. Aarsland, H. Kryvi, J. Bremer and N. Aarsaether, *Biochim. Biophys. Acta*, 1004 (1989) 345.
[5] Ø. Spydevold and J. Bremer, *Biochim. Biophys. Acta*, 1003 (1989) 72.
[6] R. Hovik, H. Osmundsen, R.K. Berge, A. Aarsland, S. Bergseth and J. Bremer, *Biochem. J.*, 270 (1990) 167.
[7] R.K. Berge, H. Kryvi, N. Aarsaether, A. Aarsland, J. Lillehaug and J. Skorve, in G. Gibson and B. Lake (Editors), *Peroxisomes: Biology and Importance in Toxicology and Medicine*, Taylor and Francis, London, 1993, pp. 277-297.
[8] E. Hvattum, S. Bergseth, C.N. Pedersen, J. Bremer, A. Aarsland and R.K. Berge, *Biochem. Pharmacol.*, 41 (1991) 945.
[9] E. Hvattum, S. Skrede, J. Bremer and M. Solbakken, *Biochem. J.*, 286 (1992) 879.
[10] S. Skrede, M. Narce, S. Bergseth and J. Bremer, *Biochim. Biophys. Acta*, 1005 (1989) 296.
[11] S.-M. Lau, R.K. Brantley and C. Thorpe, *Biochemistry*, 28 (1989) 8255.
[12] J. Skorve, A. Al-Shurbaji, D. Asiedu, I. Björkhem, L. Berglund and R.K. Berge, *J. Lipid Res.*, 34 (1993) 1177.
[13] P. Way and D.J. Hanahan, *J. Lipid Res.*, 5 (1964) 318.
[14] J. Folch, M. Lees and G.H.S. Stanley, *J. Biol. Chem.*, 226 (1957) 497.
[15] M.A. Kaluzny, L.A. Duncan, M.V. Merritt and D.E. Epps, *J. Lipid Res.*, 26 (1985) 135.
[16] W.W. Christie and K. Stefanov, *J. Chromatogr.*, 392 (1987) 259.
[17] W.W. Christie, in *Gas Chromatography and lipids. A Practical Guide*, The Oily Press, Air, UK, 1989, p. 76.
[18] F.J. Debbrecht, in R.L. Grob (Editor), *Modern Practice of Gas Chromatography*, 2nd ed., John Wiley and Sons, New York, NY, 1985, pp. 390-412.

[19] R.H.St. Louis and H.H. Hill, in R.E. Clement (Editor), *Gas Chromatography. Biochemical, Biomedical and Clinical Applications, Chemical Analysis Vol. 111*, John Wiley and Sons, New York, NY, 1990, pp. 123-126.
[20] S.T. Ingalls, M.S. Kriaris, Y. Xu, D.W. DeWulf, K.-Y. Tserng and C.L. Hoppel, *J. Chromatogr.*, 619 (1993) 9.
[21] D.J. Harvey, in W.W. Christie (Editor), *Advances in Lipid Methodology-Vol. 1*, The Oily Press, Dundee, UK, 1992, pp. 19-80.
[22] W.W. Christie, E.Y. Brechany, M.S.F. Lie Ken Jie and O. Bakare, *Biol. Mass Spectrom.*, 20 (1991) 629.
[23] J.H. Zar, *Biostatistical Analysis*, 2nd ed., Prentice-Hall, Englewood Cliffs, NJ, 1984, pp. 292-305.
[24] W.W. Christie, in *Gas Chromatography and Lipids. A Practical Guide*, The Oily Press, Air, UK, 1989, pp. 65-66.
[25] J.J. Kabasa, *Meth. Biochem. Anal.*, 10 (1962) 264.
[26] A. Aarsland, N. Aarsaether, J. Bremer and R.K. Berge, *Biochim. Biophys. Acta*, 1033 (1990) 176.
[27] D.K. Asiedu, J. Skorve, N. Willumsen, A. Demoz and R.K., Berge, *Biochim. Biophys. Acta*, 1166 (1993) 73.
[28] Q.T. Yu, B.N. Liu, J.Y. Zhang and Z.H. Huang, *Lipids*, 23 (1988) 804.
[29] K. Hall, P.K.F. Yeung and K.K. Miha, *J. Chromatogr.*, 231 (1982) 200.
[30] T.-J. Kim and J.-S. Park, *J. Chromatogr.*, 575 (1992) 295.