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Short Communication

Improved and simplified tissue extraction method for quantitating long-chain acyl-coenzyme A thioesters with picomolar detection using high-performance liquid chromatography

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ABSTRACT

A method has been developed that permits rapid and easy tissue extraction of long-chain acyl-coenzyme A (acyl-CoA) thioesters with sensitive quantitation by reversed-phase high-performance liquid chromatography (RP-HPLC). Tissue homogenants are extracted using a reserve Bligh–Dyer technique, and long-chain acyl-CoA esters are harvested in the methanolic aqueous phase. Complex lipids and phospholipids are removed in the chloroform-rich organic Bligh–Dyer second phase, and long-chain acyl-CoA compounds are further purified from the methanolic aqueous Bligh–Dyer first phase on C_{18} extraction columns after removal of the methanol. The eluted and purified acyl-CoA esters are then quantitated by RP-HPLC using heptadecanoyl-CoA as an internal standard resulting in a detector sensitivity of about 12 pmol. Ten long-chain acyl-CoA esters from $C_{12:0}$ to $C_{20:4}$ were identified and separated from canine renal cortex and murine liver samples. The predominant acyl-CoA peaks from both kidney and liver were 14:0, 16:1, 16:0, 18:1, 18:2 and 20:4. Murine liver also produced 18:0 and all peaks disappeared after alkaline hydrolysis of the samples. This extraction and quantitation technique can successfully be used for tissue samples as small as 20 mg, and many samples can be processed in a short period of time. The simplicity of the extraction procedure and the sensitivity of the assay make this an attractive alternative approach to quantitating long-chain acyl-CoA thioesters from complex biological samples such as tissues.

INTRODUCTION

Long-chain acyl-coenzyme A (acyl-CoA) thioesters are activated fatty acids that participate in the intermediary metabolism of all cells and tissues. The long-chain fatty acyl thioester linkage with coenzyme A results in a group of intracellular compounds possessing amphipathic detergent properties. Under normal physiologic circumstances, these activated intermediary metabolites are rapidly oxidized, and the intracellular concentration of these compounds is kept low. Under ischemic or pathologic situations, however, reductions in the β-oxidation of the fatty acid moiety of long-chain acyl-CoA thioesters result in the accumulation of relatively high concentrations of these intracellular detergents, with the possibility of cellular and membrane disruption [1–3]. Therefore, quantitating and elucidating the synthesis and metabolism of these compounds in many physiologic and pathophysiologic states is of significant importance.

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A number of assays to quantitate long-chain acyl-CoA esters have been described [4–11]. High-performance liquid chromatography (HPLC) has been used by many investigators with sensitivity in the nanomolar range [4–8]. These described procedures suffer from the lack of clean and sensitive chromatograms, and acceptable methods of extracting these compounds from tissues have not been developed. Current tissue extraction procedures entail complex and time-consuming protocols that may require days to complete [4–9]. We describe an easy and highly sensitive assay for extracting and quantitating long-chain acyl-CoA esters from tissues.

EXPERIMENTAL

Materials

All compounds, including long-chain acyl-CoA standards, were obtained from Sigma (St. Louis, MO, USA). Organic solvents were purchased from EM Science (Gibbstown, NJ, USA).

Tissue extraction

Samples of canine renal cortex or murine liver were immediately frozen in liquid nitrogen, and about 1 g of frozen tissue was added to 4 ml of methanol–chloroform (2:1, v/v) in a ground-glass Tenbroek tissue homogenizer (Bellco, Vineland, NJ, USA). At this time, 1 μg of heptadecyl-CoA was added as an internal standard and the samples were homogenized until all tissue was uniformly disrupted. This homogenate was centrifuged at 1300 *g* for 15 min and the supernatant was saved. The tissue pellet was rehomogenized in 4 ml of methanol–chloroform (2:1, v/v), centrifuged and the supernatant was added to the first supernatant. In order to affect phase separation, 2100 μl of 10 m*M* KH_2PO_4 buffer (pH 5.3) and 2100 μl of chloroform were added, and the samples were vigorously vortex-mixed. The lower chloroform-rich layer was removed and discarded. The methanolic upper phase containing long-chain acyl-CoA esters (see Results) was washed again by the addition of 2100 μl of chloroform. The lower chloroform layer was again removed and discarded. The methanol in the upper phase was removed by evaporation under nitrogen for 15 min. The resulting buffer was then applied to a C_{18} extraction column (Prep-Sep, Fisher, Springfield, NJ, USA) to further purify the samples. Before application of the samples, the columns were activated with 5 ml of methanol, 5 ml of water and 5 ml of 10 m*M* KH_2PO_4. After applying the samples, the columns were washed with 5 ml of 10 m*M* KH_2PO_4–acetonitrile (4:1, v/v). Long-chain acyl-CoA esters are then eluted from the columns with 2 ml of 10 m*M* KH_2PO_4–acetonitrile (3:7, v/v) and 2 ml of methanol–10 m*M* KH_2PO_4 (3:1, v/v). This combined eluate is concentrated to 1.0 ml under nitrogen to remove the organic solvents and subsequently subjected to reversed-phase HPLC. We have also found that overnight lyophilization of the samples at this point works well if time and resources permit.

High-performance liquid chromatography

The actual separation and quantitation of individual long-chain acyl-CoA compounds is accomplished by reversed-phase HPLC. Chromatography is carried out on a Varian Model 5000 liquid chromatograph (Varian Instruments, Walnut Creek, CA, USA) with a 5-μm Nucleosil C_{18} analytical column (250 mm × 4.6 mm I.D., Alltech, Deerfield, IL, USA). The following solvents were delivered at a flow-rate of 1.0 ml/min: (A) acetonitrile and (B) 10 m*M* KH_2PO_4, pH 5.3. The chromatograph was programmed to deliver the solvents as follows: initially 24% A, 76% B with linear gradient to 4 min to 34% A, 66% B; a linear gradient to 14 min to 45% A, 55% B; a linear gradient to 23 min to 57% A, 43% B; and a linear gradient to 31 min to 70% A, 30% B. After 35 min, the system was re-equilibrated to the initial time zero solvent composition for 10 min before injecting another sample. Long-chain acyl-CoA compounds were monitored by the UV absorbance at 254 nm. The detector output signal was directed to an integrator (Varian Model 4290), and peak areas of acyl-CoA compounds were automatically calculated and used to estimate the amount of material based on standard curves performed with authentic standards of

long-chain CoA compounds. The ratio of the areas of various long-chain acyl-CoA esters to the area obtained from the internal standard (heptadecanoyl-CoA, retention time of 29.5 min) was used to calculate the mass of analyte in the samples from equations generated with authentic standards (standard curves). The extracts of some samples were purified as previously described and subjected to alkaline hydrolysis before HPLC. The pH of the samples was adjusted to 12.0 with 1 *M* KOH and incubated at 40°C for 30 min. The samples were then neutralized and analyzed by HPLC.

RESULTS AND DISCUSSION

One major objective of this study was to develop an easy yet efficient procedure to extract long-chain acyl-CoA compounds from complex biological sources such as tissue. Table I compares the recoveries of authentic long-chain acyl-CoA standards (palmitoyl-CoA) with the recoveries of other related lipids (phosphatidylcholine and protaglandin E_2) at various stages in the processing of the tissue. Most interfering lipids remain in the chloroform-rich layer in the first Blight–Dyer extract with long-chain acyl-CoA esters partitioning in the aqueous–methanol layer. The amphipathicity of these compounds probably explains this unique property and allows the chromatographer to extract and purify samples with an overall recovery of greater than 60% (Table I). This recovery of long-chain acyl-CoA esters is average when compared to the described procedures. Woldegiorgis *et al.* [4] and Molaparast-Saless *et al.* [5] reported 80 and 85% recoveries, respectively. The methods described by Watmough *et al.* [8] and Wolf *et al.* [11] found overall recoveries of 69 and 22%, respectively. The ability to easily separate long-chain CoA esters from phospholipids, expecially phosphatidylcholine, is an attractive feature of this assay. Recent techniques for quantitating long-chain acyl-CoA esters depend on chemical reduction of the thioester followed by gas chromatographic analysis of the corresponding fatty alcohol [10,11]. However, contamination of the sample with phospholipids has been a problem since the chemical reduction procedure also reduces the oxoester of phospholipids that frequently follow long-chain acyl-CoA compounds in most tissue extraction schemes [8,11]. Under these conditions, analysis of liberated fatty alcohol groups overestimates the actual long-chain CoA ester levels due to the contribution of phospholipids to the fatty alcohol content in the samples. This reverse Bligh–Dyer technique appears to solve this problem by effectively extracting long-chain CoA compounds without extracting other lipids (Table I). In this study, however, phospholipid contamination would not be a problem because acyl-CoA compounds were quantitated as whole thioesters using HPLC and did not depend on quantitation of the liberated fatty alcohol groups after chemical reduction of thioester.

Table II shows the relative and absolute reten-

TABLE I

RECOVERY OF VARIOUS LIPID STANDARDS DURING TISSUE EXTRACTION, PURIFICATION, AND HPLC QUANTIFICATION

Data represent the average of three experiments with standard deviations less than 10% of the means. Radiolabeled myristoyl-CoA, phosphotidylcholine, and prostaglandin E_2 were used to represent the three different classes of lipids.

	Recovery (%)		
	Long-chain acyl-CoA	Phospholipids	Acidic lipids
Blight–Dyer aqueous phase	96.7	6.6	28.5
C_{18} column eluate	84.4	0.6	13.3
Overall recovery after HPLC	61	0	0

TABLE II

LONG-CHAIN ACYL-CoA STANDARDS AND ACYL-CoA EXTRACTS FROM CANINE RENAL CORTEX AND MURINE LIVER

Acyl-CoA levels from tissue represent the mean ± S.D. of six experiments; N.D. = not detected; numbers in parentheses next to acyl-CoA standards represent the carbon length and double bond saturation of the respective standards. Heptadecanoyl-CoA (17:0) served as the internal standard in all experiments.

Acyl-CoA	Retention time of standards (min)	Canine renal cortex (nmol/g)	Murine liver (nmol/g)	Murine liver[a] (nmol/g)
Lauroyl-CoA (12:0)	14	N.D.	N.D.	0.48
Myristoleoyl-CoA (14:1)	16	N.D.	N.D.	0.94
Myristoyl-CoA (14:0)	20	0.05 ± 0.014	0.110 ± 0.09	1.16
Palmitoleoyl-CoA (16:1)	22	0.175 ± 0.07	0.544 ± 0.8	1.32
Linoleoyl-CoA (18:2)	24	0.717 ± 0.27	1.44 ± 0.24	5.60
Arachidonyl-CoA (20:4)	24.5	0.729 ± 0.22	1.44 ± 0.24	N.A.
Palmitoyl-CoA (16:0)	27	0.417 ± 0.07	0.612 ± 0.49	4.00
Oleoyl-CoA (18:1)	28	1.27 ± 0.29	1.86 ± 1.3	4.40
Heptadecanoyl-CoA (17:0)	29.5	N.D.	N.D.	N.A.
Stearoyl-CoA (18:0)	31	N.D.	0.30 ± 0.06	0.96

[a] Data are reported from ref. 6 for comparison; N.A. = not available.

tion times of ten long-chain acyl-CoA standards that are commonly found in vertebrate tissues. This system produces baseline separation of all standards except for linoleoyl- and arachidonyl-CoA. These two standards, however, separate enough to quantitate unknown material in tissue samples (Table I and Fig. 1). Table II also shows the results of experiments designed to quantitate long-chain acyl-CoA esters in canine renal cortex and murine liver. In both tissues, at least six peaks could be identified as coeluting with authentic standards using these extraction and chromatographic procedures. The predominant CoA ester was oleoyl-CoA in both kidney and liver samples. These data are consistent with the findings of others [4,6] and serve to verify this technique.

A sample of the output from a chromatographic run is shown in Fig. 1. This tissue sample from canine renal cortex produced seven clearly defined peaks with the last one attributable to the internal standard. These peaks perfectly coeluted with authentic acyl-CoA standards (Fig. 2) and disappeared following alkaline hydrolysis of the sample (Fig. 1B). The identity of the early UV-absorbing peaks (Fig. 1) is unknown but may

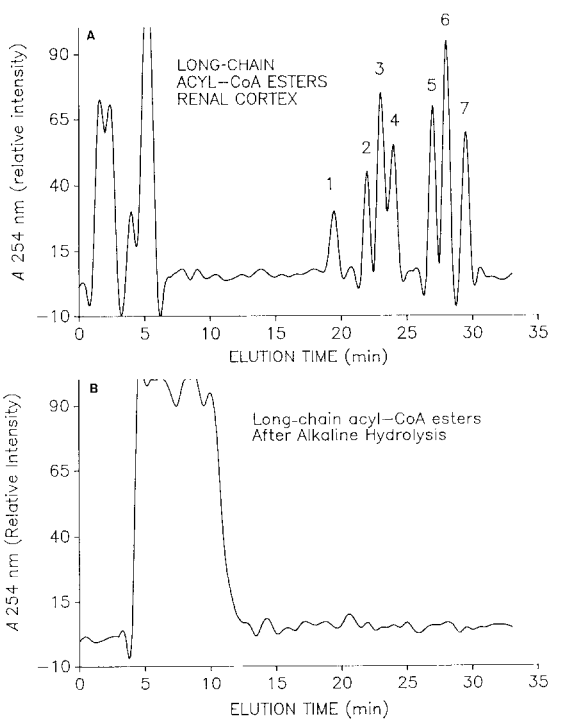

Fig. 1. Analysis of long-chain acyl-CoA esters from canine renal cortex both before (A) and after (B) alkaline hydrolysis. The numbers correspond with elution times of the following authentic standards: (1) myristoyl-CoA; (2) palmitoleoyl-CoA; (3) linoleoyl-CoA; (4) arachidonyl-CoA; (5) palmitoyl-CoA; (6) oleoyl-CoA; (7) heptadecanoyl-CoA, internal standard.

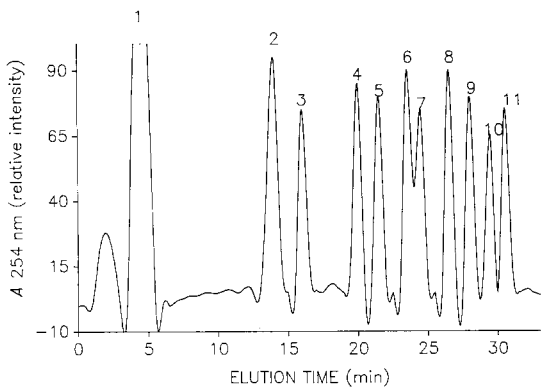

Fig. 2. HPLC separation and identification of authentic long-chain acyl-CoA esters. The numbering of HPLC peaks corresponds to the following standards: (1) unesterified CoA; (2) lauroyl-CoA; (3) myristoleoyl-CoA; (4) myristoyl-CoA; (5) palmitoleoyl-CoA (6) linoleoyl-CoA; (7) arachidonyl-CoA; (8) palmitoyl-CoA; (9) oleoyl-CoA; (10) heptadecanoyl-CoA; (11) stearoyl-CoA.

consist of nucleotides and unesterified CoA. In addition to the high degree of purification of samples using these procedures, they are technically easier and less costly to perform, when compared to the assays previously described [4–11].

Fig. 2 shows the separation of ten long-chain acyl-CoA standards after extraction, purification and HPLC. All standards separate cleanly, and the mass of material injected is linearly related to the intensity of the UV absorbance signal. This relationship is evident in the linear regression equations and coefficients of variation generated from HPLC standard curves comparing the ratio of UV absorption areas of standard CoA esters to the $C_{17:0}$ internal standard (x-axis) *versus* the total mass of authentic standard injected (y-axis). The mass of standard used ranged from 50 to 2000 ng, and the mass of the internal standard was held constant at 1000 ng for each injection. Linear regression equations and coefficients of variation of four out of ten standard curves that were performed are as follows: $y = 190x + 36.7$, $r^2 = 0.99$ for palmitoleoyl-CoA; $y = 369x + 33.1$, $r^2 = 0.98$ for linoleoyl-CoA; $y = 113x + 64.4$, $r^2 = 0.99$ for palmitoyl-CoA; $y = 81.1x + 8.3$, $r^2 = 0.99$ for myristoyl-CoA. Although strict linearity was observed with standard curves performed with all authentic standards used, the absolute relationship between the mass of standard and peak intensity was dramatically different between different chain lengths of standards. This is probably due to different molar absorptivities for the various standards since the UV absorber (CoA) is stoichiometrically different between individual acyl-CoA compounds. More specifically, 1 mol of CoA accompanies 1 mol of acyl group, and the weight of individual acyl groups varies depending on the carbon chain length. Thus, it is essential that standard curves for each acyl-CoA chain length and double-bond saturation be performed with this assay because of the utilization of UV absorbance of the CoA moiety to quantitate unknowns.

In conclusion, this report describes an easy and effective method for extracting and quantitating long-chain acyl-CoA thioesters from animal tissues. These methods are much less complicated than other described methods and achieves a high degree of sensitivity. The extraction procedure can also be used to purify samples for other methods of detection of long-chain acyl-CoA esters such as gas chromatography–mass spectrometry [10,11].

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