Novel isolation procedure for short-, medium- and long-chain acyl-coenzyme esters from tissue

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Abstract

A novel procedure for the quantitative isolation and purification of acyl-coenzyme A esters is presented. The procedure involves two steps: 1) tissue extraction using acetonitrile/2-propanol (3+1, v+v) followed by 0.1M potassium phosphate, pH 6.7, and 2) purification using 2-(2-pyridyl)ethyl functionalized silica gel. Recoveries determined by adding radiolabelled acetyl-, malonyl-, octanoyl-, oleoyl-, palmitoyl- or arachidonyl-coenzyme A to powdered rat liver varied from 93% to 104% for tissue extraction and 83% to 90% for solid phase extraction. The procedure described allows for isolation and purification, with high recoveries, of acyl-coenzyme A esters widely differing in chain-length and saturation.

Published methods for the analysis of tissue acyl-coenzyme A content are focused on either short- [1,2], medium- [3] or long-chain acyl-coenzyme A esters [4,5], with the isolation of one acyl-coenzyme A subgroup to the exclusion of others. Recovery of acyl-coenzyme A esters from tissue specimens is often disappointing, with documented recoveries between 30 and 60% [1,5,6]. A general procedure for the isolation of a wide range of acyl-coenzyme A esters, with good documented recoveries from tissues, is presently unavailable.

Despite their widely different polarities, we have shown that acylcarnitines (short-, medium-, and long-chain) can be isolated, in a single fraction, from biological samples using organic solvent extraction followed by ion-exchange solid phase extraction (SPE) [7]. This is a highly selective approach, since it combines two orthogonal procedures [8] of isolation: organic solvent extraction and ion-exchange. Extraction procedures for short-chain acyl-coenzyme A esters often use acid precipitation [1,2], but these methods would exclude long-chain acyl-coenzyme A esters. Therefore, we investigated extraction procedures originally developed for long-chain acyl-coenzyme A esters, using a mixture of acetonitrile, isopropanol, and aqueous buffer [4]. An SPE anion-exchange column is needed that would be uncharged at pH 7, since elution at high pH would cause hydrolysis of acyl-coenzyme A esters. Finding none commercially available, we ordered custom SPE columns from Supelco, (Bellefonte, PA), packed with 100 mg of 2-(2-pyridyl)ethyl functionalized silica gel. The pKa of these SPE

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[1]*Abbreviations used:* SPE, solid-phase extraction.

columns is about 5. These packed columns now are available from Sigma-Aldrich (St. Louis, MO, product number 54127-U).

All chemicals were purchased from Fisher Scientific, (Cleveland, OH) or Sigma –Aldrich unless otherwise specified. Radiolabelled acyl-coenzyme A esters were purchased from Moravek Biochemicals (Brea, CA): [acetyl-1-^{14}C]-coenzyme A, (54 mCi/mmol), [malonyl-2-^{14}C]-coenzyme A (55 mCi/mmol), [octanoyl-1-^{14}C]-coenzyme A (57 mCi/mmol), [palmitoyl-1-^{14}C]-coenzyme A (53 mCi/mmol), [oleoyl-1-^{14}C]-coenzyme A (52 mCi/mmol), and [arachidonyl-1-^{14}C]-coenzyme A (54 mCi/mmol). Powdered rat liver was prepared under liquid nitrogen [1]. Rat liver was used because in our previous work with malonyl-coenzyme A [1], we analyzed rat heart, skeletal muscle, and liver and we found that the recovery of malonyl-coenzyme A from liver was much worse than for skeletal muscle and heart (28.8 ± 0.9 for liver, versus 48.5 ± 1.8 for heart and 44.7 ± 4.4 for skeletal muscle). Therefore, we chose liver as the example, since from our experience it posed the greatest challenge. In a 12×75 mm glass tube was placed powdered rat liver (20 to 26 mg) and radiolabelled acyl-coenzyme A standards ranging from 44,440 to 55,000 dpm and 0.35 to 0.46 nmoles. These amounts of added radiolabelled acyl-coenzyme A esters are in the concentration ranges reported in the literature. Next, 1.5 ml of acetonitrile/isopropanol (3+1, v+v) was added and a 30 sec homogenization was performed using an OMNI 2000 tissue homogenizer, followed by addition of 0.5 ml of 0.1M KH_2PO_4 (pH 6.7) and a second 30 sec homogenization. The resulting homogenate was vortex-mixed (5 sec), and two 200 µl aliquots were transferred to scintillation vials for radioactivity determination (100% recovery). The remainder was transferred to a microcentrifuge tube and centrifuged for 5 min. at $16,000 \times g$. Two 200 µl aliquots were removed from the supernatant for determination of recovery by radioactivity counting, and 1 ml of the remaining supernatant was transferred to a 12×75 glass tube and acidified by adding 0.25ml of glacial acetic acid and vortex mixing.

The SPE column was conditioned with 1 ml of acetonitrile/isopropanol/water/acetic acid (9+3 +4+4, v+v+v+v). This solution ensures protonation of the pyridyl functional group, so that it will function as an anion-exchanger. Following application and flow through of the supernatant (collected in 625 µl aliquots), the SPE column was washed with 1 ml of acetonitrile/ isopropanol/water/acetic acid (9+3+4+4, v+v+v+v), to remove unretained species (collected in 500 µl aliquots). Acyl-coenzyme A esters were then eluted with 2 ml of methanol/250 mM ammonium formate (4+1, v+v; collected in 500 µl aliquots). This eluent has a pH of 7, which neutralizes the pyridyl functional group.

All aliquots had their radioactivity content determined by liquid scintillation counting. This was performed, following the addition of 4 ml/vial of Ultima Gold scintillation cocktail (Perkin Elmer, Waltham, MA), using an LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). Recoveries were calculated from the determined radioactivity using correction factors for the percent of the volume that was counted. These data are shown in Table 1 and Table 2.

The data presented in Table 1 document a 93% to 104% recovery for the extraction from rat liver of all acyl-coenzyme A esters examined: acetyl-, malonyl, octanoyl-, palmitoyl-, oleoyl-, and arachidonyl-coenzyme A. Table 2 shows high recoveries from the elution step of the SPE isolation, varying from 83% to 90%. Small amounts of radioactivity were found in the flow-through and wash steps, with the least amounts seen using arachidonyl-coenzyme A. The radiolabelled arachidonyl-coenzyme A was freshly prepared for us by the manufacturer, while the other acyl-coenzyme A esters were from the manufacturer's stock. We suggest that the slightly increased radioactivity in the flow-through and wash of the other acyl-coenzyme A esters may represent radioactive contaminants rather than acyl-coenzyme A esters, and recoveries of acetyl-, malonyl, octanoyl-, palmitoyl- and oleoyl-coenzyme A may be understated.

In conclusion, the sample simplification procedure presented results in the isolation and purification, with high recoveries of acyl-coenzyme A esters with widely differing chain-length and saturation and with high recoveries. The procedure should be of general applicability, and appropriate for quantification regardless of the subsequent detection method.

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Table 1

Percent recovery of radioactivity in the supernatant from tissue homogenate. Values are the mean ± SD of three separate experiments.

Radioactivity[a]	Acetyl-coenzyme A	Malonyl-coenzyme A	Octanoyl-coenzyme A	Palmitoyl-coenzyme A	Oleoyl-coenzyme A	Arachidonyl-coenzyme A
Added	100	100	100	100	100	100
Recovered	101.2 ± 1.2	93.2 ± 2.6	104.3 ± 0.6	98.6 ± 0.5	99.4 ± 3.1	100.7 ± 0.6

[a] Radioactivity (dpm) recovered in the supernatant is expressed as percent of radioactivity (dpm) added to the tissue.

Table 2

Percent recovery of radioactivity from the solid-phase extraction (SPE) column in the flow-through, wash and eluted fractions and of total radioactivity. Values are the mean ± SD of three separate experiments.

Radioactivity[a]	Acetyl-coenzyme A	Malonyl-coenzyme A	Octanoyl-coenzyme A	Palmitoyl-coenzyme A	Oleoyl-coenzyme A	Arachidonyl-coenzyme A
Applied	100	100	100	100	100	100
Flow-through	2.0 ± 0.5	4.4 ± 0.5	3.7 ± 0.6	1.4 ± 0.7	2.5 ± 0.2	0.6 ± 0.1
Wash	0.4 ± 0.06	1.9 ± 0.03	3.1 ± 0.5	0.5 ± 0.05	0.6 ± 0.06	0.2 ± 0.06
Elution	86.3 ± 0.5	82.8 ± 0.8	86.3 ± 1.3	87.2 ± 0.3	86.5 ± 0.8	89.7 ± 0.3
Sum	88.7 ± 0.8	89.1 ± 0.9	93.1 ± 0.7	89.1 ± 0.4	89.6 ± 1.0	90.5 ± 0.4

[a]Radioactivity (dpm) recovered in the different fractions is expressed as percent of radioactivity (dpm) added to the column.