Validated Comprehensive Analytical Method for Quantification of Coenzyme A Activated Compounds in Biological Tissues by Online Solid-Phase Extraction LC/MS/MS

ARTICLE *in* ANALYTICAL CHEMISTRY · AUGUST 2008

Impact Factor: 5.64 · DOI: 10.1021/ac800031u · Source: PubMed

7 AUTHORS, INCLUDING:

Christoph Magnes
Joanneum Research Forschungsgesellscha…
41 PUBLICATIONS **1,261** CITATIONS
SEE PROFILE

Thomas R Pieber
Medical University of Graz
441 PUBLICATIONS **7,811** CITATIONS
SEE PROFILE

Tarek Moustafa
Medical University of Graz
47 PUBLICATIONS **1,447** CITATIONS
SEE PROFILE

Guenter Haemmerle
Karl-Franzens-Universität Graz
75 PUBLICATIONS **5,597** CITATIONS
SEE PROFILE

Anal. Chem. **2008,** *80,* 5736–5742

Validated Comprehensive Analytical Method for Quantification of Coenzyme A Activated Compounds in Biological Tissues by Online Solid-Phase Extraction LC/MS/MS

Christoph Magnes,[†] Maria Suppan,[†] Thomas R. Pieber,[†,‡] Tarek Moustafa,[‡] Michael Trauner,[‡] Guenter Haemmerle,[§] and Frank M. Sinner*,[†]

Institute of Medical Technologies and Health Management, Joanneum Research, Auenbruggerplatz 20, 8036 Graz, Austria

We report a robust, reliable, and comprehensive analytical method for the identification and quantification of the entire class of coenzyme A (CoA) activated substances, particularly short-, medium-, and long-chain acyl-CoAs derived from various biological tissues. This online SPE-LC/MS/MS-based method is characterized by a simple three-step sample preparation: (1) addition of buffer, organic solvents, and internal standards; (2) homogenization; and (3) centrifugation. The supernatant is injected directly into the SPE-LC/MS/MS system. Identification of CoA activated compounds is performed by accurate mass determination within the HPLC run. Method validation for short-, medium-, and long-chain acyl-CoA fatty acids revealed excellent quality. Accuracy was found to be between 87 and 107% and precision was between 0.1 and 12.8% in mouse skeletal muscle. The lower limit of quantification for all investigated compounds was well below 3.1% of estimated physiological levels in 200 mg of mouse tissue. Comparable results were obtained for mouse liver, mouse brown white adipose tissue and rat liver. For all investigated tissues, no matrix effect was observed.

Coenzyme A (CoA) activated compounds are a key class of substances involved in many essential metabolic pathways such as the Krebs cycle, lipid, carbohydrate, and amino acid metabolism, cholesterol and porphyrin synthesis, and the metabolic detoxification of xenobiotics. Mounting evidence moreover indicates that the modulatory impact of CoA activated compounds on multiple metabolic pathways is much greater than previously thought. A significant impact on metabolic regulation is also reflected by their possible role in several severe illnesses. Malonyl-CoA[1–3] and long-chain acyl-CoAs (LCACoAs) [4–6] are for instance involved in the development of metabolic syndrome whereas medium-chain acyl-CoAs are correlated with the most common fatty acid oxidation disorder, medium-chain acyl-CoA dehydrogenase deficiency.[7,8]

Activation with CoA is furthermore a possible first step for the metabolism of xenobiotics,[9,10] an essential pathway for the elimination of these substances from the human body. In general, these reactions introduce a hydrophilic moiety (e.g., amino acids) that decreases lipophilicity and so facilitates renal elimination (detoxification). Some xenobiotic substances may however become toxic following activation with coenzyme A.[9]

In order to investigate their widespread impact on physiology, quantitative investigation of coenzyme A activated substances is needed. Since these CoA activated substances represent a heterogeneous class of compounds, a comprehensive, robust, and reliable quantitative analytical method suitable for the analysis of different biological matrices is a prerequisite for this.

We present an online SPE-LC/MS/MS method capable of simultaneously quantifying short-, medium-, long-chain and other CoA activated substances originating from different biological tissues. The method is characterized by high analytical quality, a short run time of 17 min, the option of performing structural investigations, and a simple and rapid sample preparation procedure that can be summarized in three words: homogenize, centrifuge, and inject. The high degree of sensitivity and large dynamic range of this method allow the simultaneous quantification of low-abundance CoAs such as malonyl-CoA in the presence of highly abundant CoAs such as long-chain acyl-CoAs or acetyl-CoA. The structural characterization of unknown CoA activated substances within the HPLC run by accurate mass determinations

* To whom correspondence should be addressed. E-mail: frank.sinner@joanneum.at.

[†] Joanneum Research.

[‡] Current address: Department of Internal Medicine, Medical University of Graz, Graz, Austria.

[§] Current address: Institute of Molecular Bioscience, University of Graz, Graz, Austria.

(1) Luo, Z.; Saha, A. K.; Xiang, X.; Ruderman, N. B. *Trends Pharmacol. Sci.* **2005**, *26*, 69–76.
(2) Ruderman, N. B.; Saha, A. K. *Obesity (Silver Spring)* **2006**, *14*, 25S–33S.
(3) Folmes, C. D.; Lopaschuk, G. D. *Cardiovasc. Res.* **2007**, *73*, 278–87.
(4) Shulman, G. I. *J. Clin. Invest* **2000**, *106*, 171–76.
(5) An, J.; Muoio, D. M.; Shiota, M.; Fujimoto, Y.; Cline, G. W.; Shulman, G. I.; Koves, T. R.; Stevens, R.; Millington, D.; Newgard, C. B. *Nat. Med.* **2004**, *10*, 268–74.
(6) Ellis, B. A.; Poynten, A.; Lowy, A. J.; Furler, S. M.; Chisholm, D. J.; Kraegen, E. W.; Cooney, G. J. *Am. J. Physiol. Endocrinol. Metab.* **2000**, *279*, E554–E560.
(7) Gregersen, N.; Bross, P.; Andresen, B. S. *Eur. J. Biochem.* **2004**, *271*, 470–82.
(8) Roe, C. R.; Millington, D. S.; Maltby, D. A.; Kinnebrew, P. *J. Pediatr.* **1986**, *108*, 13–18.
(9) Knights, K. M.; Drogemuller, C. J. *Curr. Drug Metab.* **2000**, *1*, 49–66.
(10) Boelsterli, U. A. *Curr. Drug Metab.* **2002**, *3*, 439–50.

10.1021/ac800031u CCC: $40.75
Published on Web 07/09/2008

is furthermore possible. Through accurate mass measurements, five CoA activated substances were identified in liver using a triple quadrupole mass spectrometer with accurate mass option. The simple sample preparation, combined with the analytical quality in terms of precision, accuracy, and specificity, make this new method an ideal research tool.

EXPERIMENTAL SECTION

Chemicals. Solvents, reference standards, and isotopically labeled standards of the various CoA activated compounds were obtained from Sigma-Aldrich Handels GmbH (Vienna, Austria). Stock solutions and internal standard solutions were prepared in 50% aqueous methanol.

Preparation of Standard Solutions. All acyl-CoA stock solutions were prepared in 1:1 methanol/water and stored at −80 °C. Final concentrations of spike solutions and internal standard were prepared by dilution with 1:1 methanol/water. Acyl-CoA free matrix tissue was obtained by storing tissue aliquots for more than 1 h at room temperature. After 1 h, no acyl-CoA could be detected with the online SPE-LC/MS/MS system.

All calibration (C) and quality control (QC) standards for the validation process were prepared by spiking the acyl-CoA-free matrix with specific concentrations of nine different acyl-CoAs including short-chain, medium-chain, and long-chain acyl-CoAs: acetyl-CoA, malonyl-CoA, C6:0-CoA, C16:0-CoA, C16:1-CoA, C18:0-CoA, C18:1-CoA, C18:2-CoA, and C20:4-CoA. Six nonzero calibration standards prepared in 1:1 dilution steps with acyl-CoA free homogenate, and one zero and blank sample were used for calibration in each batch (C1 100%, C2 50%, C3 25%, C4 12.5%, C5 6.25%, C6 3.125%, 100% levels for each compound are shown in Table 3). Levels of C and QC samples were adjusted to the estimated physiological amounts in 200 mg of tissue. Four different levels of QCs were used for precision and accuracy validation in five different tissues (mouse liver, skeletal muscle, white and brown adipose tissue, and rat liver). Detailed information concerning the QC levels for each compound is presented in Table 4. Matrix effects were investigated by comparing the slopes of five calibration curves in different tissue homogenates. There were three replicate calibrations for each tissue homogenate.

Extraction Method. All solutions and solvents used for extraction were precooled at 4 °C. A 0.5-mL aliquot of 0.1 M KH_2PO_4, and 0.5 mL of 2-propanol were added to the weighed, frozen tissue aliquots (50−400 mg). A 0.5-nmol aliquot of C17:0-CoA, 0.6 nmol of $^{13}C_2$-acetyl-CoA, and 0.3 nmol of $^{13}C_3$-malonyl-CoA in 50% aqueous methanol were added per sample as internal standards. The sample was homogenized using a Heidolph Diax 100 for 30−60 s. A 60-μL aliquot of saturated aqueous ammonium sulfate and 1 mL of acetonitrile were added to the homogenate and vortexed. After centrifugation at 2500*g* for 10 min, the supernatant was transferred to autosampler vials and analyzed. Sample extracts were stored at −80 °C before analyzing.

Animals. C57/BL6J mice were housed with a 12:12-h light/dark cycle and permitted ad libitum consumption of water and a standard mouse diet (Sniff, Soest, Germany). The experimental protocols were approved by the local Animal Care and Use Committee, according to criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences, as published by the National Institutes of Health (Publication 86−23, revised 1985). Male mice of 8−10 weeks of age were fasted overnight with free access to water. The animals were assigned to two different treatment groups ($n = 3$). The first group received standard chow, while the second group was fasted overnight. Following mouse sacrifice, the organs of interest were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis.

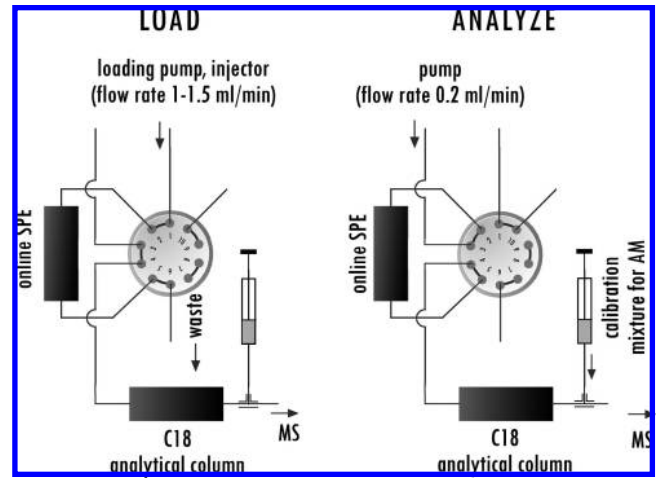

Figure 1. HPLC setup for the investigation of CoA activated substances. Load: Sample is loaded onto the polymeric SPE column with high flow rate (1−1.5 mL/min) using an eluent with low pH (0.1% aqueous formic acid). Analyze: Polymeric SPE column is switched in serial to the analytical column. Compounds are separated with lower flow rate (0.2 mL/min) and an eluent with high pH containing water, acetonitrile, and ammonium hydroxide. Separation conditions are shown in detail in Table 2. For accurate mass determinations, internal calibration standards are mixed via T-junction into the LC postcolumn flow at a flow rate of 5 μL/min with a syringe pump.

After an overnight fast, male Wistar rats were sacrificed, and the livers excised immediately, placed in liquid nitrogen, and stored at −80 °C until further analysis.

HPLC/MS Conditions. All experiments were carried out on an Ultimate 3000 System (Dionex, LCPackings) comprising an autosampler with cooled tray and a column oven with switching unit coupled to a Quantum TSQ Ultra AM (Thermo-Finnigan). The system was controlled by Xcalibur Software 1.4. An Oasis HLB column 2.1 × 20 mm cartridge was used for online SPE. The system setup is shown in Figure 1. The sample volume of 40 μL was loaded onto the SPE column with a flow rate of 1000 μL/min using 0.1% aqueous formic acid. After 48 s, acetontrile content of the eluent was increased to 40% by the gradient mixing system and the flow rate was increased to 1500 μL/min. At 3.5 min, the acetonitrile content was decreased to zero. At 6 min, the valve was switched to the analysis position to connect the trap column to the analytical column (Zorbax C18 Extend, 2.1 × 150 mm, 3 μm; see Figure 1). Separation was performed using high pH eluents beginning isocratically with 0.9% acetonitrile and 100 mM ammonium hydroxide (pH 11.1) for 1 min. Thereafter, the acetonitrile content was linearly increased to 80% over 5 min. After 2 min, acetonitrile content was decreased to 0.9% for column equilibration. The valve was switched to the loading position, and the SPE column was flushed with 60% acetontirile containing 15 mM ammonium hydroxide with a 1.5 mL/min flow rate for further cleaning. After 0.5 min, the SPE column was flushed with 0.1% formic

Table 1. Detailed Chromatographic Conditions of Online-SPE-HPLC Method for Acyl-CoA Separation[a]

		online SPE		analytical column		
time (min)	valve position	flow rate (mL)	eluent (%)	flow rate (mL)	eluent (%)	comment
0	L	1	100 A_{PE}	0.2	99 A 1 B	SPE acidic loading step
0.8	L	1	100 A_{SPE}			
0.9	L	1.5	60 A_{SPE} 40 B_{SPE}			SPE washing step
3.5	L	1.5	60 A_{SPE} 40 B_{SPE}			
3.6	L	1.5	100 A_{SPE}			SPE flushing with water
6.0	A	1.5	100 A_{SPE}			switch valve to analyze position
6.1	A	1.5	100 C_{SPE}			
7.0	A	1.5		0.2	99 A 1 B	
7.1	A	0.2				
12.0	A	0.2		0.2	20 A 80 B	
14.0	A			0.2	20 A 80 B	
14.4	A	1.5	100 C_{SPE}			
14.5	L	1.5	100 C_{SPE}			switch valve to loading position
15.0	L	1.5		0.2	99 A 1 B	
15.4	L	1.5	100 C_{SPE}			
15.5	L	1.5	100 A_{SPE}			

[a] Eluents: A_{SPE}, 0.1% formic acid; B_{SPE}, 0.1% formic acid in acetonitrile; C_{SPE}, 15 mM HH_4OH in 40% acetonitrile; A, 100 mM NH_4OH; B, 100 mM NH_4OH in 90% acetonitrile.

Table 2. MRM Scans for CoA Activated Compounds[a]

compound	MRM scans (m/z)
acetyl-CoA	810.1→ 303.1
malonyl-CoA	854.1→ 347.1
C6:0 CoA	866.2 → 359.1
C16:0 CoA	1006.3 → 499.3
C16:1 CoA	1004.3 → 497.3
C18:0 CoA	1034.4 → 527.4
C18:1 CoA	1032.4 → 525.4
C18:2 CoA	1030.4 → 523.4
[$^{13}C_2$]-acetyl-CoA	812.1 → 305.1 (internal standard for acetyl-CoA
[$^{13}C_3$]-malonyl-CoA	857.1 →350.1 (internal standard for malonyl-CoA)
C17:0 CoA	1020.4 → 513.4 (internal standard for medium- and long-chain acyl-CoAs)

[a] All scans correspond to a neutral loss of 507 Da. MS parameters, spray voltage 5.5 kV; CID, 40 eV.

acid for equilibration. Separation of all acyl-CoAs was performed within 17 min. A detailed description of the chromatographic conditions is given in Table 1.

Positive ESI-MS/MS mass spectrometry was performed using the following parameters: spray voltage 5.5 kV, capillary temperature 300 °C, sheath gas pressure 30 AU, auxiliary gas 3 AU, optimized collision energy for neutral loss and MRM 40 eV, source CID was set to 12 eV. Neutral loss scans were obtained by scanning from 800 to 900 m/z in 700 ms with a neutral loss mass of 507 Da. Quantitation was performed by MRM (Table 2).

Accurate mass determinations were performed in MRM mode using methylmalonyl-CoA (868.1 → 361.1 m/z, lock mass 361.14278 m/z) and acetyl-CoA (810.1 → 303.1 m/z, lock mass 303.13730 m/z) as lock masses. For this internal mass calibration, a mixture of methylmalonyl-CoA and acetyl-CoA (concentration ~3 pmol/μL) in acetonitrile/water/ammonium hydroxide, 50/50/15 mM, was mixed via T-junction into the LC postcolumn flow at a flow rate of 5 μL/min using a syringe pump (see Figure 1). Resolution of quadrupole three was set to high-resolution mode (FWHW 0.1). Accurate masses were acquired using scan rates of 1 amu/s. Accurate mass calibration of the mass spectrometer was performed with PEG standard solution within a range of 300−500 m/z.

RESULTS AND DISCUSSION

Mass spectrometry coupled to online SPE-liquid chromatography is a powerful approach for the simultaneous analysis of whole classes of metabolites. Mass spectrometry provides both sensitive detection and the opportunity to generate selective MS^2 scan modes for each single analyte or for whole classes of structurally related substances (e.g., using neutral loss, parent ion scan).

We have previously shown that a neutral loss of 507 Da corresponding to a unique fragment of CoA occurs for all LCACoAs under the specific MS CID conditions used and enables the facile quantitation of these CoA activated metabolites in liver extracts.[11] An example is shown in Figure 2 which presents a full MS/MS spectrum of oleoyl coenzyme A. As shown in this example, the ion current is generally carried by a small subset of fragment ions. In the positive ESI mode, the predominant ion in MS/MS spectra is the acylpantetheine fragment (oleoyl-pantheteine, m/z 525), which is derived by cleavage between the ADP and pantetheine residues with charge retention on the fatty acyl portion corresponding to the latter described neutral loss of 507 Da from $[M + H]^+$. Since in all cases the remaining acylpantheteine fragment retains the entire structural information of the activated substance, it can be used for selective quantification of specific CoA activated compounds or even to identify unknown activated compounds by, for example, accurate mass determination. The present report describes valuable enhancements and improvements to this method: (i) an extension to the analysis of all CoA activated compounds is shown, (ii) samples are now loaded via automated SPE, bringing significant sample processing simplification, (iii) online accurate mass determination is per-

(11) Magnes, C.; Sinner, F. M.; Regittnig, W.; Pieber, T. R. *Anal. Chem.* **2005**, *77*, 2889–94.

Table 3. Identification of Seven Unknown CoA Activated Compounds in Mouse Liver[a]

compound	chemical formula of acylpantheteine ion	calculated monoisotopic mass (Da)	measured accurate mass (Da)	error (mmu)	proposed chemical formula of R R-(CO)-S-CoA
acetyl-CoA (lock mass)	[C13 H23 N2 O4 S]$^+$	303.13730			
propionyl-CoA	[C14 H27 N2 O5 S]$^+$	317.15295	317.1550	2.55	C2 H5
butenoyl-CoA	[C15 H27 N2 O5 S]$^+$	329.15295	329.1540	1.05	C3 H5
butyl-CoA	[C15 H27 N2 O5 S]$^+$	331.16860	331.1712	2.6	C3 H7
pentanoyl-CoA	[C16 H29 N2 O5 S]$^+$	345.18425	345.1844	0.1454	C4 H9
malonyl-CoA	[C14 H23 N2 O6 S]+	347.12713	347.1255	−1.63	C2 H3 O2
β-hydroxybuturyl-CoA	[C15 H27 N2 O6 S]+	347.16352	347.1648	1.28	C3 H7 O1
hexanoyl-CoA	[C17 H31 N2 O5 S]$^+$	359.19990	359.2025	2.6	C5 H11
methylmalonyl-CoA (lock mass)	[C15 H25 N2 O6 S]$^+$	361.14278			

[a] All chemical formulas of the acyl portion can be proposed with the achieved mass accuracy.

Table 4. Method Validation for Nine Compounds Representing Different CoA Classes (Short-Chain, Medium-Chain, and Long-Chain Acyl-CoAs) in Five Different Biological Tissues (Mouse Skeletal Muscle, Brown Adipose Tissue, White Adipose Tissue, Mouse Liver, and Rat Liver)[a]

		RSD (%)							
		intrabatch accuracy, precision (mouse skeletal muscle, 5 replicate analyses)				intermatrix accuracy, precision (mouse skeletal muscle, brown adipose tissue, white adipose tissue, liver, and rat liver, 1 analysis in each matrix)			
	nominal[b] 100% level (nmol)	80% level	20% level	10% level	5% level	80% level	20% level	10% level	5% level
acetyl-CoA	6.18	4.907	1.220	0.559	0.330	4.994	1.257	0.614	0.293
		99 (10.4)	99 (1.4)	90 (12.8)	107 (0.9)	101 (14.2)	102 (1.5)	99 (2.3)	102 (2.1)
malonyl-CoA	0.586	0.480	0.123	0.053	0.030	0.476	0.121	0.055	0.028
		102 (1.7)	105 (0.5)	90 (0.8)	104 (0.4)	102 (3.3)	104 (0.5)	95 (0.2)	97 (0.5)
C6−0-CoA	1.93	1.388	0.347	0.172	0.086	1.462	0.362	0.174	0.085
		90 (2.9)	89 (1.0)	89 (0.4)	89 (0.1)	94 (10.7)	93 (2.0)	90 (1.1)	88 (1.0)
C16−0	1.99	1.609	0.406	0.199	0.099	1.653	0.436	0.203	0.093
		101 (3.6)	102 (1.7)	100 (0.7)	99 (0.6)	104 (8.8)	109 (2.9)	102 (1.0)	94 (1.0)
C16−1	1.99	1.588	0.376	0.182	0.093	1.580	0.392	0.188	0.092
		100 (4.0)	95 (0.8)	92 (0.5)	93 (0.3)	99 (4.9)	98 (2.7)	95 (0.9)	93 (0.8)
C18−0	1.93	1.496	0.384	0.185	0.097	1.566	0.416	0.203	0.103
		97 (4.7)	99 (2.2)	95 (0.4)	100 (0.8)	101 (10.1)	108 (0.8)	105 (0.8)	106 (1.4)
C18−1	1.94	1.604	0.401	0.195	0.096	1.584	0.405	0.199	0.093
		103 (3.7)	103 (0.7)	101 (0.3)	99 (0.3)	102 (5.8)	104 (4.1)	103 (1.3)	96 (0.9)
C18−2	1.94	1.571	0.381	0.187	0.093	1.574	0.399	0.190	0.087
		101 (5.0)	98 (0.8)	96 (0.4)	96 (0.3)	101 (8.8)	103 (4.1)	98 (1.2)	89 (0.8)
C20−4	1.88	1.539	0.366	0.171	0.082	1.535	0.389	0.176	0.085
		102 (4.3)	97 (1.4)	91 (1.9)	87 (1.1)	102 (7.3)	103 (4.3)	93 (2.5)	90 (0.6)

[a] Intrabatch accuracy and precision in mouse skeletal muscle and intermatrix accuracy and precision are shown. [b] Approximately 2-fold of expected levels in 200 mg of tissue.

formed by triple quadrupole MS/MS, and (iv) the applicability of the procedure to a broad range of different matrixes is demonstrated.

Online SPE/LC. For reproducible ionization efficiency and to separate isobaric acyl-CoAs, sample pretreatment and separation by HPLC are crucial. By using a setup consisting of an online SPE coupled to a C-18 analytical column, we were able to eliminate liquid extraction and evaporation pretreatment steps (Figure 1). An Oasis HLB column 2.1 × 20 mm cartridge was used as the online SPE column because, due to its polymeric nature and to the high particle diameter (20 μm) of the packing material, it can tolerate sample matrixes that would block other column types. Oasis HLB columns are resistant to clogging even when nonfiltered samples are injected. Tissue homogenates consequently need only be centrifuged before injection onto the SPE column; i.e., additional sample filtering was rendered unnecessary. Indeed, we have never observed column clogging, even after more than 1000 injections. All CoA activated compounds were, moreover, efficiently retained on the SPE-column.

Briefly, online SPE was performed using the following scheme: (i) loading of the extract at low pH (0.1% formic acid) to increase the lipophilicity of the CoA activated substances through protonation of the phosphate esters.

Despite the fact that the extraction sample consisted of strong solvents (25% 2-propanol and 50% acetontrile at pH 4.9), no effect on the retention of CoA activated compounds was observed. All CoA activated compounds, including highly hydrophilic compounds such as malonyl-CoA, were retained on the column head. This can be attributed to dilution into the loading flow of 1 mL/min of the injected 40 μL sample.

(ii) A washing step with increased acetonitrile content in the eluent was performed without any loss of CoA activated compounds. This washing step increased the robustness of our system, enabling us to apply our method to different biological matrixes. Before switching the valve to the analysis position, the acetonitrile

(12) Hankin, J. A.; Murphy, R. C. *Int. J. Mass Spectrom. Ion Processes* **1997**, *165/166*, 467–74.

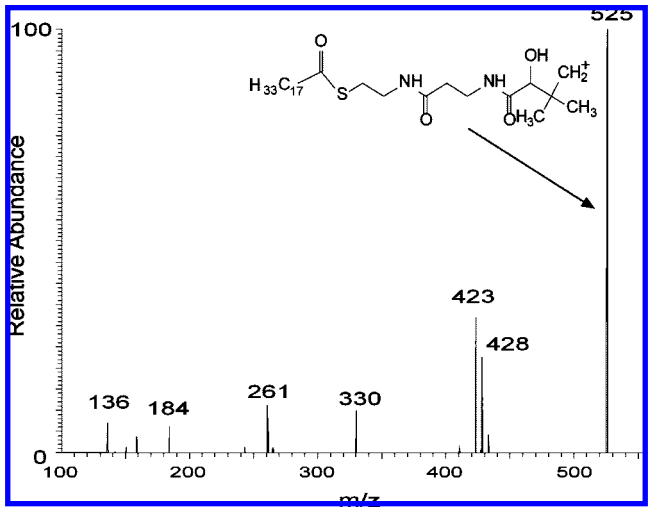

Figure 2. Full MS/MS spectrum of oleoyl coenzyme A (parent ion $[M + H]^+$, *m/z* 1032, collision energy 40 eV). In the positive ESI mode, the predominant ion in MS/MS spectra is the acyl-pantetheine fragment (oleoyl-pantheteine, *m/z* 525). There is one further ion common to CoA esters corresponding to the neutral loss of 609 from $[M + H]^+$ at *m/z* 423 for oleoyl-CoA. The abundant ion with *m/z* 428 corresponds to the protonated 3′-phospho-AMP. The lesser abundant ion at *m/z* 330 corresponds with the loss of the 3′-phosphate group from *m/z* 428.[12]

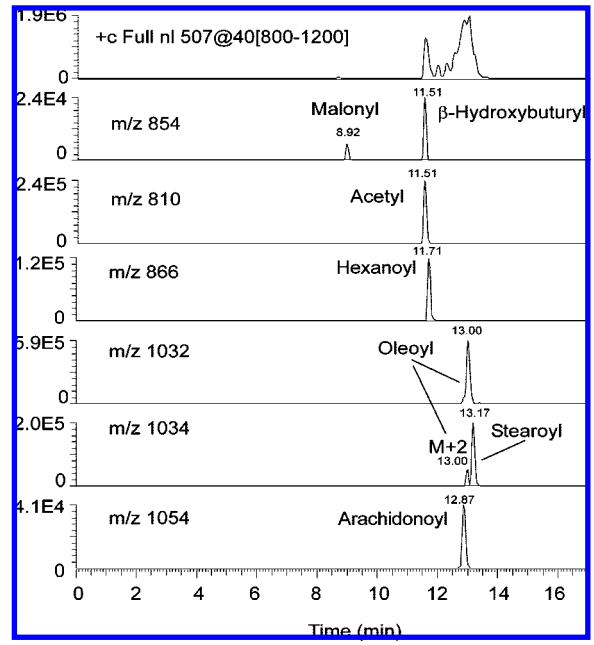

Figure 3. Neutral loss scan of skeletal muscle extract (mouse, neutral loss 507 Da, collision energy 40 eV, scan range 800−1200 *m/z*). Parent ions of different CoA activated compounds are shown. Isobaric compounds malonyl- and hydroxybutryl-CoA and M + 2 natural isotopic peak of oleoyl-CoA and stearoyl-CoA are baseline separated. HPLC conditions are described in the Experimental Section.

content was decreased to zero. This was necessary because the HPLC separation process is performed under basic conditions, which leads to deprotonation of the CoA phosphate esters and thus reduced retention of these compounds.

(iii) The next step was elution of activated CoA compounds from the SPE column and separation of the latter beginning isocratically with 0.9% acetonitrile. The isobaric compounds malonyl-CoA and the often more abundant hydroxybutryl-CoA must be separated by LC for quantification, as also mentioned by Gao[13] et al. The current new method separates these compounds very efficiently as shown in Figure 3. Hydroxybutryl-CoA was identified by accurate mass determination as shown in Table 3. Malonyl-CoA eluted during the initial isocratic conditions, and hydroxybutryl-CoA eluted after the gradient was started. As expected for RP-HPLC; retention times increased with increasing carbon number and decreased in proportion to the number of double bonds in activated fatty acids. In summary, by applying these HPLC conditions an excellent separation of CoA activated compounds was achieved (Figure 3).

Online Accurate Mass Determination. As mentioned above, fragment ions can be used for selective compound quantification or structural determination. One powerful tool for the latter purpose is accurate mass determination. Accurate mass measurements for determining elemental compositions are mainly performed by TOF or FTMS[18] with both types of instrument providing excellent mass accuracy. Quantification using TOF instruments, however, suffers from a low dynamic range and FTMS instruments are very expensive. A key feature of the present report is the use of a triple quadrupole mass spectrometer. This type of machine is only used infrequently for accurate mass determinations, which is reflected by the fact that there are only a few reports published using this technique.[14–17] Triple quadruple spectrometers are, however, relatively inexpensive and provide an excellent dynamic range for quantification. Thus, the method we present is both effective, enabling the online determination of accurate masses in high-resolution mode, and affordable, enabling more general use. The accurate mass measurements presented here were performed using internal mass calibration. Paul et al.[15] reported an external calibration method for MS accurate mass determinations of product ions of cabergoline using PEG standards. In general, however, external calibration with substances differing significantly from the analytes cannot achieve the accuracy and robustness of internal calibration. A number of different approaches for internal mass calibration in MS/MS mode using triple quadrupole mass spectrometry have been published. One method for obtaining internal mass calibration in MS/MS mode for example uses identified product ions as lock masses. Marull et al.[19] used this approach to determine accurate masses of product ions derived from imatinib and imatinib metabolites. This method, however, requires the use of product ions with known accurate masses similar to the mass range of the ions of interest. The positive ESI MS/MS spectra of acyl-CoAs are

(13) Gao, L.; Chiou, W.; Tang, H.; Cheng, X.; Camp, H. S.; Burns, D. J. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* In press.

(14) Hughes, N.; Winnik, W.; Dunyach, J. J.; Amad, M.; Splendore, M.; Paul, G. *J. Mass Spectrom.* **2003**, *38*, 743–51.

(15) Paul, G.; Winnik, W.; Hughes, N.; Schweingruber, H.; Heller, R.; Schoen, A. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 561–68.

(16) de Rijke, E.; Ruisch, B. J.; Bouter, N.; Konig, T. *Mol. Nutr. Food Res.* **2006**, *50*, 351–55.

(17) Marull, M.; Rochat, B. *J. Mass Spectrom.* **2006**, *41*, 390–404.

(18) Bristow, A. W.; Webb, K. S. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 1086–98.

(19) Marull, M.; Rochat, B. *J. Mass Spectrom.* **2006**, *41*, 390–404.

Table 5. Investigation of Matrix Effect by Comparing the Slopes of Calibration Curves Spiked in Five CoA Free Homogenates Obtained from Different Tissues (Mouse Skeletal Muscle, Mouse WAT, Mouse BAT, Mouse Liver, and Rat Liver, Three Replicate Analyses of Each C-Sample)[a]

compound	average slope (area ratio/ng)	RSD of slopes (%)
acetyl CoA	0.6820	3.35
malonyl CoA	3.2374	3.79
C6−0 CoA	4.5361	5.56
C16−0 CoA	1.6295	2.26
C16−1 CoA	2.1296	3.67
C18−0 CoA	1.6295	1.45
C18−1 CoA	2.9089	2.06
C18−2 CoA	2.8231	2.38
C20−4 CoA	2.0408	2.19

[a] Relative standard deviations (RSD) of the slopes in the different tissues for short- and long-chain acyl-CoAs were between 1.45 and 5.56%.

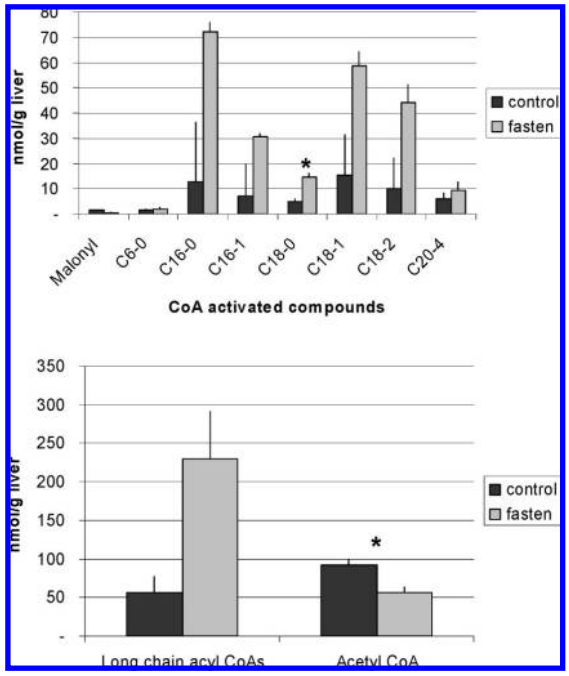

Figure 4. Application of the online SPE-LC/MS/MS method for CoA activated compounds in a study with two groups of mice. The first group (gray) was fasted overnight, and the second group was fed standard food (black). Levels of all long-chain acyl CoAs in liver tend to increase after fasting because of the raised β-oxidation of fatty acids in the hepatic mitochondria. Malonyl-CoA is decreased because fatty acid synthesis is downregulated. Additionally, a significant reduction of acetyl-CoA levels in the fasted group can be observed. The values are means ± SE*. ($p < 0.05$).

dominated by the fattyacyl-pantetheine fragment ion. The CoA portion yields only a few product ions (i.e., m/z 428 3′-phospho-AMP or m/z 330 pantetheine ADP ion.[12] See also Figure 2.) suitable for general use for all CoA activated compounds. Their low abundance and lower m/z however make them unsuitable for use as lock masses. Optimum accuracies by internal mass calibration in MS/MS mode can be achieved by using homologous substances with similar dissociation reactions with comparable dissociation energies. For this reason, a mixture of acetyl-CoA and methylmalonyl-CoA (concentration ~3 pmol/μL) in acetonitrile/water/ammonium hydroxide 50/50/15 mM was used for mass calibration and was mixed via T-junction into the LC postcolumn flow at a flow rate of 5 μL/min with a syringe pump. The homologous product ions generated by the neutral loss of 507 Da (acetyl-CoA 303.137 854 Da, methylmalonyl-CoA 361.143 334 Da) were used as lock masses. This approach is very flexible, as a wide range of different CoA activated substances are commercially available and can be used to spread or change the mass ranges for accurate mass determinations of CoA activated substances. In order to demonstrate the suitability of this method for analysis of biological samples, accurate mass determinations were carried out for seven different endogenous acyl-CoAs in mouse liver extract. The results are summarized in Table 3. The chemical formulas of the activated compounds were proposed by Xcalibur 1.4 Software. The molecular weights of the acyl portions were calculated by subtraction of the calculated accurate mass of the pantheteine portion (288.114 379 Da) from the measured accurate mass. The software calculated possible chemical formulas using the accurate mass determined for the acyl portion. Possible atoms were limited to hydrogen, carbon, nitrogen, phosphorus, and sulfur. The correct chemical formulas for all substances were determined in this manner, in each case with an error margin less than 2.6 mmu. The results for propionyl-, β-hydroxybuturyl-, malonyl-, and hexanoyl-CoA were confirmed by comparing the identified peaks with the retention times of standards. The identification of butenoyl-, butyl-, and pentanoyl-CoA peaks was confirmed by their expected retention time shifts compared to propionyl-CoA and hexanoyl-CoA.

Quantification of CoA Acitivated Compounds. As described earlier, the new method we describe is not only capable of identifying unknown CoA activated compounds but can also accurately and precisely quantify known or identified metabolites. In order to illustrate the quality of quantification, nine compounds representing different CoA classes (short-chain, medium-chain, and long-chain acyl-CoAs) were quantified in five different biological tissues. This validation study demonstrated excellent quality in terms of both accuracy and precision. Accuracy for all compounds in mouse skeletal muscle using five replicate analyses was between 87 and 107% and precision was between 0.1 and 12.8%. Detailed data for intrabatch accuracy and precision for skeletal mouse muscle is shown in Table 4. Comparable results were generated for mouse brown adipose tissue, white adipose tissue, liver, and rat liver (data not shown). The sample matrix can greatly influence the quality of results obtained by MS due to possible different ion suppression effects during the electrospray ionization. An MS method that is matrix-independent and that can thus be applied to a broad range of tissue would constitute a powerful tool for metabolite analysis. To assess the matrix dependency of our method, intermatrix precision and intermatrix accuracy were evaluated from the first analysis of each intrarun batch in each tissue. Thus, five analyses were used to determine the average concentration and corresponding relative standard deviation (RSD) values. The detailed results are shown in Table 4. Intermatrix accuracy was between 88 and 109% for all compounds whereas intermatrix precision was between 0.2 and 14.2%. Lowest QC (5% level) and lowest C (3.125% level) met all FDA acceptance criteria for limit of quantitation for all compounds. According to FDA criteria for the lower limit of quantitation

(LLoQ), the signal-to-noise ratio must be above 10, accuracy must be between 80 and 120%, and precision must be below 20% RSD. For all compounds, the signal-to-noise ratio was above 200. The LLoQ for all investigated compounds was therefore well below 3.1% of the estimated physiological levels in mouse. The matrix effect was also investigated by comparing the slopes of calibration curves for five spiked, CoA free homogenates obtained from different tissues (three replicate analyses of each C-sample). RSDs of the slopes for short- and long-chain acyl-CoAs in the different tissues were between 1.45 and 5.56% (see Table 5). These results show clearly that no matrix effect was observed using this new method. The absence of a matrix effect is also supported by the observation that accuracy and precision were not improved when isotopically labeled internal standards were used. In the presence of a matrix effect, isotopically labeled standards would compensate for ion suppression effects of the matrix during electrospray ionization leading to better quality results. Our data thus show that the concentrations of CoA activated compounds in different tissues determined by this method can be directly compared to each another. In order to illustrate the usefulness of this method, a study with two groups of mice was carried out. The first group was fasted overnight and the second group was fed standard food. The levels of short-, medium-, and long-chain acyl-CoAs in liver were quantified. The results are shown in Figure 4. As expected, the levels of all long-chain acyl-CoAs tended to increase after fasting because of the raised β-oxidation of fatty acids in the hepatic mitochondria. Furthermore, malonyl-CoA is decreased because fatty acid synthesis is downregulated. Additionally, a significant reduction in acetyl-CoA levels was observed in the fasted group.

CONCLUSION

This validated online SPE-LC/MS/MS method is a robust, reliable, and comprehensive analytical way in which to identify and quantify the entire class of CoA activated substances, particularly short-, medium-, and long-chain acyl-CoAs derived from different biological tissues such as liver, skeletal muscle, brown adipose tissue, and white adipose tissue. The method is furthermore characterized by simple sample preparation. The extraction procedure consists of three steps: (1) adding buffer, organic solvents, and internal standards, (2) homogenization, and (3) centrifugation. The supernatant is injected directly into the SPE-LC-MS/MS system. Identification of CoA activated compounds is performed by accurate mass determinations within the HPLC run. The structures of seven different endogenous acyl-CoAs in a mice liver extract were determined using this method.

Validation for short-, medium-, and long-chain acyl-CoA fatty acids revealed excellent quality. Accuracy was found to be between 87 and 107% and precision was between 0.1 and 12.8% in skeletal mouse muscle. The LLoQ for all investigated compounds was therefore well below 3.1% of estimated physiological levels in mouse. Comparable data were found for mouse and rat liver, mouse brown adipose tissue, and mouse white adipose tissue. For all investigated tissues, no matrix effect was observed. The combination of an extremely simple sample pretreatment with fast separation times of ~17 min make this method ideal for the investigation of CoA activated substances in clinical research.

ACKNOWLEDGMENT

This study was supported by the Austrian Federal Ministry for Transport, Innovation and Technology.

Received for review January 7, 2008. Accepted June 5, 2008.

AC800031U