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Simultaneous quantification of malonyl-CoA and several other short-chain acyl-CoAs in animal tissues by ion-pairing reversed-phase HPLC/MS

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Abstract

Malonyl-CoA is a key intermediate involved in lipid synthesis and lipid oxidation. Here, we report on a novel method for the quantification of malonyl-CoA and seven other short-chain acyl-CoAs in various rat and mouse tissues using ion-pairing reversed-phase HPLC/MS. This method is capable of measuring malonyl-CoA, free coenzyme A (CoASH), acetyl-CoA, β-hydroxyl-butyryl-CoA (HB-CoA), 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), propionyl-CoA, succinyl-CoA, and isobutyryl-CoA simultaneously with a dynamic linear range over two orders of magnitude in a 7.0 min HPLC gradient run. The lower limit of quantification (LLOQ) was 0.225 pmol for all acyl-CoAs studied, except for HMG-CoA which had a higher LLOQ of 0.90 pmol. The interference of HB-CoA on the quantification of malonyl-CoA in animal tissues was also explored for the first time.

Keywords: Coenzyme A (CoA); Short-chain acyl-CoA; Malonyl-CoA; HPLC; LC/MS; Mass spectrometry; β-Oxidation; Lipid metabolism; Acetyl-CoA; β-Hydroxybutyryl-CoA; Succinyl-CoA; 3-Hydroxy-3-methylglutaryl-CoA; *n*-Propionyl-CoA; Isobutyryl-CoA

1. Introduction

Malonyl-CoA, a short-chain acyl-CoA, is formed by the addition of a carboxyl group to acetyl-CoA in an enzymatic reaction catalyzed by acetyl-CoA carboxylase (ACC), a committed step in fatty acid synthesis. Malonyl-CoA is a known substrate of fatty acid synthase. A decrease in intracellular malonyl-CoA concentration in response to fasting is expected to decrease fatty acid synthesis in lipogenic tissues, such as liver and fat. In addition, malonyl-CoA also functions as a potent inhibitor of carnitine palmitoyltransferase-I (CPT-I) [1,2], a mitochondrial membrane-associated enzyme responsible for transporting long-chain acyl-CoA into mitochondria, a rate-limiting step for β-oxidation [3]. Therefore, a decrease in malonyl-CoA level should lead to increase in fatty acid oxidation by activating CPT-1. The low level of malonyl-CoA in tissues and limited tissue amounts available from certain animals necessitate a sensitive method for detecting malonyl-CoA levels. Since acetyl-CoA is the precursor for malonyl-CoA, and many other short-chain acyl-CoAs are involved in the formation and consumption of acetyl-CoA, the quantitative determination of other short-chain acyl-CoAs will also provide useful information in dissecting the complex lipid metabolism pathways involving malonyl-CoA and acetyl-CoA regulation.

Previously published methods for short-chain acyl-CoA detection include radioactive-based enzymatic assay [4–6], high-performance liquid chromatography (HPLC) [7–11] and capillary electrophoresis [12] with UV detection. However, these approaches are limited in their sensitivities, sample throughput, and dynamic ranges. In addition, fatty acid synthase, an enzyme required for the enzymatic assay, is not commercially available. Thus, current HPLC methods either cannot positively identify acyl-CoAs or require long HPLC run time (>20 min). Because UV detectors cannot distinguish malonyl-CoA from other compounds, long HPLC run time is often required to achieve a baseline separation for all acyl-CoAs. Although there are reports describing sensitive detection of acyl-CoA levels using gas chromatography/mass spectrometry (GC/MS) [13–15], these methods are tedious and require the hydrolysis and derivatization of acyl-CoAs, which can seriously impact the accuracy of quantitative measurements.

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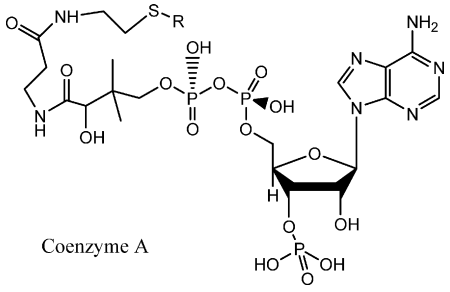
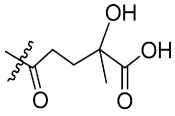
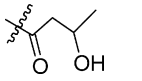
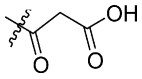
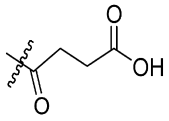
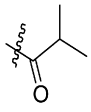
Table 1
Short-chain acyl-coenzyme as studied

	Compound	R	Formula	Exact mass
 Coenzyme A	CoASH	H	$C_{21}H_{36}N_7O_{16}P_3S$	767.1152
	Acetyl-CoA		$C_{23}H_{38}N_7O_{17}P_3S$	809.1258
	3-Hydroxy-3-methyl-glutaryl-CoA		$C_{27}H_{44}N_7O_{20}P_3S$	911.1575
	β-Hydroxybutyryl-CoA		$C_{25}H_{42}N_7O_{18}P_3S$	853.1520
	Malonyl-CoA		$C_{24}H_{38}N_7O_{19}P_3S$	853.1156
	Succinyl-CoA		$C_{25}H_{40}N_7O_{19}P_3S$	867.1313
	Propionyl-CoA		$C_{24}H_{40}N_7O_{17}P_3S$	823.1414
	Isobutyryl-CoA		$C_{25}H_{42}N_7O_{17}P_3S$	837.1571

A recent report on quantification of tissue malonyl-CoA levels using HPLC/MS [16] addressed many shortcomings found in the methods described above. However, care should be taken to separate β-hydroxyl-butyryl-CoA (HB-CoA) from malonyl-CoA; as HB-CoA ($C_{25}H_{42}N_7O_{18}P_3S$) and malonyl-CoA ($C_{24}H_{38}N_7O_{19}P_3S$) have very close exact masses (853.1520 and 853.1156 Da, respectively) that cannot be distinguished using a typical mass spectrometer. Additionally, HB-CoA and malonyl-CoA have similar physicochemical properties, and it is extremely difficult to separate them using reversed-phase HPLC without the use of an ion-pairing agent in the mobile phase. This is due to the fact that short-chain acyl-CoAs have the same highly hydrophilic core structure and are poorly retained on typical reverse phase HPLC columns. The addition of a basic ion-pairing agent to the mobile phase can greatly enhance the retention of hydrophilic analytes on reverse phase columns [17–19]. However, the addition of a basic ion-pairing agent often hampers the electrospray ionization (ESI) process, and can significantly decrease the MS sensitivity.

In this work, we developed a novel LC/MS method by employing a very low concentration of the basic ion-pairing agent, dimethylbutyl amine (DMBA), to improve the separation of short-chain acyl-CoAs. In addition, a post-column addition of acetonitrile was used to improve the sensitivity. Since this method did not require any organic or solid phase extraction of tissue homogenates prior to LC/MS analysis, the short-chain acyl-CoAs in those tissue homogenates were very well preserved. Finally, the potential interference between the isobaric compounds, HB-CoA and malonyl-CoA, was evaluated to confirm that this would not impact the quantification of malonyl-CoA. This novel LC/MS method was capable of separating the isobaric interference at baseline and provided a sensitive and reproducible method for the simultaneous quantification of eight short-chain acyl-CoAs (Table 1) with a throughput of 7.0 min/sample.

2. Experimental

2.1. Materials

Malonyl-CoA, acetyl-CoA, *n*-propionyl-CoA, succinyl-CoA, free coenzyme A (CoASH), isobutyryl-CoA, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), β-hydroxyl-butyryl-CoA (HB-CoA), 1,4-dithioerythritol (DTE), and 5-sulfosalicylic

acid (SSA) were purchased from Sigma (St. Louis, MO, USA). Acetic acid, ammonium acetate (NH_4OAc), triethylamine (TEA), and dimethylbutyl amine (DMBA) were obtained from Sigma–Aldrich (Milwaukee, WI, USA). Acetonitrile was purchased from EMD Chemicals (Gibbstown, NJ, USA). Water was treated with Millipore purification system (Billerica, MA, USA). Aqua C18 columns (50 × 1.0 mm, 5 μm, 125 Å) were purchased from Phenomenex (Torrance, CA, USA).

2.2. Tissue sample preparation

Sprague-Dawley (SD) rats and CD-1 mice were purchased from Charles Rivers Laboratories (Wilmington, MA, USA), and the animal maintenance and tissue collections were carried out in accordance with the Abbott Laboratories guidelines on animal care and usage. CD-1 mice were grouped ($N = 4$) into three treatment groups; freely fed on the standard chow, fasted for 24 h, or fasted for 24 h followed by re-fed with a high sucrose diet (46% kilo calories from carbohydrate) for an additional 24 h. At the end of the treatments, the animals were anesthetized in 60% CO_2/40% air, and heart, liver, and soleus muscle were immediately removed and snap frozen in liquid nitrogen. The tissues were homogenized with a Polytron in the extraction solution (ice-cold 5% SSA containing 50 μM DTE) with the ratio 1:10 (w/v) [9]. The tissue homogenates were centrifuged at 14,000 × *g* for 15 min at 4 °C. The clear supernatants were then filtered through 0.22 μm pore size filtering discs before being injected into the LC/MS system for the detection of the short-chain acyl-CoAs.

2.3. Preparation of calibration standards

Acyl-CoA standards were prepared as 1.0 mM stock in extraction solution (5% SSA containing 50 μM DTE) and stored at −80 °C. The acyl-CoA stock solutions were periodically tested and found to be stable for at least three months at −80 °C. Right before sample analysis, an aliquot of every acyl-CoA stock solution was thawed at 4 °C for 2 h and then mixed in the extraction solution to the desired concentration. Propionyl-CoA was used as the internal reference for quantification and was added to the calibration standards or samples at a ratio of 1:20 to achieve a final concentration of 5.0 μM, except otherwise specified.

2.4. Chromatography

A Shimadzu HPLC system was used for the sample analysis. The system consisted of one SIL-10Advp autosampler, one SCL-10Avp controller, and three LC-10Advp pumps. The HPLC was running in the binary gradient mode with the third pump delivering 100% pure acetonitrile isocratically as post-column mixing solvent. The samples were kept at 4 °C inside the closed chamber of the autosampler. The HPLC analysis was carried out at ambient temperature. Solvent A for HPLC consisted of 5.0 mM NH_4OAc, 5.0 mM DMBA, and was adjusted to pH 5.6 with acetic acid (around 6.5 mM). Solvent B contained 0.1% formic acid in acetonitrile. At initial stage, the LC was running at flow rate 200 μL/min with 2.5% B. The details of HPLC gradient conditions are described in Table 2. From 0 to 2.0 min, the flow was diverted to waste. At 2.0 min, HPLC eluent was directed to MS probe and data acquisition was started. Pump C delivered acetonitrile to mix with HPLC eluent post-column, before MS probe, which improved the ESI desolvation efficiency (for approximately 10 times). The autosampler was programmed to prepare the next sample injection before the end of current LC analysis. Once the current LC run was finished, the controller would initiate the injector valve and start the next LC run. The sample throughput was 7.0 min/sample.

Table 2
HPLC conditions for short-chain acyl-coenzyme as analysis

Time (min)	Total flow rate (Pump A + B) (μL/min)	*B%*	Comments
0	200	2.5	Divert flow to waste
0.50		2.5	
0.55		12.5	
2.00			Divert flow to MS. Start MS acquisition
2.50	200		
2.55	100		
5.00	100	25	
5.05	200	2.5	Divert flow to waste
6.50			Start preparing for next injection
7.00			Finish current run. Inject and start HPLC gradient for next sample

2.5. Mass spectrometry

The LC eluent was introduced into either an Applied Biosystem API 3000 or a Pulsar I mass spectrometer through a turbo ionspray (electrospray ionization) probe. The data were acquired using positive ion mode Q1MS or TOFMS scanning from 450 to 1300 *m/z* range with 1.0 s scan time. The turbo ionspray assisting gas temperature was set at 450 °C. The gas flow rates and electric lens settings were optimized using the Analyst automatic optimizing wizard by mixing the flow of malonyl-CoA standards (5 μM) at 5 μL/min with the initial HPLC mobile phase.

3. Results and discussions

Due to the high hydrophilicity of the short-chain acyl-CoAs, these compounds were not adequately retained and separated under typical reverse-phase HPLC conditions. The addition of an ion-pairing agent to the mobile phase has been known to improve the retention and separation of acyl-CoA compounds [20]. TEA is the most commonly used ion-pairing agent for separating acidic analytes on reverse phase HPLC columns. We systematically tested LC conditions using TEA as the ion-pairing agent at the concentrations ranging from 2.5 to 10.0 mM and NH_4OAc at the concentrations ranging from 2.5 to 15.0 mM, with pH 4.5–6.5. However, we failed to achieve a baseline separation for malonyl-CoA and HB-CoA, the mass/charge isobaric species, based on these LC conditions. Due to the negative effects

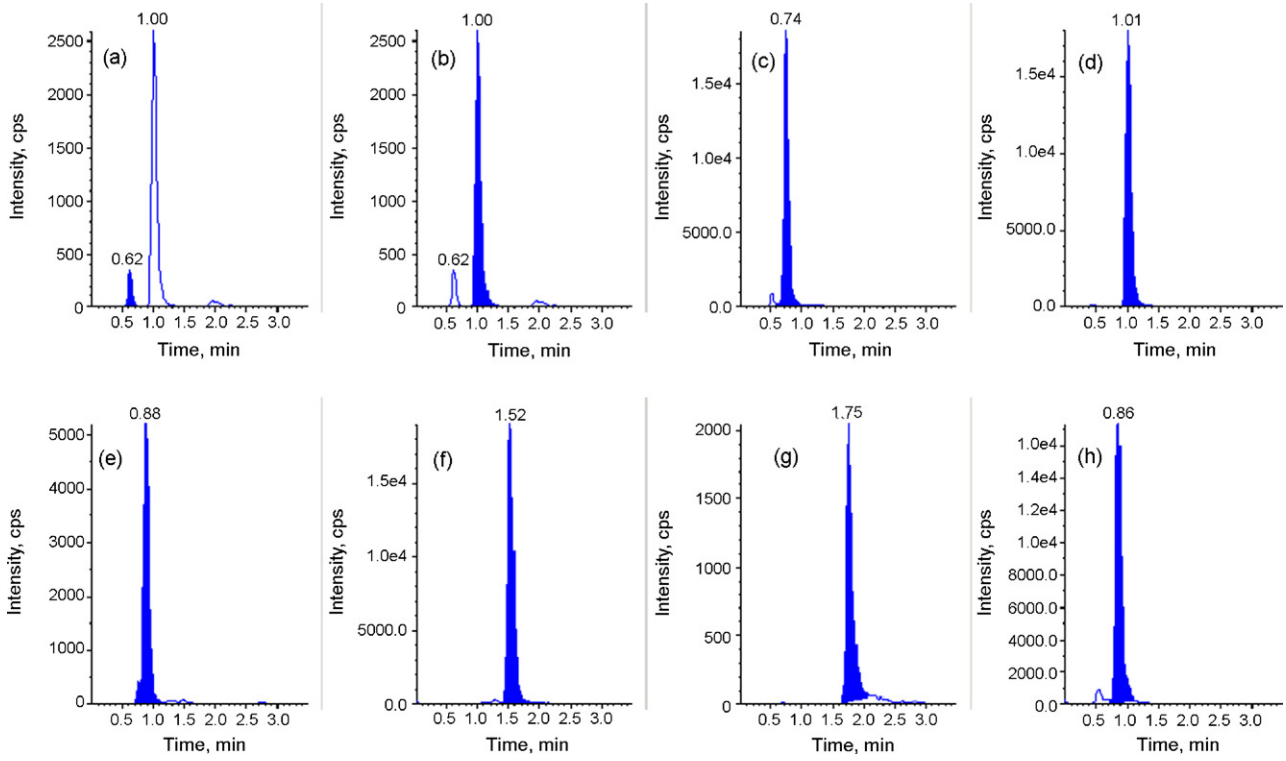


Fig. 1. Selected ion chromatograms of short-chain acyl-CoAs from SD rat liver extract (filled peaks): (a) *m/z* 854.15; malonyl-CoA; (b) *m/z* 854.15, HB-CoA; (c) *m/z* 768.12, CoASH; (d) *m/z* 810.13, acetyl-CoA; (e) *m/z* 868.13, succinyl-CoA; (f) 824.14, propionyl-CoA; (g) *m/z* 838.16; isobutyryl-CoA; and (h) 912.16, HMG-CoA.

of TEA and NH_4OAc for ESI-MS analysis sensitivity, conditions with higher concentrations of TEA and NH_4OAc were not tested. It has been reported that increasing the alkyl chain length of the ion-pairing agent could improve the retention of hydrophilic analytes [21], so we replaced TEA with DMBA as the ion-pairing agent in our subsequent LC optimization studies. After testing various concentrations of DMBA and NH_4OAc between pH 5.0 and 6.5, we were able to separate these two isobaric species with an optimized solvent A containing 5.0 mM NH_4OAc, 5.0 mM DMBA, and pH 5.6 (adjusted with acetic acid). Acyl-CoAs HPLC separations were stable when the pH of solvent A was kept between 5.5 and 5.8. The detailed HPLC conditions are described in the Section 2. Because short-chain acyl-CoAs are very hydrophilic, they all eluted out with high aqueous content (>80%), even with the addition of DMBA, the ESI ionization efficiencies were low. Higher organic composition tends to increase the ESI-MS sensitivity [22]. We used a third HPLC pump to deliver 100% acetonitrile at 50 μL/min to mix with the HPLC eluent post-column before MS analysis. The ESI-MS sensitivity for acyl-CoAs was increased about 10× after increasing the organic contents of the mobile phase.

Fig. 1 shows the HPLC chromatograms of eight short-chain acyl-CoAs from SD rat liver extracts. Malonyl-CoA and HB-CoA, which have a mass difference of 0.0364 Da, were baseline-separated. The other acyl-CoA peaks were at least partially separated. HPLC peaks were identified by comparing chromatographic retention times and exact *m/z* values of corresponding ions with those of authentic acyl-CoAs spiked into tissue extracts.

3.1. Baseline separation of HB-CoA and malonyl-CoA in HPLC is necessary for quantitative measurement of malonyl-CoA

Mass spectrometry (MS) is a highly selective and specific detector. It can differentiate interferences from compounds of interest based on their difference in mass to charge ratios; therefore, a complete HPLC separation of chemical interferences is usually not necessary if there is sufficient difference in masses for LCMS analysis. However, the analysis for short-chain acyl-CoAs presents a particular challenge, because there are many endogenous short-chain acyl-CoAs, and some of which can be isobaric analogs with very close masses. One can use either accurate mass measurement with high-resolution mass spectrometers, or MS/MS if the compounds have unique product ions, or HPLC, or any combinations of the three to distinguish the interferences from the compounds of interest.

In SD rat liver extracts, the MS signal of HB-CoA was 10 times more intense than that of malonyl-CoA (Fig. 1). The exact mass difference between these two compounds is 0.0364 Da (853.1520 − 853.1156 = 0.0364 Da). If we solely relied on the accurate mass measurement to distinguish these two compounds, it would require an instrument that has a mass resolving power greater than 23,000 (853/0.0364 = 23,434) when their LCMS peaks were at equal intensity. Since HB-CoA signal was 10 times more intense than malonyl-CoA, it would require resolving these two compounds at 5% peak height level of HB-CoA in MS spectra to obtain reliable quantification of malonyl-CoA. This would require a mass resolution of greater

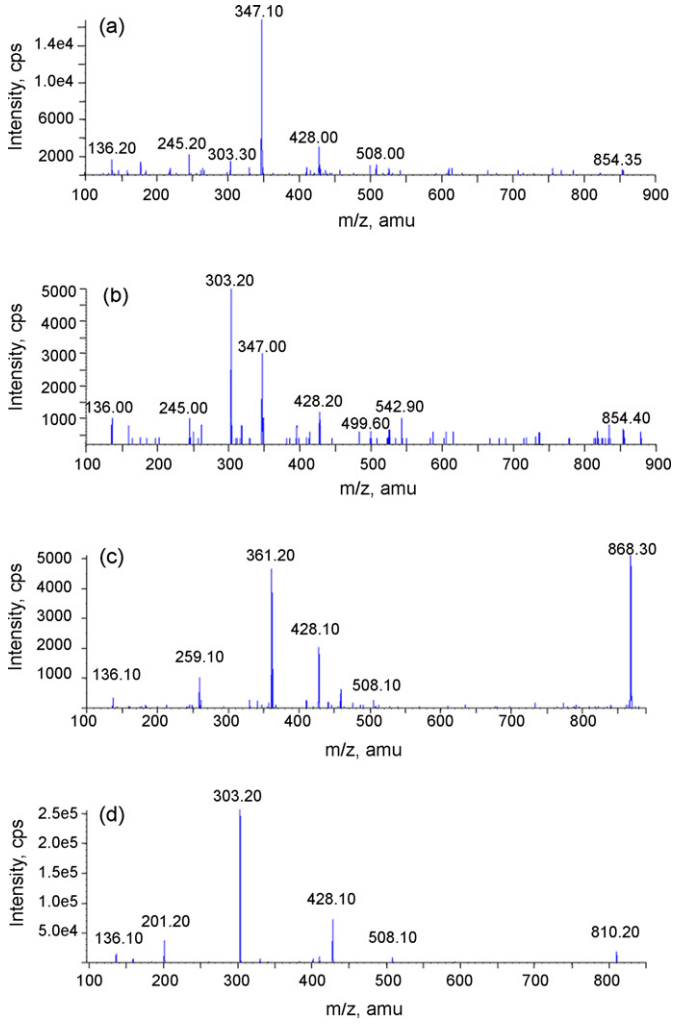


Fig. 2. MS/MS spectra obtained on API 3000 triple-quadrupole mass spectrometer with collision energy of 52 eV: (a) HB-CoA (854.1); (b) malonyl-CoA (854.1); (c) succinyl-CoA (868.1); and (d) acetyl-CoA (810.1).

than 100,000, which is far beyond the capabilities of the Applied Biosystem Q-Star (Quadrupole-Time-of-Flight) and API 3000 (triple quadrupole) mass spectrometers available in our laboratory.

Collision induced dissociation (CID) fragmentation is frequently used in quantitative analysis to improve signal-to-noise ratio and to differentiate potential interferences from compounds of interest. If HB-CoA and malonyl-CoA have unique product ions with reasonable intensities, then the specific multiple reaction-monitoring (MRM) technique can be utilized to distinguish these two compounds. CID MS/MS spectra were obtained on Applied Biosystem API 3000 for HB-CoA and malonyl-CoA with various collision energies. Fig. 2a and b shows MS/MS spectra of both HB-CoA and malonyl-CoA at collision energy of 52 eV. The top seven product ions (*m/z* values for these singly charged ions are: 136, 245, 303, 347, 428, 508, and 543 Da) are common for both compounds. According to our analysis, no unique product ion with reasonable intensity could be identified for MRM to distinguish HB-CoA from malonyl-CoA.

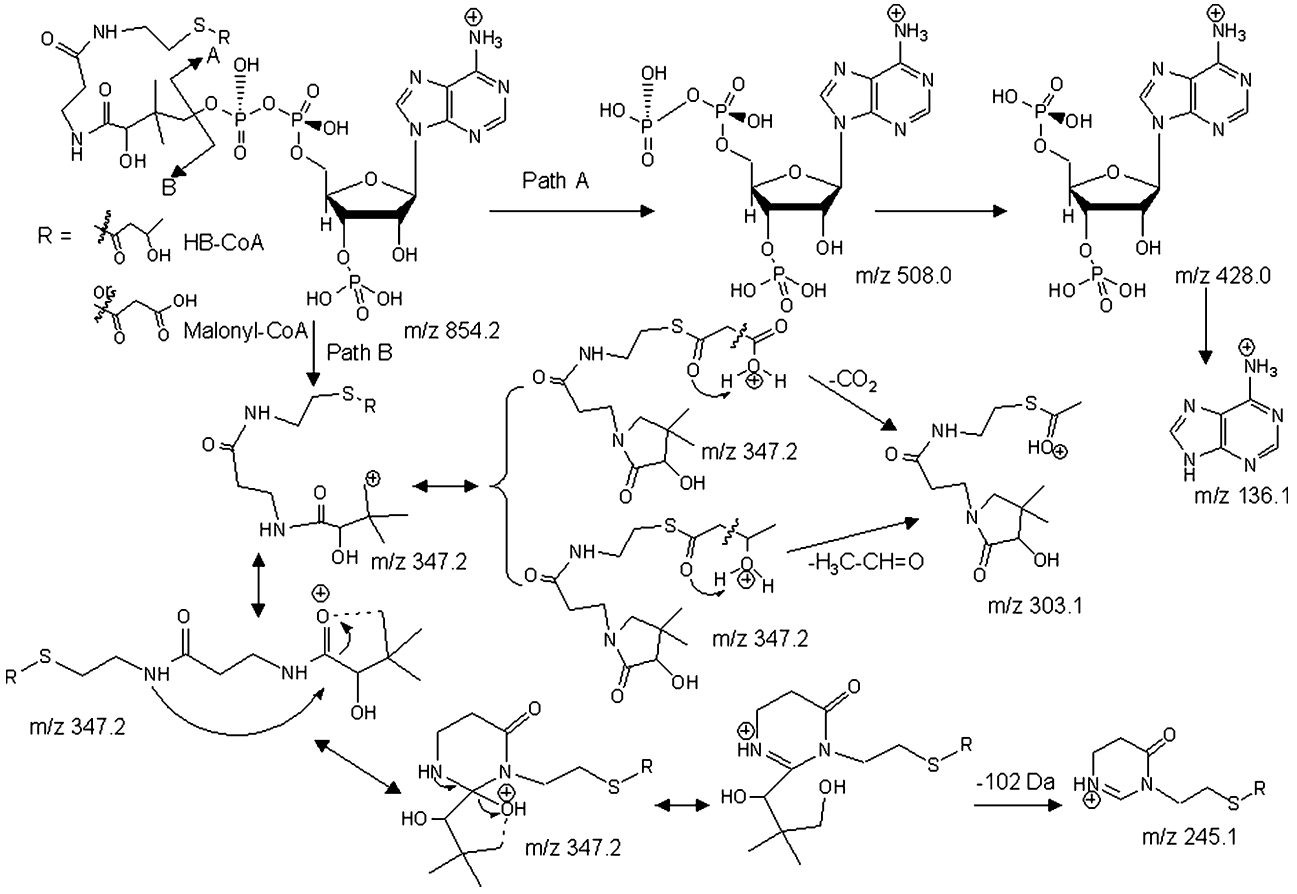
The product ion with *m/z* value of 303 is the dominant ion for malonyl-CoA, while the ion 347 is the dominant product ion for HB-CoA. As illustrated in Scheme 1, acyl-CoA can form common product ions 508 through path A by breaking the C–O bond next to the phosphate group. Further fragmentation of ion *m/z* 508 leads to the formation of product ions of *m/z* 428 and 136. This is a common fragmentation pathway for all acyl-CoAs, and it can be utilized in parent ion scan or neutral loss scan modes for improving the sensitivity of all acyl-CoAs monitoring. Product ions of *m/z* 347, 303 and 245 are formed from alkyl side chain after breaking the C–O bond through path B. Potentially, the ion 347 can go through a series of structure rearrangement before finally losing a neutral fragment of 102 Da to form the product ion of *m/z* 245, which still contains the specific acyl side chain. Malonyl-CoA and HB-CoA can both lose a neutral fragment of 44 Da to form the ion of *m/z* 303. The β-position hydroxyl group seems to be important to facilitate the cleavage of CO_2 or CH_3–CHO moiety through a six-member ring transition state (Scheme 1). Increasing the space between the α-carbonyl group and the end hydroxyl group decreases the efficiency of cleaving the end CO_2 or CH_3CHO group. This was evident in the MS/MS spectrum of succinyl-CoA, which has an γ-OH group (Fig. 2c). Since the major product ions for HB-CoA and malonyl-CoA are common (same or very close *m/z*), it is impossible to distinguish these two compounds by simple MRM techniques on a conventional triple quadrupole mass spectrometer, such as API3000.

The proposed fragment pathway for HB-CoA and malonyl-CoA was largely confirmed in the CID MS/MS studies of succinyl-CoA and acetyl-CoA (Fig. 2c and d) under similar conditions. The major product ions are *m/z* 136, 259, 361, 428 and 508 for succinyl-CoA, and the major product ions are *m/z* 136, 201, 303, 428 and 508 for acetyl-CoA. The acyl-CoA characteristic product ions of *m/z* 136, 428 and 508 containing the adenine were observed in the MS/MS spectra of HB-CoA and malonyl-CoA as well. The product ions of *m/z* 259 and 361 for succinyl-CoA and the product ions of *m/z* 201 and 303 for acetyl-COA are formed in a similar manner, following path B as illustrated in Scheme 2.

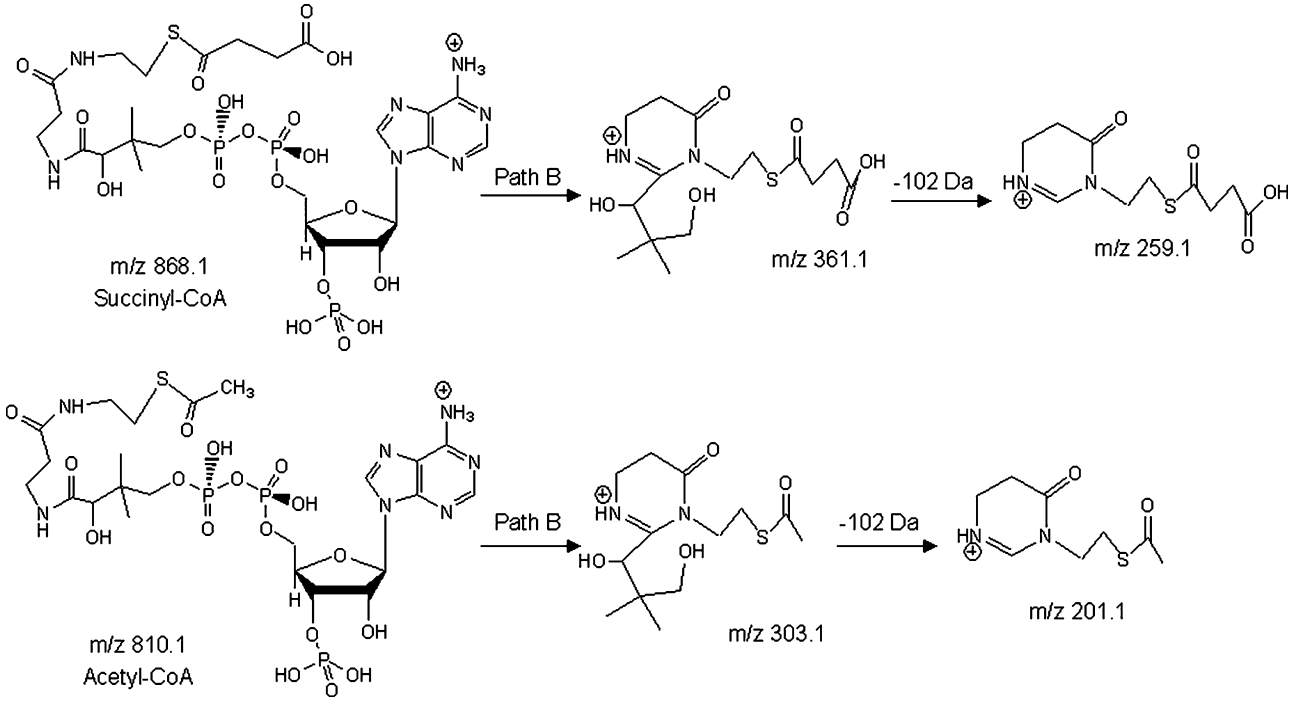
Since neither accurate mass measurement, nor MS/MS would be sufficient to differentiate HB-CoA and malonyl-CoA, it was necessary to separate them at the baseline by chromatography. Thus, the strong MS signal of HB-CoA would not interfere with the quantification of malonyl-CoA. In a recent paper describing the quantification of tissue malonyl-CoA using LC/MS [16], the authors did not discuss the potential interferences from HB-CoA and other short-chain acyl-CoAs. It is not clear whether or not the malonyl-CoA signals might have been affected by HB-CoA signals in their report.

3.2. Validation of quantification methods

All short-chain acyl-CoAs examined in this study are found endogenously in various tissues. It is therefore impossible to prepare a real "blank" sample mimicking all the background matrix of tissue extract samples. To address the possible background matrix effects, we used pooled SD rat liver extracts to



Scheme 1. Collision induced dissociation pathways for HB-CoA and malonyl-CoA with singly charged positive ions.



Scheme 2. Collision induced dissociation pathways for succinyl-CoA and acetyl-CoA with singly charged positive ions.

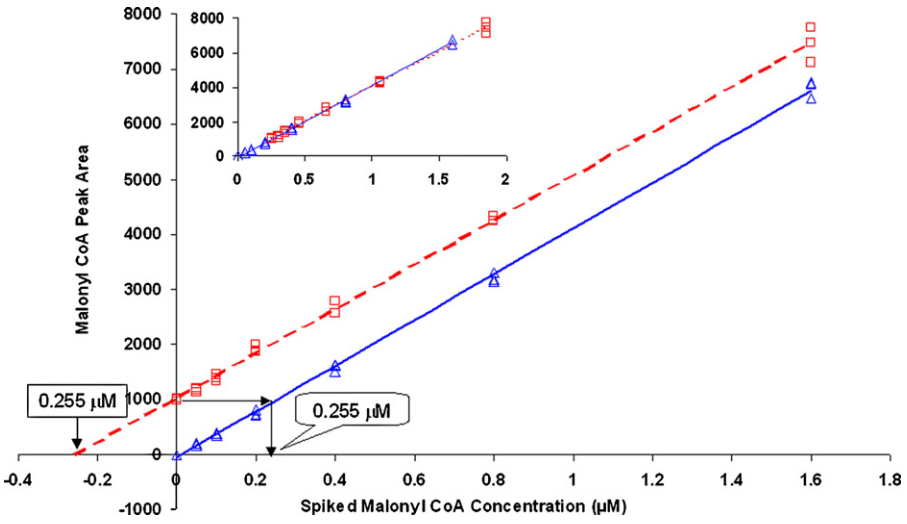


Fig. 3. Comparison of LC/MS quantification methods for malonyl-CoA in SD rat liver extracts. Method A (open triangle) and method B (open square) generated parallel linear regression lines. The two regression lines became super-imposable when the intrinsic malonyl-CoA concentration (0.255 μM) was added to the spiked concentrations in the method B (the insert plot).

study the background effect on the standard curve calibrations. Two calibration methods were evaluated extensively and their results carefully compared. In method A, the working calibration standards were prepared in the extraction solution. Fig. 3 shows that there is a linear relationship between the malonyl-CoA signal intensity and its concentration. The concentration of malonyl-CoA in the pooled SD rat liver extract was determined to be 0.255 ± 0.012 μM from the regression function of the calibration curve in method A. In method B, the calibration standards were spiked into the pooled SD rat liver extracts. A linear correlation between the malonyl-CoA signals and the spiked malonyl-CoA concentrations was found (Fig. 3). The intrinsic concentration of malonyl-CoA in the pooled SD rat liver extracts was obtained by extrapolating the linear regression line to the X-axis where it intercepted. The malonyl-CoA concentration was therefore determined to be 0.255 ± 0.10 μM by using method B. This value matches very well with the value obtained using the method A.

When malonyl-CoA signals obtained from method B was plotted against the true concentration, the spiked malonyl-CoA concentrations in the pooled SD rat liver extract added the determined intrinsic concentration, the calibration line is super-imposable with that of method A (insert of Fig. 3). This result demonstrates that there was no significant matrix background effect in the liver extracts and that tissue malonyl-CoA can be quantified by using either method A or B. The rest of the data presented thereafter were obtained by using method A for quantitative measurement of the short-chain acyl-CoAs.

3.3. Reproducibility, linearity, lower limit of quantification, and carry-over

We conducted a comprehensive study for the reproducibility, linearity and detection limit of this method. The reproducibility of peak areas, acquired from acyl-CoA standards at concentrations of 0.05, 0.5 and 5.0 μM for a total of 9 consecutive injections (18 μL/injection), was expressed as a percentage of relative standard deviation (RSD). As shown in Table 4, all peak area RSD values were less than 3.0%, except for HMG-CoA, which had a slightly higher RSD value of 3.41%. The primary reason for a higher average RSD (3.41%) for HMG-CoA could be that its LC peak overlaps partially with the front end of HB-CoA peak. Plus, the DMBA adduct of HB-CoA (*m/z* 912.261) has very close *m/z* values to the protonated ion of HMG-CoA (*m/z* 912.165) and might interfere with the HMG-CoA quantification. The maximum RSD, obtained from this study, ranged from 2.66% to 5.24%, which is well within the generally acceptable value of 15% for this type of study. The reproducibility of HPLC retention times was expressed as the variation of HPLC retention times at the maximum peak height. The maximum retention time variations were determined from 9 consecutive analyses of acyl-CoA standards at the concentrations of 0.05, 0.5 and 5.0 μM with 18 μL injections. The maximum retention time shift ranged from 0.01 to 0.05 min for all acyl-CoAs studied. The results indicated that the LC method was highly reproducible in peak area and retention time for the short-chain acyl-CoA analysis.

The linear dynamic range of quantification for each acyl-CoA was determined by injecting 18 μL of authentic acyl-CoA standards prepared in the extraction solution in the range of 0.013–5.00 μM. The least square curve fitting method was used to generate the linear regression function. The correlation coefficient (R^2) ranged from 0.9961 to 0.9999 (Table 3), which demonstrates good linearity over two and a half orders of magnitude. The lower limits of quantification (LLOQ) were determined as the signal to noise ratio greater than 10. The LLOQ for malonyl-CoA studied was determined to be 0.225 pmol, or 0.0125 μM with 18 μL of sample injection, as shown in Fig. 4a. The LLOQ of all other acyl-CoAs (Table 3) were 0.225 pmol or lower, except for HMG-CoA (0.90 pmol). The 0.225 pmol LLOQ can be translated into the amount of acyl-CoA at the level of 0.125 nmol/g in 1.8 mg of animal tissues. The achieved sensitivity for tissue malonyl-CoA detection is especially critical to the studies that use tissues with low intrinsic level of

Table 3
Reproducibility, linearity and lower limit of quantification (LLOQ)

Compound	Retention time (min)		RSD of peak area		Linearity			LLOQ	
	Retention time (min)	Maximum variation (min)	Average (RSD)	Maximum (RSD)	Slope	Intercept	R^2	(μM)	(pmol)
CoASH	2.74	0.04	2.34	5.24	0.1367	0.01371	1.000	0.0125	0.225
Acetyl-CoA	3.01	0.04	2.87	4.67	0.1640	0.00673	0.999	0.0125	0.225
HMG-CoA	2.86	0.05	3.41	5.10	0.1331	−0.00032	1.000	0.050	0.900
HB-CoA	3.00	0.04	1.52	3.28	0.2088	0.00897	0.998	0.025	0.450
Succinyl-CoA	2.88	0.04	2.29	3.17	0.3446	0.00001	0.996	0.0125	0.225
Malonyl-CoA	2.62	0.04	2.55	3.78	0.0884	−0.00160	1.000	0.0125	0.225
Propionyl-CoA	3.52	0.02	1.93	3.06	0.3300	0.00159	0.999	0.025	0.450
Isobutyryl-CoA	3.75	0.01	1.70	2.66	2.3982	−0.00373	1.000	0.0125	0.225

The calibration standard concentration ranges from 0.0125 to 5.0 μM. Triplicate injections of 18 μL standard samples were analyzed for every concentration point. The LLOQs were determined by the lowest concentration of sample with signal-to-noise ratio greater than 10, or the lowest concentration of sample tested if signal-to-noise ratio were all above 10. The reproducibility was obtained from nine consecutive analysis of standards at concentrations of 0.05, 0.5 and 1.5 μM. RSD: percentage of relative standard deviation.

malonyl-CoA, and/or have limited amount of tissues (such as soleus muscle in mice). To survey the carry-over of the acyl-CoAs, blank control samples (extraction solution containing internal reference) were injected immediately after the injections of the highest concentrated acyl-CoA standards (5.0 μM). The resulting selected ion chromatogram indicates that there is no apparent carry-over (Fig. 4b).

3.4. Sample stability

It has been reported in the literature that the stability of some short-chain acyl-CoAs are pH- and temperature-dependent [8]. Higher pH tends to accelerate hydrolysis of acyl-CoAs. For instances, after 12 h storage at 8 °C, about 21% of succinyl-CoA was decomposed at pH 3, while 77% was decomposed at pH 6 [8]. In our studies, the samples were kept in acidic extraction solutions (pH < 2) in the presence of 50 μM DTE, and the HPLC mobile phase was buffered at pH 5.6 to prevent acyl-CoAs from degrading before and during the sample analysis.

To test the acyl-CoA stability under the conditions in our studies, SD rat liver extracts from 10 different animals were pooled and then divided into multiple aliquots immediately after the extraction and filtration. One aliquot sample was analyzed immediately. The rest of aliquots were stored at −80 °C for 25 days and 55 days, respectively, for the subsequent analyses. The frozen tissue extracts were thawed in 4 °C ice-water bath for 2 h

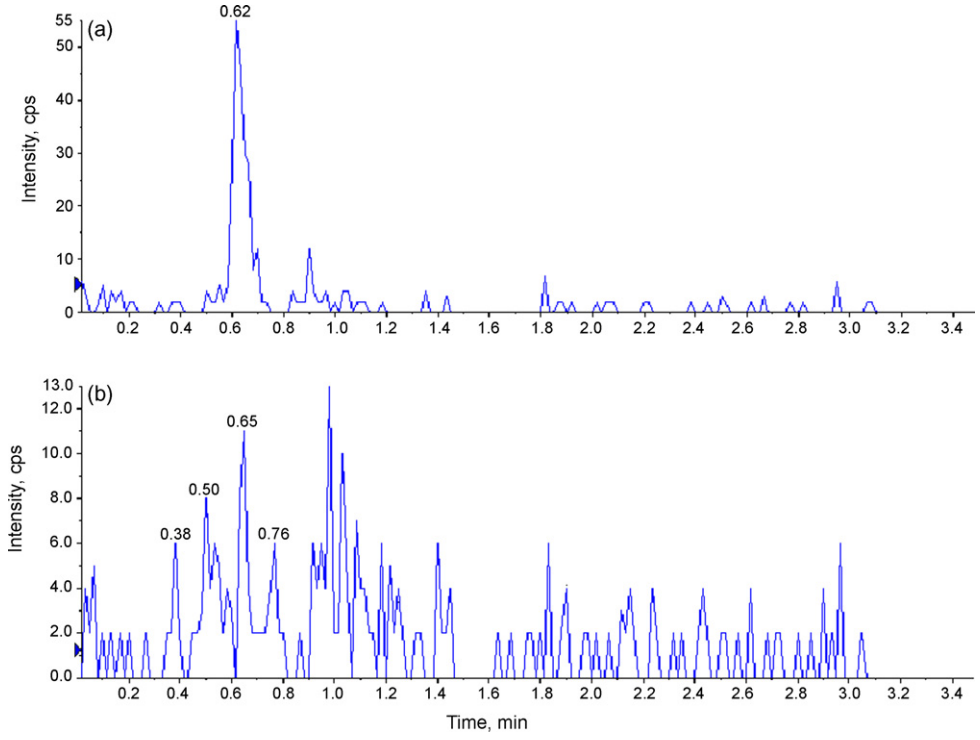


Fig. 4. Selected ion chromatogram of malonyl-CoA (*m/z* 854.12): (a) 0.0125 μM of malonyl-CoA in extraction solution; and (b) blank control injected immediately after the injections of 5.0 μM malonyl-CoA standards.

Table 4
Short-chain acyl-CoA stability in 5% sulfosalicylic acid and 50 μM 1,4-dithioerythritol

Compound	Time 0	Day 25 (−80 °C)			Day 55 (−80 °C)			Day 25 + 12 h (4 °C)		
	Conc. (μM)	Conc. (μM)	Deviation from *T*0		Conc. (μM)	Deviation from *T*0		Conc. (μM)	Deviation from T25	
			(μM)	%		(μM)	%		(μM)	%
Acetyl-CoA	4.61 ± 0.04	5.24 ± 0.08	0.623	13.5%	4.61 ± 0.06	0.00	−0.1%	5.09 ± 0.26	−0.147	−3.2%
CoASH	4.76 ± 0.23	4.40 ± 0.18	−0.360	−7.6%	4.51 ± 0.19	−0.25	−5.2%	4.65 ± 0.22	0.248	5.2%
HB-CoA	0.22 ± 0.004	0.24 ± 0.01	0.017	7.5%	0.19 ± 0.01	−0.03	−14.8%	0.22 ± 0.01	−0.021	−9.4%
Isobutyryl-CoA	0.29 ± 0.01	0.30 ± 0.01	0.012	4.1%	0.23 ± 0.06	−0.06	−22.1%	0.32 ± 0.02	0.014	4.8%
Malonyl-CoA	0.115 ± 0.01	0.12 ± 0.00	0.009	7.8%	0.11 ± 0.00	−0.01	−4.6%	0.12 ± 0.00	−0.004	−3.4%
Succinyl-CoA	1.28 ± 0.04	1.50 ± 0.07	0.220	17.2%	1.24 ± 0.08	−0.04	−3.2%	1.44 ± 0.08	−0.058	−4.5%

Concentrations of various acyl-CoA from pooled SD rat liver extracts were measured after different storage periods. Triplicate samples were analyzed for every data point (mean ± standard deviation). Time 0 (*T*0) samples were analyzed immediately after homogenization; T25 represents samples analyzed after 25 days storage at −80 °C.

before sample analysis. The internal reference (isobutyryl-CoA) was added to the samples before loading into a cooled Shimadzu 10-Advp autosample (4 °C) for LC/MS analyses. Samples were injected either immediately, or 12 h after loading in the autosampler. The results are listed in Table 4.

A new HPLC column and a new set of calibration standards prepared in the extraction solution were used for every new batch of samples. The deviations shown in Table 4 reflected the combined effects of sample stability, reproducibility of sample preparation, and instrument performance variations (including HPLC column variations). For most of the acyl-CoAs, the measured concentrations were within 10% variation after 25 or 55 days of storage at −80 °C. Though acetyl-CoA and succinyl-CoA showed increases of 14% and 17%, respectively, in concentrations after 25 days of storage at −80 °C, further storage to 55 days showed decreases of these acyl-CoAs for −0.1% and −3.2%, respectively, from the original contents. The lack of a consistent trend of concentration changes with increasing storage time suggests that the variation in concentrations were most likely due the instrument performance, different columns, and sample preparations, rather than the sample decomposition during storage. The above data indicates that: (1) samples were stable for at least 12 h at 4 °C during sample analysis; (2) samples were stable for at least 55 days of storage at −80 °C.

3.5. Choice of internal reference for LCMS analysis

In order to control the quality of LC/MS quantification, an internal reference is often added to compensate for the variations during sample analysis. A stable isotopic labeled acyl-CoA would be the ideal internal reference for the quantification of short-chain acyl-CoAs. However, due to the unavailability of stable isotopic labeled acyl-CoAs, we screened a list of commercially available short-chain acyl-CoAs to select an appropriate internal reference for our study.

Three short-chain acyl-CoAs, HMG-CoA, isobutyryl-CoA and propionyl-CoA were initially considered to be the internal standards. Their natural abundances in SD rat liver extract were determined to be 0.22, 0.35, and 0.22 μM, respectively. HMG-CoA was barely separated from acetyl-CoA (Fig. 1). Thus, the integration of HMG-CoA may not be reliable when there is a slight decrease in the performance of HPLC column. In contrast, isobutyryl-CoA and propionyl-CoA were well separated from other short-chain acyl-CoAs by HPLC. Isobutyryl-CoA has been used as an internal reference for HPLC/UV quantification of short-chain acyl-CoAs in rat liver tissues [10]. However, we observed that the level of liver isobutyryl-CoA was highly influenced by the metabolic states of the animals in the fasting/refeeding cycles in both CD-1 mice (Table 5) and SD rat. In comparison, tissue propionyl-CoA level was much more stable during the fasting/refeeding cycles of animals (from 0.22 to 0.24 μM in SD rat liver extracts). The maximum fluctuation of internal reference's signal caused by the variation of intrinsic propionyl-CoA level was less than 0.4% when 5.0 μM propionyl-CoA was spiked into the samples as internal reference for LC/MS analysis. Therefore, propionyl-CoA was the best choice as the internal reference for the acyl-CoA quantification for our studies.

Table 5
Abundance of short-chain acyl-CoAs (nmol/g) in animal tissues from CD-1 mice

Compound	Liver			Heart			Muscle	
	Normal	Fasted	Fasted/re-fed	Normal	Fasted	Fasted/re-fed	Normal	Fasted
Acetyl-CoA	78.84 ± 7.29	84.94 ± 14.63	95.74 ± 23.01	29.78 ± 2.78	21.51 ± 2.02	38.35 ± 3.00	3.30 ± 0.37	0.00
CoASH	65.58 ± 9.42	125.00 ± 13.12	44.00 ± 5.35	36.96 ± 3.42	27.93 ± 3.41	50.73 ± 5.12	8.54 ± 3.14	3.65 ± 2.37
HB-CoA	3.46 ± 0.43	7.68 ± 2.23	1.68 ± 0.44	1.13 ± 0.21	1.46 ± 0.18	1.01 ± 0.41	0.00	0.00
Isobutyryl-CoA	8.03 ± 1.90	4.66 ± 1.46	14.79 ± 4.46	0.82 ± 0.21	1.09 ± 0.17	0.82 ± 0.12	0.00	0.00
Malonyl-CoA	0.78 ± 0.11	0.25 ± 0.02	0.62 ± 0.16	2.67 ± 0.17	2.23 ± 0.17	2.81 ± 0.19	1.12 ± 0.15	0.60 ± 0.14
Succinyl-CoA	21.81 ± 2.28	10.37 ± 1.31	15.78 ± 4.40	10.11 ± 1.29	11.54 ± 2.18	11.24 ± 1.76	6.24 ± 1.39	3.08 ± 0.58

Mean ± standard deviation, $n = 3$.

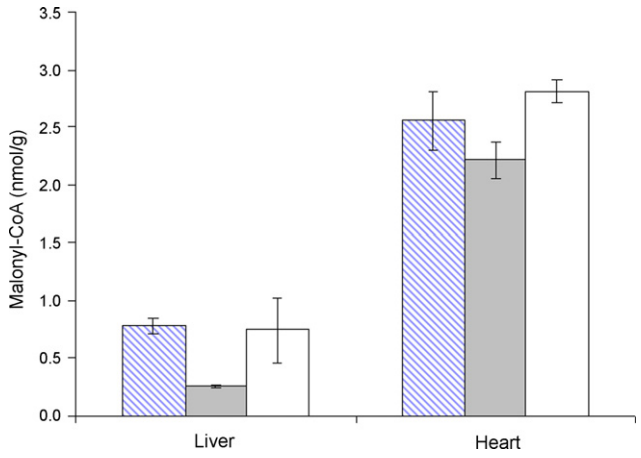


Fig. 5. Malonyl-CoA levels in CD-1 mouse liver and heart from the normally fed (slanted line shaded column), fasted (filled column), and fasted/re-fed (open column) animals.

3.6. Quantification of short-chain acyl-CoAs in animal tissues

The tissue levels of malonyl-CoA and several other short-chain acyl-CoA in CD-1 mouse liver, heart and muscle were quantified. The CD-1 mice were grouped into three treatments, fed controls, 24 h fasted, and fasted then re-fed with high sucrose diet. There were four animals in each group. Upon sacrifice, liver, heart, and soleus muscle were homogenized and extracted as indicated in the Section 2. The extract samples were either analyzed immediately or stored under −80 °C until LC/MS analysis. Fig. 5 shows the levels of malonyl-CoA in liver and heart extracts of normally fed, fasted, and fasted/re-fed CD-1 mice. As expected, the malonyl-CoA level was substantially decreased by 68% from 0.78 ± 0.07 to 0.25 ± 0.01 nmol/g in the liver upon 24 h fasting, and returned to its basal (pre-fasting) level (0.74 ± 0.28 nmol/g) upon re-feeding with high sucrose diet. The observed decrease of malonyl-CoA in the liver after fasting is consistent with literature reports [23]. In our experiments there was a slight trend in the reduction of malonyl-CoA levels in the heart upon fasting (reduction from 2.55 ± 0.25 to 2.21 ± 0.16 nmol/g). This is not too surprising as that heart relies predominantly on fatty acids as a major fuel source compared to glucose, and therefore it is possible that regulatory steps that modulate malonyl-CoA levels in the heart are quite different from those in the liver. The averaged quantitative measurements of other short-chain acyl-CoAs are listed in Table 5. In liver the levels of free CoA, isobutyryl-CoA, HB-CoA and succinyl-CoA also fluctuated significantly under the different feeding paradigms. Interestingly, the acetyl-CoA level remained relatively constant during the feeding and fasting cycle.

4. Conclusion

We describe a simple and reliable method for the simultaneous quantification of several short-chain acyl-CoAs in animal tissue using ion-pairing reversed-phase HPLC/MS method. The short-chain acyl-CoAs from mouse or rat liver, heart, and muscle were processed in a single extraction step using 5% SSA containing 50 μM DTE. The tissue extracts were then subjected to LC/MS analysis without further purification to minimize sample loss and compound decomposition. Our results show that CoASH, acetyl-CoA, HB-CoA, malonyl-CoA, succinyl-CoA, and isobutyryl-CoA were stable in the extraction solution for at least 12 h at 4 °C and 55 days at −80 °C. The potential interferences between isobaric compounds (HB-CoA and malonyl-CoA) in the LC/MS (or LC/MS/MS) analysis were studied for the first time. The results indicate that it was necessary to completely separate HB-CoA and malonyl-CoA for reliable measurements of their endogenous concentrations. The quantification accuracy was verified by using two calibration methods wherein standards were prepared for both the extraction solution and the pooled SD rat liver extracts. Our analytical method has a much better LLOQ value (0.225 pmol) than other methods reported in the literature, for example, the LLOQ values of 50 pmol using LC/MS [16], 1.0 nmol using CE-UV [12], and 1.0 pmol using HPLC followed by post-column fluorescent derivatizations [24]. Additionally, our method has a much shorter total sample analysis time (7.0 min) compared to other reported HPLC methods (20 min or longer) [16]. We attributed the better LLOQ value and the shorter sample analysis time mostly to our use of the ion-pairing agent DMBA, rather than the more common TEA. The post-column mixing of HPLC eluent with acetonitrile was also a contributing factor to the better performance of our methodology. In summary, we have developed and validated an HPLC/MS method employing a low concentration of DMBA to minimize the ion suppression for LC-ESI-MS analysis, and coupled with post-column mixing of HPLC eluent with acetonitrile to improve the sensitivity of detecting short-chain Acyl-CoAs by a magnitude of 10 folds compared to existing protocols. This method provided a valuable tool for studying lipid metabolism by monitoring the levels of multiple acyl-CoAs in tissues. Finally, we have successfully used this method to profile several lead compounds for their ability to inhibit ACC2 activity in mice and rat animal models.

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