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Corresponding Author:	Suzanne Jackowski St. Jude Children's Research Hospital Memphis, TN UNITED STATES
Corresponding Author's Institution:	St. Jude Children's Research Hospital
Corresponding Author E-Mail:	suzanne.jackowski@stjude.org
Order of Authors:	Suzanne W. Jackowski
	Suzanne Jackowski
	Chitra Subramanian
	Charles Rock
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TITLE:

Quantification of Coenzyme A in Cells and Tissues

AUTHORS AND AFFILIATIONS:

Matthew W. Frank, Chitra Subramanian, Charles O. Rock and Suzanne Jackowski

Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA

Email addresses of co-authors:

Matthew W. Frank	matthew.frank@stjude.org
Chitra Subramanian	chitra.subramanian@stjude.org
Charles O. Rock	charles.rock@stjude.org

Corresponding author:

Suzanne Jackowski	suzanne.jackowski@stjude.org

KEYWORDS:

Coenzyme A, cultured cells, animal tissues, monobromobimane, high-pressure liquid chromatography, solid phase extraction

SUMMARY:

This method describes sample preparation from cultured cells and animal tissues, extraction and derivatization of coenzyme A in the samples, followed by high pressure liquid chromatography for purification and quantification of the derivatized coenzyme A by absorbance or fluorescence detection.

ABSTRACT:

Emerging research has revealed that the cellular coenzyme A (CoA) supply can become limiting with a detrimental impact on growth, metabolism and survival. Measurement of cellular CoA is a challenge due to its relatively low abundance and the dynamic conversion of free CoA to CoA thioesters that, in turn, participate in numerous metabolic reactions. A method is described that navigates through potential pitfalls during sample preparation to yield an assay with a broad linear range of detection that is suitable for use in many biomedical laboratories.

INTRODUCTION:

Coenzyme A (CoA) is an essential cofactor in all living organisms and is synthesized from pantothenic acid, also called pantothenate (the salt of pantothenic acid) or vitamin B5. CoA is the major intracellular carrier of organic acids, including short-chain acids such as acetate and succinate, branch-chain acids such as propionate and methylmalonate, long-chain fatty acids such as palmitate and oleate, very long-chain fatty acids such as polyunsaturated fatty acids, and xenobiotics such as valproic acid. The organic acid forms a thioester linkage enzymatically with CoA to enable its use as a substrate in over 100 reactions in intermediary metabolism[1]. CoA thioesters are also allosteric regulators and transcriptional activators. It is now appreciated[2] that the cellular total CoA supply is regulated[3,4]; thus, CoA availability can be

limiting, and that CoA deficiencies can be catastrophic, as exemplified by inherited genetic disorders that impact CoA biosynthesis[5]. Pantothenate kinase catalyzes the first step in CoA biosynthesis (**Figure 1**) and Pantothenate Kinase Associated Neurodegeneration, called PKAN, is caused by mutations in the *PANK2* gene[6]. CoA synthase, encoded by the *COASYN* gene, catalyzes the last two steps in CoA biosynthesis (**Figure 1**) and COASY Protein-Associated Neurodegeneration, called CoPAN, is caused by a mutation in the *COASYN* gene[7]. Both PKAN and CoPAN are inherited neurodegenerative diseases associated with iron accumulation in the brain and CoA deficiencies underly the disease pathologies.

Cellular levels of total CoA vary among tissues[8] and total CoA can increase or decrease under a variety of physiological, pathological and pharmacological states. Liver CoA increases during fuel switching from the fed to the fasted state[9], and liver CoA levels are abnormally high in leptin-deficient obese mice[10]. Liver CoA decreases in response to chronic ethanol ingestion[11]. Brain CoA levels in the *Pank2* knockout mouse model are depressed during the perinatal period, but later in the adult stage brain CoA content is equivalent to wild-type levels, indicating an adaptive CoA response during development[12]. Manipulation of tissue CoA content by transgenesis or gene delivery methods impacts metabolic and neural functions[13-15]. Preclinical development of potential therapies for PKAN or CoPAN includes cell or tissue CoA measurements as indicators of efficacy[16-20]. Evaluation of all of these conditions and their metabolic or functional consequences requires a quantitative method for measurement of total CoA.

An accurate, reliable assay for measuring CoA in biological samples is a technical challenge in many labs. Unfortunately, there are no probes available to evaluate or quantify CoA or CoA thioesters in intact cells, although analogs of natural CoA thioesters have been widely used as mechanistic probes in studies of CoA ester utilizing enzymes[21]. The conversion of CoA, with a free sulfhydryl (-SH) moiety, to a CoA thioester (or vice versa) is rapid in cells or animal tissues during transfer to a different environment and during cell lysis. Numerous acyl-CoA synthetases and acyl-CoA thioesterases in cells mediate the interconversions within the CoA pool, and additional enzymes that utilize CoA thioesters as substrates remain active in biological samples until quenched by chemical or physical means. The off-loading of acyl-groups from CoA to carnitine by acyl-transferases is one example within the network of reactions that can alter the CoA/CoA thioester distribution. Radioactive tracers can be used to measure rates of CoA synthesis in cells. Current methods for measuring CoA and CoA derivatives in biological samples have been reviewed[22] and include coupled enzymatic spectrophotometric assays, high-pressure liquid chromatography and mass spectrometry-based procedures. However, these methods are often focused on particular CoA molecular species and are blind to variation of the total CoA pool. The coupled enzymatic assays generally require larger amounts of input material due to low detection sensitivities and have a limited range of linearity.

Our laboratory has developed a reliable procedure for quantification of total CoA in cultured cells and animal tissues. The strategy includes hydrolysis of all CoA thioesters to yield only free CoA during sample preparation, rather than making efforts to maintain and analyze the entire spectrum of CoA species. The procedure is a compilation of individual published methods for

sample preparation, CoA derivatization, purification and identification following high-pressure liquid chromatography (HPLC), and quantification of the derivatized CoA by absorbance or fluorescence detection[23-25]. The CoA determinations obtained using this procedure have enabled our understanding of CoA regulation and the development of a therapeutic approach for treatment of CoA deficiencies.

PROTOCOL:

The animal procedure referred to in this protocol was performed according to protocols 323 and 556 and specifically approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee.

1. Preparation of solutions

NOTE: Use ultrapure water for all solutions and when stated in procedures.

1.1. Prepare 1 mM potassium hydroxide (KOH) in water.

1.2. Prepare 0.25 M KOH in water.

1.3. Prepare 1 M Trizma-HCl in water and adjust to pH 8.0.

1.4. Prepare 100 mM monobromobimane (mBBr) in acetonitrile (Optima Grade). Stock solutions of mBBr are stable at -20 °C for many months provided they are kept in the dark as exposure to light can photolyze the monobromobimane to bimane[26].

1.5. Prepare Wash Buffer for solid phase extraction (SPE) column: 50% methanol (Optima Grade) + 2% acetic acid.

1.6. Prepare Elution Buffer for SPE column: 95% ethanol (HPLC Grade) containing 50 mM ammonium formate.

2. Preparation of CoA-bimane standard

2.1. Purchase a high-quality CoA standard from a reputable source (e.g., Avanti Polar Lipids, Inc.).

2.2. Prepare a high concentration stock solution of CoA in 20 mM Tris, pH 8. The amount of CoA in the high concentration stock is determined or confirmed by spectrophotometric absorbance at λ260 nm (ε = 16,800 $M^{-1} \cdot cm^{-1}$). Add 4-fold molar excess of mBBr; vortex on high for 10 s. For example, for 10 mM CoA in buffer, add 40 mM mBBr. The mBBr is added in excess to ensure that all the CoA is derivatized.

2.3. Incubate at room temperature for 4 h in the dark without shaking to derivatize the CoA

with mBBr. mBBr reacts with the thiol group of the CoA, and it is pH and temperature dependent[27]. Addition of the mBBr converts CoA to a water-soluble fluorescent (or ultraviolet absorbant) CoA-bimane (CoA-bimane; **Figure 2**).

2.4. Add 100 µL acetic acid and vortex on high for 10 s to stop the reaction.

2.5. Centrifuge at 2,000 x *g* for 15 minutes to remove any precipitant.

2.6. Clean-up the supernatant using an SPE-column to remove the unreacted mBBr (described below). Filtration and centrifugation of the eluate is not necessary after the SPE column clean-up (Section 5.8).

2.7. Collect the eluate from the SPE column containing the CoA-bimane in a pre-weighed tube, dry under nitrogen gas, weigh and construct a standard calibration curve with known quantities of CoA-bimane.

2.7.1. Check the CoA-bimane standard for purity by HPLC (see below) with absorbance detection at both λ260 (adenine moiety) and λ393 (bimane moiety) and coincidence of both peaks in the chromatogram.

2.7.2. Confirm the quantity of the CoA-bimane standard in the high concentration stock using λ260 nm (Ɛ = 16,800 $M^{-1} \cdot cm^{-1}$).

2.8. Register the HPLC retention time for identification of CoA-bimane in experimental samples using the CoA-bimane standard.

2.9. Store aliquots of the high concentration stock of the CoA-bimane standard protected from light and stored at -20 °C for up to 2 years. Avoid repeated thawing and re-freezing.

3. Extraction and derivatization of CoA in cultured cells

3.1. Harvest adherent cells in culture when approaching late subconfluent densities. For example, human HepG2/C3A cells are grown to a density of 6-8 x 10^6, or HEK 293T cells are grown to a density of ~1.3 x 10^7 per 100 mm dish.

3.1.1. Use duplicate or triplicate cultures routinely for CoA determinations. Incubate separate culture dishes in parallel with the sample cell cultures to determine viable cell number. Calculate the amount of CoA per 10^6-10^7 cells after HPLC purification.

3.2. Aspirate culture medium off the dish. Quickly wash cells on the dish with ice-cold phosphate-buffered saline (PBS) to remove any residual medium and aspirate the PBS off the dish. Quickly wash cells with ice-cold water to remove residual PBS and aspirate the water off the dish quickly. Avoid disturbing the adherent cells when adding the PBS or water.

3.3. Add 1 mL of ice-cold water to the culture dish. Scrape cells into the cold water in the dish and transfer the cell suspension to a glass test tube containing 400 µL of 0.25 M KOH and 1.5 mL of water.

3.3.1. Mix the suspension vigorously by vortex on high for 10 s. Then cover tightly with paraffin film and incubate at 55 °C for 1 h without shaking in a water bath. The pH of the sample should be ≥ 12. The high pH is important to hydrolyze the CoA thioesters and can be adjusted with additional aliquots of KOH if necessary.

3.4. Harvest cultured cells growing in suspension by centrifugation at low speed: 136 x *g* for 6 min at 4 °C. Wash once with ice-cold PBS, then briefly wash again with cold water, and finally resuspend in 2.5 mL of cold water plus 400 µL 0.25 M KOH. Mix vigorously and incubate at 55 °C as described above.

3.5. Add 160 µL of 1 M Trizma-HCl and 10 µL of 100 mM mBBr and mix by vortex on high for 10 s. This brings the pH to approximately 8 to support the mBBr reaction with free CoA. Cover and incubate samples at room temperature for 2 h in the dark for the mBBr to react with the thiol of CoA.

3.6. Add 100 µL of acetic acid and mix by vortex on high for 10 s to stop the reaction

3.7. Centrifuge at 2,000 x *g* for 15 min to remove precipitated cell debris.

3.8. Remove and save supernatant in a glass test tube for the SPE column clean-up that is described below.

4. Extraction and derivatization of CoA in tissues

4.1. Dissect animal tissue pieces, about 0.5 cm diameter or smaller, and blot briefly (1-2 s) on absorbent paper and then flash freeze in liquid nitrogen and store at -80 °C until processing. CoA in tissues is hydrolyzed or converted to a thioester if not flash frozen. This step is important to obtain the maximum yield of CoA in tissues.

4.2. Weigh frozen tissue pieces (5 – 60 mg) quickly prior to starting the analysis, and record the weight for each sample. This wet weight determination is used for calculation of the CoA content following HPLC purification. Avoid complete thaw of the tissue pieces.

4.3. Add 2 mL of 1 mM KOH to a glass test tube (e.g., 13 mm x 100 mm disposable) destined for insertion of a probe and tissue disruption with a rotor-stator homogenizer. Keep the tube on ice until use. This is done to ensure that the samples are homogenized at a cold temperature and CoA is not destroyed due to the heat generated during homogenization.

4.4. Transfer and homogenize tissue (30 – 40 mg) in the glass test tube (containing cold 1 mM KOH) for 30 s. Avoid complete thawing of the tissue.

4.5. Add 500 µL of 0.25 M KOH; vortex on high for 10 s and keep on ice until all samples are homogenized. This will bring the pH of the sample above 12 to hydrolyze the CoA thioesters to yield total CoA (free CoA + hydrolyzed CoA thioesters).

4.6. Incubate samples at 55 °C for 2 h in a water bath without shaking to support hydrolysis.

4.7. Add 150 µL of 1 M Trizma-HCl and 10 µL of 100 mM mBBr; vortex on high for 10 seconds. This brings the pH to about 8 to support the mBBr reaction with free CoA.

4.8. Incubate samples at room temperature for 2 h in the dark to ensure all the CoA is derivatized with mBBr.

4.9. Add 100 µL of acetic acid and vortex on high for 10 s to stop the reaction.

4.10. Centrifuge at 2,000 x *g* for 15 min to pellet precipitated cell debris.

4.11. Remove and save supernatant in a glass test tube for the SPE column clean-up (described below).

5. Sample clean-up with solid phase extraction (SPE) column

5.1. At room temperature, equilibrate each disposable 2-(2-pyridyl)-ethyl silica gel column (1 mL size) with 1 mL of Wash Buffer to ensure that the pyridyl functional group is protonated and will function as an anion-exchanger. 2-(2-pyridyl)-ethyl silica gel is a weak anion exchanger which is ideal for CoA-bimane at a basic pH. 2-(2-pyridyl)-ethyl silica gel has a pKa of 6 and the elution is done pH ≥ 7.

5.2. Add sample supernatant (step 4.11) to column and collect eluate.

5.3. Wash column twice with 1 mL of Wash Buffer to remove any unretained species.

5.4. Wash column once with 1 mL of water.

5.5. Wash column twice with 1 mL of Elution Buffer into a separate glass test tube (12 mm x 75 mm) to collect the CoA-bimane.

5.6. Dry CoA-bimane sample in tube under nitrogen gas to dryness. The dried sample is stable at room temperature until further use. Seal and completely cover the tube and store.

5.7. When ready for HPLC analysis, resuspend sample in 300 µL of water and mix vigorously by vortex on high for 10 s.

5.8. Transfer resuspended sample to a centrifuge tube filter (0.22 µm cellulose acetate, 2 mL

size) and centrifuge at 5,000 x *g* for 10 min to remove any precipitant.

5.9. Transfer filtered sample to a glass vial suitable for HPLC injection.

6. HPLC purification and measurement of CoA-bimane

6.1. Prepare buffers. Buffer A: 50 mM KH_2PO_4, pH 4.6; Buffer B: Acetonitrile (Optima Grade).

6.2. Power up the HPLC system.

NOTE: The HPLC system in our laboratory is a Waters e2695 Separations Module equipped with a 2489 Ultraviolet-Visible (UV-Vis) absorbance detector and a 2475 fluorescence detector controlled with Empower 3 software. The system also has an automated sample injector.

6.3. Inject each sample (e.g., 20 µL) for separation on a Gemini C18, 3 µm 100 Å, 4.6 mm x 150 mm column using the program in **Table 1** with a flow rate of 0.5 mL/min. The column temperature is 25 °C. Measure the UV/Visible detector absorbance at λ393 nm, and fluorescent detection at λex = 393 nm, λem = 470 nm. The retention time is determined by running a CoA-bimane standard curve before each set of samples.

6.4. Record the area under the CoA-bimane peak for each sample and compare with the standard curve to calculate pmol of CoA-bimane injected onto the HPLC column. The CoA-bimane absorbance standard curve is used for tissue samples, and the CoA-bimane fluorescence standard curve is used for tissue culture samples.

6.5. As the CoA-bimane values represent total CoA for each sample, normalize to the number of viable cells for culture samples, or mg wet weight for tissue samples (**Figure 6**). Alternately, calculate the total CoA values following normalization to DNA or protein content for each sample. DNA or protein content can be determined separately using sample aliquots that are removed during sample preparation prior to KOH addition.

REPRESENTATIVE RESULTS:

A relatively fast and reliable method for the detection of total CoA in cultured cells and tissues has been developed by derivatizing the thiol of CoA to a fluorescent agent using mBBr, and then purifying the derivatized CoA-bimane using reverse phase HPLC. A standard curve is first generated, where known and increasing amounts of the CoA-bimane standard are injected individually and the areas under the peaks in the CoA-bimane chromatograms are plotted as a function of the input CoA-bimane (**Figure 4**). CoA-bimane has an absorbance maximum at λ393 nM and a representative HPLC profile shows the retention time of the CoA-bimane standard on a C18 HPLC column (**Figure 3**) using the elution program in **Table 1**. Representative standard curves of CoA-bimane, detected by measuring absorbance or fluorescence units, are shown in **Figure 4A** and **Figure 4B**, respectively. The standard curve in **Figure 4A** reflects the magnitude of absorbance of CoA-bimane at λ393 nm and **Figure 4B** represents the fluorescence of CoA-bimane (λex = 393 nm and λem = 470 nm) plotted versus the input amount of CoA-bimane. The

CoA-bimane standard can be detected from 0.01 to 12,000 pmol and covers a 10^6-fold range when detection using both absorbance and fluorescence is combined. While the lower limit of detection of the standard is 0.01 pmol, the lower limit of CoA-bimane quantitation in experimental samples is 0.2 pmol which is about 5-fold greater than the baseline or background fluorescence in the chromatogram. The choice of detection by absorbance or fluorescence of CoA-bimane depends on the amount of CoA normally present in tissues or cells, together with the practicality of working with larger or smaller sample sizes. Absorbance is generally useful for tissue samples because handling 40-50 mg of starting material yields better recovery than 5 mg tissue samples, whereas fluorescence is generally useful for cultured cell samples which are smaller in size and there is greater confidence in interpolation of values at the lower end of the fluorescence standard curve. Laboratories with only absorbance detection may consider increasing or decreasing the starting sample size, increasing the HPLC injection volume or decreasing the volume for sample resuspension (Section 5.9 above) for measurements in cultured cells.

The conditions for hydrolyzing acyl-CoAs from tissues and cultured cells were optimized by adjusting the KOH concentration, and the time and temperature of subsequent incubation (data not shown). The optimum condition was found to be 0.25 M at 55 °C for 2 hours. The thiol group of free CoA plus any CoA liberated from thioesters were derivatized by reaction with mBBr following adjustment of the pH. Subsequent HPLC separation and typical detection profiles for mouse liver or human cultured C3A cells are indicated in **Figure 5** as red peaks, with a retention time between 11 and 12 minutes using the elution program described in **Table 1**. Typical amounts of biological starting material are 30-40 mg of murine liver (wet weight), 6-8 x 10^6 cells for human "liver-like" C3A cells, or ~1.3 x 10^7 cells for human HEK293T cells. The C3A cells were treated with a PanK experimental drug PZ-2891 at 10 uM which elevates CoA[17] (**Figure 6**). The CoA measurements in HEK293T cells when PanK isoforms are overexpressed show CoA measurements over a wide range (431-6925 pmoles/µL) (**Figure 6**). The sample sizes required for this methodology are practical for application to many experimental contexts.

The area under the CoA-bimane peak was calculated using software provided with the HPLC. The peak limits can be determined automatically by the HPLC software pending proper adjustment of input values for baseline correction and peak definition, but our laboratory prefers to manually designate the CoA-bimane peak in each chromatogram, particularly for unfamiliar biological samples.

FIGURE AND TABLE LEGENDS:

Table 1: HPLC Program for CoA-bimane Separation. Buffer A: 50 mM KH_2PO_4, pH 4.6; Buffer B: Acetonitrile. The mixing of Buffer A and Buffer B follows Curve 6 which is a linear gradient, with an intervening Curve 8 which is a medium concave gradient.

Figure 1. Coenzyme A biosynthesis pathway. Pantothenate kinase (PanK) catalyzes the phosphorylation of pantothenate (vitamin B_5) to 4'-phosphopantothenate in the first step of CoA biosynthesis. Formation of phosphopantothenate is followed by condensation with cysteine catalyzed by 4'-phosphopantothenoylcysteine synthase and then decarboxylation to

form 4'-phosphopantetheine by 4'-phosphopanthenoylcysteine decarboxylase. 4'-Phosphopantetheine is converted to Coenzyme A (CoA) in a two-step process catalyzed by CoA Synthase.

Figure 2. mBBr Reaction with CoA. Monobromobimane (mBBr) is mixed with CoA-SH (free CoA) and incubated at room temperature for 2 hours in the dark. Non-fluorescent mBBr becomes fluorescent when bound to CoA to form CoA-bimane.

Figure 3: Absorbance HPLC Trace of CoA-bimane Standard. CoA-bimane was separated on a Gemini C18 column using the HPLC program in **Table 1**. Typical retention time (min) is indicated by aborbance units (AU).

Figure 4: CoA-bimane standard curves detected by absorbance or fluorescence. (**A**) The standard curve measured using absorbance detection units for CoA-bimane was measured at λ393 nm. Tissue samples are usually evaluated using the absorbance standard curve. (**B**) The standard curve measured using fluorescence detection units for CoA-bimane at λex = 393 nm, λem = 470 nm. Cultured cells are usually evaluated for CoA content using the fluorescence standard curve. Duplicate standards (and samples) are routinely evaluated.

Figure 5: HPLC traces of typical liver or cultured cell samples. (**A**) CoA-bimane identification and quantification of content in mouse liver extract. Absorbance units (AU) are indicated. (**B**) CoA-bimane identification and quantification of content in HepG2/C3A cultured cells. Fluorescence emission units (EU) are indicated. The CoA-bimane peaks are shown in red.

Figure 6. CoA levels in mouse liver, cultured C3A and HEK293T cells. (**A**) CoA measurement in mouse liver from pantothenate kinase knockout (*Pank1*$^{-/-}$) and matched wild-type (WT) animals. *Pank1*$^{-/-}$ animals have lower CoA in the liver compared to WT animals due to the absence of *Pank1* expression[9]. The data were obtained using 5 male mice per genotype and are represented as the mean ± SEM. (**B**) CoA levels in HepG2/C3A cells treated with either dimethylsulfoxide (vehicle control) or PZ-2891, an experimental drug that modulates PanK activity at 10 uM[17]. The cellular CoA level is elevated following 24-hour incubation with PZ-2891. (**C**) CoA levels in HEK293T cells transfected with either empty vector pcDNA3.1, or cDNAs encoding human PANK isoforms: PANK1β, PANK2m which is the mature, processed isoform of human PANK2, or PANK3. CoA levels are elevated by overexpression of all active PANK isoforms. The data in (**B**) and (**C**) are from independent triplicate samples and are represented as the mean ± SEM. The statistical analysis was done using the unpaired t-test and the values of significance are shown in red. The data in panels (**B**) and (**C**) are adapted from Sharma *et al*.[12]

DISCUSSION:

Here we demonstrate a reliable, step-by-step procedure for quantifying total CoA in cells and animal tissues with a wide range of linear detection that is accessible in many laboratories that have an HPLC with either an absorbance or fluorescence output detector. Alternatively, mass spectrometry is a common technique for evaluating CoA and CoA thioesters, but is not widely available due to the cost of the instrumentation and the specialized knowledge required for

development of methodology and interpretation of data. Isotopically labeled CoA that is suitable for use as an internal standard for quantification of free CoA in mass spectrometry is not commercially available, and free CoA is not detected by the instrument with the same sensitivity as CoA thioesters such as acetyl-CoA. Thus, free CoA levels are often grossly underestimated by mass spectrometry unless the output data are compared with a CoA standard calibration curve.

We compared the method described here with a different method previously used in our laboratory and found greater CoA recovery from mouse liver, 123.4 ± 7.9 pmol/mg wet weight, with this current method compared to ~80 pmol/mg wet weight using an enzymatic assay that derivatized free CoA with a radioactive tag[28]. The method for measuring total CoA that is described here is considerably less cumbersome, faster and easier to control compared to the method previously used in our lab[28]. Formerly, CoA was determined following hexane extraction of the cell lysate and subjected to enzymatic conversion to radioactive [^{14}C]lauryl-CoA mediated by the *Escherichia coli* acyl-CoA synthetase (FadD). The acid-soluble short-chain acyl-CoAs and the acid-insoluble long-chain acyl-CoAs in the cell lysate were hydrolyzed with KOH to yield free CoA and then subjected to hexane extraction prior to the enzymatic conversion to free CoA[29]. Although this previous procedure had good specificity and sensitivity, in practice the task required 2 working days to complete and the recombinant *E. coli* acyl-CoA synthetase had to be prepared, purified and measured for enzymatic specific activity prior to the CoA analysis. It also required instrumentation capable of detecting and quantifying radioactive isotopes which is much less common in biomedical laboratories in more recent history. The current method as described here is most suitable for our research interest in pantothenate kinase (PanK). PanK controls the flux through the CoA biosynthetic pathway and so the total CoA level is the readout for the PanK activity in biological samples.

Quenching of metabolic reactions in a biological sample to maintain a true amount of CoA requires care and rapidity and is critical for this procedure. Rapid deproteinization with an acid or organic solvent, or flash-freezing of samples are two methods that have been used in the past[30,31]. In the present method, ice-cold ultrapure water is quickly added to ice-cold PBS-washed cells from cultures, and adherent cells plus water are then scraped off the culture dish before transfer to KOH. Freezing of the cultured cell suspension in [KOH + water] for storage at -80 °C prior to sample preparation is an acceptable stopping point. However, CoA values from frozen cell samples should be compared to those from freshly prepared samples to determine if there is substantial loss of the CoA signal. Loss of ≤ 10% CoA has occurred in our hands following cell sample freezing and may be related to the extended time of the sample in the KOH which needs to be controlled. Animal tissue pieces are quickly frozen on a small foil 'raft' floating on LN_2 immediately following excision of the tissue from a euthanized animal. Sample sizes from 5 mg to 60 mg of frozen tissue are appropriate for this method with linear CoA yields. Once frozen, tissue samples stored at -80 °C are stable for at least 3 months, providing that intermittent thaw does not occur.

The sample preparation prior to the HPLC analysis is not high throughput and requires a full working half-day. It is recommended that the operator handles a maximum of 30 samples at

one time, starting with homogenization of a set of 15 samples followed by homogenization of the second set of 15 samples during the 2 h incubation with KOH for the first set. The KOH incubation period was first optimized using purchased CoA thioesters with incubation times ranging from 30 min to 4 h, and then the 2 h incubation was chosen to accommodate the time for complete CoA thioester hydrolysis in a variety of tissue samples, ranging from skeletal muscle to liver [8]. Incubation for CoA derivatization with mBBr is set at 2 h at approximately pH 8 and a 20-fold excess of mBBr is added to completely convert the CoA in the biological samples. Sulhydryl moieties of proteins and metabolites other than CoA such as carnitine or glutathione in the sample will be derivatized in addition to CoA, and the 20-fold excess was calculated on the basis of the expected amount of total CoA liver which has the highest level among tissues. The pH is reduced prior to incubation with mBBr because bromobimanes in general are less reactive towards other nucleophiles like amines and carboxylates in more neutral aqueous solutions. The time of derivatization should not exceed 2 h to avoid or lessen reaction with protein thiols[26]. One needs to use nonnucleophilic buffers to reduce and maintain the pH because the presence of buffer anions at high concentrations can interfere with the mBBr derivatization of CoA.

Sample clean-up on the SPE column is done manually in our laboratory and can be performed with the aid of a vacuum manifold to shorten the time required for this step. Good recovery of derivatized CoA from the SPE column is routinely attainable and this was determined separately using radioactive [^{13}C]CoA thioesters which are also retained on the SPE column. Recovery of [^{13}C]acetyl-CoA was 97.6% and recovery of [^{13}C] palmitoyl-CoA was 96.1%. Evaporation of the SPE-column eluate is usually performed under nitrogen gas overnight in our laboratory as a matter of convenience, but drying can be completed within 4 hours under nitrogen gas or using a speedvac concentrator. The most critical steps in the method are related to pH adjustment and can be checked with pH paper strips along the way. For the KOH hydrolysis, the pH needs to be ≥ 12 to achieve complete release of the thioester from CoA. The pH needs to be 7.8 – 8.5 to support the reactivity of the mBBr-derivatization, and after the derivatization is complete, the pH should be adjusted to become very acidic, about pH 1-2, in order for the CoA-bimane to bind to the SPE column. The SPE column cleans up the sample to eliminate excess unreacted mBBr and some unrelated biological substances.

The HPLC program is designed so that washing and re-equilibration of the C18 column is sufficient to eliminate background and carryover signals between samples. Water blank samples are inserted after every 5 samples in the experimental sample set as a precaution to monitor possible carryover between sets and to ensure column cleanliness. An occasional error from improper sample injection can occur when using an automated sample injector for the HPLC, and this can easily be checked by comparing the volumes remaining in the sample vials after injection or inspection of the non-CoA related peaks in the output chromatogram. If the CoA values exceed the linear range of the calibration curve for fluorescence detection, the values will most likely be in range for ultraviolet absorbance detection. If not, dilution of the sample with a known volume of water can reduce the signal to be in the linear range and calculations should take any dilution factor into account.

CoA levels will vary among inbred mouse strains with different genetic backgrounds, although the values are reproducible when biological samples are obtained from the same strain under the same conditions. We have estimated CoA in several different mouse lines in various studies [10,12,16,17]. CoA was also measured in several cultured cell lines[16,17] using either primary or immortalized cells.

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DISCLOSURES:

MWF, CS and SJ wrote the paper, MWF and CS provided the data and figures, COR designed the experiments and critically reviewed the manuscript. SJ and COR are inventors on a pending patent application (PCT/US17/39037) “Small molecule modulators of pantothenate kinases” held by St. Jude Children’s Research Hospital that covers the PZ-2891 compound referenced in this article.

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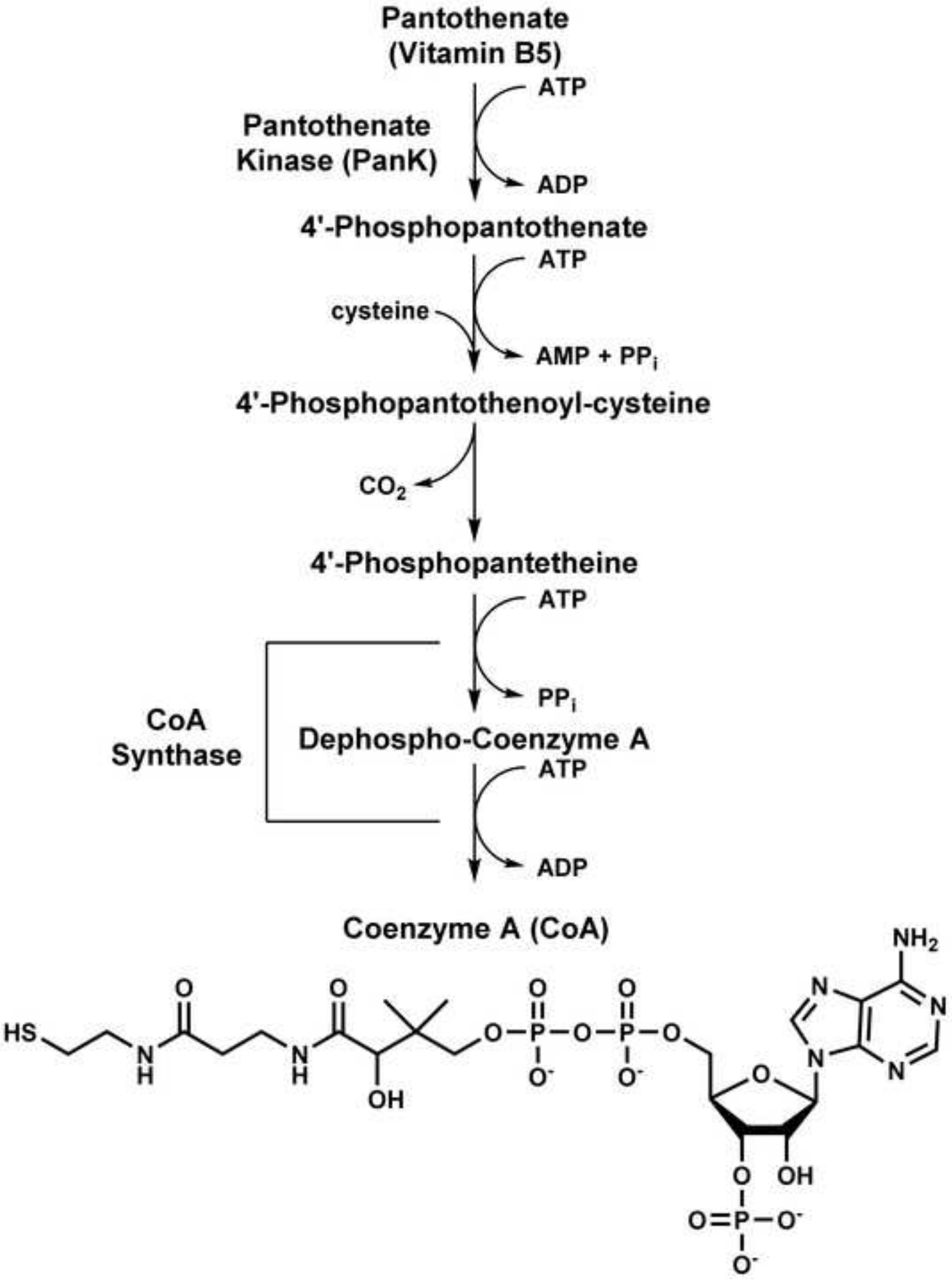

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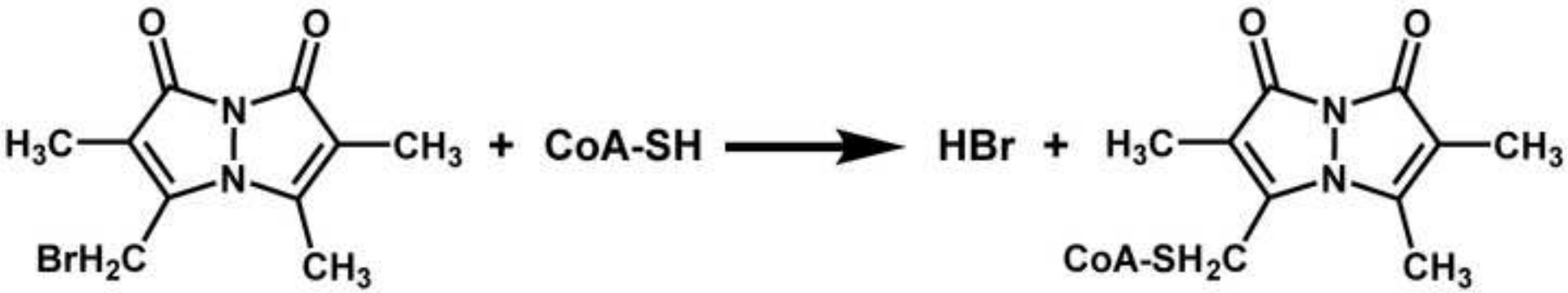

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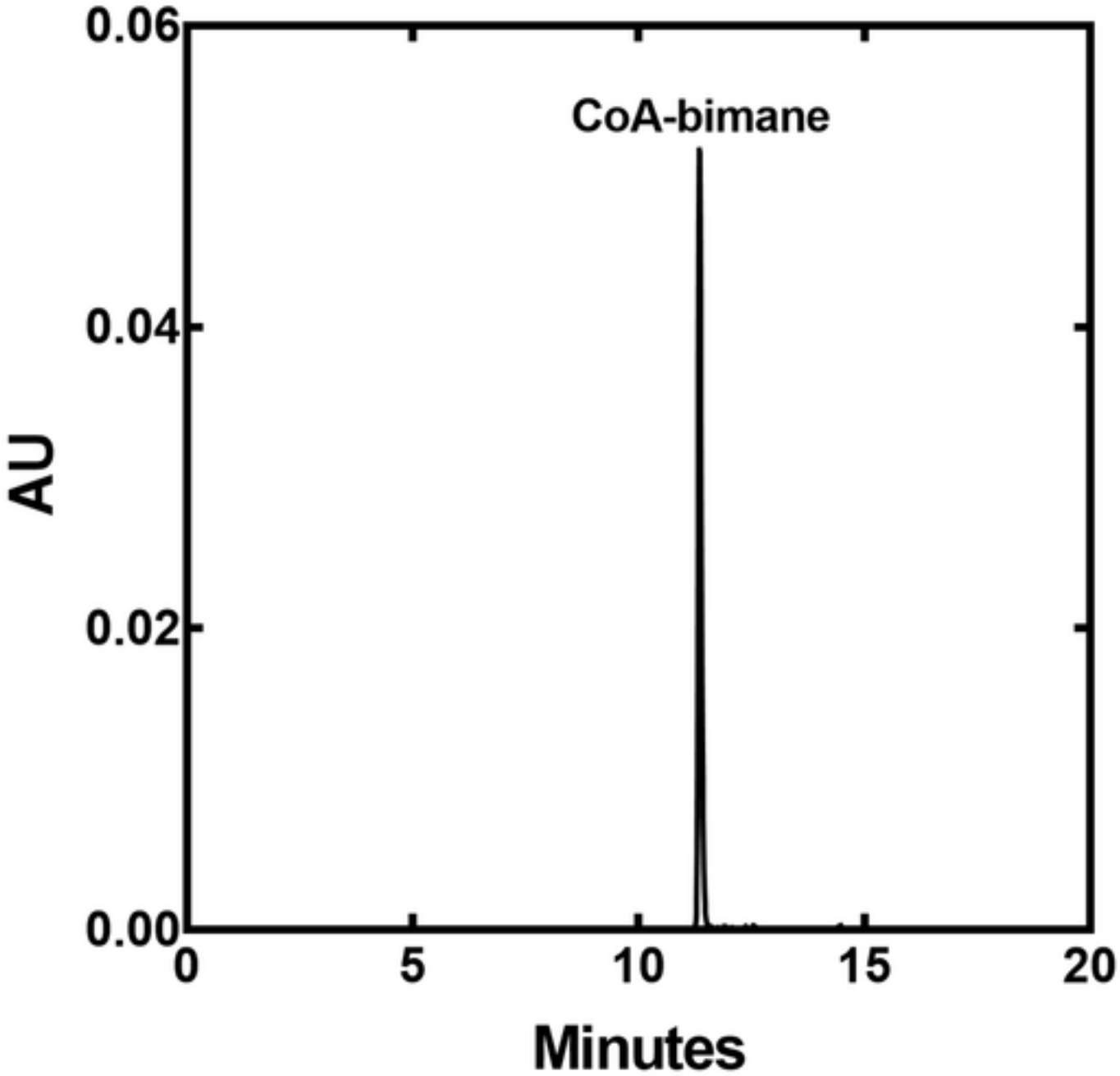

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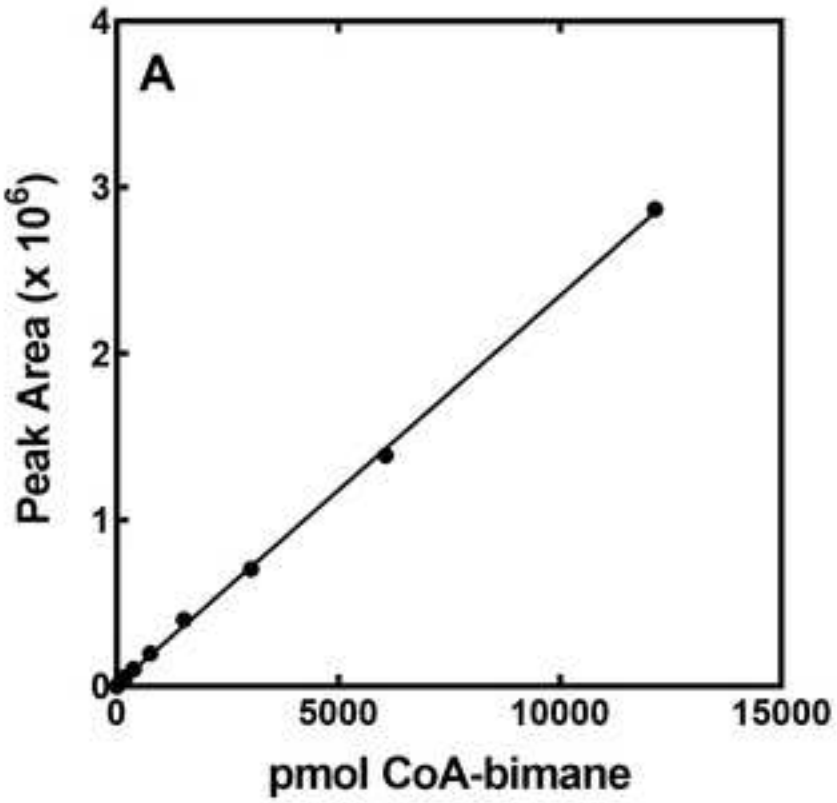

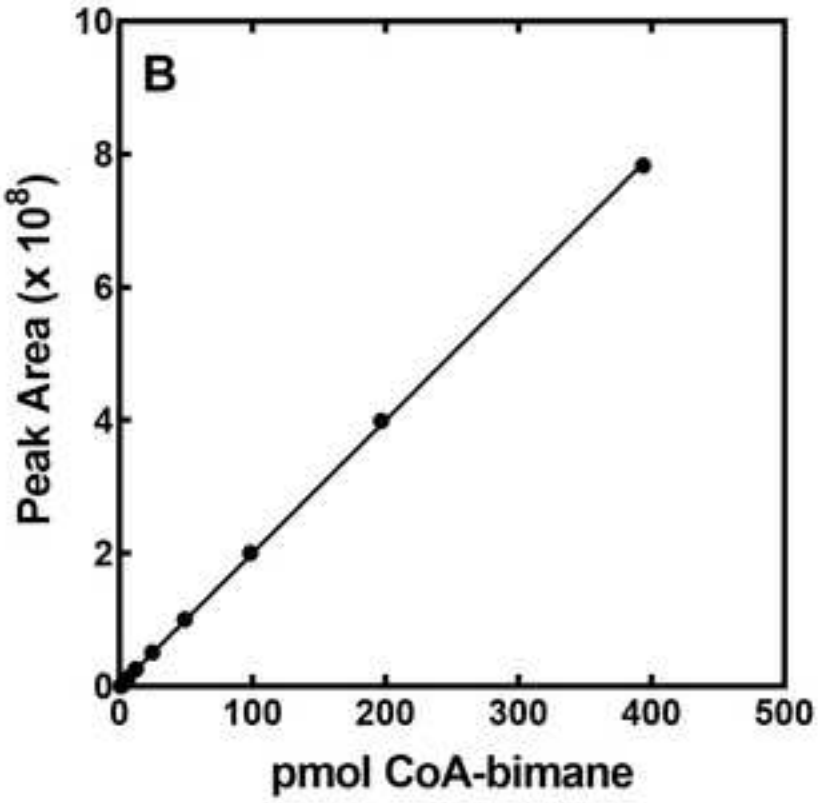

Figure

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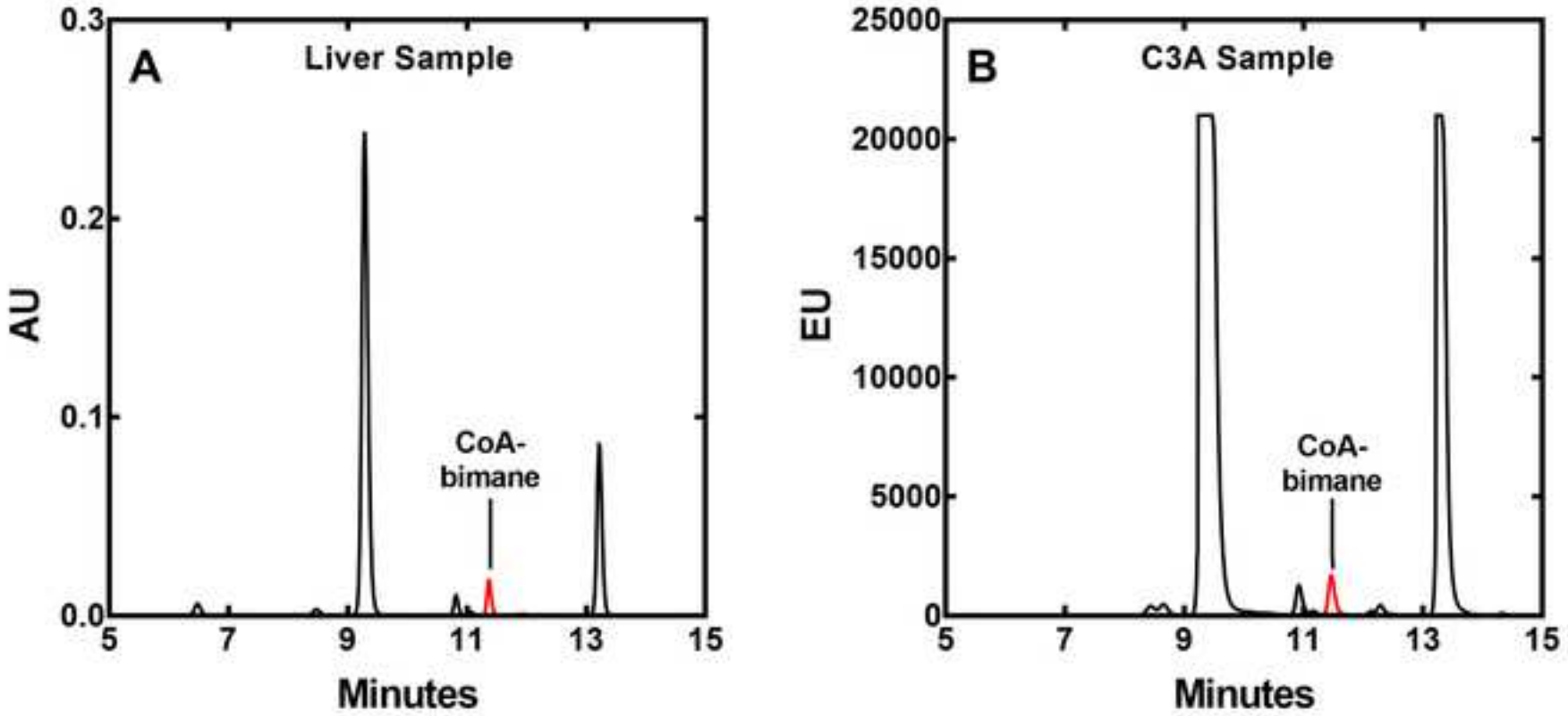

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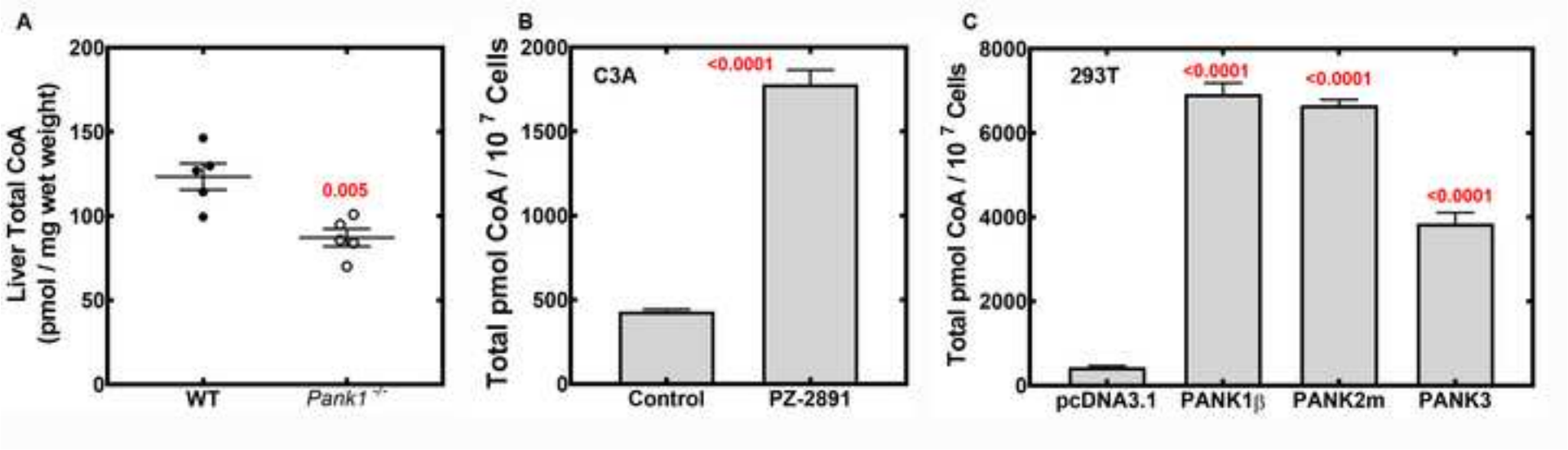

Table 1

Time (min)	Flow Rate (	% A	% B	Curve
0	0.5	90	10	0
2	0.5	90	10	6
6	0.5	85	15	6
18	0.5	60	40	6
23	0.5	60	40	6
25	0.5	90	10	6
30	0.5	90	10	66
				6

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Name of Material/ Equipment	Company
2-(2-pyridyl)-ethyl silica gel SPE column	Millipore-Sigma
coenzyme A	Avanti Polar Lipids
Gemini C18 3 µm 100 Å HPLC column	Phenomenex
monobromobimane	ThermoFisher Scientific
Omni-Tip probe tissue disrupter	Omni International
Parafilm	Fisher
PowerGen 125 motorized rotor stator homogenizer	ThermoFisher Scientific
Spin-X centrifuge tube filter	CoStar
Trizma-HCl	Fisher
Waters 2475 fluorescence detector	Waters
Waters 2489 UV-Vis detector	Waters
Waters e2695 separations module	Waters

Catalog Number	Comments/Description
54127-U	
870700	
00F-4439-E0	
M-1378	
32750H	
S37440	
NC0530997	
8161	
T395-1	
2475	
2489	
e2695	

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Name:	Suzanne Jackowski
Department:	Infectious Diseases
Institution:	St. Jude Children's Research Hospital
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We thank the reviewers for a thorough evaluation of the manuscript. Corrections and additions to the text and figures were made in response and are detailed below. The responses refer to lines in the text in the revised manuscript.

Protocol Comments

2.5-2.6: A line has been added to 2.6 to clarify.
"It is not necessary to filter and centrifuge the eluate from the SPE column (Section 5.8) when preparing the CoA-bimane standard."

The Protocol has been revised to reflect the imperative case consistently.

Larger steps in the Protocol with ≥ 4 sentences are divided into 2 steps.

Figures and Tables Question

Figure 6 Legend states: "The data in panels (B) and (C) are adapted from Sharma et al.[12] "
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A screen shot of the journal policy is provided in a separate file.

The items have been added to the Table of Materials and Reagents: Parafilm, Trizma-HCl

Reviewer #1 – Minor concerns

Line 111.
The phrase "4-fold molar excess" is substituted.

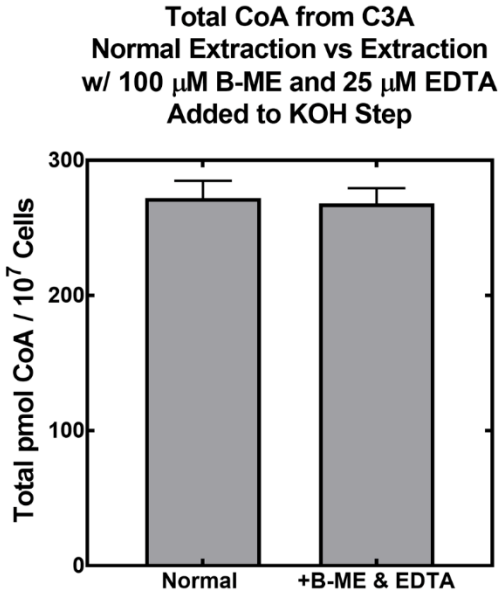
Line 232 moved to Line 118
Description of the quantification of the CoA and CoA-bimane standard is moved to Step 2. "Preparation of CoA-bimane Standard" in the Protocol section.

Figure 5 (and all of the figures) was reviewed and the software used to transfer the figure is changed. Hopefully the figures are improved.

Reviewer #2 - Major concerns

1. *Accounting for/preventing the formation of CoA disulfide ….*
This is a good point raised by the reviewer. The addition of KOH to cells and tissues raises the pH to 12 which can potentially promote oxidative CoA-disulfide formation. It is stated in the reference provided by the reviewer that "Loss of thiol due to oxidation …. is minimized by using heavy metal chelators and limiting the exposure of the thiol to oxygen while at high pH." We do not shake or stir the samples during the 2-hour incubation in KOH and this is now pointed out specifically in the protocol (Lines 129, 175, 222). Handling of samples in the presence of KOH is done at ≤ 4 ºC prior to the addition of mBBr which minimizes oxidative reactions. Nevertheless, we decided to test directly whether addition of a reducing agent and metal chelator would change the CoA quantification results. We performed an experiment in which 100 µM β-mercaptoethanol and 25 µM EDTA were added to the 2-hour KOH incubation of cell

samples. The CoA quantification under those conditions was compared, side-by-side, with CoA quantification obtained using the original conditions in the Protocol. The amount of mBBr added to the cell lysates samples containing mercaptoethanol was doubled to maintain this reagent in excess. The data below show that there is no difference in the amount of CoA-bimane that is detected at the end of the protocol, plus or minus the [100 μM β-mercaptoethanol and 25 μM EDTA].



Step 3.3. and Step 4.5 in the Protocol outline are modified to include a phrase "…incubated at 55 º C …without shaking in a water bath."

2. *Drawbacks of using mBBr as label*

A. *Background fluorescence of mBBr*

The mBBr compound itself is only weakly fluorescent and thus the background fluorescence of unreacted mBBr is minimal. When the mBBr is converted to bimane, fluorescence intensity increases substantially. It is stated in Line 138: "Clean-up the supernatant using an SPE-column to remove the unreacted mBBr (described below)." And in Line 465: "The SPE column cleans up the samples to eliminate excess unreacted mBBr and some unrelated biological substances." Any remaining background fluorescence of non-CoA-bimane components in biological samples is separated from the CoA-bimane during the HPLC step.

B. *Non-specific binding to amines, carboxylates and phosphates*

Excess mBBr (20-fold molar excess based on liver CoA measurements) is added to samples to take into account not only the reactivity with thiols other than CoA, but also the possibility that amines, carboxylates and phosphates may react slowly with the mBBr reagent. The labeling of cellular components other than CoA is expected. The SPE column and HPLC separation of CoA-bimane from non-CoA-bimane components results in a clean determination of CoA-bimane. The "CoA-bimane" peak has been checked several times by mass spectrometry in our lab to confirm this statement.

Line 446 addresses this issue: "The pH is reduced prior to incubation with mBBr because bromobimanes in general are less reactive towards other nucleophiles like amines and carboxylates in more neutral aqueous solutions." In addition, it is pointed out in Line 450: "One needs to use nonnucleophilic buffers to reduce and maintain the pH because the presence of buffer anions at high concentrations can interfere with the mBBr derivatization of CoA."

C. *Photodegradation*

Line 116: It is stated “Incubate at room temperature for 4 hours in the dark….”
Line 256. Step 6 has been modified to read “Seal and completely cover the tube and store.”

3. *Preparation of standard*

A. *Purity of the CoA-bimane standard*

High quality CoA to be used as the standard is purchased from Avanti Polar Lipids, purity >99%. Section 2.1. has been added to indicate this important point.
We failed to state that after the CoA-bimane standard is cleaned up on the SPE column to remove unreacted mBBr, the standard is checked by HPLC with absorbance detection at both λ260 and λ393 to assess purity by coincidence of both peaks in the chromatogram, as well as confirm the retention time and the amount of CoA-bimane standard in the high concentration stock. (In our experience, there is only 1 peak as shown in Figure 3.) The purity of the CoA-bimane standard can also be checked by mass spectrometry, depending on availability of the instrumentation. This information has been added to the Protocol, section 2.7.

B. *Using weight to prepare stock standard solutions*

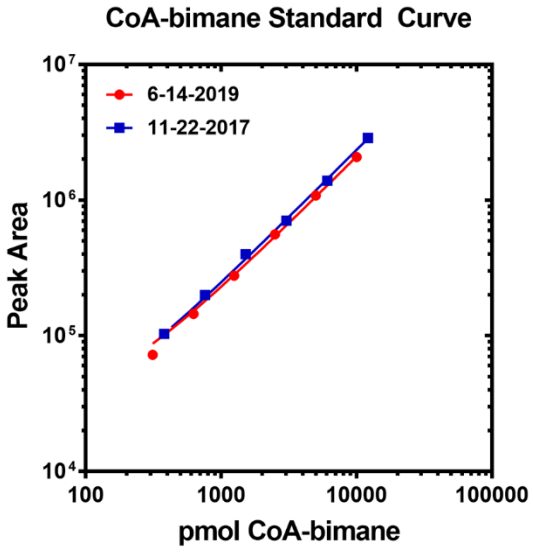
The reviewer is correct and the information above is added to the protocol.

C. *Using absorbance in the absence of a purity check*

The information regarding a purity check is added to the protocol.

D. *Assessment of stability of the CoA-bimane*

The stability of the CoA-bimane is addressed in the new section 2.8. in the Protocol. We compared the standard curves using aliquots of CoA-bimane made in 2017 with those made last week. The data are shown below.



4. Analytical parameters of standard curve (Line 258)

A. *Limit of detection (LOD)*

The limit of detection is indeed 0.01 and it is linear to 12,000 pmol when one measures the CoA-bimane standard using detection by fluorescence and absorbance.

B. *Limit of quantification (LOQ)*

The limit of quantification is defined as 5-fold above baseline noise. A sentence has been added to Results: “The CoA-bimane standard can be detected from 0.01 to 12,000 pmol and covers a 10^6-fold range when detection using both absorbance and fluorescence is combined. While the lower limit of detection of the standard is 0.01 pmol, the lower limit of CoA-bimane

quantitation in experimental samples is 0.2 pmol which is about 5-fold greater than the baseline or background fluorescent noise in the chromatogram."

5. *Enzyme quenching …*
Addition of KOH to cells and tissues raises the pH to 12 and denatures/inactivates the enzymes that may alter the amounts of CoA and CoA thioesters. This is the quenching step that stops metabolic re-alignment during sample preparation in addition to hydrolyzing CoA thioesters. Reducing the sample temperature with ice-cold buffer/water in the case of cultured cells, or with flash freezing of small pieces in the case of animal tissues, is the best one can practically do prior to denaturation and quenching with KOH. Complete thawing of tissue pieces is avoided and this point is now included in section 4.3.

Reviewer #2 - Minor concerns:

1. The term "mBBr-CoA" has been replaced with "CoA-bimane" throughout the text and the figures.

2. Line 115. Item has been changed to "… 95% ethanol (HPLC Grade) containing 50 mM ammonium formate."

3. Line 200.
Weighing fresh or frozen samples is a common way of normalizing data from animal tissues. The amount of liquid (not water) per mg in each tissue type is consistent for each particular tissue type. During preparation of tissue prior to freezing, each piece (0.5 cm approx. diameter) is blotted gently 1-2 seconds to remove excess blood if present. Most often, animals are exsanguinated prior to tissue harvest. The key to consistent CoA measurements is flash-freezing almost immediately after dissection. Sample drying is lengthly and requires too much time that might lead to loss of reduced CoA, and lyophilization requires specialized instrumentation to maintain a cold temperature during the lengthy drying process. In our experience, drying promotes CoA-disulfide and CoA-GSH formation.

4. Line 472.
The "occasional error from improper sample injection" is indeed an instrument error. This phrase was written to warn the investigator to check the vials in the autosampler when an unexpectedly low value is obtained upon evaluation of the endpoint data, although we agree that not all vial configurations would make this possible. When one is running hundreds of samples, as we do in our lab, even the best instrumentation can be imperfect on rare occasions. We have added a phrase …… The amounts of non-CoA components of the HPLC profile are another indicator of possible instrument malfunction. The phrase has been added "… or inspection of the non-CoA related peaks in the output chromatogram." Line 475

5. A review of recent methods for measuring CoA or CoA thioesters was pointed out with a citation in Line 72. We think that these lines and this citation in INTRODUCTION are sufficient. This article is not a critical review of the field.

Reviewer #3

Line 33.

We understand that pantothenate is the salt of pantothenic acid, but the terms are often used interchangeably in colloquial discussion. The qualifying phrase "…(the salt of pantothenic acid)…" was inserted.

Line 39.
References inserted.

- Abiko Y. Metabolism of coenzyme A, p. 1-25. In D. M. Greenberg (ed.), Metabolic Pathways, vol. 7, 3rd ed. Academic Press, Inc., New York. 1975.

In addition, another source has been inserted "(www.biochemical-pathways.roche.com)". The numerous metabolic pathways that utilize CoA thioesters are described in multiple sections in multiple monographs. This web site illustrates the multiple pathways in a single figure and is widely available.

Line 40.
A review was inserted to support the statement that "It is now appreciated …"

- Leonardi R, Zhang Y-M, Rock CO, Jackowski S. Coenzyme A: Back in action. *Prog Lipid Res*. 2005;44:125-153.

Two primary references were inserted to support the statement that "…CoA supply is regulated…"

- Jackowski S,Rock CO. Regulation of coenzyme A biosynthesis. *J Bacteriol*. 1981;148:926-932.
- Robishaw JD, Berkich DA, Neely JR. Rate-limiting step and control of coenzyme A synthesis in cardiac muscle. *J Biol Chem*. 1982;257:10967-10972.

Citation of a discussion of the inherited genetic disorders that impact CoA biosynthesis is inserted at the end of the statement.

- Di Meo I, Carecchio M, Tiranti V. Inborn errors of coenzyme A metabolism and neurodegeneration. *J Inherit Metab Dis*. 2019;42:49-56.

Line 74.
The statement has been modified to read:
"Radioactive tracers can be used to measure rates of CoA synthesis in cells."

Line 169 (unrevised version).
Extra "this" removed.

Line 391 (unrevised), now Line 406 (revised version).
The general statement regarding the recovery of CoA derivatives following SPE column clean-up is removed. The specific recovery data for two CoA thioesters are now included in the text.

Line 408 (unrevised version).
Grammar corrected "…be…" removed.