analytical chemistry

ARTICLE

pubs.acs.org/ac

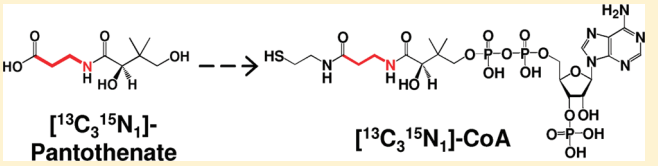
# Stable Isotope Labeling by Essential Nutrients in Cell Culture for Preparation of Labeled Coenzyme A and Its Thioesters

Sankha S. Basu, Clementina Mesaros, Stacy L. Gelhaus, and Ian A. Blair*

Centers of Excellence in Environmental Toxicology and Cancer Pharmacology, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, Pennsylvania 19104, United States

Supporting Information

**ABSTRACT:** Stable isotope dilution mass spectrometry (MS) represents the gold standard for quantification of endogenously formed cellular metabolites. Although coenzyme A (CoA) and acyl-CoA thioester derivatives are central players in numerous metabolic pathways, the lack of a commercially available isotopically labeled CoA limits the development of rigorous MS-based methods. In this study, we adapted stable isotope labeling by amino acids in cell culture (SILAC) methodology to biosynthetically generate stable isotope labeled CoA and thioester analogues for use as internal standards in liquid chromatography/multiple reaction monitoring mass spectrometry (LC/MRM-MS) assays. This was accomplished by incubating murine hepatocytes (Hepa 1c1c7) in media in which pantothenate (a precursor of CoA) was replaced with [$^{13}C_3{}^{15}N_1$]-pantothenate. Efficient incorporation into various CoA species was optimized to >99% [$^{13}C_3{}^{15}N_1$]-pantothenate after three passages of the murine cells in culture. Charcoal−dextran-stripped fetal bovine serum (FBS) was found to be more efficient for serum supplementation than dialyzed or undialyzed FBS, due to lower contaminating unlabeled pantothenate content. Stable isotope labeled CoA species were extracted and utilized as internal standards for CoA thioester analysis in cell culture models. This methodology of stable isotope labeling by essential nutrients in cell culture (SILEC) can serve as a paradigm for using vitamins and other essential nutrients to generate stable isotope standards that cannot be readily synthesized.




Coenzyme A (CoA), a ubiquitous and essential cofactor, is recognized to play a central role in numerous metabolic pathways[1] from fatty acid metabolism to xenobiotic acetylation.[2] More recently, it has been shown that levels of CoA species are perturbed in many pathological settings including diabetes,[3,4] hyperoxia,[5,6] seizures,[7] and various inherited metabolic disorders.[8−13] Therefore, robust and specific methods are required in order to accurately quantify CoA and its thioester derivatives in cell and tissue samples. Stable isotope dilution liquid chromatography/multiple reaction monitoring mass spectrometry (LC/MRM-MS) represents the gold standard for the quantification of intracellular metabolites.[14] Unfortunately, a broad stable isotope dilution method to measure a wide variety of CoA thioesters does not exist due to the lack of a commercially available isotopically labeled CoA.[15] As a result, stable isotope methods to quantify CoA and its derivatives have been limited to those in which an isotopically labeled thioester is available.[16,17]

We have developed a biosynthetic method to generate isotopically labeled CoA and its thioester derivates using an approach similar to the stable isotope labeling by amino acids in cell culture (SILAC) method, which was developed by Ong et al.[18] SILAC methodology involves incubating cells through multiple passages in the presence of isotopically labeled essential amino acids,[19,20] to generate labeled protein internal standards. The use of SILAC methodology to prepare stable isotope labeled proteome standards has significantly improved the field of quantitative proteomics.[14,21−23] Analogous to essential amino acids, there are also essential nutrients such as pantothenate (vitamin $B_5$), a water-soluble vitamin precursor of CoA, that cannot be biosynthesized by mammals. After cellular uptake of pantothenate, a five-step enzymatic pathway is used to synthesize CoA (Figure 1).[1] Since mammals are incapable of pantothenate synthesis, it is a required cofactor in mammalian cell culture media. By incubating cells in media containing [$^{13}C_3{}^{15}N_1$]-pantothenate instead of unlabeled pantothenate, we have developed the stable isotope labeling by essential nutrients in cell culture (SILEC) method for efficiently labeling CoA and its thioester derivatives analogous to the SILAC method used for labeling proteins.

## EXPERIMENTAL SECTION

**Chemicals and Reagents.** Reduced CoA (CoASH), acetyl-CoA, succinyl-CoA, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), propionyl-CoA, 5-sulfosalicylic acid (SSA), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 pantothenate-omitted media was purchased from Athena Environmental Sciences (Baltimore, MD). Undialyzed fetal bovine serum (uFBS), dialyzed FBS (dFBS), and charcoal−dextran-stripped FBS (csFBS) were purchased from Gemini Bio-Products (West Sacramento, CA). All solvents used were Optima grade (Fisher Scientific, Pittsburgh, PA). [$^{13}C_3{}^{15}N_1$]-pantothenate was purchased from IsoSciences (King of Prussia, PA).

**Received:** October 16, 2010
**Accepted:** December 28, 2010
**Published:** January 26, 2011

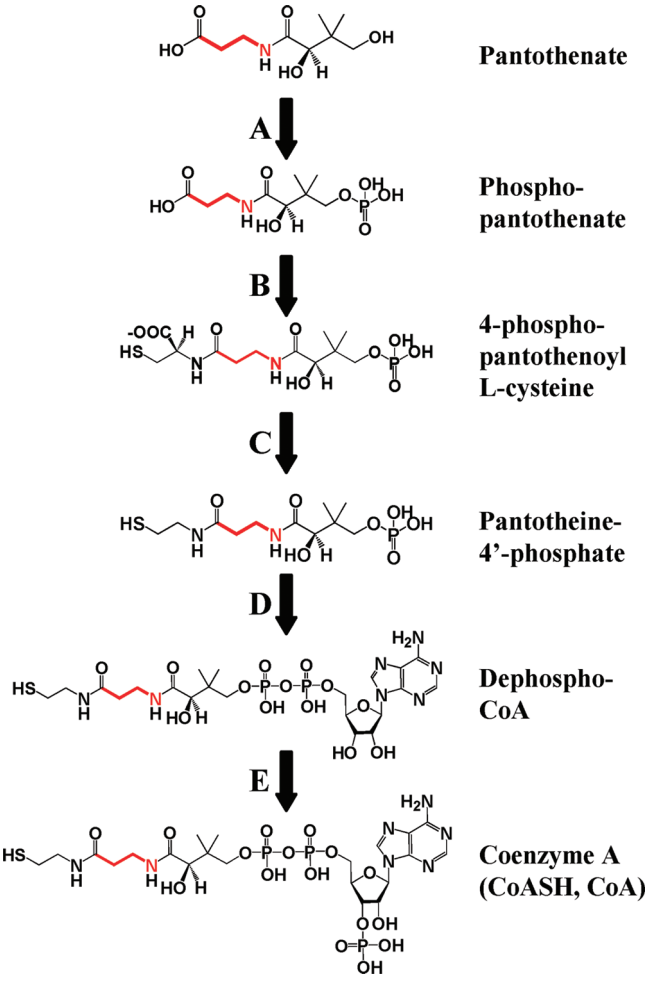



**Figure 1.** Metabolic pathway for CoA biosynthesis from pantothenate (vitamin B5). Enzymes: (A) pantothenate kinase (rate-limiting); (B) phosphopantothenoyl cysteine synthetase; (C) phosphopantothenoyl cysteine decarboxylase; (D) phosphopantetheine adenylyltransferase; (E) dephospho-CoA kinase. [$^{13}$C] and [$^{15}$N] atoms are marked in red.

**Cell Culture and Extraction of Short-Chain CoA Thioesters.** Murine hepatocytes (Hepa 1c1c7, ATCC no. CRL-2026) were maintained in RPMI 1640 media supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin at 37 °C and 5% $CO_2$. Extraction of CoA species was performed using modifications to similar methods.[15,16,24] Briefly, cells were washed twice with 10 mL of ice-cold phosphate-buffered saline (PBS). Cells were harvested by scraping into 1 mL of PBS. A 100 $\mu$L aliquot was saved for protein quantification, and the remainder was pelleted at 1000*g*. The cell pellet was resuspended in 1 mL of ice-cold 10% TCA and pulse-sonicated for 30 s on ice using a sonic dismembranator (Fisher), followed by a 5 min centrifugation at 15 000*g*. The supernatant was transferred to a fresh tube, and the pellet was discarded. The supernatant was purified by solid-phase extraction as follows: Oasis HLB 1 cm$^3$ (30 mg) SPE columns (Waters) were conditioned with 1 mL of methanol followed by 1 mL of water. The collected supernatant was applied, washed with 1 mL of water, and finally eluted using three subsequent applications of 0.5 mL of methanol containing 25 mM ammonium acetate. Eluted compounds were dried down under nitrogen and resuspended in 100 $\mu$L of 5% 5-SSA. Injections of 10 $\mu$L were made for liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) analysis.

**Table 1. MRM Transitions for Short-Chain CoA Thioesters and Stable Isotope Labeled Analogues Prepared by SILEC**

| compound | parent ($m/z$) | daughter ($m/z$) |
|---|---|---|
| CoASH | 768.1 | 261.1 |
| [$^{13}C_3{}^{15}N_1$]-CoASH | 772.1 | 265.1 |
| acetyl-CoA | 810.1 | 303.1 |
| [$^{13}C_3{}^{15}N_1$]-acetyl-CoA | 814.1 | 307.1 |
| propionyl-CoA | 824.1 | 317.1 |
| [$^{13}C_3{}^{15}N_1$]-propionyl-CoA | 828.1 | 321.1 |
| succinyl-CoA | 868.1 | 361.1 |
| [$^{13}C_3{}^{15}N_1$]-succinyl-CoA | 872.1 | 365.1 |
| HMG-CoA | 912.1 | 405.1 |
| [$^{13}C_3{}^{15}N_1$]-HMG-CoA | 916.1 | 409.1 |

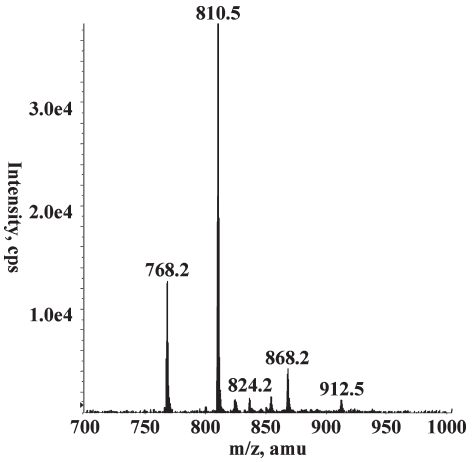



**Figure 2.** LC/constant neutral loss MS analysis of hepatocytes extracts with a neutral loss of $m/z$ 507 showing CoA ($MH^+$, $m/z$ 768), acetyl-CoA ($MH^+$, $m/z$ 810), propionyl-CoA ($MH^+$, $m/z$ 824), succinyl-CoA ($MH^+$, $m/z$ 868), and HMG-CoA ($MH^+$, $m/z$ 912).

**LC/MS.** Analytes were separated using a reversed-phase Phenomenex HPLC Luna C18 column (2.0 mm × 150 mm, pore size 5 $\mu$m) with 5 mM ammonium acetate in water as solvent A, 5 mM ammonium acetate in 95/5 acetonitrile/water (v/v) as solvent B, and 80/20/0.1 (v/v/v) acetonitrile/water/formic acid as solvent C. Gradient conditions were as follows: 2% B for 1.5 min, increased to 25% over 3.5 min, increased to 100% B in 0.5 min and held for 8.5 min, washed with 100% C for 5 min, before equilibration for 5 min. The flow rate was 200 $\mu$L/min. Samples were analyzed using an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) in the positive ESI mode. Samples (10 $\mu$L) were injected using a Leap CTC autosampler (CTC Analytics, Switzerland) where they were maintained at 4 °C, and data was analyzed with Analyst 1.4.1 software. The column effluent was diverted to the mass spectrometer from 8 to 13 min and to waste for the remainder of the run. The mass spectrometer operating conditions were as follows: ion spray voltage (5.0 kV), nitrogen as curtain gas (15 units), ion source gas 1 (8 units), gas 2 (15 units), and collision-induced dissociation (CID) gas (5 units). The ESI probe temperature was 450 °C, the declustering potential was 105 V, the entrance potential was 10 V, the collision energy was 45 eV, and the collision exit potential was 15 V. Isotopic labeling using [$^{13}C_3{}^{15}N_1$]-pantothenate resulted in

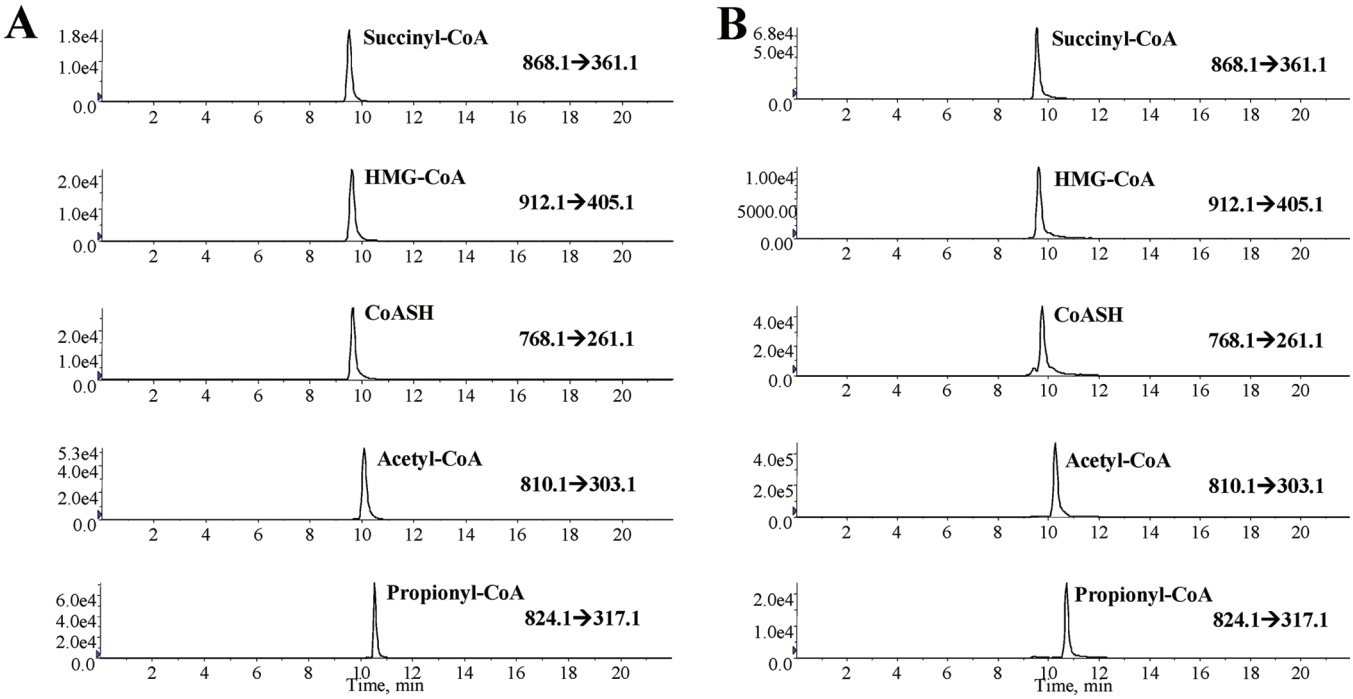



**Figure 3.** Analysis of short-chain CoA thioesters. LC/MRM-MS chromatograms of (A) CoA thioester standards (1 pmol each) and (B) acidic extracts from 1 plate of Hepa 1c1c7 cells.

labeled CoA and thioester derivatives with a mass shift of 4 amu. Transitions employed for LC/MRM-MS analyses are shown in Table 1.

**Stable Isotope Labeling Using [$^{13}C_3{}^{15}N_1$]-Pantothenate.** Labeling of CoA and its thioesters was achieved using a procedure similar to SILAC except that [$^{13}C_3{}^{15}N_1$]-pantothenate was used instead of deuterated amino acids.[18] Murine hepatocytes were cultured in RPMI media containing 1 mg/L [$^{13}C_3{}^{15}N_1$]-pantothenate and 10% serum (uFBS, dFBS, or csFBS). To monitor labeling efficiency, cells were harvested at each passage and processed as described above. The ratio of unlabeled to labeled reduced CoA (CoASH) was used to monitor labeling and to calculate absolute concentrations of pantothenate (Supporting Information Supplemental Table 1). To characterize pantothenate labeling, cells were grown to confluence, washed and treated with the labeling media for 0, 1, 3, 6, 12, 18, or 24 h, followed by harvesting and extraction. CoA labeling and scale-up were optimized by varying the type of serum (uFBS, dFBS, and csFBS) as well as the concentration of [$^{13}C_3{}^{15}N_1$]-pantothenate, supplemented into the labeling media. After scale-up (as described below), cells were harvested by scraping and sonication in 10% TCA. Acidified extracts containing stable isotope labeled CoA thioesters were pooled, aliquoted, frozen, stored at −80 °C, and thawed as needed.

**Scale-Up of Stable Isotope Labeling of CoA and Its Thioesters.** Cells were split 1:5, collected, and processed each day for seven days. Although there was some minor variation among different CoA species, the peak level for each of the CoA species occurred on day 4 or 5 (Supporting Information Supplemental Table 2). Therefore, a two-step approach was employed. First, cells were grown in media supplemented with 10% csFBS and 1 mg/L [$^{13}C_3{}^{15}N_1$]-pantothenate for three passages. Second, the media was replaced on the third day of the final passage with media containing 3 mg/L labeled pantothenate and 3% csFBS. The incubation was continued overnight; cells were then harvested and processed the next day. This resulted in efficient labeling of all CoA species to >99% labeling. Typically this was conducted using 20 plates or more.

**Validation.** Cells from 20 plates were sonicated in 10% TCA (0.5 mL per plate), pooled, and aliquoted into three fractions of 3 mL, which were subsequently stored at −80 °C. Lysates from [$^{13}C_3{}^{15}N_1$]-pantothenate-labeled cells were prepared as described above and also stored at −80 °C. CoASH and short-chain acyl-CoA standards were stored in 5% SSA at −80 °C. For the standard curve, CoA standards were thawed and diluted using 10% TCA, each with a final volume of 0.5 mL. One 3 mL cell lysate aliquot was thawed each day of the validation and divided into six 0.5 mL aliquots. Each standard or fraction was mixed with 0.5 mL of the labeled CoA TCA lysate. Samples were processed as described above. Intraday and interday validation was performed for each analyte.

**Propionate Treatment.** Propionate treatment was used to demonstrate changes in intracellular CoA thioester concentrations in Hepa 1c1c7 cells. Cells were grown to approximately 90% confluence in 10 cm tissue culture dishes. Prior to treatment, cells were washed twice with PBS. Cells were then treated with HBSS supplemented with $CaCl_2$, $MgSO_4$, glucose, 25 mM HEPES buffer, and 10 mM propionate. Control cells were treated with the same media, excluding propionate. Cells were incubated in the treatment media for 1 h and processed as described previously.

## RESULTS AND DISCUSSION

**LC/ESI-MS/MS and CoA Extraction.** Although many LC/MS methods have been developed to analyze CoA thioester species in plant[25,26] and animal tissues,[15−17,27−29] the focus of our study is using mammalian cell culture models, which often require increased sensitivity.[24,30,31] CoA and short-chain CoA thioesters were identified in cellular extracts using a constant neutral loss scan of $m/z$ 507 amu corresponding to the ATP moiety (Figure 2).


dx.doi.org/10.1021/ac1027353 |*Anal. Chem.* 2011, 83, 1363–1369

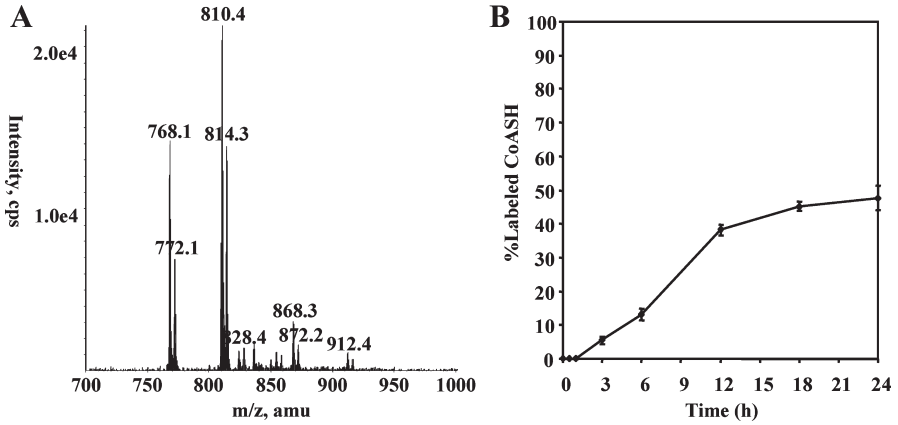



**Figure 4.** Stable isotope labeling of CoA thioesters in Hepa 1c1c7 cells grown in [$^{13}C_3{}^{15}N_1$]-pantothenate (1 mg/L). (A) LC/constant neutral loss MS analysis with a neutral loss of $m/z$ 507 from hepatocytes extracts after 24 h of pantothenate labeling. (B) Time course of pantothenate incorporation into CoASH using LC/MRM-MS analysis.

**Table 2. Effect of Different Sera on Stable Isotope Labeling of CoA**[a]

| | passage no. | | | | |
|---|---|---|---|---|---|
| serum | 1 | 2 | 3 | 4 | 5 |
| uFBS | 59.3 | 59.8 | 55.9 | 58.7 | 63.4 |
| dFBS | 93.5 | 97.6 | 97.7 | 97.4 | 97.9 |
| csFBS | 94.6 | 98.4 | 98.9 | 99.0 | 99.1 |

[a] Percentage of labeled CoASH in Hepa 1c1c7 cells serially passaged in pantothenate-omitted RPMI 1640 media supplemented with 1 mg/L [$^{13}C_3{}^{15}N_1$]-pantothenate and 10% serum: uFBS, dFBS, csFBS.

MRM transitions were developed for five unlabeled and labeled CoA thioesters (Table 1) in order to conduct sensitive quantitative LC/MS analysis (Supporting Information Supplemental Figure 1). However, this SILEC technique can be extended to other CoA thioesters by making modifications to the extraction procedure.[32] Due to the similarity of the various short-chain CoA thioesters, LC/MRM-MS analysis was employed for specific identification as well as accurate and precise quantification of the various CoA species without requiring absolute baseline separation as is needed for HPLC-UV methods.[33,34] CoA standards and acid-extracted CoA thioesters from hepatocytes were separated and quantified using LC/MRM-MS (Figure 3).

**Stable Isotope Labeling with [$^{13}C_3{}^{15}N_1$]-Pantothenate.** Stable isotope labeled CoA thioester derivatives were generated by growing cells in pantothenate-omitted media supplemented with [$^{13}C_3{}^{15}N_1$]-pantothenate. To verify and characterize the incorporation of labeled pantothenate into acyl-CoA species, cells were grown in labeled media, harvested at different times, and processed (Figure 4). Various CoA species and their heavy-labeled isotopes were monitored to assess the relative amounts of labeling. The ratio of labeled to unlabeled CoA was consistent throughout all analyzed CoA thioesters. Labeled CoASH could be detected as early as 3 h, replacing approximately 4% of the CoASH in the cells every hour. After 12 h, there appeared to be a labeling "plateau", likely the result of an active or sequestered CoA pool unavailable for turnover.

Like SILAC methodology, labeling efficiency is dependent on the relative concentration of labeled to unlabeled substrate in the media. As such, minimizing unlabeled pantothenate in the media is critical for the production of pure isotopically labeled CoA standards. Since circulating vitamins represent a major source of contaminating unlabeled pantothenate in serum-supplemented cell culture, three different types of sera were tested: undialyzed (uFBS), dialyzed (dFBS), and charcoal−dextran-stripped (csFBS). Although dFBS and csFBS are both often used in many cell culture applications to decrease interbatch variation, dialysis uses only size filtration, whereas stripping serum with activated charcoal more specifically binds and depletes certain small molecules such as steroids,[35] peptide hormones, and vitamins.[36] We found that maximal labeling was achieved by the third passage with minimal increases in labeling with additional passages (Table 2). csFBS was determined to be the most efficient for our application due to its lower pantothenate content. The maximal achievable steady state of labeled CoA represents the percentage of labeled pantothenate to total pantothenate in the media. By determining the percentage of CoA that is labeled within the cells, the approximate concentration of unlabeled contaminating pantothenate was calculated and determined to be the following: uFBS (5.7 mg/L), dFBS (0.21 mg/L), and csFBS (0.092 mg/L) (Supporting Information Supplemental Table 1).

**Scale-Up of Stable Isotope Labeling of CoA and Its Thioesters.** To increase the purity of the labeled CoA standards, two techniques were employed: (A) increasing the amount of [$^{13}C_3{}^{15}N_1$]-pantothenate in the media or (B) decreasing the amount of serum. Decreasing the FBS concentration resulted in significantly slowed growth characteristics, while further increases in media supplementation of [$^{13}C_3{}^{15}N_1$]-pantothenate resulted in limiting returns on labeling. By utilizing a two-step approach using 10% csFBS initially followed by a final incubation with 3% csFBS, efficient scale-up was achieved while limiting the amount of labeled pantothenate that was required.

Other approaches to generating heavy-labeled CoA are possible, though less optimal. Since CoA contains pantothenate, cysteine, and ATP, labeling with cysteine or a heavy-labeled carbon source would also result in labeled CoA. One limitation of cysteine labeling is that this can be synthesized de novo, which would result in decreased isotopic purity of the compound.

Since only a small fraction of cysteine or carbon from isotopic nutrient sources is incorporated into CoA, these methods are less efficient than the described method, where nearly all of the pantothenate is incorporated into CoA. Furthermore, preparation of [$^{13}C_3{}^{15}N_1$]-analogues avoids problems that can arise through the metabolic instability of deuterium and the separation

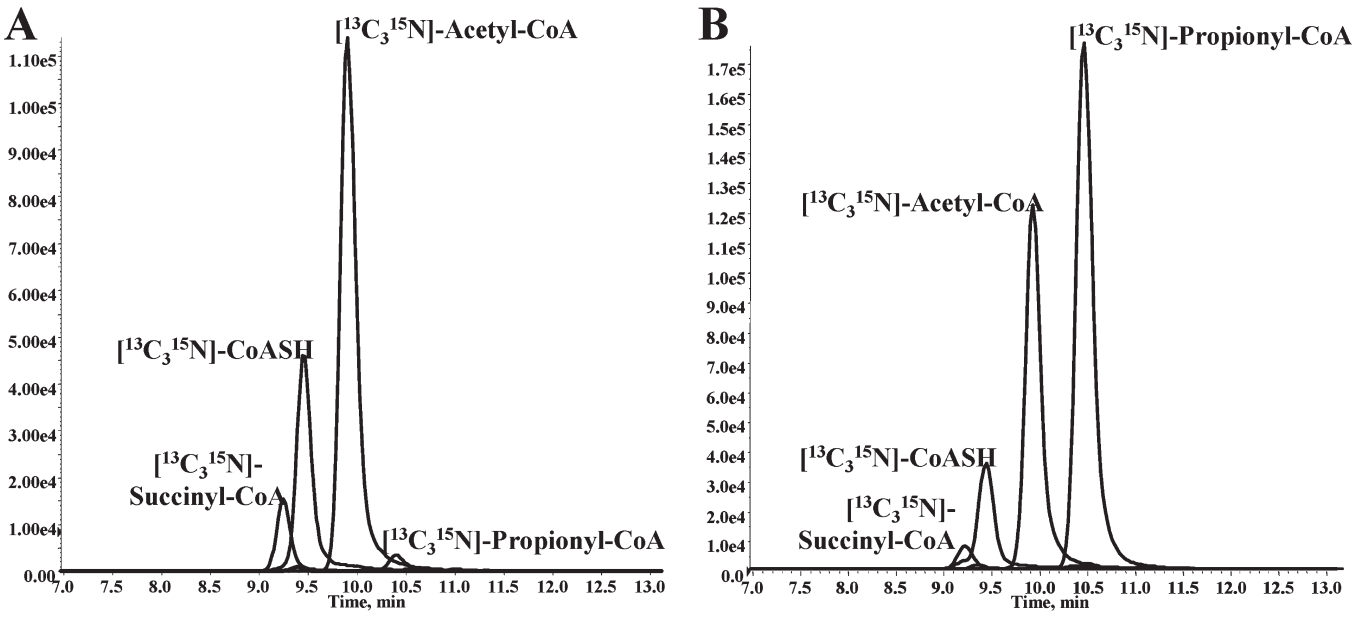



**Figure 5.** Preparation of a specific stable isotope labeled CoA thioester stable isotope standard: CoA extracts from stable isotope labeled cells (A) untreated and (B) treated with 10 mM propionate for 60 min.

**Table 3. Assay Validation**[a]

| | | precision (RSD) | | |
|---|---|---|---|---|
| | linearity ($R^2$) | intrabatch | interbatch | LOQ (pmol) |
| acetyl-CoA | 0.9990 | 2.4% | 2.6% | 0.1 |
| succinyl-CoA | 0.9996 | 5.6% | 1.6% | 0.2 |
| CoASH | 0.9966 | 6.8% | 2.6% | 0.1 |
| propionyl-CoA | 0.9999 | 9.1% | 13.9% | 0.05 |
| HMG-CoA | 0.9939 | 7.1% | 10.4% | 0.2 |

[a] Standard curves were generated by spiking increasing concentrations of different CoA standards with 0.5 mL of acid-extracted CoA. Pooled frozen extracts from Hepa cells were thawed and mixed with internal standard mixture and extracted. Limit of quantitation (LOQ) was determined to be the level at which the signal-to-noise ratio was 5:1 compared to a blank (Supporting Information Supplemental Figure 1).

of deuterium and protium analogues that occurs during LC/MS analysis.[37−39] Finally, methods using stable isotope CoA compounds with labeled acyl moieties are limited to those in which such labeled thioesters are available.[16] If a large number of CoA species are quantified, this would require labor-intensive preparation of each standard individually. Also, labeled CoASH could not be produced using this methodology. In contrast, biosynthetic generation of CoA thioesters provides simultaneous synthesis of every labeled thioester standard in a particular cell type in a naturally occurring profile in the appropriate concentration range to be used in the study. Moreover, different internal standard mixtures could be readily generated for specific applications by treating the labeled cells with a precursor fatty acid to reflect more appropriate thioester concentrations (Figure 5).

**Assay Validation.** Validation was performed on various CoA thioesters using our SILEC-derived internal standards (Table 3). Extracted samples were quantified with and without SILEC standards. Specificity was also improved since the compounds were coeluting with their standards. More importantly, it was found that using stable isotope analogues as internal standards provided a significant improvement in accuracy and precision when compared with nonidentical internal standards (Supporting Information Supplemental Table 3). The approach more commonly employed involves using a nonlabeled compound that is similar, but distinct, from the analyte of interest as the internal standard. There are several drawbacks to using this type of method. First, using an internal standard that is different than the analyte of interest assumes that both analyte and internal standard will behave equally throughout the assay. Although this may be a reasonable assumption for closely related compounds, as compounds diverge chemically, they also will show a greater disparity in their behavior. Second, ESI is particularly susceptible to matrix effects, due to the limited amount of ionization that can occur at the source. These effects can lead to an under- or overestimation of certain compounds as they coelute in a complex mixture.[40−42] Matrix effects are nonlinear and can vary dramatically throughout a gradient run, particularly when analyzing tissue samples. Finally, analyte stability and loss during sample preparation can vary considerably for different CoA species. Therefore, the assumption that the measured analyte will have the same stability as the analogue internal standard may also lead to over- or underestimation of the compound. Additionally, the stable isotope can act as a carrier for trace analytes and improve sensitivity.

**Application of SILEC Standards for CoA Thioester Quantification in Cell Culture.** Propionate incorporation into propionyl-CoA in Hepa 1c1c7 cells was used as a model for CoA metabolism.[43] Due to the wide disparity of CoA thioesters concentrations in treated compared to untreated cells, an optimal "customized" internal standard mixture was generated by combining the CoA extracts from untreated SILEC cells and an equal number of propionate-treated SILEC cells (Figure 5). This internal standard contained an acyl-CoA profile more applicable to both experimental groups. An equal amount of these extracts was added into both groups as well as the standard curve. Cells treated with 10 mM propionate for 1 h showed dramatic increases in propionyl-CoA levels relative to control cells, with concomitant decreases in levels of acetyl-CoA, succinyl-CoA, and CoA (Figure 6).

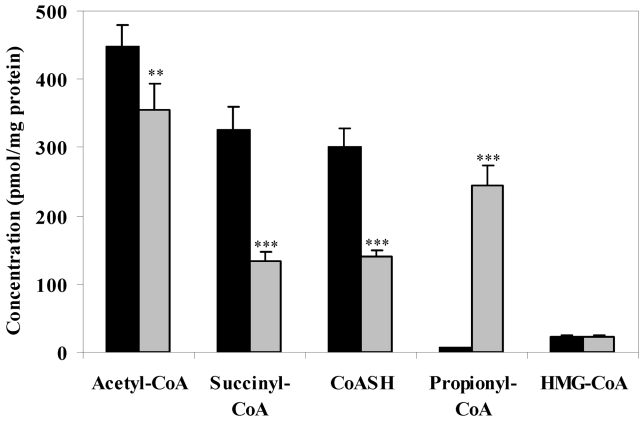



**Figure 6.** Changes in intracellular CoA thioester levels in Hepa 1c1c7 after propionate treatment: untreated (black) and 10 mM propionate for 1 h (gray). CoA thioesters extracted from control and treated labeled cells were combined and used as stable isotope internal standards in measuring short-chain acyl-CoA species. ** $p < 0.005$, *** $p < 0.00005$.

## CONCLUSIONS

A SILEC method has been developed for generating labeled CoA and CoA thioester standards by adapting SILAC methodology to utilize an essential vitamin and an endogenous biosynthetic pathway. Isotopically labeled substrates have been employed previously in tracer experiments to monitor cellular metabolic flux.[44,45] However, our study has focused on the optimization and scale-up of stable isotope labeled standards with low levels of the endogenous unlabeled analogues for use in quantitative assays. By using charcoal-stripped FBS instead of dialyzed or undialyzed FBS, more effective incorporation of pantothenate was achieved. The resulting SILEC methodology employing [$^{13}C_3{}^{15}N_1$]-pantothenate (instead of labeled amino acids used in SILAC methodology) made it possible to rigorously quantify short-chain CoA thioesters in cell culture. Although our analysis was limited to short-chain acyl-CoA species, this approach could be adapted to analyze medium- or long-chain CoA thioesters and other CoA-containing species with appropriate modifications to the extraction protocol.[32] The availability of labeled CoASH will also facilitate the development of methodology for the quantification of this important mitochondrial thiol and its oxidized mixed disulfide derivatives such as CoA−glutathione.

## ASSOCIATED CONTENT

**Supporting Information.** Supplemental Tables 1−3 and Supplemental Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

### Corresponding Author

*Phone: 215-573-9880. Fax: 215-53-9889. E-mail: ianblair@mail.med.upenn.edu.

## ACKNOWLEDGMENT

The support of NIH Grants RO1CA130038, P30ES013508, F32ES016683, 5T32HL007439, and 5T32GM007170 is gratefully acknowledged.

## REFERENCES

(1) Robishaw, J. D.; Neely, J. R. *Am. J. Physiol.* **1985**, *248*, E1–E9.
(2) Sim, E.; Walters, K.; Boukouvala, S. *Drug Metab. Rev.* **2008**, *40*, 479–510.
(3) Beinlich, C. J.; Robishaw, J. D.; Neely, J. R. *J. Mol. Cell. Cardiol.* **1989**, *21*, 641–649.
(4) Li, L. O.; Klett, E. L.; Coleman, R. A. *Biochim. Biophys. Acta* **2010**, *1801*, 246–251.
(5) Wong, Y. L.; Smith, C. V.; McMicken, H. W.; Rogers, L. K.; Welty, S. E. *Toxicol. Lett.* **2001**, *123*, 179–193.
(6) O'Donovan, D. J.; Rogers, L. K.; Kelley, D. K.; Welty, S. E.; Ramsay, P. L.; Smith, C. V. *Pediatr. Res.* **2002**, *51*, 346–353.
(7) Liang, L. P.; Patel, M. *Free Radical Biol. Med.* **2006**, *40*, 316–322.
(8) Bennett, M. J.; Hale, D. E. *N. J. Med.* **1992**, *89*, 675–678.
(9) Feliz, B.; Witt, D. R.; Harris, B. T. *Arch. Pathol. Lab. Med.* **2003**, *127*, e325–e328.
(10) Van Hove, J. L.; Grunewald, S.; Jaeken, J.; Demaerel, P.; Declercq, P. E.; Bourdoux, P.; Niezen-Koning, K.; Deanfeld, J. E.; Leonard, J. V. *Lancet* **2003**, *361*, 1433–1435.
(11) Boneh, A.; Andresen, B. S.; Gregersen, N.; Ibrahim, M.; Tzanakos, N.; Peters, H.; Yaplito-Lee, J.; Pitt, J. J. *Mol. Genet. Metab.* **2006**, *88*, 166–170.
(12) Mitchell, G. A.; Gauthier, N.; Lesimple, A.; Wang, S. P.; Mamer, O.; Qureshi, I. *Mol. Genet. Metab.* **2008**, *94*, 4–15.
(13) van Maldegem, B. T.; Wanders, R. J.; Wijburg, F. A. *J. Inherited Metab. Dis.* 2010, *33*, 507–511.
(14) Ciccimaro, E.; Blair, I. A. *Bioanalysis* **2010**, *2*, 311–341.
(15) Gao, L.; Chiou, W.; Tang, H.; Cheng, X.; Camp, H. S.; Burns, D. J. *J. Chromatogr., B* **2007**, *853*, 303–313.
(16) Minkler, P. E.; Kerner, J.; Kasumov, T.; Parland, W.; Hoppel, C. L. *Anal. Biochem.* **2006**, *352*, 24–32.
(17) Magnes, C.; Suppan, M.; Pieber, T. R.; Moustafa, T.; Trauner, M.; Haemmerle, G.; Sinner, F. M. *Anal. Chem.* **2008**, *80*, 5736–5742.
(18) Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. *Mol. Cell. Proteomics* **2002**, *1*, 376–386.
(19) Ong, S. E.; Mann, M. *Nat. Protoc.* **2006**, *1*, 2650–2660.
(20) Ong, S. E.; Mittler, G.; Mann, M. *Nat. Methods* **2004**, *1*, 119–126.
(21) Yan, Y.; Weaver, V. M.; Blair, I. A. *J. Proteome Res.* **2005**, *4*, 2007–2014.
(22) Shah, S. J.; Yu, K. H.; Sangar, V.; Parry, S. I.; Blair, I. A. *J. Proteome Res.* **2009**, *8*, 2407–2417.
(23) Yu, K. H.; Barry, C. G.; Austin, D.; Busch, C. M.; Sangar, V.; Rustgi, A. K.; Blair, I. A. *J. Proteome Res.* **2009**, *8*, 1565–1576.
(24) Park, J. W.; Jung, W. S.; Park, S. R.; Park, B. C.; Yoon, Y. J. *qJ. Mass Spectrom.* **2007**, *42*, 1136–1147.
(25) Hayashi, O.; Satoh, K. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 2676–2681.
(26) Perera, M. A.; Choi, S. Y.; Wurtele, E. S.; Nikolau, B. J. *J. Chromatogr., B* **2009**, *877*, 482–488.
(27) Kasuya, F.; Oti, Y.; Tatsuki, T.; Igarashi, K. *Anal. Biochem.* **2004**, *325*, 196–205.
(28) Mauriala, T.; Herzig, K. H.; Heinonen, M.; Idziak, J.; Auriola, S. *J. Chromatogr., B* **2004**, *808*, 263–268.
(29) Magnes, C.; Sinner, F. M.; Regittnig, W.; Pieber, T. R. *Anal. Chem.* **2005**, *77*, 2889–2894.
(30) Dalluge, J. J.; Gort, S.; Hobson, R.; Selifonova, O.; Amore, F.; Gokarn, R. *Anal. Bioanal. Chem.* **2002**, *374*, 835–840.
(31) Haynes, C. A.; Allegood, J. C.; Sims, K.; Wang, E. W.; Sullards, M. C.; Merrill, A. H., Jr. *J. Lipid Res.* **2008**, *49*, 1113–1125.
(32) Zhang, G. F.; Kombu, R. S.; Kasumov, T.; Han, Y.; Sadhukhan, S.; Zhang, J.; Sayre, L. M.; Ray, D.; Gibson, K. M.; Anderson, V. A.; Tochtrop, G. P.; Brunengraber, H. *J. Biol. Chem.* **2009**, *284*, 33521–33534.
(33) Hosokawa, Y.; Shimomura, Y.; Harris, R. A.; Ozawa, T. *Anal. Biochem.* **1986**, *153*, 45–49.
(34) King, M. T.; Reiss, P. D.; Cornell, N. W. *Methods Enzymol.* **1988**, *166*, 70–79.

(35) Iwasaki, K.; Underwood, B.; Herman, M.; Dinda, S.; Kodali, S.; Kloosterboer, H. J.; Hurd, C.; Moudgil, V. K. *Mol. Cell. Biochem.* **1999**, *198*, 141–149.
(36) Cao, Z.; West, C.; Norton-Wenzel, C. S.; Rej, R.; Davis, F. B.; Davis, P. J.; Rej, R. *Endocr. Res.* **2009**, *34*, 101–108.
(37) Zhang, R.; Sioma, C. S.; Thompson, R. A.; Xiong, L.; Regnier, F. E. *Anal. Chem.* **2002**, *74*, 3662–3669.
(38) Remane, D.; Wissenbach, D. K.; Meyer, M. R.; Maurer, H. H. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 859–867.
(39) Mesaros, C.; Lee, S. H.; Blair, I. A. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 3237–3247.
(40) Bonfiglio, R.; King, R. C.; Olah, T. V.; Merkle, K. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1175–1185.
(41) King, R.; Bonfiglio, R.; Fernandez-Metzler, C.; Miller-Stein, C.; Olah, T. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 942–950.
(42) Matuszewski, B. K. *J. Chromatogr., B* **2006**, *830*, 293–300.
(43) Brass, E. P.; Beyerinck, R. A. *Metabolism* **1987**, *36*, 781–787.
(44) Evans, J.; Wang, T. C.; Heyes, M. P.; Markey, S. P. *Anal. Biochem.* **2002**, *306*, 197–203.
(45) Bennett, B. D.; Yuan, J.; Kimball, E. H.; Rabinowitz, J. D. *Nat. Protoc.* **2008**, *3*, 1299–1311.