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Reversed-phase high-performance liquid chromatography procedure for the simultaneous determination of *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine in mouse liver and the effect of methionine on their concentrations

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Abstract

An improved reversed-phase high-performance liquid chromatography (HPLC) procedure with ultraviolet detection is described for the simultaneous determination of *S*-adenosyl-L-methionine (SAM) and *S*-adenosyl-L-homocysteine (SAH) in mouse tissue. The method provides rapid resolution of both compounds in a 25-μl perchloric acid extract of the tissue. The limits of detection in 25-μl injection volumes were 22 and 20 pmol for SAM and SAH, respectively. The limits of quantitation in 25-μl injection volumes were 55 and 50 pmol for SAM and SAH, respectively, with recovery consistently >98%. The assay was validated over linear ranges of 55–11 000 pmol for SAM and 50–10 000 pmol for SAH. The intra-day precision and accuracy were ≤6.4% relative standard deviation (RSD) and 99.9–100.0% for SAH and ≤6.7% RSD and 100.0–100.1% for SAM. The inter-day precision and accuracy were ≤5.9% RSD and 99.9–100.6% for SAH and ≤7.0% RSD and 99.5–100.1% for SAM. Compared to earlier procedures, the HPLC method demonstrated significantly better separation, detection limit and linear range for SAM and SAH determination. The assay demonstrated applicability to monitoring in mice the time-course of the effect of methionine on SAM and SAH levels in the liver. Administering methionine to mice increased by 10-fold the liver concentration of SAM and SAH within 2 h, which then rapidly decreased to the control levels by 8 h. This indicated that methionine was promptly converted to SAM and then rapidly catabolized into SAH. Thus, the metabolism of methionine to SAM should be considered in the supplementation of methionine to maintain SAM levels in the body. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *S*-Adenosyl-L-methionine; *S*-Adenosyl-L-homocysteine; Methionine

1. Introduction

S-Adenosyl-L-methionine (SAM) is an important methyl donor that is found in all living organisms. The liver plays a central role in the homeostasis of SAM as the major site of its synthesis and degradation [1–3]. Methionine adenosyltransferase is the enzyme responsible for the synthesis of SAM using the essential amino acid methionine and ATP [1–3]. In this reaction, the adenosyl moiety of ATP is

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transferred to methionine, forming a sulfonium ion that can transfer its methyl group to a large variety of acceptor substrates including nucleic acids, proteins, phospholipids, biologic amines, and a long list of small molecules [1]. By donating the methyl group, SAM is converted into *S*-adenosyl-L-homocysteine (SAH) [1–4]. SAH is a potent competitive inhibitor of transmethylation reactions; both an increase in SAH level as well as a decrease in SAM level or in the SAM-to-SAH ratio inhibits transmethylation reactions. SAH can be further metabolized by SAH hydrolase to homocysteine and adenosine [4].

SAM is involved in over 40 biochemical reactions in the body. Most notably, SAM functions in tandem with vitamin B_6, folic acid and vitamin B_{12} in a series of methylation reactions involving the transfer of a single carbon from one molecule to another [1–3]. Methylation reactions are essential for the synthesis of numerous physiological agents including neurotransmitters, glutathione and cartilage, the formation of 5-methylcytosine in DNA (approximately 5% of the 5-methylcytosine in DNA) and for the detoxification of harmful metabolites [1–3]. SAM is also the precursor of the aminopropyl groups utilized in polyamine biosynthesis and the precursor for cysteine via the *trans*-sulfuration pathway [1–3]. Given the critical role of SAM in many metabolic processes an alteration in its tissue level could have profound consequences.

Tissue levels of SAM and SAH, especially in the liver, can be decreased by toxic and some carcinogenic chemicals and by a diet deficient in choline and methionine or in folic acid and vitamin B_{12}, and can be increased by methionine, a precursor for the synthesis of SAM [5,6]. Furthermore, methionine can prevent the activity of these toxic chemicals purposely by increasing the tissue level of SAM [5,6]. Thus, accurate, sensitive and reproducible determination of SAM and SAH levels in the liver is necessary for the understanding of the toxicity and carcinogenic activity of chemicals. Various methods for the determination of SAM and SAH in tissues, plasma, blood and cerebrospinal fluid have been reported [7–17]. These methods use HPLC with detection by fluorescence [7,8], electrochemical [9], photodiode array [10], mass spectrometer [11] and ultraviolet [12–17]. Many of these methods, including those that used ultraviolet detection, suffered from the use of complex and tedious procedures for sample preparation [7,8,11–16] and from a narrow linear range of detection [9,11,12]. A significant improvement in sample preparation for animal tissues that involves perchloric acid extraction has been reported by She et al. [17]. Separation of SAM and SAH was accomplished by using a TSKgel ODS-80TM column with isocratic elution and ultraviolet detection. Although they reported levels of SAM and SAH in rat tissues, their level of detection was low and the separation of peaks for SAM and SAH was not complete and too wide [17]. We report here an improved HPLC method using a C_{18} reversed-phase column, gradient elution and ultraviolet detection for the simultaneous determination of SAM and SAH in mouse tissues. This method demonstrated significantly better separation, detection limits and linear ranges for both compounds. Furthermore, the applicability of the method for measuring SAM and SAH levels in mouse tissues is reported.

2. Experimental

2.1. Chemicals and reagents

SAM iodide salt, SAH, L-methionine and 1-heptanesulfonic acid sodium salt were obtained from Sigma (St. Louis, MO, USA). Methanol (HPLC grade) and $HClO_4$ solution (70% saturated) were from Aldrich (Milwaukee, WI, USA). All other chemicals were HPLC grade or the highest purity available.

2.2. HPLC apparatus and chromatographic conditions

A Waters HPLC system (Milford, MA, USA) was used that consisted of a Model 510 pump, a Model U6K universal injector, a Model 481 Lambda Max UV/visible LC spectrophotometer, and a Model 730 Data Module. Separation was carried out with a Whatman PartiSphere C_{18} reversed-phase analytical column (250 mm×4.6 mm I.D., 5 μm particle) (Clifton, NJ, USA). To protect the analytical column, a Whatman guard pre-column was fitted between the analytical column and the injector. The mobile phase consisted of two solvents: Solvent A, 8 m*M* oc-

tanesulfonic acid sodium salt and 50 m*M* NaH_2PO_4 adjusted to pH 3.0 with H_3PO_4; and Solvent B, 100% methanol. Before use, Solvent A was filtered through a 0.2-μm membrane filter. The HPLC column was equilibrated with 80% Solvent A and 20% Solvent B. The sample was injected and separation was obtained using a step gradient. The gradient consisted of 8 min at the equilibration conditions, 30 s to increase Solvent B to 40%, 12.5 min at the new condition, and 30 s to return to the equilibration conditions and a minimum of 10 min before a subsequent injection. The flow-rate was 1 ml/min and detection was monitored at 254 nm. The HPLC was performed at room temperature. SAM and SAH were identified according to their retention times and co-chromatography with SAM and SAH standards. Quantification was based on integration of peak areas and compared to the standard calibration curves of SAM and SAH. The results are expressed in nanomoles (nmol)/gram wet tissue.

2.3. Standards

SAM and SAH were dissolved in water at a concentration of 1 m*M* and then diluted with 0.4 *M* $HClO_4$ to the final concentration used during HPLC analysis. Aliquots of 25 μl of standard solutions containing 50 –11 000 pmol were injected onto the HPLC.

2.4. Animals and treatments

VAF (viral antibody free) female B6C3F1 mice were purchased from Charles River Breeding Laboratories (Portage, MI, USA). At 7–8 weeks of age, the mice were administered by intraperitoneal injection a single dose of 450 mg/kg methionine or the same volume of saline as control. The mice were euthanized by carbon dioxide asphyxiation at 0, 2, 5, 8 and 24 h after administering the methionine. At necropsy, the liver was rapidly excised, weighed, frozen in liquid nitrogen and stored at −70°C.

2.5. Sample preparation

Approximately 100 mg of liver tissue was homogenized in four volumes of 0.4 *M* $HClO_4$. After centrifugation at 10 000 *g* for 15 min at 4°C, the supernatant was filtered through a 0.2-μm polypropylene syringe filter (0.4 mm diameter, Whatman, Clifton, NJ, USA). A 25-μl aliquot of the acid extract was applied directly onto the HPLC.

2.6. Validation

The linearity of the calibration curves was checked for triple injections of SAM or SAH at each concentration. Five different concentrations in the range of 20–14 000 pmol were used for calibration.

The detection limit was determined by varying the amount of SAM or SAH in the 25-μl injection volume of water. The detection limit is defined as a signal-to-noise ratio (S/N) equal to 5. The limit of quantitation (LOQ) for a compound is defined as the lowest concentration in the linear ranges of the calibration curve.

The recovery from tissue samples was determined by the standard addition method. Known amounts of SAM and SAH standards were added to aliquots of mouse liver homogenate. The samples were then treated and chromatographed as described above. The recovery of SAM and SAH was determined by subtracting the mean value obtained for blank tissue preparations from that found for the preparation with added standards. Five replicates of blank tissue preparations and five of tissue preparations with added standards of SAM and SAH were analyzed.

The method was validated on the basis of intra- and inter-assay variations. Control liver homogenate was spiked with either SAM (110, 1100 and 11 000 pmol) or SAH (100, 1000 and 10 000 pmol), respectively. Five aliquots of each compound at each concentration were extracted and analyzed on Day 1 to determine intra-assay variation. The acid extracts were stored at −20°C until use for evaluating inter-assay variations. One aliquot of each compound at each concentration was analyzed per day for the next 5 days to determine inter-assay variation.

2.7. Statistics

The statistical significance among means was determined at $P<0.05$ by an ANOVA followed by a Tukey test using SigmaStat software, version 2.03 (Jandel, San Rafeal, CA, USA).

3. Results and discussion

3.1. Assay characteristics

A representative chromatogram of SAH and SAM standards is presented in Fig. 1A, a chromatogram of mouse liver extract is presented in Fig. 1B and chromatograms of mouse liver extract spiked with 550 pmol SAM or SAH are presented in Fig. 1C and D, respectively. The retention times for SAH and SAM were 16 and 19 min, respectively. The chromatograms of mouse liver extracts do not contain any interfering peaks. The simultaneous determination of SAH and SAM has been reported in the literature [7–9,11–17]. However, under those conditions the chromatographic peaks of SAH and SAM were not completely separated and peak waves were wider than 1.5 and 2.0 min for SAH and SAM, respectively [17]. Therefore, we used a PartiSphere C_{18} reversed-phase analytical column and a gradient mobile phase to improve the separation. As depicted in Fig. 1A–D, this improvement in the HPLC offered rapid resolution and complete separation of SAH and SAM. The peak widths were 0.7 and 0.8 min for SAH and SAM, respectively, and thus the peaks were narrower and sharper than reported by She et al. [17].

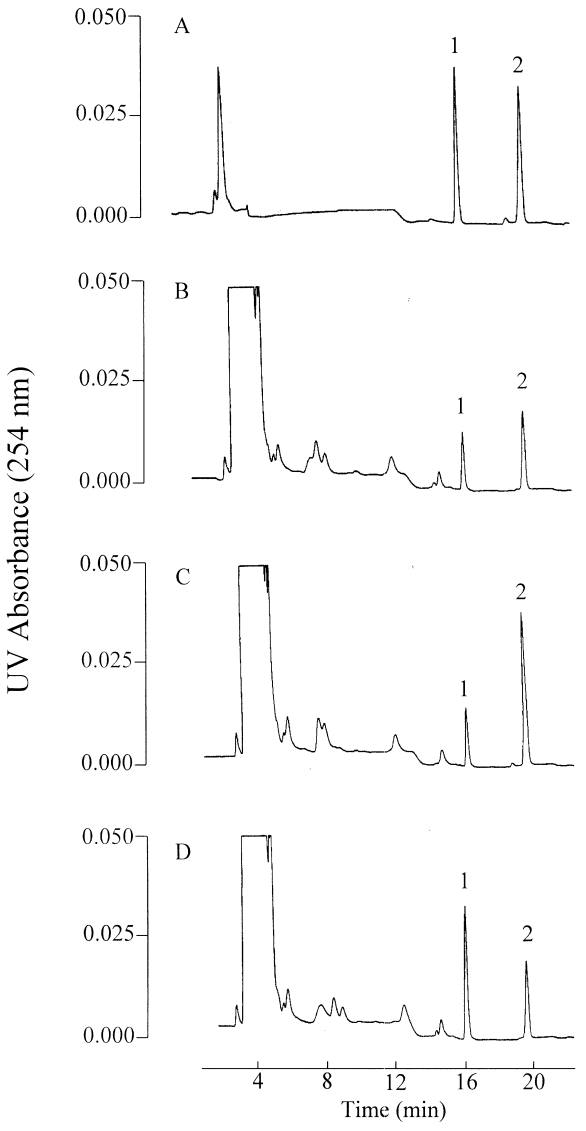

Fig. 1. (A) Chromatogram of a 25-μl injection of standards containing 1000 pmol SAH and 1100 pmol SAM. (B) Chromatogram of a 25-μl injection of control mouse liver extract. (C) Chromatogram of the control mouse liver extract spiked with 550 pmol of SAM. (D) Chromatogram of the control mouse liver extract spiked with 550 pmol of SAH. Peaks: 1=SAH, 2=SAM.

3.2. Limits of quantification, calibration curves and recoveries

The limit of detection was 22 and 20 pmol/25-μl injection for SAM and SAH, respectively. The quantitation limits were 55 and 50 pmol/25-μl for SAM and SAH, respectively. These quantitation limits had S/N ratios of approximately 12.5. The level of SAM and SAH in mouse liver was 111.9 ± 4.7 and 50.5 ± 2.7 nmol/g liver (mean$\pm$SD, $n=25$), respectively. The levels of SAM and SAH are the average of all the time points during 24 h in control mice (Fig. 2). Thus, it is possible to analyze the amount of SAM and SAH in a 1 mg sample of liver.

The calibration curves for SAM and SAH were prepared by dissolving them in water at a concentration of 1 mM and then diluting them with 0.4 M $HClO_4$ to the final concentrations used for HPLC analysis. The equations of the calibration curves were $y = 604x + 27\,129$ ($r^2 = 0.99952$) and $y = 611x + 20\,792$ ($r^2 = 0.99954$) for SAM and SAH, respectively, where x represents the analyte concentration in pmol and y represents the corresponding peak area. The calibration curve for SAM was linear over the concentration range 50–11 000

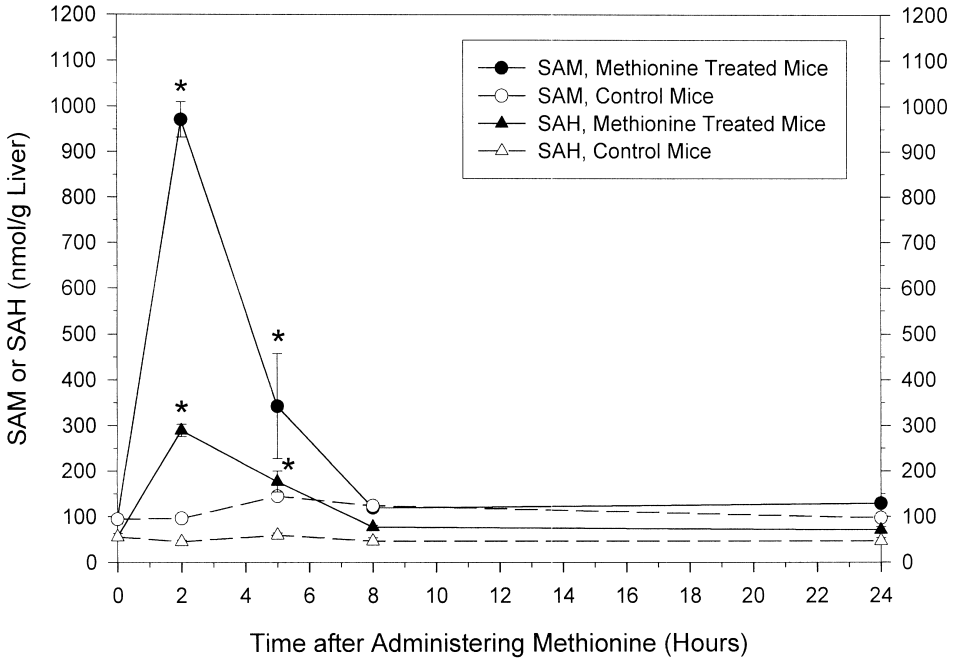

Fig. 2. Effect of methionine on the concentrations of SAH and SAM in mouse liver. The mice were administered by intraperitoneal injection 450 mg/kg methionine and were euthanized 0, 2, 5, 8 and 24 h later. The liver was homogenized in four volumes of 0.4 *M* $HClO_4$. A 25-μl aliquot of the acid extract was applied directly onto the HPLC. Results are mean±SE for five or six mice. *Significant difference from control mice by ANOVA followed by the Tukey test ($P<0.05$).

pmol/25-μl aliquot, and for SAH the curve was linear over the range 50–10 000 pmol/25-μl aliquot. Since the 25-μl aliquot contained the extract from 5 mg of liver, the linear ranges were 10–2200 and 10–2000 pmol/mg liver for SAM and SAH, respectively. Thus, the concentration of SAM and SAH was expressed as

$$\text{nmol/g liver} = x \text{ pmol/25 μl} \times \text{total Volume of homogenate (μl)/liver weight (g)} \div 1000$$

The recovery of SAH and SAM from tissue was determined by adding known amounts of their standards to an aliquot of mouse liver homogenate. The spiked homogenates were then extracted and chromatographed. The recovery of SAH and SAM was 98.8±8.4 and 99.4±3.6%, respectively, for concentrations ranging between 50 and 11 000 pmol/25 μl. This indicated that the recovery of SAH and SAM from the homogenate was almost complete. Furthermore, it indicated that both compounds were stable throughout sample preparation and chromatography. Therefore, an internal standard and correction for recovery would appear not to be needed when determining the concentration of SAH and SAM in liver tissue.

3.3. Intra- and inter-assay variations

Table 1 shows the intra-day precision and accuracy for SAH and SAM. The relative standard deviation (RSD) was 1.4–6.4% and the accuracy was 99.9–100.0% in the range 100–10 000 pmol SAH ($n=5$). The RSD was 2.0–6.7% and the accuracy was 100.0–100.1% in the range 110–11 000 pmol SAM ($n=5$). Table 2 shows the inter-day precision and accuracy for SAH and SAM. The RSD was 4.6–5.9% and the accuracy was 99.9–100.6% in the range 100–10 000 pmol SAH ($n=5$). The RSD was 5.2–7.0% and the accuracy was 99.5–100.1% in the range 110–11 000 pmol SAM ($n=5$).

The data suggest that this HPLC method is very consistent, accurate and reliable. Furthermore, SAH

Table 1
Intra-day precision and accuracy for SAH and SAM ($n=5$)

SAH				SAM			
Theoretical conc. (pmol)	Mean conc. found (pmol)	RSD (%)	Accuracy (%)	Theoretical conc. (pmol)	Mean conc. found (pmol)	RSD (%)	Accuracy (%)
100	100	1.4	100.0	110	110	2.0	100.0
1000	1000	4.6	100.0	1100	1101	4.8	100.1
10 000	9997	6.4	99.9	11 000	11 000	6.7	100.0

and SAM were found to be stable in the $HClO_4$ extract for at least 5 days when stored at $-20°C$.

3.4. Applications

This HPLC method was used to study the effect of methionine on the concentrations of SAM and SAH in mouse liver. Mice were administered by intraperitoneal injection a single dose of 450 mg/kg methionine and the concentrations in the liver of SAM and SAH were monitored 0, 2, 5, 8 and 24 h later. Within 2 h after administering methionine, the concentrations of SAM and SAH in the liver were increased by 10-fold (Fig. 2). Their concentrations in the liver then rapidly decreased so that by 8 h they were back at control levels. Thus methionine was promptly converted to SAM and rapidly metabolized to SAH. Thus, the metabolism of methionine to SAM should be considered in the supplementation of methionine to maintain SAM levels in the body.

The method has been used to determine the level of SAM and SAH in mouse kidney and lung. This was done in order to determine whether the method would be sensitive enough to analyze the affect of toxic chemicals on SAM and SAH levels in these organs and the ability of methionine to prevent the alterations in their levels. The concentrations of SAM and SAH were 62.4 ± 4.5 and 21.6 ± 3.8 nmol/g wet weight (mean$\pm$SD, $n=10$) in the kidney and 27.6 ± 2.5 and 12.7 ± 5.3 nmol/g wet weight (mean$\pm$SD, $n=10$) in the lung of untreated mice, respectively. Thus, the method was sensitive enough to determine the concentrations of SAM and SAH in the lung where their concentrations were only 25% of that in the liver.

4. Conclusions

An improved method using reversed-phase HPLC in gradient mode with ultraviolet detection (254 nm) was developed for the simultaneous determination of SAM and SAH in mouse tissues, including the liver, kidney and lung. The HPLC method demonstrated significantly better separation, detection limits and linear ranges for both compounds when compared to previously published procedures. Thus, the method makes it possible to more accurately determine SAM and SAH levels in tissue. The reliability and applicability of the method to determine SAM and SAH concentrations in tissue was demonstrated by the determination of the effect of administering

Table 2
Inter-day precision and accuracy for SAH and SAM ($n=5$)

SAH				SAM			
Theoretical conc. (pmol)	Mean conc. found (pmol)	RSD (%)	Accuracy (%)	Theoretical conc. (pmol)	Mean conc. found (pmol)	RSD (%)	Accuracy (%)
100	100.5	5.4	100.6	110	110.0	7.0	99.9
1000	999.8	5.9	99.9	1100	1100.0	5.2	100.1
10 000	9992.5	4.6	99.9	11 000	10 952.8	6.7	99.5

methionine to mice on the concentrations of SAM and SAH in the liver. Methionine increased both their concentrations in the liver by 10-fold. The results also demonstrated that methionine is rapidly converted to SAM and metabolized to SAH.

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