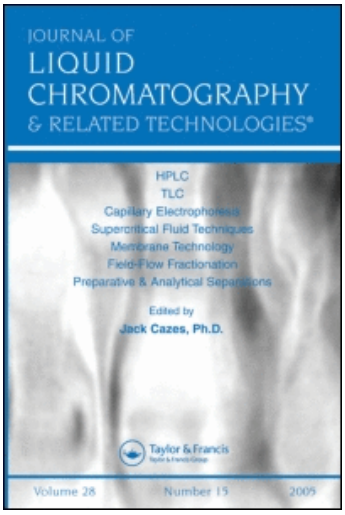

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Analytical Procedure for Determination of S-Adenosylmethionine, S-Adenosyl-Homocysteine, and S-Adenosylethionine in Same Isocratic HPLC Run, with a Procedure for Preparation and Analysis of the Analog S-Adenosylhomocysteine Sulfoxide

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ANALYTICAL PROCEDURE FOR DETERMINATION OF S-ADENOSYLMETHIONINE, S-ADENOSYL-HOMOCYSTEINE, AND S-ADENOSYLETHIONINE IN SAME ISOCRATIC HPLC RUN, WITH A PROCEDURE FOR PREPARATION AND ANALYSIS OF THE ANALOG S-ADENOSYLHOMOCYSTEINE SULFOXIDE

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ABSTRACT

Virtually all methyltransferase enzymes are regulated largely by the relative levels of S-adenosylmethionine (SAM) to its metabolic product, S-adenosylhomocysteine (SAH). Ethionine is the hepatocarcinogenic antimetabolite of methionine, and has been found to produce hypomethylation of hepatic DNA when fed to rats in acute doses. The hypomethylation apparently results from the accumulation in the liver of S-adenosylethionine (SAE), the sulfur activation product of ethionine, which is a competitive inhibitor of DNA methylase. Researchers seeking to measure SAM and SAH levels by HPLC in the past have experienced numerous analytical problems because of their separation characteristics. Previous methods have either required two separate HPLC runs or used gradient elution to measure the two compounds. The method outlined here, is an accurate and precise method, that measures SAM and SAH as well as SAE in a single isocratic HPLC run. S-Adenosyl-l-homocysteine sulfoxide

(SAHO), the sulfoxide of SAH is known to be formed by spontaneous oxidation of SAH during sample preparation and storage. We have prepared the SAHO using a 4 hour process. Since SAHO is not readily available commercially, the present method could be very beneficial to researchers who need to verify whether SAH oxidation has occurred in analytical samples, or whether oxidation of the SAH has occurred in tissues.

INTRODUCTION

S-adenosylmethionine (SAM), the substrate of DNA methylase, is a major methyl group donor used in all tissues by all methyltransferase enzymes except the enzymes that synthesize methionine. These enzymes are regulated largely by the relative levels of SAM to its metabolic product, S-adenosylhomocysteine (SAH), a DNA methylase inhibitor (1). A decrease in the SAM/SAH ratio, either by reduction in SAM or by increase in SAH, can inhibit the activity of methyltransferase enzymes (2). Studies with rats and mice fed either amino acid-defined diets lacking methionine and choline or choline-devoid diets containing proteins with low methionine content (3-8), have shown decreased levels of hepatic SAM (9,10) and suppressed level of hepatic DNA methylation (14). These methyl-deficient diets have also caused increased hepatic levels of SAH, further decreasing SAM availability (10). Further, the chronic administration of these methyl-deficient diets has been shown to produce liver cancer in male F344 rats and B6C3F1 mice (3-8). Studies have shown that in rats the hepatic contents of SAM and SAH are more dramatically altered by chronic dietary methyl deprivation than are SAM and SAH levels in other tissues (10). Such results indicate that levels of DNA methylation are subject to dietary manipulation and support the postulated relationship between methyl deficiency and hepatocarcinogenicity (5,6 and 15-19).

In eukaryotic cells, the extent and patterns of DNA methylation appear to regulate the expression of genetic information (20-22). Any circumstance that causes a decrease in the SAM/SAH ratio results in inhibition of DNA methylation. Thus, it has been suggested that resulting alterations in gene expression might induce tumor formation.

Physiological methyl insufficiency may also play a causative role, at least in part, in a variety of toxic and pathologic endpoints produced by a multiplicity of circumstances. Recently, there has been increasing interest in this area of research, as evidenced by the literature and reports at scientific meetings. Thus, convenient methodologies for monitoring such studies are necessary.

Ethionine is the hepatocarcinogenic antimetabolite of methionine and has been found to produce hypomethylation of hepatic DNA when fed to rats in acute doses (11). The hypomethylation apparently results from the accumulation in the liver of S-adenosylethionine (SAE), the sulfur activation product of ethionine, which is a competitive inhibitor of DNA methylase (11-13). Thus, ethionine is a convenient material for use in hypomethylation studies. Researchers are often interested in determining SAM and SAH and sometimes SAE in the same sample. Therefore, it would be most convenient to determine the levels of all of the compounds in the same HPLC run.

Researchers seeking to measure SAM and SAH levels by HPLC are aware that the compounds are rather unique and cause special analytical problems because of their separation characteristics. For example, HPLC retention times are greatly affected by small pH changes. Molloy *et al.* (23) have reported an HPLC method that measured SAM and SAH in the same run, but their method used a gradient. The method outlined here accomplishes the same results using only one buffer and can be used to measure SAE as well. The stable conditions of isocratic chromatography have the advantages of being more convenient and efficient as well as giving more accurate quantification than a gradient, since the baseline of a chromatogram from a gradient usually drifts, mechanical errors such as pump variations are eliminated, and the HPLC does not have to return to initial conditions and equilibrate between each sample.

S-Adenosyl-l-homocysteine sulfoxide (SAHO), the sulfoxide of SAH, is known to be formed by spontaneous oxidation of SAH during sample preparation and storage. Duerre *et al.* (24) developed a method for preparation and characterization of SAHO from SAH. The method they reported required a 10

to 12 hour waiting period, and the addition of crystalline beef liver catalase. Their characterization used paper chromatography and ultraviolet light absorption. We have prepared the SAHO using a 4 hour process, without addition of catalase. Since SAHO is not readily available commercially, the present method could be very beneficial to researchers who need to verify whether SAH oxidation has occurred in analytical samples, or whether oxidation of the SAH has occurred in tissues.

MATERIALS AND METHODS

Extraction Procedure

Approximately 0.7 g (0.4-1.0 g) of tissue was weighed immediately after sacrifice, diced, and transferred to a 10 ml culture tube. The SAM, SAH and SAE were extracted from the tissue using the method of Shivapurkar and Poirier (10), with modifications. A volume of 0.1 M sodium acetate, pH 6.0, (ice cold) equal to two times the weight of the tissue was added. The resulting solution can be stored in ice up to 1 hour while other samples are being prepared. The samples were then homogenized with a Tissuemizer (Tekmar, Cincinnati, OH) at setting of 45 for 30 (3 x 10) seconds (samples must be kept cold). The protein was precipitated using 40% (w/v) trichloroacetic acid (a volume 1.5 times the original weight of the tissue). The solution was mixed well on vortex and placed in ice for 30 minutes. To remove the precipitated protein, the tubes were spun in a CRU-5000 centrifuge (International Equipment Co., Needham Heights, MA), maintained at 5°C, at 1000 x g for 10 minutes. The supernatant containing SAM, SAH, and SAE was decanted into a conical shape glass centrifuge tube and the volume was recorded. If necessary the procedure could be interrupted at this point. Supernatant should be stored at −70°C until assayed and should not be stored more than one week. An equal volume of ice-cold peroxide-free diethyl ether was added (to adjust to pH to 3.3). The ether must be peroxide-free (1 ppm or less), or the SAH will be oxidized rapidly to form SAHO. The tubes were

vortexed for 20 seconds and then were spun in a centrifuge to accomplish separation of the phases. The unwanted top layer was aspirated off, using a pasteur pipette attached to vacuum. Any solid matter at the interface was also removed when possible. The extraction steps were repeated once. The samples were filtered through a millipore filter (0.45 μm). The samples were then ready for HPLC analysis for SAM, SAH and SAE. Samples should be kept cold at all times until analyzed and should be injected onto the HPLC within 24 hours in order to minimize decomposition of the SAM and SAH content.

HPLC Analysis

Twenty (20) μl (or an appropriate volume) of the extracted samples were injected onto a Hewlett-Packard 1090 HPLC (Wilmington, DE), equipped with a Beckman Ultrasphere ODS, 4.5 mm x 25 cm (5μm particle size) column (Fullerton, CA) maintained at 25.5°C using a column heater, a pre-column filter (#C-751, ChromTech, Apple Valley, MN), and a Hewlett-Packard photodiode array detector operated at 260 nm. The mobile phase was 50 mM NaH_2PO_4, 10 mM Heptanesulfonic acid (sodium salt, 1-hydrate, HPLC grade, Eastman Kodak, Rochester, NY), 20% methanol adjusted to pH 4.38 with phosphoric acid, with a flow rate of 0.9 ml/min. The mobile phase was degassed for at least 1 hour before the pumps were started. The column was equilibrated for 1 hour before injections were started.

Tissue concentration calculation

External analytical standards of SAM (#102407, Boehringer Mannheim, Indianapolis, IN), SAH (#102393, Boehringer Mannheim, Indianapolis, IN), and SAE (#A-2758, Sigma, St. Louis, MO) were dissolved in 0.001 N HCl. A series of concentrations of each compound was injected onto the HPLC and concentration response curves were constructed. Standards of SAM, SAH, and SAE were injected at concentration ranges of 1-10 ng/μl.

The following calculation was used to determine the concentration of SAM, SAH or SAE in tissue samples:

$$\frac{\mu g}{g \text{ tissue}} = \frac{\text{Area of peak}}{\text{slope}} \times \frac{\text{total vol. (ml)}}{\text{sample wt (g)}}$$

Preparation of S-adenosyl-l-homocysteine sulfoxide (SAHO)

Using the analytical method outlined above, we discovered that over a period of one week the SAH in both the standards and samples may be completely converted to another product by the presence of even a minute quantity of a strong oxidizing substance, such as peroxide (>1ppm). The HPLC peak for the SAH gradually diminished as a new peak appeared at an earlier retention time and increased nearly proportional in area. The new product was identified to be SAHO using Mass Spectoral Analysis by Fast Atom Bombardment (FAB) and NMR.

Duerre *et al.* (24) reported a procedure for preparation of the SAHO in 1970; we have developed a faster and simpler procedure to produce the chemical in pure form. SAH was oxidized with hydrogen peroxide (H_2O_2) (#H 341-500 Fisher, Pittsburgh, PA) to produce SAHO. SAH standard, 20 mg, was placed in a test tube with 1 ml of methanol. The SAH did not dissolve readily in methanol, leaving some of the solid material on the bottom of the test tube. A 300 μl volume of 50% H_2O_2 was added, and the tube was vortexed, and sonicated briefly. As the H_2O_2 reacted, the SAH was dissolved. After the solution was allowed to stand at room temperature for $3^1/_2$ hours, a 5 μl aliquot was diluted with 200 μl of water and injected onto the HPLC to confirm that all of the SAH had been converted to the new product. When the SAH peak had disappeared, the resulting product was precipitated using 5 ml of cold (-20°C) absolute ethanol (it was necessary that the ethanol be very dry or the product which is very soluble in water would redissolve). The test tube was allowed to stand in the freezer for about 30 minutes and spun in a CRU-5000 Centrifuge at 1000 x g for 10 minutes

at 5°C. The ethanol which contained the H_2O_2 was decanted off. The product in the bottom of the test tube was rinsed with an additional 5 ml of cold absolute ethanol, vortexed and centrifuged. The ethanol was poured off and the precipitate was dried in the Speed Vac (Savant, Farmingdale, NY). The final product, which was a dry white solid, was stored in the freezer over a desiccant. The yield was about 90% by weight. A small amount of the material was dissolved in water for injection onto the HPLC. Injections were made onto HPLC using two different mobile phases to obtain two separate chromatograms. One of the mobile phases was the one described above, used to separate SAM, SAH and SAE. A single peak with a retention time of 3.4-3.6 minutes (Figure 1A) was obtained, compared to a 5.4-5.6 minutes (Figure 1B) retention time for SAH. The second mobile phase used was 15% methanol in water which also showed a single peak, this time with a retention time of 4.6 minutes, compared to 8.1 minutes for SAH. The identity of the product (SAHO) and the SAH were confirmed by NMR and FAB.

RESULTS AND DISCUSSION

We have developed a precise and accurate HPLC method for measuring SAM, SAH, and SAE in the same run. The advantage of the new method was that we could measure all three compounds in only 15 minutes (Figure 2). The retention times were as follows:

Compound	Standards	Samples
SAH	~ 5.5 min.	~ 5.5 min.
SAM*	~ 9.5 min.	~ 10.5 min.
SAE*	~ 10.5 min.	~ 11 min.

*The differences in retention times between samples and neat standards were due to pH differences of the material injected. Standards taken through the reaction process, have the same RT as the samples. (Figures 2 A-D).

We compared our previous method of measuring SAH on the HPLC, using 0.05M KH_2PO_4 in 8% methanol (14), with an approximate retention time

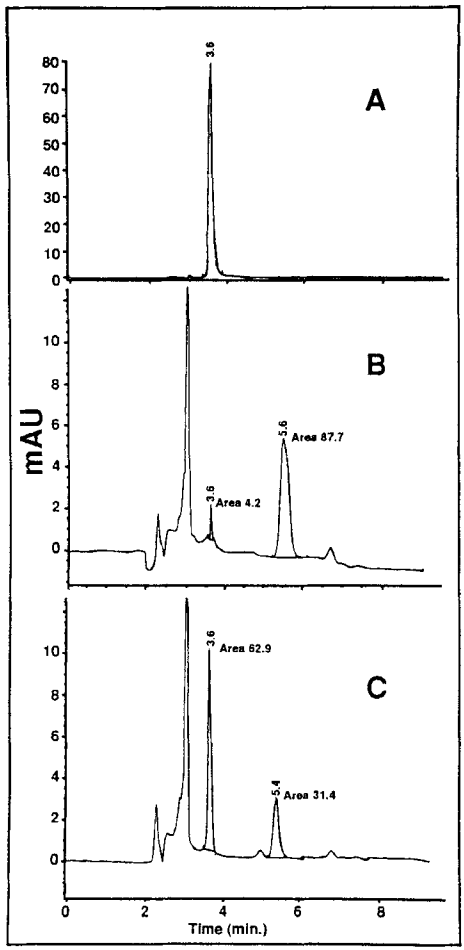

FIGURE 1

HPLC of: A. Purified oxidation product of SAH, identified to be SAHO by NMR and FAB, B. Standard SAH (3.0 ng/μl) treated as a sample using ether containing over 1 ppm. peroxide, and C. Sample B. run again 7 days later. SAH peak has diminished, and SAHO peak has grown proportionally over a period of time.

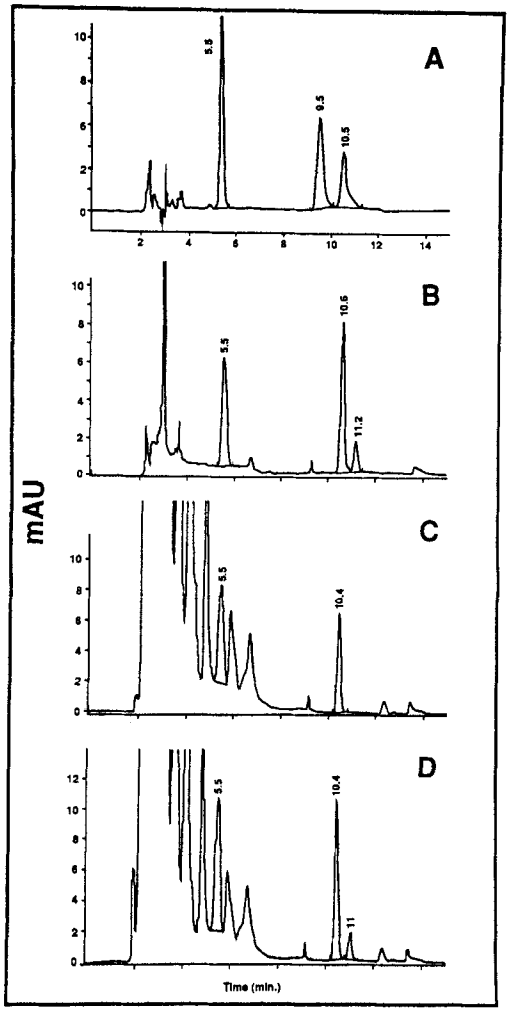

FIGURE 2

Isocratic separation using the present HPLC method of: A. Standards of 3 ng/μl SAH (5.4 min.), SAM (9.5 min.) and 1.5 ng/μl SAE (10.5 min.) in 1 x 10^{-4} N HCl, B. Standards of 3 ng/μl SAH (5.5 min.), SAM (10.6 min.) and 0.75 ng/μl SAE (11.2 min.), processed as samples, C. Rat liver sample, SAH (5.5 min.) and SAM (10.4 min.). (Normal rat liver does not contain SAE.), IID. Rat liver sample with standards of 1.5 ng/μl SAH (5.5 min.), SAM (10.4 min.) and 0.75 ng/μl SAE (11.0) added.

of 8.5 minutes and a total run time of 25 minutes with our new method. Both methods measured the SAH accurately and precisely when we ran the same sample by the two different methods.

Using the present method we measured the amount of SAM and SAH in rat liver tissue (Figure 2C). We ran the same extract sample again after small amounts of the standards SAM, SAH and SAE had been added (Figure 2D). The size of the SAM and SAH peaks increased proportionately to the amount of standard added, and the SAE peak appeared. Since normal rat liver does not contain SAE, it will only be found in the liver of treated rats. We have used this method in our laboratory to measure SAM and SAH in various tissue, including liver, spleen, brain, kidneys and in whole rat embryos. We have been able to measure amounts as small as 2.5 μg per gram of tissue, and have found that SAH was not present at detectable levels in some tissue, such as some rat brain samples. This lack of SAH in the rat brain tissue was confirmed by running the samples again using our previous method of measuring SAH.

Experiments have shown accurate pH and ionic concentrations of the mobile phase to be very important. Higher pH gave shorter retention times while higher concentration of NaH_2PO_4 moved SAM and SAE closer to the SAH. Lower methanol content moved SAM and SAE together. These facts are useful if one of the peaks needs to be moved to avoid interfering peaks.

Using the HPLC method describe here, we have shown that samples should be analyzed quickly to avoid oxidation of SAH to SAHO. We have described a procedure for preparing SAHO from SAH in just four hours with a yield of approximately 90% by weight. Samples of the product and the SAH were analyzed by NMR. The hydrogens on the carbons adjacent to the sulfur atom showed the greatest shift, as would be expected if the sulfur was bonded to an oxygen atom. The NMR indicated nearly equal amounts of 2 very similar products, possibly the two stereoisomers of the sulfur atom being made asymmetric by oxidation. A sample was subjected to FAB to determine whether there was 1 or 2 oxygens attached to the sulfur atom. The molecular weight of

the product was found to be 400, which is the molecular weight of the SAHO ($C_{14}H_{20}N_6O_6S$). The product was determined to be 99% pure. The melting point of the solid material was 185-187°, where it decomposed to a brown product. This agrees with reports in the literature which states the SAHO decomposes at 185-188° (25).

Experiments were also performed to determine whether it would be possible to identify SAHO if it were present in a liver sample using the HPLC method described above. We were able to find a peak in liver samples with a retention time that corresponded with SAHO. The peak area increased in size when SAHO was added to the sample. It was also determined that when SAH was added to a sample that had been extracted with ether containing >1 ppm peroxide, and aliquots injected onto the HPLC over a period of 2 days, the SAH peak decreased and the SAHO peak increased. This indicated the importance of analyzing the prepared samples as soon as possible, since strong oxidants in the samples may cause errors in measuring the SAH. When samples extracted with ether containing >1ppm peroxide were stored in the refrigerator for more than 2 months, the SAH peak completely disappeared.

As researchers become more and more interested in studying DNA methylation, it is important that methods are available to accurately measure the various components used in the methylation process. The method described above, is an accurate and precise method, that has the advantages of measuring three compounds SAM, SAH and SAE.

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