A SIMPLE HPLC METHOD FOR THE DETERMINATION OF S-ADENOSYLMETHIONINE AND S-ADENOSYLMETHIONINE IN RAT TISSUES: THE EFFECT OF VITAMIN B6 DEFICIENCY ON THESE CONCENTRATIONS IN RAT LIVER

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SUMMARY: A simple method using isocratic HPLC with ultraviolet detection was established for the simultaneous measurement of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) in rat tissues. The method provides rapid resolution of both compounds in a single run by direct injection of the perchloric acid extract of tissue, so that sampling procedures and analytical errors can be reduced in determining the SAM/SAH ratio, a measure of transmethylation reactions. The assay has a detection limit of 25 pmol and is linear in the range 50-2000 pmol. This procedure was applied to determine the change of SAM/SAH ratio in liver caused by vitamin B6 deficiency. The marked reduction of the SAM/SAH ratios in vitamin B6-deficient rats were due to a drastic rise in SAH concentration with a concurrent striking decrease in SAM concentration. © 1994 Academic Press, Inc.

S-Adenosylmethionine (SAM) is the most widely recognized methyl donor in a number of transmethylation reactions involving nucleic acids, proteins, phospholipids, catecholamines and other biogenic amines (1). The demethylated product, S-adenosylhomocysteine (SAH), regulates transmethylation by its strong inhibition of many SAM-dependent methyltransferases (2-6), including phosphatidylethanolamine methyltransferase as described previously (7). It has become clear that obtaining the ratio of intracellular SAM/SAH is important for a more meaningful evaluation of the methylation capacity of cells. In mammalian liver, SAH is reversibly catalyzed by SAH hydrolase (EC 3.3.1.1) to adenosine and L-homocysteine (8). The further catalysis of L-homocysteine to cysteine is catalyzed by two pyridoxal 5'-phosphate (PLP)-dependent

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Abbreviations: HPLC, high-performance liquid chromatography; PLP, pyridoxal 5'-phosphate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.
enzymes: cystathionine β-synthase (EC 4.2.1.22) and γ-cystathionase (EC 4.4.1.1) (9). Consequently, the concentrations of adenosine and L-homocysteine directly influenced SAH concentration and transmethylation (10). An increase in plasma L-homocysteine concentration has been reported in dietary deficiency of vitamin B6 (11).

Various methods for the analysis of SAM and SAH in tissues have been described, but they involve either separate analysis by radiochemical methods (12,13), separation by HPLC using gradient elution (14,15) or a complex and tedious procedures for sample preparation prior to HPLC analysis (16-18). More recently, a significant improvement in the analysis of these compounds was reported by Guattari (19). However, it was necessary for the tissue extract to be incubated in water at 40°C for 90 min before HPLC analysis. In addition, the analysis of these compounds in liver have not been presented.

This paper reports a simple HPLC-UV isocratic method for the determination of SAM and SAH in rat liver with minimal sample handling. It is also suitable for a wide variety of tissue types including brain, kidney and adrenal. The effective utilization of the method is demonstrated by a study of the effects of vitamin B6 deficiency on SAM and SAH levels in rat liver.

**MATERIALS AND METHODS**

**Chemicals:** SAM iodide salt, SAH and 1-heptanesulfonic acid sodium salt were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NH₄H₂PO₄, HClO₄ solution (70% saturation), methanol (HPLC grade) and all the other chemicals were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

**HPLC system:** A Hitachi Model L-6000 pump (Tokyo, Japan) equipped with a Model 7161 injector (Rheodyne Co., Cotati, California, U.S.A.), a Hitachi Model 638 Variable Wavelength UV Monitor operating at 254 nm, a Shimadzu Model C-R5A Chromatopac (Kyoto, Japan) and a TSKgel ODS-80TM column (25 cm × 4.6 mm I.D., Tosoh Co., Tokyo, Japan) were used. The mobile phase consisted of 40 mM NH₄H₂PO₄, 8 mM 1-heptanesulfonic acid sodium salt and 18% (v/v) methanol; the pH was adjusted to 3.0 by addition of HCl, and then the mobile phase was filtered through a 0.45-μm membrane filter (Toyo Roshi Co., Tokyo, Japan) and degassed under vacuum. The isocratic elution was carried out using a flow-rate of 1 mL/min at 35°C. During HPLC analysis, SAM and SAH in biological samples were identified according to their retention times and co-chromatography with authentic standards.

**Standards:** SAM and SAH were dissolved in water at a concentration of 1 mM and then diluted with 0.4 M HClO₄ to the final concentration used during HPLC analysis. Aliquots of these standard solutions (10-100 μL) were injected to a column.

**Sample preparation:** All procedures of sample preparation prior to chromatography were done at 0-4°C. Rat tissues were weighed, homogenized with an HG 30 homogenizer (Hitachi Co., Tokyo, Japan) in 5, 2, 2 and 10 volumes of 0.4 M HClO₄ for liver, brain, kidney and adrenal, respectively. After centrifugation at 10,000×g for 20 min, each supernatant was filtered through a Millipore membrane (0.45 μm). Aliquots of the acid extracts (25-100 μL) were directly applied to HPLC analysis.

**Recovery experiments:** Known amounts of SAM and SAH standards were added to an aliquot of the homogenate from rat liver. The samples were treated and chromatographed as described above. The recoveries of SAM and SAH were determined by subtracting the values obtained from the original homogenates.
Animals and diets: Male Wistar rats weighing 80-90 g were used for studying the effect of vitamin B6 deficiency on SAM and SAH levels in liver. The method of feeding and the composition of the diet were the same as described previously (20). At the end of experimental feeding, the blood was drawn from the heart under pentobarbital anesthesia (Nembutal, Dainabot Co., Osaka, Japan). The liver was then immediately excised, perfused, and used for SAM and SAH determination as described above.

Statistics: The statistical significance among means was estimated at $p<0.05$ according to standard ANOVA and Duncan's multiple-range test, using a PC-9801 personal computer (Nippon Electric Co., Tokyo, Japan) (21).

RESULTS

HPLC elution profiles. Careful selection of the ionic strength of the buffer, concentration of the ion-pair reagent, 1-heptanesulphonic acid, amount of methanol and the pH of the mobile phase was found to be effective for the separation of SAM and SAH. Under the conditions, the retention times for SAH and SAM were 15.7 min and 17.8 min, respectively (Fig. 1A). Fig. 1B shows a chromatogram of rat liver extract with peaks corresponding to the elution position of SAM and SAH standards, and the positions of the SAM and SAH peak were also confirmed by spiking the same sample with the authentic standards (Fig. 1C and 1D). The SAM and SAH were well resolved from numerous unknown compounds and all the UV-absorbing compounds were eluted within 20 min of injection. Similar chromatograms of extracts obtained from rat brain, kidney and adrenal are given in Fig. 2. It was clearly demonstrated that the method described here was satisfactory for analysis of SAM and SAH in rat tissues other than liver.

![Fig. 1](image-url)
Sensitivity and reproducibility. The limit of detection for the assay was estimated to be 25 pmol for both SAM and SAH. Calibration curves were based on peak area and linearity of response was obtained between 50 and 2000 pmol for SAM or SAH with correlation coefficients greater than 0.999 for each curve (Fig. 3). The wide linear range for SAM and SAH was over the range investigated in rat tissues. Excellent reproducibility of retention times and peak areas was always obtained. The mean coefficient of variation for five consecutive injections of standard solutions or tissue extracts was found to be about 2% for SAH and 3% for SAM.
Recovery and stability. The recoveries of SAM and SAH in homogenate of rat liver were found to be near 100%, which indicated that SAM and SAH were completely recovered and stable throughout the sample preparation as well as during chromatography steps. Therefore, no internal standard was added and no correction for recovery was performed in the determination of SAM and SAH concentrations. In addition, SAM and SAH were found to be stable in HClO₄ extract for at least 5 days if stored at -40 °C (data not shown).

Changes of SAM and SAH levels in rat liver induced by vitamin B₆ deficiency. The effect of a 5 week deficiency of vitamin B₆ on SAM and SAH concentrations in the liver of rats was monitored. Fig. 4 shows typical chromatograms of liver extracts obtained from the control, pair-fed control and vitamin B₆-deficient rats. The SAM peak in the vitamin B₆-deficient or pair-fed control rat was much smaller than the peak in the control rat, whereas the SAH peak greatly increased in the vitamin B₆-deficient rat than in the pair-fed control as well as in control rats. The changes of SAM and SAH concentrations in rat liver upon vitamin B₆ deficiency were confirmed to be similar as reported previously (7). These results are presented in Table 1. The concentration of SAM of the vitamin B₆-deficient group decreased about 1/3 of the control group, but not being significantly different from that of the pair-fed control group. On the other hand, the vitamin B₆-deficient group had a SAH concentration of about three fold higher than that for the pair-fed control or control group. Thus, the ratio of SAM/SAH dropped from 2.1 in the control group to 0.85 in the pair-fed control group or to 0.34 in the vitamin B₆-deficient group.

![Chromatograms](image-url)
Table 1. Concentrations of SAM and SAH in liver of rats fed on the vitamin B6-deficient or control diets for 5 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Pair-fed control</th>
<th>Vitamin B6-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>(nmol/g of liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAM</td>
<td>95.6 ± 11.4a</td>
<td>28.6 ± 2.6b</td>
<td>34.2 ± 4.6b</td>
</tr>
<tr>
<td>SAH</td>
<td>46.7 ± 2.4b</td>
<td>33.7 ± 5.1c</td>
<td>109.5 ± 25.1a</td>
</tr>
<tr>
<td>SAM/SAH ratio</td>
<td>2.10 ± 0.16a</td>
<td>0.85 ± 0.18b</td>
<td>0.34 ± 0.04c</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE for six rats in each group. 
abc Values without a common superscript letter in the same horizontal row are significantly different at p<0.05.

DISCUSSION

The present method described allows the simultaneous analysis of SAM and SAH in tissues using a simple isocratic HPLC system, and further, offers a rapid and excellent separation of both compounds with high sensitivity and reproducibility. One person can analyze 25 or more samples daily and less to 0.05 g of tissues are sufficient for analysis. This method has several advantages over previous methods (12-18). Those methods involve radiochemical analysis (12,13), gradient elutions (14,15) or a preparative clean-up procedure (16-18), which result in time-consuming or losses of SAM and SAH. The direct injection of tissue extract makes us promise minimal sample handling in this method. Thus, any stability and recovery errors encountered when using an extraction procedure prior to HPLC can be avoided. We have also determined the levels of SAM and SAH in tissues with the method as described by Guattari (19), who incubated the tissue extract in water at 40°C for 90 min before injection onto the column. We found that his method resulted in about 20% loss of SAM during the heating procedure comparing with our results (data not shown), presumably because of the degradation of SAM.

The in vivo chang of SAH level in rat liver induced by vitamin B6 deficiency is in agreement with that reported by Loo and Smith (22), who used a radiochemical method (12). However, they found that the SAM level in the vitamin B6-deficient rats was not significantly different from that of the control or pair-fed control rats. There might have been differences in methodology used for analyzing SAM, although the strain of rats, type of diet and length of deficiency were somewhat different from those used in the present experiment. The decreased SAM level of vitamin B6-deficient rat liver observed in the present study might be due to the reduced activity of methionine adenosyltransferase (EC 2.5.1.6) (23). Using the present method for the analysis of SAM and SAH, we have recently reported that, upon vitamin B6 deficiency, the marked reduction of the SAM/SAH ratio induced an effective inhibition of methylation of phosphatidylethanolamine to phosphatidylcholine in liver microsomes (7). We have also
demonstrated that the marked accumulation of liver SAH during vitamin B6 deficiency is obviously due to leading to the decreased activities of two PLP enzymes: cystathionine \( \beta \)-synthase and \( \gamma \)-cystathionase.

It becomes now possible in the present method to obtain an accurate determination of the SAM/SAH ratio in tissues since the clear resolution of both SAM and SAH is performed in a single chromatographic run without any losses of these compounds. This method is suitable for analysis of diet-related changes in tissue SAM and SAH. Furthermore, this analytical system can also be employed for the assay of enzymatic activities where SAM and SAH are involved as products, \textit{i.e.} methionine adenosyltransferase and SAH hydrolase. It is anticipated that this system will be of use in studying the many other problems involving transmethylation or methyl (C1) metabolism.

REFERENCES