Measurement of Plasma and Intracellular S-Adenosylmethionine and S-Adenosylhomocysteine Utilizing Coulometric Electrochemical Detection: Alterations with Plasma Homocysteine and Pyridoxal 5′-Phosphate Concentrations

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Background: The relative changes in plasma and intracellular concentrations of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) may be important predictors of cellular methylation potential and metabolic alterations associated with specific genetic polymorphisms and/or nutritional deficiencies. Because these metabolites are present in nanomolar concentrations in plasma, methods of detection generally require time-consuming precolumn processing or metabolite derivitization.

Methods: We used HPLC with coulometric electrochemical detection for the simultaneous measurement of SAM and SAH in 200 µL of plasma, 10⁶ lymphocytes, or 10 mg of tissue. Filtered trichloroacetic acid extracts were injected directly into the HPLC system without additional processing and were eluted isocratically.

Results: The limits of detection were 200 fmol/L for SAM and 40 fmol/L SAH. In plasma extracts, the interassay CV was 3.4–5.5% and the intraassay CV was 2.8–5.6%. The analytical recoveries were 96.8% and 97.3% for SAM and SAH, respectively. In a cohort of healthy adult women with mean total homocysteine concentrations of 7.3 µmol/L, the mean plasma value was 156 nmol/L for SAM and 20 nmol/L for SAH. In women with increased homocysteine concentrations (mean, 12.1 µmol/L), plasma SAH, but not SAM, was increased (P < 0.001), and plasma pyridoxal 5′-phosphate concentrations were reduced (P < 0.001). Plasma SAM/SAH ratios were inversely correlated with homocysteine concentrations (r = 0.73; P < 0.01), and the SAM/SAH ratio in plasma was directly correlated with the intracellular SAM/SAH ratio in lymphocytes (r = 0.70; P < 0.01).

Conclusions: Increased homocysteine in serum is associated with an increase in SAH and a decrease in the SAM/SAH ratio that could negatively affect cellular methylation potential. Accurate and sensitive detection of these essential metabolites in plasma and in specific tissues should provide new insights into the regulation of one-carbon metabolism under different nutritional and pathologic conditions.

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Chronic imbalance in the integrated pathways of one-carbon metabolism as a consequence of genetic aberrations and/or nutritional insufficiencies underlies the pathogenesis of many chronic human disease states (1–5). Heterozygous or homozygous mutations in genes coding for enzymes that participate directly or indirectly in one-carbon metabolism, such as methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase, or cystathionine β-synthase (CBS), can interact with inadequate dietary intake to exacerbate metabolic pathology (3, 6, 7). Gene-nutrient interactions that compromise normal one-carbon metabolism are associated with increased risk of cardiovascular disease (2), certain cancers (8), birth defects (9, 10), recurrent early pregnancy loss (11), central nervous system demyelination (12), and neuropsychiatric disease (13, 14). In many cases, appropriate nutritional intervention can normalize metabolic imbalance and re-

1 Nonstandard abbreviations: MTHFR, methylenetetrahydrofolate reductase; CBS, cystathionine β-synthase; EC, electrochemical; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; TCA, trichloroacetic acid; PLP, pyridoxal 5′-phosphate; and tHcy, total homocysteine.
tard the progression of pathology. Early diagnosis and preventive intervention strategies depend on the development of simple, sensitive, and reproducible methodologies to monitor plasma concentrations of homocysteine and the multiple thiols involved in homocysteine and methionine metabolism. For example, a new method utilizing HPLC with coulometric electrochemical (EC) detection for the simultaneous measurement of plasma methionine, homocysteine, cysteine, cystathionine, cysteinylglycine, and glutathione could aid in differential diagnoses and in the design of intervention strategies (15).

An abbreviated overview of one-carbon metabolism with emphasis on essential cellular methylation reactions is presented in Fig. 1. Methionine is converted to S-adenosylmethionine (SAM), the major intracellular methyl donor, by methionine adenosyltransferase (EC 2.5.1.6) and subsequently to S-adenosylhomocysteine (SAH) by a variety of cellular methyltransferases present in all cells. This one-way reaction is subject to competitive product inhibition by SAH because SAH has a higher affinity for the methyltransferase active site than does its precursor, SAM (17). The pathologic accumulation of SAH can lead to a decrease in the SAM/SAH ratio and inhibition of most cellular methyltransferases (17–19).

SAH is hydrolyzed to homocysteine and adenosine by SAH hydrolase (EC 3.3.1.1), a reversible reaction with thermodynamics that actually favor SAH synthesis (20, 21). Accumulation of SAH and the associated inhibition of cellular methyltransferases will therefore occur under metabolic conditions that interfere with product removal of homocysteine or adenosine (17, 22–25).

Although the regulatory role of SAM on the enzyme activities of MTHFR, CBS, and methionine synthase has been emphasized (21, 22, 26), the regulatory importance of SAH in the maintenance of balanced one-carbon metabolism may be underestimated. Under physiologic conditions, SAH concentrations generally are several-fold lower than SAM concentrations, and SAH concentrations in plasma have only recently become detectable with newer methods (27, 28). Alterations in cytosolic SAH have tissue-specific bioregulatory functions and have been reported to up-regulate CBS activity (18), decrease betaine-homocysteine methyltransferase activity (18), and decrease MTHFR activity (29). Inhibition of the several methyltransferase reactions by SAH would be expected to spare the substrate, SAM. Consistent with this notion, an increase in SAM with increased intracellular SAH has been observed in end stage renal patients (30) and in cultured mouse lymphoma cells (31). Homozygous mutations in the CBS gene (32) or the adenosine deaminase gene (33) lead to

Fig. 1. Overview of one-carbon metabolism with emphasis on the reversible SAH hydrolase reaction.

The hydrolysis of SAH is dependent on product removal of homocysteine and adenosine. In the absence of efficient product removal, SAH accumulation can inhibit methyltransferase reactions by high affinity binding to the enzyme active site. THF, tetrahydrofolate; 5CH₃THF, 5-methyltetrahydrofolate; DMG, dimethylglycine.
substantial increases in homocysteine and SAH without affecting methionine synthase activity. By contrast, drugs or nutritional deficiencies that negatively affect the methionine synthase reaction are generally associated with a decrease in SAM and an increase in homocysteine and SAH (34,35). The extent of reduction in SAM concentrations with folate or B12 deficiencies, however, does not approach the...

Although several HPLC methods exist for the simultaneous measurement of SAM and SAH, most of these methods rely on precolumn derivatization and ultraviolet detection, which are more time-consuming and less sensitive than direct injection using coulometric EC detection (36–39). In addition, these methods often require the use of internal standards to correct for sample losses during preparative procedures (27). In this report, we present a new method in which neutralized trichloroacetic acid (TCA) extracts are injected directly into the HPLC system without any further manipulation. SAM and SAH are separated by isocratic elution and coulometric EC detection to provide femtomolar detection limits with exceptional reproducibility and recovery.

**Materials and Methods**

**REAGENTS**

SAM, SAH, TCA, sodium phosphate monobasic, monohydrate, and 1-heptanesulfonic acid were obtained from Sigma. HPLC grade methanol was purchased from J.T. Baker. Deionized HPLC-grade water was prepared by passage through a Sybron/Barusted NANOpure II filtration system and subsequent passage through a C18 Sep-Pak cartridges (Millipore).

**SUBJECTS AND SAMPLE PREPARATION**

Subjects were 58 healthy adult females with a mean age of 37.2 years (range, 19–53 years) who had participated in a previous clinical trial (40). Fasted blood samples were collected into EDTA Vacutainer Tubes, chilled immediately in an ice-water bath, and centrifuged at 4000 g for 15 min at 4 °C. Aliquots of the plasma layer were transferred into multiple cryostat tubes and stored at −20 °C until analysis. Separate aliquots were thawed for determination of plasma homocysteine, pyridoxal 5'-phosphate (PLP), SAM, and SAH. In a subset of women, mononuclear cells were isolated by carefully layering whole blood onto an equal volume of Histopaque®-1077 (Sigma Diagnostics) and centrifuged at 400 g for 30 min. Mononuclear cells were recovered from the interface and washed several times as described by the manufacturer. Samples of liver tissue (10–15 mg wet weight) or isolated lymphocytes (106 cells) were homogenized with 200 μL of phosphate-buffered saline. To precipitate protein, 40 μL of 400 g/L TCA was added to 200 μL of plasma or cell extract, mixed well, and incubated on ice for 30 min. After centrifugation for 15 min at 18 000g at 4 °C, supernatants were filtered through a 0.2 μm filter, and 20 μL was injected into the HPLC system.

**CHROMATOGRAPHY**

Separation of SAM and SAH in plasma and cell extracts was accomplished by HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reversed-phase C18 column (5 μm bead size; 4.6 × 150 mm; MCM) obtained from ESA. Isocratic elution with a mobile phase consisting of 50 mmol/L sodium phosphate monobasic, monohydrate; 10 mmol/L heptanesulfonic acid; and 75 mL/L methanol adjusted to pH 3.4 with concentrated phosphoric acid, was performed at ambient temperature at a flow rate of 1.0 mL/min and a pressure of 100–110 kfg/cm² (1500–1800 psi). Extracts were injected directly onto the column using a Beckman autosampler (model 507E). To assure standardization between sample runs, calibration and reference plasma samples were interspersed at intervals during each run. Total homocysteine (tHcy) and cysteine concentrations were quantified using HPLC and coulometric EC detection as described previously in detail (15).

**COULOMETRIC EC DETECTION**

After HPLC separation, detection of SAM and SAH was accomplished using a model 5200A Coulomem II electrochemical detector (ESA) equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). A guard cell was placed in line before the injector to remove oxidizable impurities present in the mobile phase that might compromise baseline stability. The dual analytical cell contained two porous graphite electrodes in series. The first electrode (E1) is used as an oxidative screen and was set at a lower voltage than the second electrode (E2) to remove interfering compounds that oxidize at lower potentials than the compounds of interest. For selectivity, E2 was set at or above the established oxidation potential of the compounds of interest. For optimum detection of SAM and SAH, the electrode potentials for the guard cell, electrode E1, and electrode E2 were set at +1000 mV, +400 mV, and +920 mV, respectively. The current generated at E2 represents the oxidation of the active species between +400 mV and +920 mV, which encompasses the peak oxidative range for both SAM and SAH. These potentials provide peak area response with minimum background and are the basis for quantification. Peak area analysis was provided by GOLD Nouveau software (Beckman Instruments) based on calibration curves generated for each compound. Each day, electrode sensitivity and baseline stability were confirmed by the application of −400 mV at each electrode for 30 min with the mobile phase at 1.5 mL/min, followed by a 30-min water rinse at 1 mL/min with the electrodes turned off, and a final 30-min rinse with 500 mL/L methanol (electrodes off). Before injection of the first sample, the potential at the electrodes was increased in a stepwise fashion to the final working potential and the HPLC-EC system was equili-
Cathodically polarized for ~1 h with the mobile phase at 1 mL/min. The use of an autosampler is highly recommended and allows continuous sampling overnight. When not running samples, the mobile phase was set at a rate of 0.2 mL/min with lower voltages of +50 mV, +100 mV, and +200 mV at E1, E2, and the guard cell, respectively.

CURRENT/VOLTAGE CURVES, CALIBRATION CURVES, AND LIMITS OF DETECTION
Curves reflecting the current generated by the oxidation of SAM and SAH with increasing voltage at E2 were used to estimate the respective voltage requirements for peak sensitivity and reproducibility. Linear calibration curves for SAM and SAH in 100 μmol/L HCl were generated in the following physiologic ranges for each compound: 1–600 nmol/L for SAM, and 0.2–100 nmol/L for SAH. Before each analysis, calibration curves were generated from aliquots of frozen calibrators and examined for reproducibility.

RECOVERY, PRECISION, AND STATISTICAL ANALYSIS
To determine analytical recovery, known concentrations of SAM and SAH within the physiologic range were added to plasma and mouse liver tissue extracts. The concentrations of SAM and SAH in the supplemented samples were determined in five independent samples, and the mean quantitative recoveries were calculated. To determine the intraassay precision, 10 replicates of the same sample were analyzed in a single analytical run. The interassay precision was determined by analyzing aliquots from a single sample on 10 different days over a 1-month period. The CV was calculated as the standard deviation expressed as a percentage of the mean values. Statistical differences between means were calculated using the Student t-test and Sigmastat software (Jandel Scientific).

PLP
Concentrations of PLP were determined in fasting plasma by the radioenzymatic assay described previously in detail (41).
Results

Details of the theory and advantages of coulometric EC detection over amperometric EC detection have been described previously (15). Based on the current/voltage curve in Fig. 2A, the voltage was set at 400 mV and 920 mV for electrodes E1 and E2, respectively, for maximum sensitivity and reproducibility without damage to the electrodes. In Fig. 2, B and C, the linearity of detection within the physiologic range for each compound was confirmed. Typical chromatograms from calibrators, plasma extracts, and mouse liver extracts are presented in Fig. 3, A, B, and C, respectively. The concentration of the ion-pairing reagent, heptanesulfonic acid, was critical for optimum separation. The retention times were strongly affected by the pH and methanol concentration in the mobile phase. Under the conditions selected, the retention times for SAH and SAM were ~10.5 and 20.5 min, respectively. Excellent separation without interfering peaks was achieved in both plasma and cell extracts, as shown in Fig. 3. With EC detection, the selectivity for SAM and SAH was greatly enhanced at E2 by the oxidation and elimination of interfering compounds at E1. Biological samples required ~35 min between injections to eliminate a large unidentified peak that elutes at ~30 min.

Limits of Detection, Precision, and Recovery

The limit of detection for the calibrators, defined as the concentration that produced a signal-to-noise ratio $>5$, was 200 fmol/L for SAM and 40 fmol/L for SAH. These limits of detection are several-fold lower than those reported previously with methods using derivatization and ultraviolet detection (27, 28, 38). The interassay/intraassay precision and product recovery are presented in Table 1. In plasma extracts, the mean within-run (intraassay) CV was 3.4% for SAM and 5.5% for SAH, with mean recoveries of 96.8% for SAM and 97.3% for SAH. In mouse liver extracts; the mean intrassay CV was 6.1% for SAM and 8.8% for SAH, with mean recoveries of 95.2% for SAM and 97.1% for SAH. The mean between-run (interassay) CV in plasma extracts was 2.8% for SAM and 5.6% for SAH; for mouse liver extracts, the mean interassay CV was 7.3% for SAM and 7.6% for SAH.

<table>
<thead>
<tr>
<th>Table 1. Precision and recovery of assays.</th>
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### A. Precision of the assay

<table>
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<th>Intraassay (n = 10)</th>
<th>Interassay (n = 10)</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>Plasma extract, nmol/L</td>
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<tr>
<td>SAM</td>
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<td>SAH</td>
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<td>Mouse liver extract, pmol/mg protein</td>
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<td>SAM</td>
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<td>SAH</td>
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### B. Recovery of the assay*

<table>
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<th>SD</th>
<th>Mean recovery, %</th>
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<td>Plasma extract, nmol/L</td>
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<tr>
<td>SAM</td>
<td>150.9</td>
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<td>42.2</td>
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<tr>
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<td>824.7</td>
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<td>80</td>
<td>166.5</td>
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*Mean of five independent experiments.
The intracellular concentrations of SAM and SAH, as the substrate and product of essential cellular methyltransferase reactions, are important metabolic indicators of the cellular methylation capacity (17, 30, 44). Although emphasis has been placed on the toxicity of increased tHcy and depressed SAM concentrations in metabolic pathology of various diseases (22, 26, 45), a chronic increase in SAH, secondary to the homocysteine-mediated reversal of the SAH hydrolase reaction, may also have significant, albeit indirect, pathologic consequences. Because SAH can bind to and inhibit multiple cellular methyltransferases, increased SAH concentrations can lead to DNA hypomethylation and altered chromatin configuration, reduced membrane phosphatidylcholine concentrations, reduced protein and RNA methylation, and reduced neurotransmitter synthesis (17, 19, 22, 30, 46). These alterations have been associated with functional abnormalities, including inappropriate gene expression, altered signal transduction, immune deficiency, and cytotoxicity (21, 31, 47–49).

The results presented in Table 2 indicate that increased plasma tHcy was associated with a twofold increase in SAH, no change in SAM, and a twofold decrease in the SAM/SAH ratio in this cohort of women. Whether the decrease in SAM/SAH is of sufficient magnitude to affect methyltransferase activity and cellular methylation reactions cannot be determined from the present data. None-
theless, these observations are consistent with a homocysteine-mediated reversal of the SAH hydrolase reaction. The regression analysis in Fig. 4 indicates that plasma tHcy concentrations are inversely correlated with SAM/SAH ratios and suggests the possibility that tHcy could have indirect effects on cellular methylation potential. The decreases in plasma PLP concentrations in these women further suggest the possibility that nutritional vitamin B₆ deficiency may have contributed to the increase in tHcy; the decrease in cysteine concentrations is consistent with this interpretation. Similar increases in SAH concentrations associated with B₆ deficiency previously have been observed in rat liver (28). In Fig. 5, the significant positive correlation between plasma and lymphocyte SAM/SAH ratios suggests that the plasma SAM/SAH ratio may be a reasonable reflection of the intracellular ratio. In lymphocytes, export of SAH has been well documented and appears to be carrier-mediated and largely unidirectional (50). The kidney appears to be the only route for SAH removal from plasma (51). The origin of SAM in plasma is still an open and interesting question because export from the hepatocytes does not occur despite the fact that more SAM is used for creatine synthesis in the liver than in all extracellular tissues combined (52).

The mean plasma concentration of SAM detected with the present HPLC-EC method in women with normal tHcy was 156 nmol/L, which is considerably higher than previously published values of 26.5 nmol/L (38), 60 nmol/L (30), and 102 nmol/L (27) in plasma from healthy individuals obtained with other methods of detection. The higher values with the present method most likely reflect the direct injection of the extract without the need for precolumn processing and derivatization, which can lead to significant losses. The SAM peak was completely eliminated by boiling the extract for 5 min before injection, suggesting that coelution of a contaminating metabolite is unlikely, although this possibility cannot be definitively ruled out. Adenosine elutes before the SAH peak, at ~8 min. The mean plasma concentration of SAM obtained with the present method was 20 nmol/L and is within the ranges reported previously using other methods (27). The limits of detection using the coulometric EC detector were 200 fmol/L for SAM and 40 fmol/L for SAH. These values far exceed previous ultraviolet detection limits of 10 nmol/L (30), 25 pmol (28), and 5 pmol/L (27) for both SAM and SAH. Using the present method, we obtained a SAM/SAH ratio of 8.5 for plasma from healthy individuals, which is considerably higher than previously reported values (38). A disadvantage of the present method is that 40 min is required for complete elution of the accompanying peaks in biological samples; however, the precolumn processing time is minimal, and with the use of an autoinjector, ~20 samples per day can be processed. Because of the relatively long elution times, this method for measurement of SAM and SAH is best suited for research purposes and refinement of diagnoses rather than routine clinical analyses that require high throughput methodology. The ability to sensitively and reproducibly detect these important metabolites in plasma should provide new insights into the differential regulation of one-carbon metabolism with specific nutritional deficiencies and specific genetic polymorphisms.

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