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A simple rapid immunoassay for *S*-adenosylhomocysteine in plasma☆

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Abstract

The measurement of plasma *S*-adenosylhomocysteine is a more sensitive indicator of the risk for vascular disease than is plasma homocysteine. Because the level of *S*-adenosylhomocysteine is normally in the nanomolar range, it has been difficult to measure and necessitated the development of complex fluorometric and mass-spectrophotometric methods. We have now adapted an existing immunoassay used for the measurement of homocysteine to the measurement of *S*-adenosylhomocysteine in plasma. This assay is sensitive down to the level of less than 0.1 pmol, and there is no interference by *S*-adenosylmethionine. The assay is carried out in microplates, allows the measurement of 12 samples per plate and can easily be carried out in a 4-h period. The method is applicable to plasma samples having *S*-adenosylhomocysteine concentrations ranging from 10 to 150 nM without dilution. The mean value for 16 normal subjects by this method was 18.9±1.4 nM (S.E.M.), compared with 17.8±1.4 nM obtained by a previously described method using two high-performance liquid chromatography columns with fluorescence derivatization. Mean values for seven cirrhotic patients were 46.5±3.3 nM by this new method compared with 44.6±5.3 by the former method. The ease and speed of this method should allow the widespread measurement of this important metabolite in laboratories without access to sophisticated equipment.
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1. Introduction

S-adenosylhomocysteine (SAH) is the product of most methyltransferase reactions that are carried out in living organisms with *S*-adenosylmethionine (SAM) as the methyl donor. It is the metabolic precursor of homocysteine (Hcy), which has been identified as a risk factor for vascular disease in many studies [1] and has also been suggested to be a risk factor in a number of neuropsychiatric disorders [2–4]. The measurement of plasma Hcy is relatively straightforward since the normal level in human plasma is in the micromolar range, a number of methods have been published and several commercial kits are available for its measurement. On the other hand, plasma SAH is in the nanomolar range, and the published methods for its measurement are complicated usually requiring specialized equipment. A method we had developed several years ago for the measurement of SAH in plasma employs two high-performance liquid chromatography (HPLC) steps and derivatization with naphthalene dicarboxaldehyde to produce a fluorescent isoindole [5]. Although time-consuming, we have used this method successfully to show that plasma SAH is a more sensitive indicator of the risk for cardiovascular disease [6] and for renal disease than is plasma Hcy [7]. More recently, we have shown that in children with renal disease, uncomplicated by other risk factors, plasma SAH is highly correlated with the glomerular filtration rate, while plasma Hcy is not [8]. For this

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reason, a simple, rapid method for the measurement of plasma SAH would permit its use to determine whether elevated SAH is a risk factor in other conditions that may be involved in vascular disease.

Because plasma SAH is in the nanomolar range, a method of high sensitivity as well as specificity is needed. A number of methods for the measurement of SAH in plasma or serum have been developed. For the most part, they are cumbersome or require highly specialized equipment. Loehrer et al. [9] adapted a method utilizing conversion of the adenosine ring to a fluorescent derivative that was then subjected to HPLC chromatography. This required an 8-h period for the derivation. Castro et al. [10] were able to shorten the reaction time to 4 h. A number of other methods used tandem mass spectrometry [11–13]. A competitive immunoassay for total plasma Hcy was developed by Frantzen et al. [14]. Plasma Hcy was first converted to SAH using the enzyme, *S*-adenosylhomocysteine hydrolase, and was then quantitated with anti-SAH antibody and adapted for microtiter plates. We eliminated the first part of this procedure involving the enzymatic conversion of Hcy to SAH and then modified the conditions of the procedure so that the range of the method runs from 0.05 to 0.5 pmol of SAH in the assay. Although this is a narrow range, different amounts of sample can be used, and those with higher concentrations can be diluted. At this level of sensitivity, SAM does not interfere. Using this method, we obtained a mean value of plasma SAH of 18.9±1.4 nM for 16 normal individuals. This is compared with a value of 17.8±1.4 nM for the same samples analyzed using our fluorescence method. When plasma samples from 7 patients with liver disease were analyzed using this method, a mean value of 44.6±5.3 nM was obtained.

2. Methods and materials

2.1. Subjects

Blood was drawn from normal human volunteers or from patients having Child-Pugh Class C cirrhosis using EDTA as anticoagulant. In two cases, samples were drawn from a normal individual at different times after different oral doses of SAM were administered (1600 and 800 mg). The plasma was obtained by centrifugation within 1 h of collection. Studies were approved by the institutional review boards, as appropriate.

2.2. Materials

SAH, casein and bovine serum albumin (BSA) were obtained from Sigma. The microtiter plates were Maxisorp and obtained from Nunc. The ultrafiltration units were ULTRAFREE-MC from Amicon. The SAH–BSA conjugate, anti-SAH antibody, horseradish peroxidase (HRP)-conjugated rabbit antimouse antibody and the HRP substrate were obtained from Axis-Shield, Dundee, Scotland. Pooled plasma from 13 normal individuals was from Innovative Research, Southfield, MI, USA.

2.3. Reagents

The assay buffer contains 100 mM Na_2HPO_4, 150 mM NaCl and 14 mM NaN_3 adjusted to pH 8.5. The wash solution was 100 mM Na_2HPO_4, 150 mM NaCl and 0.1 g/L merthiolate, adjusted to pH 7.4, and then 2 g/L BSA and 0.5 ml/L Tween 20 were added. The dilution buffer was 5.0 mM K_2HPO_4 (pH 7.0).

2.4. Sample preparation

One hundred microliters of plasma were added to an Amicon ULTRAFREE-MC filter unit (#UFC3LGCOO) and centrifuged at 18,000*g* for 45 min at 5°C. The filtrate (~70–80 μl) was used for assay.

2.5. Coating of microtiter plates

To each well was added 250 μl of a solution (0.1 μg/mL) of SAH–BSA conjugate in phosphate-buffered saline (PBS) containing 2 μg/ml BSA and incubated overnight at 4°C. The plates were emptied by inversion and the excess liquid removed by tapping the plates on paper towels. Then, to each well was added 250 μl of blocking solution containing 25 g/L sodium caseinate in PBS and incubated overnight at 4°C. The plates were emptied by inversion, blotting as before, and washed 4 times with 250 μl of 10-fold-diluted Assay Buffer. The plates were then dried by inverting for 30 min on paper towels, stored inverted and wrapped in aluminum foil in a sealed plastic bag at −20°C. They are stable for 5–6 months in this condition.

2.6. Preparation of standards

A 10-μM stock solution of SAH in 5 mM potassium phosphate (pH 7.0) was diluted 100-fold in assay buffer to give a working solution of 0.1 μM. Further dilutions are made to give working solutions of 0.05, 0.02 and 0.01 μM. These solutions are stable for at least 1 month at −20°.

2.7. Samples

Routinely, aliquots of 5, 10 and 15 μl of an undiluted ultrafiltered plasma sample are assayed in duplicate. This usually ensures that at least two of the aliquots fall on the useful portion of the standard curve, giving four measurements that are used to obtain a mean value. In this way, a range of concentrations from 3 to 100 nM can be measured without dilution. Samples with higher concentrations of SAH can be measured by dilution of the ultrafiltered plasma samples in 5 mM potassium phosphate buffer, pH 7.0.

2.8. Assay procedure

The standards or the samples were diluted to 50 μl in assay buffer in the coated microtiter wells, followed by 100 μl of anti-SAH antibody solution. The plates were incubated in the dark at room temperature on a shaking platform for 1 h. Each well was then washed 4 times with 250 μl of the wash solution. Each well then received 50 μl of HRP-conjugated rabbit antimouse antibody and the plates

incubated again for 20 min in the dark at room temperature with shaking. The wells were washed again four times with 250 μl of wash solution, and 50 μl of HRP substrate solution was added. The plates were incubated for 10 min at room temperature and the reaction stopped by adding 50 μl of H_2SO_4 (0.8 M). The yellow color is read at 450 nm in a Bio-RAD microplate reader within 5 min. The values were fitted to a quadratic equation as provided by the instrument.

2.9. Statistical analysis

This was carried out using GraphPad PRISM. All values presented are means±S.E. unless otherwise indicated. Figures were drawn with Kaliedagraph (Synergy Software).

3. Results

Standards providing 0.0, 0.1. 0.2, 0.3, 0.4, 0.5, 0.75 and 1.00 pmol of SAH per well were run in duplicate on each microtiter plate. The results of a typical standard curve is shown in Fig. 1. The useful portion of the curve is from 0.05 to 0.5 pmol.

The recovery was measured after the addition of 10, 20 and 50 nM SAH to 5 individual plasma samples. The recovery of 10 nM (1 pmol) added to the samples was 90.2%±3.3% (% CV=8.1). The recovery of 20 nM (2 pmol) was 81.3±6.6% (% CV=18.2) and the recovery of 50 nM (5 pmol) was 107.8±7.9% (% CV=16.5).

The between-assay precision was determined using plasma pooled from 13 normal individuals. Seven individual measurements were carried out in quadruplicate over a period of 2 weeks. A mean value of 31.7±1.5 was obtained with a % CV=12.5. Within-assay precision was carried out also using pooled plasma from 13 individuals. Eleven separate assays were carried out on the same microtiter plate. A mean value of 29.6±1.2 was obtained with % CV=13.6.

Table 1
Effect of added SAM on plasma SAH values

[a]SAM added (nM±S.E.M.)					
0	50	100	500	1000	2000
35.0±0.7	35.2±4.4	35.4±4.7	35.2±4.1	36.1±0.5	37.5±4.6

[a] The indicated amount of SAM was added to different aliquots of the same plasma and processed as indicated in Methods and materials. The values are the means of duplicate determinations.

The original paper by Frantzen et al. [14] indicated that SAM was recognized to a slight extent by the anti-SAH antibody and could result in falsely high values for Hcy when SAM was present at concentrations greater than 10 μM. Surprisingly, addition of as much as 2000 nM SAM to a plasma sample containing 35 nM SAH did not change the measured value of SAH (Table 1). This is possibly because the amount of anti-SAH antibody has been greatly reduced in this method.

The values obtained by the immunoassay were compared with the values obtained using our original fluorescence assay [5]. We compared values for normal individuals, an individual at several times after receiving a single oral dose of SAM as well as individuals with cirrhotic liver disease. The values for 16 control subjects were 18.9±1.4 and 17.8±1.4 nM, respectively, for this new method and for the older HPLC/isoindole fluorescence assay. The values for seven subjects with cirrhotic liver disease were 44.6±5.3 and 46.5±3.3 for the former and current methods, respectively. The results are shown in Fig. 2 for 40 individual samples covering a long range of values. There is good agreement between the two methods, and there is no

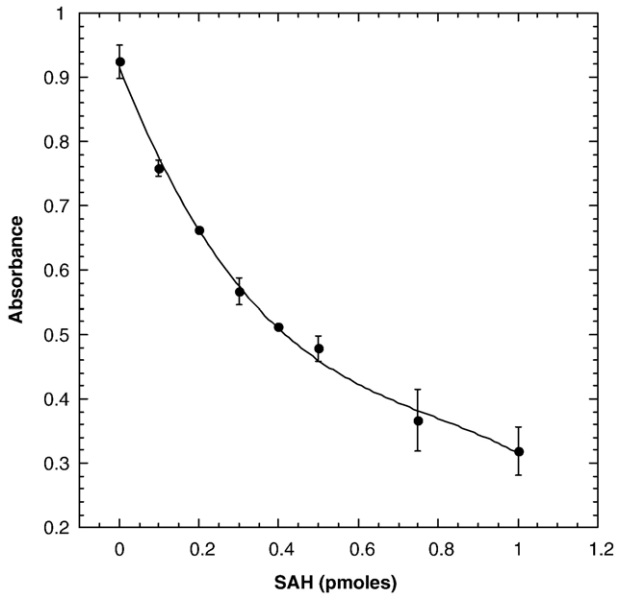

Fig. 1. Standard curve for measurement of SAH. The standard curve was measured as described in the text, and absorbance was measured in a microplate reader.

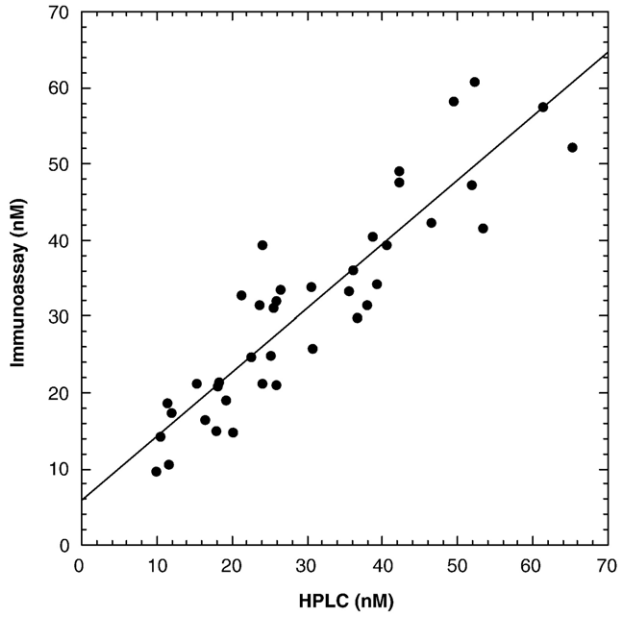

Fig. 2. Comparison of values for plasma SAH obtained by the HPLC/fluorescence method and the immunoassay described here. R=.91; slope=.97.

Table 2
Comparison of plasma normal SAH values

Method	nM	Reference
Fluorescence 1,N^6-etheno conversion plus HPLC	24±1 (S.E.M.)	[15]
Modified etheno conversion plus HPLC	28±3 (S.D.)	[10]
Fluorescence 1,N^5-etheno conversion plus capillary electrophoresis	29±2 (S.D.)	[16]
Fluorescence isoindole formation plus HPLC	23±3 (S.E.M.)	[5]
Coulometric electrochemical detection with HPLC	20±6 (S.D.)	[17]
Stable isotope dilution Tandem mass-spectrometry	12±4 (S.D.)	[13]
Stable isotope dilution LC/MS	15 (8–26, 95% CI)	[12]
Tandem mass spectrometry	26±6 (S.D.)	[11]
Immunoassay	19±4 (S.E.M.)	This study

HPLC, high performance liquid chromatography; MS, mass spectrometry.

significant difference between the values obtained with either of the two methods. Table 2 shows a comparison of the published values for plasma or serum SAH of normal individuals. The values obtained using this method are comparable to the others and were obtained in less than 4 h.

4. Discussion

Elevation of plasma or serum Hcy is generally accepted as an independent risk factor for vascular disease [1,18,19]. Several studies have indicated that elevation of plasma SAH may be a more sensitive indicator of the risk of vascular disease [6–8]. Measurement of plasma or serum total SAH, however, is challenging because of the low levels present in normal subjects (approximately 20 nM). A number of methods have been developed. Most methods provided results for both SAM and SAH. Loehrer et al. [9] developed a method based upon the formation of the fluorescent 1,N^6-etheno derivatives of SAH and SAM followed by HPLC separation. This method required a long period of time for the reaction to proceed, although the results for measurement of SAH compared favorably with other methods. More recently, Castro et al. [10] was able to shorten the derivatization time. Other methods involved the use of highly specialized equipment such as tandem mass spectrometry [11–13] and coulometric electrochemical detection [17]. We had developed a reliable assay for the measurement of both SAM and SAH based on the conversion of these compound to their fluorescent isoindoles. Although reliable, the method required two HPLC separations with derivatization before the second column [5].

Using this method, we showed that measurement of plasma SAH was a more sensitive indicator of the risk for cardiovascular disease than measurement of plasma total Hcy [6]. This was also the case for renal disease [7]. Because adults with cardiovascular disease or renal disease are likely to have other confounding factors such as diabetes and hypertension, which can result in elevated Hcy, we compared plasma Hcy and plasma SAH in a group of children with varying degrees of renal insufficiency but no other risk factors [8]. It was very clear that there was a strong and significant inverse association of SAH with glomerular filtration rate but no such association with plasma Hcy. Yi et al. [20] found a positive correlation of plasma SAH levels and plasma Hcy levels in healthy young women, but there was no correlation of Hcy with plasma SAM. This was also associated with lymphocyte DNA hypomethylation. SAH is a potent product inhibitor of most methyltransferases [21]. This group speculated on the toxic effects of elevated SAH on DNA methyltransferase and the effects this might have on DNA methylation and gene expression [22].

Because SAH is the direct metabolic precursor of Hcy, and the enzyme catalyzing this conversion, SAH hydrolase, is reversible, it might be expected that changes in the plasma concentrations of these two metabolites might follow each other. A discrepancy between changes in plasma SAH and Hcy has been shown by the study by Becker et al. [23]. They showed that changes in folate, cobalamin and vitamin B_6 concentrations did not affect plasma SAH concentrations, although plasma Hcy *was* affected. The fact that SAH and Hcy are not equally affected in the case of the children with decreased renal function [24] and that SAH is more sensitive to changes in vascular function suggests that it may be more appropriate to monitor plasma SAH values. It should be noted, also, that there is no generally accepted mechanism for the pathophysiology of elevated Hcy while the potent end product inhibition of SAM-mediated methyltransferases by SAH is well known [21]. For these reasons, this rapid and simple immunoassay for the measurement of SAH in plasma may be a useful tool in the study of methionine metabolites in vascular disease.

References

[1] HS Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. JAMA 2002;288:2015–22.
[2] Bottiglieri T, Hyland K. *S*-adenosylmethionine levels in psychiatric and neurological disorders: a review. Acta Neurol Scand Suppl 1994; 154:19–26 [Review] [53 refs].
[3] McIlroy SP, Dynan KB, Lawson JT, Patterson CC, Passmore AP. Moderately elevated plasma homocysteine, methylenetetrahydrofolate reductase genotype, and risk for stroke, vascular dementia, and Alzheimer disease in Northern Ireland. Stroke 2002;33:2351–6.
[4] Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002;346:476–83.
[5] Capdevila A, Wagner C. Measurement of plasma *S*-adenosylmethionine and *S*-adenosylhomocysteine as their fluorescent isoindoles. Anal Biochem 1998;264:180–4.
[6] Kerins DM, Koury MJ, Capdevila A, Rana S, Wagner C. Plasma *S*-adenosylhomocysteine is a more sensitive indicator of cardiovascular disease than plasma homocysteine. Am J Clin Nutr 2001;74:723–9.
[7] Wagner C, Stone WJ, Koury MJ, Dupont WD, Kerins DM. *S*-adenosylhomocysteine is a more sensitive indicator of renal insufficiency than homocysteine. Nutr Res 2004;24:487–94.
[8] Jabs K, Koury MJ, Dupont WD, Wagner C. Relationship between plasma *S*-adenosylhomocysteine concentration and glomerular filtration rate in children. Metabolism 2006;55:252–7.

[9] Loehrer FM, Haefeli WE, Angst CP, Browne G, Frick G, Fowler B. Effect of methionine loading on 5-methyltetrahydrofolate, *S*-adenosylmethionine and *S*-adenosylhomocysteine in plasma of healthy humans. Clin Sci 1996;91:79–86.

[10] Castro R, Struys EA, Jansen EE, Blom HJ, deAlmeida IT, Jakobs C. Quantification of plasma *S*-adenosylmethionine and *S*-adenosylhomocysteine as their fluorescent 1,*N*(6)-etheno derivatives: an adaptation of previously described methodology. J Pharm Biomed Anal 2002;29:963–8.

[11] Struys EA, Jansen EE, deMeer K, Jakobs C. Determination of *S*-adenosylmethionine and *S*-adenosylhomocysteine in plasma and cerebrospinal fluid by stable-isotope dilution tandem mass spectrometry. Clin Chem 2000;46:1650–6.

[12] Stabler SP, Allen RH. Quantification of serum and urinary *S*-adenosylmethionine and *S*-adenosylhomocysteine by stable-isotope-dilution liquid chromatography–mass spectrometry. Clin Chem 2004;50:365–72.

[13] Gellekink H, van Oppenraaij-Emmerzaal D, van Rooij A, Struys EA, den Heijer M, Blom HJ. Stable-isotope dilution liquid chromatography-electrospray injection tandem mass spectrometry method for fast, selective measurement of *S*-adenosylmethionine and *S*-adenosylhomocysteine in plasma. Clin Chem 2005;51:1487–92.

[14] Frantzen F, Faaren AL, Alfheim I, Nordhei AK. Enzyme conversion immunoassay for determining total homocysteine in plasma or serum. Clin Chem 1998;44:311–6.

[15] Loehrer FM, Angst CP, Brunner FP, Haefeli WE, Fowler B. Evidence for disturbed *S*-adenosylmethionine: *S*-adenosylhomocysteine ratio in patients with end-stage renal failure: a cause for disturbed methylation reactions? Nephrol Dial Transplant 1998;13:656–61.

[16] Sbrana E, Bramanti E, Spinetti MC, Raspi G. *S*-Adenosyl methionine/*S*-adenosyl-L-homocysteine ratio determination by capillary electrophoresis employed as a monitoring tool for the antiviral effectiveness of adenosine analogs. Electrophoresis 2004;25:1518–21.

[17] Melnyk S, Pogribna M, Pogribny IP, Yi P, James SJ. Measurement of plasma and intracellular *S*-adenosylmethionine and *S*-adenosylhomocysteine utilizing coulometric electrochemical detection: alterations with plasma homocysteine and pyridoxal 5′ -phosphate concentrations. Clin Chem 2000;46:265–72.

[18] Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, et al. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149–55.

[19] Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. [see comments] JAMA 1995;274:1049–57.

[20] Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma *S*-adenosylhomocysteine and lymphocyte DNA hypomethylation. J Biol Chem 2000;275:29318–23.

[21] Clarke S, Banfield K. *S*-adenosylmethionine-dependent methyltransferases. In: Carmel R, Jacobsen DW, editors. Homocysteine in health and disease. Cambridge, (UK): Cambridge University Press; 2001. p. 63–78.

[22] James SJ, Melnyk S, Pogribna M, Pogribny IP, Caudill MA. Elevation in *S*-adenosylhomocysteine and DNA hypomethylation: potential epigenetic mechanism for homocysteine-related pathology. J Nutr 2002;132:2361S–6S.

[23] Becker A, Smulders YM, Teerlink T, Struys EA, de Meer K, Kostense PJ, et al. *S*-adenosylhomocysteine and the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine are not related to folate, cobalamin and vitamin B6 concentrations. Eur J Clin Invest 2003;33:17–25.

[24] The VITATOPS (Vitamins to Prevent Stroke) Trial: rationale and design of an international, large, simple, randomised trial of homocysteine-lowering multivitamin therapy in patients with recent transient ischaemic attack or stroke. Cerebrovasc Dis 2002;13:120–6.