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Short communication

Measuring S-adenosylmethionine in whole blood, red blood cells and cultured cells using a fast preparation method and high-performance liquid chromatography

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Abstract

The physiological methyl donor S-adenosylmethionine (SAM) plays a key role in the maintenance of human health and in the prevention of disease. A convenient clinical test for blood SAM does not exist, even though blood SAM is increasingly seen as an important indicator of health. We have developed a simple procedure to extract SAM from small amounts of blood or cells. The extracted SAM is then measured by high-performance liquid chromatography (HPLC). This measurement is sensitive, precise and uses as little as 200 μl of blood or $0.5 \cdot 10^6$ cultured cells per determination. SAM, as tested with this method, under acidic conditions, is stable for hours and can be frozen for later analysis. The method has been used to show that blood SAM varies with species, sex and treatment. We have also measured the SAM levels in cultured cells, and have been able to detect wide variations depending upon treatments administered during the growth of those cells. In conclusion, this is a very rapid and easy method to measure SAM in biological fluids and cell culture and which could be adapted to the clinical setting.

Keywords: S-Adenosylmethionine

1. Introduction

S-Adenosylmethionine (SAM) is the physiological methyl donor for nearly all methyltransferase reactions in mammals. Pharmacologically, SAM is an effective treatment for clinical depression in humans and is often used in Europe for this purpose [1–4]. Physiological methyl donors have been associated with decreased risk of colon and liver cancer [5,6] and SAM itself is a cancer chemopreventive agent in animals [7]. These methyl donors also appear to exert a role in preventing heart disease and stroke [8–10], in addition to cancer and neurological disorders [11].

Measurement of blood SAM has great clinical potential. The small amounts of blood needed to measure SAM are easily obtained from humans by minimally invasive techniques (blood collection). A convenient clinical test is needed for SAM because blood SAM offers the possibility of being an indicator of other parameters that may be difficult to measure in solid human tissues, such as brain or

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liver. Blood SAM may be a useful adjunct in the diagnosis of depression, heart disease risk and nutritional imbalances. The method described here uses a small amount of blood, such as that obtained from a finger-prick or from a mouse. The extraction method is simple and requires only two easily obtainable chemicals. Minor adaptations in the method make it possible to measure SAM in whole blood, in fresh or frozen red blood cells (RBCs) and in cultured cells.

2. Experimental

2.1. Extraction procedure

2.1.1. Whole blood

Whole blood was collected from B6C3F1 and C57BL6N mice and F344 rats on sacrifice. A small amount of fresh whole blood, 200–500 μl, was placed in a 1.5-ml eppendorf tube. SAM was extracted as follows: The protein was precipitated by adding 40% (w/v) trichloroacetic acid (TCA) equal to one-fifth of the original volume of the blood. The mixture was vortex-mixed until it had turned a dark rust color, and the tube was placed in ice for 30 min to complete precipitation.

To remove the precipitated protein, the tubes were centrifuged at 25 000 *g* for 10 min at 5°C in an Eppendorf 5403 refrigerated centrifuge (Brinkmann Instruments, Westbury, NY, USA). The supernatant containing SAM was transferred to a 0.5-ml eppendorf centrifuge tube. The volume was noted and recorded. An equal volume of ice-cold peroxide-free diethyl ether was added (to extract lipids, to extract excess trichloroacetic acid (TCA) and to adjust the pH to 3.3). The tubes were vortex-mixed for 20 s and centrifuged to separate the phases. The top layer was drawn off and discarded using an Eppendorf pipettor. Whenever possible, any solid matter at the interface was also removed. The ether extraction steps were repeated once. The samples were filtered through an Ultrafree-MC filter (0.45 μm; Millipore, Bedford, MA, USA). The samples were then ready for HPLC analysis of SAM. The samples were kept at 0–4°C at all times until analyzed.

2.1.2. Red blood cells

When the experiment was started with frozen or fresh packed RBCs instead of whole blood, an equal volume of 0.1 *M* sodium acetate, pH 6.0 (ice cold), was added, and the mixture was vortex-mixed. TCA was added and the solution was completely mixed on a Vortex and placed on ice. For example, when frozen RBCs were analyzed for SAM, the cells were thawed, and a 100-μl sample was pipetted into a 1.5-ml eppendorf tube, 100 μl of 0.1 *M* sodium acetate, pH 6.0, was added, and the tube was vortex-mixed. Then, 40 μl of 40% TCA was added and the rest of the experiment proceeded as above.

2.1.3. Cultured cells

With slight modifications, SAM levels were also measured in cultured cells. The cells used were AHH-1 TK±, a spontaneously transformed human B lymphoblastoid cell line, by Genetest. They were grown in RPMI 1640 media with 10% equine serum. When measuring the SAM in cultured cells, 80 μl of 0.1 *M* sodium acetate buffer, pH 6.0, was added to a soft pellet containing a known number of cells. As few as $0.5 \cdot 10^6$ cells could be used. After the cells were lysed with the sodium acetate, 20 μl of 40% TCA were added. The solution was vortex-mixed and allowed to stand on ice for 30 min. The solution was centrifuged at 25 000 *g* for 10 min at 5°C, and 100 μl of the supernatant were transferred to another 0.5 ml eppendorf tube. The solution was extracted twice with an equal volume of diethyl ether, and was filtered.

2.2. HPLC analysis

The method of Wise and Fullerton [12] was used for the HPLC analysis of SAM. Specifically, 20 μl (or an appropriate volume) of the extracted sample was injected into a Waters HPLC system (Milford, MA, USA) equipped with a Beckman Ultrasphere ODS, 25×0.46 cm (5 μm particle size) column (Fullerton, CA, USA), a pre-column filter (No. C-751, ChromTech, Apple Valley, MN, USA) and a Waters 996 photodiode array detector operated at 254 nm. The mobile phase consisted of 50 m*M* NaH_2PO_4 and 10 m*M* heptanesulfonic acid (sodium salt, SigmaUltra, Sigma, St. Louis, MO, USA) in 20% methanol, adjusted to pH 4.38 with phosphoric acid, with a flow-rate of 0.9 ml/min. The column was equilibrated for 1 h before injections were started. Standard samples of SAM (No. 102407, Boehringer Mannheim, Indianapolis, IN, USA) rang-

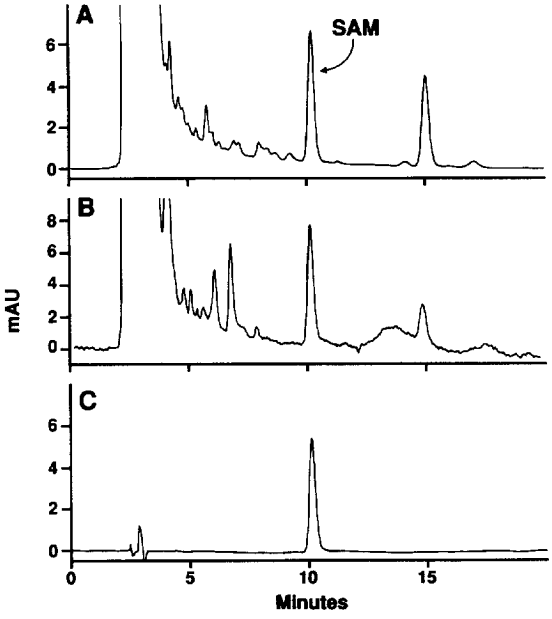

Fig. 1. SAM chromatograms of (A) whole blood sample, (B) cells in vitro ($0.5 \cdot 10^6$) and (C) standard (3 μg/ml).

ing from 0.25 to 5 μg/ml in 10^{-4} *M* HCl were run before and after the experimental samples. The retention time for the SAM was 10.5±0.8 min [12]. Representative chromatograms are shown in Fig. 1.

After completion of a series of analyses, the HPLC system was cleared with H_2O for 1 h. The gradient was then gradually increased from 0 to 100% methanol, kept at 100% for 30 min, and then decreased and left at 70% methanol. Water was pumped through the column before beginning the next analysis. When the retention time of the first injected SAM standard was significantly less than 10.5±0.8, the wash procedure was repeated before beginning subsequent analyses.

Table 2
Comparison of SAM concentrations in whole blood, RBCs and plasma samples in the same blood from one male rat

Test sample	Preparation method	SAM concentration of the test sample (nmol/ml)
Whole blood	Blood only	5.98
Whole blood	Blood+NaOAc (1:1, v/v)	5.88
RBCs	RBC+NaOAc (1:1, v/v)	11.86
RBCs	RBC+NaOAc (5:2, v/v)	10.89
Plasma	Plasma only	not detected

2.3. Statistical analysis

Results from multiple samples are reported as means±standard error of mean (SEM).

2.4. Experiments

The experiments to develop a method for measuring blood SAM were conducted using whole blood from B6C3F1 and C57BL6N mice and F344 rats (Table 1). After the initial tests were completed, 5 ml of blood were collected from a single F344 rat to determine the recovery of a standard and to study the effects of certain variations in the analysis (Table 2). Later, 5 ml of blood were collected from each of two human volunteers to determine the precision of the experiment and the results of various temperature storage methods (Table 3). A comparison was also made of SAM concentrations in plasma and RBC portions of blood (Tables 2 and 3).

Table 1
Whole blood SAM in mice and rats

Experiment	Species	Strain	Age (weeks)	Sex	Number of animals	SAM (mean±SEM) (nmol/ml)
1	Mice	C57BL6N	4	M	4	8.09±0.14
2	Mice	C57BL6N	4	M	4	8.56±0.25
3	Mice	C57BL6N	4	M	4	8.57±0.45
4	Mice	B6C3F1	52	F	8	7.91±0.20
5	Rats	Fisher 344	36	M	4	5.49±0.32
6	Rats	Fisher 344	36	M	7	5.74±0.17
7	Rats	Fisher 344	36	M	7	5.75±0.09
8	Rats	Fisher 344	36	F	6	4.93±0.05

Table 3
Effect of storage and treatment temperature on SAM in whole human blood

Treatment	Number of aliquots	SAM from donor A (mean±SD) (nmol/ml)	SAM from donor B (mean±SD) (nmol/ml)
Fresh	4	2.59±0.05	1.48±0.03
Kept at 4° overnight	2	3.07±0.05	1.95±0.02
Left at room temp. overnight	2	2.25±0.00	1.34±0.02
Left at 37° overnight	2	1.04±0.20	0.87±0.15
Plasma	2	0.17±0.00	0.11±0.00
RBCs	2	3.40±0.26	1.83±0.06

The first experiments were performed using mouse blood. Various volumes of blood from 80–500 μl were used. TCA, equivalent to one-fifth of the blood volume, was added. The HPLC area for 1 μg/ml of SAM and the retention time for the peak were established using SAM standards. The extract was injected and the SAM peak was integrated. The sample peak area was divided by the fraction of whole blood in the total volume and by the standard peak area for 1 μg/ml SAM to obtain the μg of SAM/ml of blood, which was converted to nmol/ml. This allowed comparison of results obtained to those found in the literature. In subsequent experiments, 200 μl of whole blood were used in each tube, and 40 μl of TCA were added. This facilitated direct comparison of the raw data.

Blood samples from humans were obtained in a clinical setting, 200 μl were placed in a 1.5-ml eppendorf tube, 40 μl of 40% TCA were added, the tubes were vortex-mixed and allowed to stand at 4°C for 30 min. The tubes were centrifuged at 25 000 *g* for 10 min, and then were placed in a freezer at −20°C and stored. They were transported to our laboratory in a frozen state, where they were thawed and centrifuged again for 10 min. The supernatant was transferred to another tube and extracted as described above.

Human whole blood samples were obtained from a blood bank and had been stored at 4°C for between 4–34 days. These samples were tested to determine the SAM levels as described above. Additional fresh human blood samples were obtained from volunteers and these were placed on ice within 10 min and analysed to determine the SAM concentration, for comparison with the above-mentioned samples.

3. Results

The experimental results are described in Tables 1–3. Table 1 describes the blood SAM levels of control groups of mice and rats taken from various toxicity studies. The first three groups show the whole blood SAM levels in male C57BL6 mice. Blood SAM levels ranged from 8.09±0.14 to 8.57±0.45 nmol/ml. The corresponding SAM value for B6C3F1 female mice was 7.91±0.20 (Table 1, group 4).

Blood SAM levels in rats were also measured (Table 1). In seven to nine month old male Fisher 344 rats, these ranged from 5.49±0.32 to 5.75±0.09 nmol/ml (Table 1, groups 5–7). The corresponding value in F344 females was 4.93±0.05 (Table 1, group 8). The blood SAM level was significantly lower in rats than in mice and appeared to be lower in female than in male rats.

Comparisons were then made on the SAM content in whole blood, in RBCs and in plasma from a single blood sample. The RBCs had to be treated with sodium acetate buffer in order to extract the SAM after the addition of TCA. The packed RBCs have twice the concentration of SAM as whole blood (Table 2). Plasma constitutes about half the volume of whole blood and, as indicated in Table 2, contains no detectable SAM. Thus, virtually all of the SAM is found in the RBCs (Table 2). The studies described in Tables 1 and 2 were conducted with blood samples that had been stored on ice and processed within 1–4 h.

In fifteen human blood samples collected in a clinical setting and frozen after the addition of TCA, the blood SAM level was 2.02±0.08 nmol/ml. The

range was 1.52–2.61 nmol/ml. Twenty-nine samples from the blood bank were divided into groups according to the length of time for which the samples were stored. The first group contained six samples from seventeen–eighteen year old donors, and had been stored for only four days at 4°C. The SAM level for these samples was 3.05±0.06 nmol/ml. The next group, from donors aged 25–65 years, consisted of six samples that had been stored from five–nine days; their SAM levels were 3.25±0.27 nmol/ml. In the last group, from donors aged 19–55, which had been stored from 10–34 days, the SAM levels measured were 4.04±0.47 nmol/ml with a range of 2.41–6.76 nmol/ml. Fresh samples of human blood, four aliquots of which were analysed from each of two donors, contained 2.59±0.03 nmol/ml from Donor A and 1.48±0.02 from Donor B (Table 3). The human samples were significantly lower in SAM content than were the rodent samples.

An investigation was made of the effects of other temperature ranges during sample handling on the detectable levels of SAM in human blood. The effects of such temperature treatments are described in Table 3. Samples that were placed in the refrigerator at 4°C overnight showed an increase in observed SAM levels. This corresponds to what was found when samples that had been stored for long periods of time at the blood bank were analyzed. Samples left at room temperature (22°C) overnight showed a slight decrease in SAM. Samples heated to 37°C overnight showed a greater decrease. Freezing at −20°C lysed the cells and made it more difficult to extract the SAM from whole blood. Freezing on dry ice, followed by thawing, rendered the sample too viscous for analysis. It is recommended that if frozen storage is necessary, the RBCs be separated prior to freezing. Sodium acetate buffer may then be added to each sample, to aid in extraction of the SAM. Plasma samples contained only 5–6% as much SAM as the RBCs. The RBCs were diluted 50:50 with sodium acetate buffer and the levels of SAM were higher than the levels measured in whole blood. The reason for this is that the whole human blood did not exactly 50% RBCs, but rather 42.9% for Donor A and 39.4% for Donor B, as measured by the hematocrit. The hematocrit could be used to adjust the SAM level in the RBCs to calculate the level that would be present in whole blood.

SAM levels were determined in RBCs taken from F344 rats and stored at −70°C for two months. The observed SAM level was 11.09±0.35 nmol/ml (n= 16), which was comparable to the values for RBCs reported in Table 2.

To determine the accuracy of the SAM measurement, samples of whole blood were spiked with various amounts of standard SAM. SAM standards containing the same concentrations as present in the experimental samples were also run; these data are shown in Fig. 2. The added SAM was quantitatively recovered in each sample tested.

The effect of acute i.p. doses of betaine on the blood SAM levels in mice was also examined. Betaine, a physiological methyl donor, is known to increase liver SAM when fed to rats [13]. Four-week-old C57BL6N male mice were injected i.p. with betaine (pharmaceutical grade, Finnsugar Bioproducts, Schaumburg, IL, USA) and 2 h later their blood was collected on sacrifice. In these mice, blood SAM levels increased as a function of the betaine dose (Fig. 3).

This technique was extended to measure the SAM content of cells. In experiments with $1.5 \cdot 10^6$ and $3.0 \cdot 10^6$ cells per tube, SAM levels could be measured, and the concentration of SAM was directly related to the number of cells per tube (Fig. 4). Since the volume of liquid added to each tube was the same, the amount of SAM per million cells could be to calculated. SAM could be measured in as few as $0.5 \cdot 10^6$ cells (Fig. 1C).

4. Discussion

Results of the experiments described above showed that the content of SAM in blood can be measured with a relatively small amount of sample. For example, as little as 200 μl of blood will yield a sample of sufficient volume for duplicate analyses by HPLC. This is useful when doing research on small rodents, such as mice. More importantly, the assay may have clinical use. The extraction procedure is relatively easy, using only two chemicals. A large number of samples can be processed in a day. In the acidic extract, the SAM is very stable and can be stored, if necessary, for a day, before injection into the HPLC. While no SAM was found in plasma,

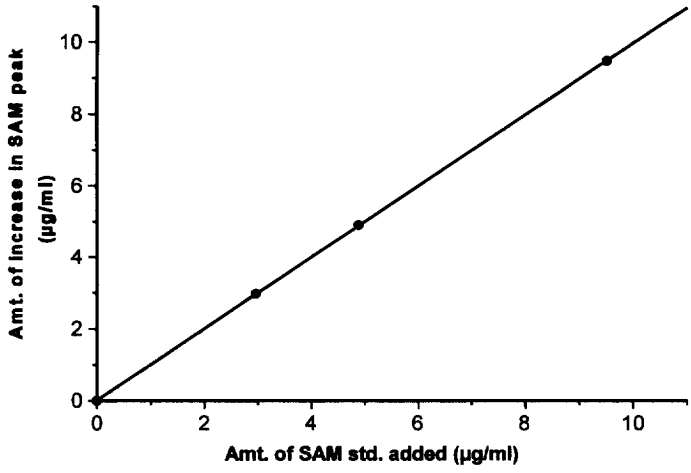

Fig. 2. Recovery of standard SAM from whole blood (r=0.999).

RBCs, which appear to contain all of the SAM found in blood, can be used for measurements, even after being frozen at −70°C for two months. Blood SAM was measured in over 100 animals and the results were found to be very consistent among comparable groups. Standard deviations are less than 10%.

The SAM levels in blood measured by the method described here are comparable to those previously reported [14–16]. Effective means to measure blood SAM have been described by others including Lagendijk et al. [14] and Perna et al. [15]. The method described here provides increased sensitivity

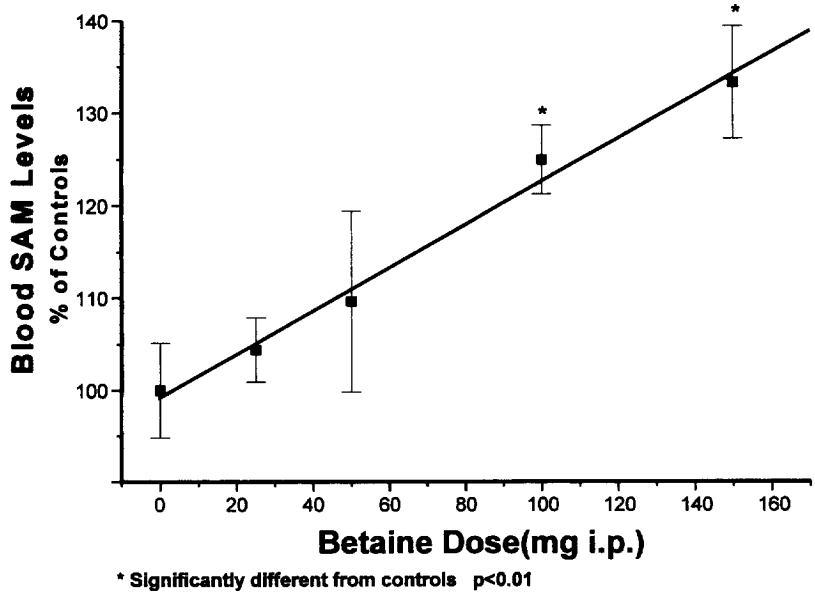

Fig. 3. Increase in whole blood SAM levels after injection of betaine solutions of increasing concentration (r=0.994).

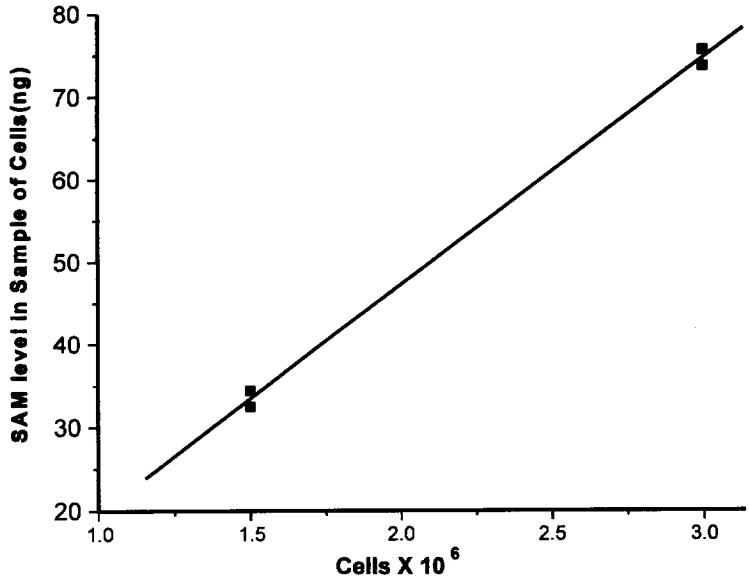

Fig. 4. SAM levels as a function of number of cells.

and applicability compared to earlier methods. The present method offers advantages over that of Lagendijk et al. [14], which requires large amounts of blood, the use of an internal standard and the exposure of SAM to alkaline conditions in which it is labile, thus necessitating quick and careful handling. The present method is also more sensitive and more versatile than that of Perna et al. [15], which uses only frozen packed RBCs. In the present study, blood SAM levels in male rats, mice and humans ranged from 5.48–5.75, 8.08–8.56 and 2.02–4.04 μM, respectively, while in female rats and mice, the comparable values were 4.93 and 7.91 μM, respectively. Lagendijk et al. [14] reported values of 5.2 ± 0.48 μM in packed RBCs from human subjects. Barber et al. [17], Castagna et al. [16] and Perna et al. [15] reported SAM levels of 3.5, 2.95 and 2.68 μM, respectively, in human RBCs. The levels of SAM in human whole blood were reported by Gaull et al. [19] as being 0.8–4.1 μM. The levels of SAM measured in human blood by the present method agree with those previously found.

SAM is the ultimate methyl donor and an excellent and often used indicator of the level of methyl availability in animal tissues. Low levels of SAM in human blood have been correlated with clinical depression, and SAM has been used to treat depression [2–4]. Low blood SAM has also been correlated with heart disease in humans [8]. Deficiencies of other physiological methyl donors have been associated in humans with heart disease and stroke [9,10], with depression and with other neurological disorders [2–4,11], and with colon cancer [5]. Physiological methyl donors, including SAM, act as chemopreventive agents against liver cancer in mice and rats [6,7]. Where it is impractical to obtain human tissue for measurement of SAM, measurement of SAM in blood may be an important indicator of nutritional status and health. Correlations between blood and tissue SAM in animals may help define the metabolic and disease conditions indicated by particular levels of SAM in blood.

Betaine or antidepressants have been shown to increase SAM in the cerebrospinal fluid and blood of humans [3,18]. With the present method, dose-dependent increases in blood SAM due to treatment of mice with the methyl donor betaine were readily detected. The amount of blood needed for such testing could be obtained with a finger prick and thus the present method has great potential for convenient human testing. The method described here for measuring SAM levels in blood is rapid, accurate

and precise. It could also be used to predict physiological methyl group deficiency without invasive procedures. It could be used in a clinical setting, or in experiments with animals, to measure methyl group status without animal sacrifice.

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