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Quantification of erythrocyte S-adenosyl-L-methionine levels and its application in enzyme studies

Jennifer Lagendijk*, Johan B. Ubbink and W. J. Hayward Vermaak

Department of Chemical Pathology, Faculty of Medicine, University of Pretoria, P.O. Box 2034, Pretoria 0001 (South Africa)

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ABSTRACT

A highly selective high-performance liquid chromatographic method for the quantification of human erythrocyte S-adenosyl-L-methionine levels is described. A strong cation-exchange sorbent with propylsulphonic acid functional groups was used to extract S-adenosyl-L-methionine and S-adenosylethionine (internal standard) from erythrocytes. Quantification of erythrocyte S-adenosyl-L-methionine levels was achieved by using reversed-phase high-performance liquid chromatography and ultraviolet detection at 254 nm. This method was adapted to measure methionine-adenosyltransferase activity in erythrocytes, which enables us to study the possible role of altered methylation in different diseases.

INTRODUCTION

S-Adenosyl-L-methionine (SAM) is a metabolite which plays a key role in the regulation of different biological processes through its involvement in various metabolic pathways [1]. In the transmethylation pathway it is the sole methyl donor involved in the metabolism of neurotransmitters, including norepinephrine, dopamine and serotonin, all which have been implicated [2–4] in the pathogenesis of affective disorders (depression). SAM is also a precursor of glutathione via the transulphuration pathway and it has been shown that an increased intake of SAM can increase the level of glutathione. This compound facilitates the conjugation of various metals and steroid hormones in the liver resulting in an increased excretion. Based on these observations, the clinical usefulness of SAM supplementation has been tested in patients with psychiatric depression [1–6], lead poisoning [7,8], ethynylestradiol-induced jaundice [9,10], porphyria cutanea tarda [11,12] and fatty degeneration of hepatocytes [13], all of which have shown encouraging results. However, only in one study were SAM levels measured and correlated with the presence or absence of depression and Alzheimer's dementia [3]. In order to investigate the causal relation between disturbed SAM metabolism and various disorders, a suitable routine method is a prerequisite.

Several methods for the analysis of SAM in various tissues have been described [14–22], however, neither of these are suitable for routine quantification of this compound as will be required in *i.e.* clinical trials to establish the efficacy of SAM therapy in various pathological conditions. The lack of suitable routine laboratory procedures is reflected by the observation that only one [3] of the quoted studies [1–13] on the pharmacological applications of SAM administration reported SAM levels in biological fluids during the trial period. Quantification of SAM in biological fluids is usually based on UV absorption at 254 nm. [14–17,19–22]. However, interference by other UV-absorbing components in the SAM assay procedure is a major problem. Acid extracts of biological tissues or fluids have been

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used in a direct high-performance liquid chromatographic (HPLC) analysis for SAM, however, the presence of a variety of UV-absorbing components necessitate the use of binary solvent programmes to separate SAM from these interfering products [14–17]. These methods are particularly suitable if information is simultaneously required on the levels of SAM metabolites. In studies where SAM is used as a drug, however, a rapid and specific assay is required to monitor patient compliance and to define appropriate therapeutic blood levels.

A direct, isocratic HPLC method for the quantification of SAM and other adenine nucleotides in acid extracts has been reported [19], but the total HPLC analysis time is in excess of 2 h. This is not acceptable in clinical trials where large numbers of samples need to be analysed. Clearly, efficient sample clean-up procedures are required if SAM is to be selectively determined.

Ion-exchange chromatography on Dowex 50 (H^+) resin has been used to purify biological samples before isocratic HPLC analysis [21–23]. This procedure is not only tedious but SAM levels quantitated after sample clean-up differed over a ten-fold concentration range [23–25], indicating interference by co-eluting UV-absorbing components. Recently, solid-phase extraction has become a popular technique to prepare samples for HPLC analysis. Two reports were published on the applications of solid-phase extraction in SAM analysis [20,26]. Fell *et al.* [20] described solid-phase C_{18} extraction of amino acid S-adenosyl derivatives, however, the recovery of SAM was only 75%. Creason *et al.* [26] successfully used a cation-exchange column to extract SAM and S-adenosylhomocysteine from soy beans and radish leaves; the applicability of this method to human blood samples has not been investigated.

We now report highly selective purification of SAM from human erythrocytes using cation-exchange solid-phase extraction. The high efficiency and selectivity of the extraction process allows SAM quantification in the column eluate by a rapid, isocratic HPLC method. This method can easily be adapted to measure erythrocyte activity levels of the enzyme methionine-adenosyltransferase (MAT; EC 2.5.1.6), which catalyzes SAM formation from the substrates methionine and ATP.

EXPERIMENTAL

Materials

SAM and adenosine-5′-triphosphate (ATP) were obtained from Boehringer Mannheim (Mannheim, Germany) while S-adenosyl-L-ethionine (SAE) was supplied by Sigma (St. Louis, MO, USA). All other reagents were obtained from Merck (Darmstadt, Germany).

Extraction of SAM from erythrocytes

Sample preparation. Venous blood was collected with EDTA as anticoagulant; plasma was removed by centrifugation, and the red cells were washed twice with saline. To 300 μl of packed erythrocytes, 900 μl of perchloric acid (6%, v/v) were added. After vigorous mixing followed by centrifugation (25 000 *g*; 15 min), 50 μl of a SAE solution (200 μM) were added as internal standard to 780 μl of supernatant.

Solid-phase extraction. Disposable extraction columns packed with 1 ml of strong cation-exchange resin [propylsulphonic acid (sodium form) functional groups bonded to silica; supplied by Analytichem, Harbor City, CA, USA] were washed with 3 ml of methanol and then with 4 ml of 0.05 *M* potassium dihydrogenphosphate buffer (pH adjusted to 2.0 with concentrated orthophosphoric acid). An aliquot (700 μl) of the supernatant, to which SAE was added as internal standard, was directly applied to the extraction column. The column was then washed with 4 ml of 0.05 *M* phosphate buffer (pH 2.0) which removed the bulk of UV-absorbing material contained in the supernatant. Bound SAM and SAE were subsequently eluted with 2 ml of 0.05 *M* disodium hydrogenphosphate buffer (pH adjusted to 11.0 with a 6 *M* sodium hydroxide solution). All solutions were eluted from the extraction column by applying vacuum to a twelve-position Supelco vacuum manifold (Supelco, Bellefonte, PA, USA).

Elution profile and column capacity. A standard solution of SAM (1 m*M*) and SAE (1 m*M*) was mixed with an equal volume of perchloric acid (6%, v/v) and 900 μl were applied to a pre-conditioned cation-exchange extraction column. The column was washed with phosphate buffer (pH 2.0) as described above. Subsequently, the extraction column was washed with eight aliquots (300 μl each) of phosphate buffer (pH 11.0). Each aliquot was analyzed for SAM and SAE, using HPLC as described below.

In a second experiment, increasing volumes (0.5–1.5 ml) of supernatent (as described in sample preparation) containing known amounts of SAM and SAE were applied to the PRS extraction column to determine the capacity of the column to retain SAM and SAE.

HPLC analysis

A Waters (Milford, MA, USA) Model 510 HPLC pump, coupled to a Spectra-Physics (San Jose, CA, USA) SP 8780 XR autosampler, was fitted with a Whatman (Clifton, NJ, USA) Partisphere C_{18} reversed-phase analytical column (100 mm × 4.6 mm I.D.). To protect the analytical column, a Whatman reversed-phase guard column was fitted between the analytical column and the autosampler, while a Whatman Solvecon pre-column was installed between the autosampler and the pump. A 0.05 *M* potassium dihydrogenphosphate buffer (pH 5.7) was used as mobile phase at a flow-rate of 1.2 ml/min. The column eluate was monitored at 254 nm with a Linear (Reno, NV, USA) UVIS 200 variable-wavelength detector, coupled to a Spectra-Physics 4290 integrator. For analysis of erythrocyte SAM levels, an injection volume of 150 μl was used, while analysis of SAM produced by MAT activity (see below) required an injection volume of 50 μl.

Quantification of MAT activity in erythrocytes

For the measurement of MAT activity in erythrocytes, 250 μl of packed erythrocytes were added to 300 μl of tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4) and 50 μl of a 10% Triton X-100 solution. The haemolysate haemoglobin content was measured by the spectrophotometric method of Drabkin and Austin [27]. The reaction mixture for MAT activity contained 500 μl of haemolysate, 200 μl of an ATP (100 m*M*), 50 μl of a magnesium chloride (400 m*M*) and 50 μl of a potassium chloride (1.2 *M*) solution. This mixture was pre-incubated at 30°C for 10 min. Subsequently, 200 μl of an L-methionine (100 m*M*) solution was added as substrate and the reaction was terminated after 1 h by addition of 1 ml of perchloric acid (6%, v/v). After centrifugation (25 000 *g*, 15 min), 80 μl of an SAE solution (200 μ*M*) was added to 1 ml of supernatant, and 900 μl of the supernatant were then directly applied to the cation-exchange extraction column for purification of SAM and SAE as described above. SAM produced by MAT activity was measured by HPLC as described above; the results were adjusted for basal erythrocyte SAM levels and expresed as nmol SAM formed per g haemoglobin per h.

Stability of the MAT enzyme in pooled human erythrocytes, when stored at −20°C in CPP buffer (30 m*M* sodium dihydrogenphosphate, 16.8 m*M* disodium hydrogenphosphate and 109 m*M* trisodium citrate containing 40% glycerol) for up to three weeks was investigated. MAT activity levels were also measured in erythrocytes obtained from 32 human subjects.

RESULTS

Fig. 1 illustrates typical chromatograms obtained after HPLC analysis of (A) a 10 μ*M* SAM standard solution, (B) a sample of human erythrocytes (basal SAM levels), (C) the same erythrocyte sample to which an additional amount (5 μ*M*) of SAM was added and (D) a haemolysate incubated with substrates for MAT in order to measure SAM formation as indicator of MAT activity. SAM elutes at 3.9 min and SAE elutes as a double peak at 6.1 and 6.5 min, respectively. This double peak probably corresponds to the two different SAE stereoisomers [19]; the sum of the two SAE peak areas was used in calculating the peak-area ratio of SAM to SAE. Fig. 2 represents the elution profiles obtained when both SAM and SAE were eluted from the strong cat-

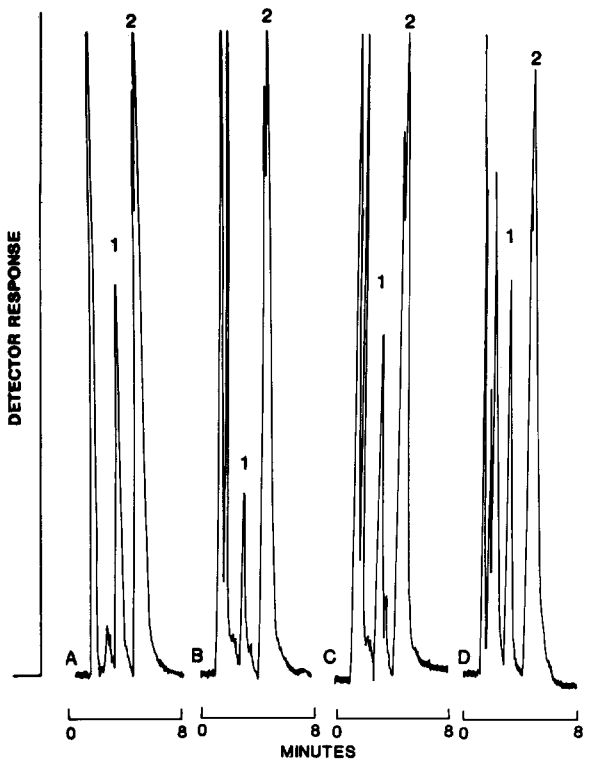

Fig. 1. Determination of S-adenosyl-L-methionine (SAM) in erythrocytes after cation-exchange solid-phase extraction, using HPLC and UV detection at 254 nm. (A) Standard solution containing 10 μM SAM and 12 μM S-adenosylethionine (SAE, internal standard); (B) pooled blood erythrocyte sample containing 5.2 μM SAM; (C) the same pooled sample spiked with 5 μM SAM; (D) erythrocyte MAT activity (94.3 nmol SAM formed per haemoglobin per h). Peaks: 1 = SAM; 2 = SAE.

ion-exchange extraction column using a phosphate buffer (pH 11.0). Complete recovery of both compounds was achieved after washing the column with 2.3 ml of phosphate buffer (pH 11.0). The first 300 μl of buffer were usually discarded since it contained less than 2% of the applied SAM and no SAE (Fig. 2B). The phosphate buffer (pH 2.0), which was used to wash the extraction column after application of the acid extracts from erythrocytes, contained no SAM or SAE.

The analytical performance of the method was satisfactory; the mean (± S.D.) erythrocyte SAM level as determined (n = 10) in a pooled blood sample was 5.2 ± 0.48 nmol/ml of packed red cells, implying a within-batch coefficient of

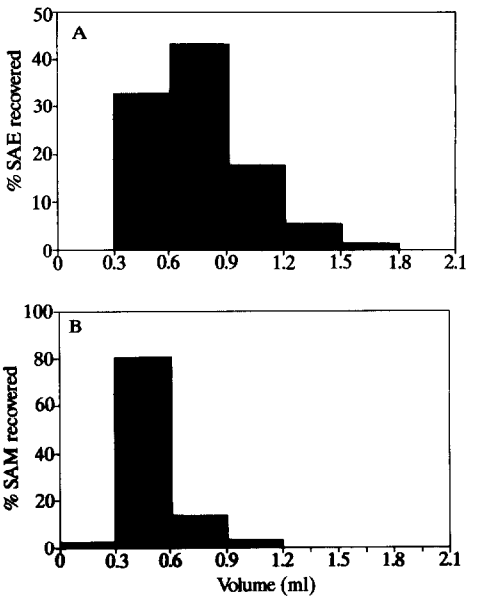

Fig. 2. Elution profile of absorbed SAM (B) and SAE (A) from a 1-ml strong cation-exchange extraction column (propylsulphonic acid functional groups). A 0.05 M disodium hydrogenphosphate–sodium hydroxide buffer (pH 11.0) was used to elute SAM and SAE from the extraction column.

variation of less than 10%. Mean recovery of SAM added (5 μmol/l) to different erythrocyte samples mounted to 107%.

A major limitation of ion-exchange solid-phase extraction is saturation of ionic binding sites by other ionic species, resulting in possible incomplete SAM or SAE recovery. Fig. 3 illustrates the capacity of the PRS extraction column to extract SAM and SAE from the haemolysate.

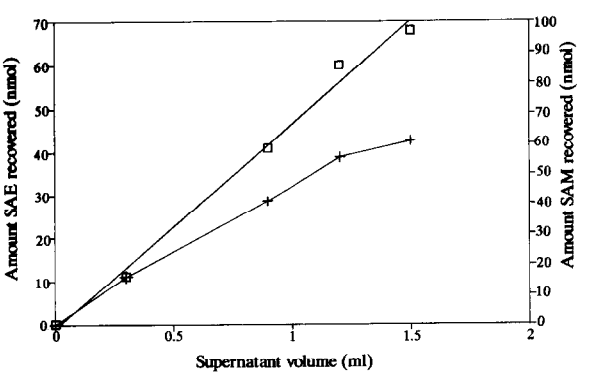

Fig. 3. Capacity of the cation-exchange extraction column for retention of SAM (+) and SAE (□) in haemolysate.

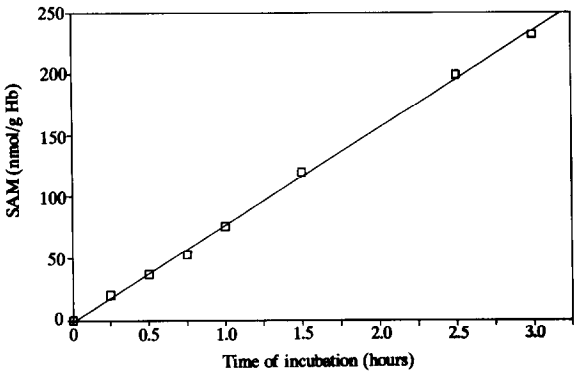

Fig. 4. Linearity of the methionine-adenosyltransferase (MAT) assay with time.

The amount of SAM extracted increased linearly with increasing supernatant volume up to 1.2 ml, while SAE extraction was linear up to 1.5 ml (Fig. 3). Therefore, we used 700 μl of supernatant for basal SAM levels and 900 μl of supernatant for MAT activity measurement; both volumes are well within the linear range for quantitative SAM and SAE extraction.

In agreement with previous assay procedures [28,29], our MAT assay is performed in presence of 20 m*M* of each of the two substrates (methionine and ATP). Under these conditions, product (SAM) formation increased linearly for up to 3 h (Fig. 4), indicating that neither substrate depletion nor product accummulation were rate-limiting factors in the assay procedure. The 1-h incubation time which is used in the enzyme assay is therefore well within the linear range. When erythrocytes were stored at -20°C in CPP buffer, MAT activity was found to be stable for at least 21 days. The mean ($\pm$ S.D.) MAT activity in erythrocytes from 32 men was 116.7 $\pm$ 29.4, with a relative wide range between 65.5 and 193.8 nmol SAM formed per g haemoglobin per h.

DISCUSSION

We have exploited the strong cationic character of SAM and SAE (internal standard) at low pH to extract these two compounds from a haemolysate. The perchloric acid extract from haemolysate has a pH < 1.5 [30]; at this pH SAM and SAE have two protonated amino groups and a positively charged sulphonium ion, while the α-carboxyl group is presumably also protonated and therefore without charge. By applying the perchloric acid extract directly to the cation-exchange extraction column, SAM and SAE are selectively bound to the column, while other UV-absorbing components can be removed from the column with a low-pH buffer. When the eluent is changed to a buffer with pH 11.0, both SAM and SAE lose their strong cationic character and are eluted with 2.0 ml of buffer from the extraction column. This extraction method is simple, but also highly selective. The high selectivity of the extraction procedure is demonstrated by the observation that a closely related compound, S-adenosylhomocysteiene, is not retained by the extraction column under the conditions described above (results not shown). The high selectivity of extraction is also shown by Fig. 1; apart from two or three faster-eluting UV-absorbing components, only peaks for SAM and SAE, with retention times of 3.9 and 6.5 min, respectively, could be demonstrated. The high efficiency of the clean-up procedure therefore allowed a simple, isocratic HPLC method to separate SAM and SAE from other UV-absorbing components. Total analysis time per sample was 8 min, which renders this method suitable for clinical trials where the measurement of SAM levels are indicated.

Although a pH 11.0 buffer is used to elute SAM and SAE from the cation-exchange extraction column, the pH of the first 2.0 ml which contains SAM and SAE is between 7.5 and 7.7 This is advantageous, because SAM is relatively more stable at pH 7.0 than at pH 11.0. However, it should be realized that even at lower pH, solutions of SAM are not stable, and a decline of about 14% can be expected to occur within 8 h at 37°C at pH 7.5 (% calculated from graph; see ref. 31). We therefore limit the amount of samples to be analyzed to twelve per batch. The HPLC analysis time for a batch of twelve samples is less than 2 h, and the loss of SAM due to degradation over this short time-span is negligible.

Recently, several publications have drawn at-

tention to the therapeutic applications of SAM. Promising results were published on the use of SAM as antidepressant, however, measurement of circulating SAM levels in these studies were notably absent [1,2,4–6]. Little is also known about the pharmacokinetic properties and possible therapeutic levels of this drug. The relatively easy HPLC method described in this paper may therefore be used to monitor patients on SAM therapy and to study the pharmacokinetic properties of this drug in more detail.

The method to measure erythrocyte SAM levels can be easily adapted to measure erythrocyte MAT activity levels. Previous methods to determine MAT activity levels used radiolabelled substrates with subsequent quantification of radiolabelled SAM [32–34]. These methods are normally laborious; not only is a laboratory suitable for work with radioactive material required, but chromatography is also needed as pre-purification step to separate SAM from the radiolabeled substrate [33,34].

In a recent review on the use of SAM as therapeutic agent, Friedel *et al.* [35] concluded that, although SAM is a promising drug in several disorders, insufficient comparative data are available to define the role of SAM in therapy. The method presented in this paper is suitable to measure both SAM levels and MAT activity in human erythrocytes. The ability to measure both SAM and MAT activity in a highly selective manner may help to elucidate the relation between altered methionine adenosylation, SAM therapy and various psychopathological disorders.

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