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Short communication

Quantification of plasma *S*-adenosylmethionine and *S*-adenosylhomocysteine as their fluorescent 1,N^6-etheno derivatives: an adaptation of previously described methodology

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Abstract

A simplified reversed phase HPLC system for the detection of fluorescent 1,N^6-etheno derivatives of SAM (*S*-adenosylmethionine) and *S*-adenosylhomocysteine (SAH) is described. The most important changes from the previously reported method are a shorter derivatization reaction time, the use of a solid-phase extraction resulting in an increase of the method's sensitivity, and the use of only one chromatographic system to separate SAM and SAH (in which the use of an ion-pairing reagent in the mobile phase is avoided). The linearity of the method was established, and the intra-assay coefficients of variation were 10.4 and 4.7% for SAM and SAH, respectively. Normal plasma values ($n = 8$), evaluated with the present methodology, were, for SAM and SAH, respectively, 57 ± 12 and 28 ± 3 nM (mean $\pm$ SD).

Keywords: *S*-adenosylmethionine; *S*-adenosylhomocysteine; HPLC; 1,N^6-etheno derivatives; Fluorometric detection

1. Introduction

Mild hyper homocysteinemia is nowadays consensually accepted as an independent risk factor for vascular disease [1–3]. However, the underlying pathogenic mechanism is unknown so far [1–3]. An interruption of the co-ordinate regulatory function of *S*-adenosylmethionine (SAM) in homocysteine metabolism, due to an elevation of *S*-adenosylhomocysteine (SAH) and a concomitant decrease of the SAM/SAH ratio, has been recently proposed [2,3]. In fact, SAH is known to be a potent inhibitor of the methyltransferases [2,3]. Therefore, an increase in its concentration

Abbreviations: SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; SPE, solid-phase extraction.

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could lead to a decrease in crucial methylation processes of the organism which have been postulated as being involved in the genesis of vascular disease. For this reason, the measurement of plasma levels of SAM and SAH is of great interest. For this purpose, recently Struys et al. [4] published an attractive method, using liquid chromatography coupled with tandem mass spectrometry. However, this method requires equipment not available in most clinical laboratories. Standard high performance liquid chromatographic (HPLC) methods using ultraviolet (UV) detection are not suitable for the analysis of plasma SAM and SAH levels, at the nanomolar range, due to lack of sensitivity [5–10]. To overcome this difficulty the classical procedure of the formation of the fluorescent 1,N^6-etheno derivatives before or after column separation has been used [11–16]. Based on the well-known reaction of chloroacetaldehyde with adenine and its analogues to give the fluorescent 1,N^6-etheno derivatives, Loehrer et al. [13–16] published an HPLC method coupled with fluorescence detection for the determination of plasma SAM and SAH levels. However, the derivatization reaction time is long (8 h) as well as the HPLC run time for SAM, which elutes as a broad chromatographic peak. Moreover, the presence of an ion-pairing reagent (heptanesulfonic acid) in the mobile phase used to increase retention for SAM decreases the reproducibility of the method. Furthermore, SAH is eluted in a second HPLC system, using a different mobile phase free of ion-pairing reagent.

In this article, a simplified reversed phase HPLC system for the detection of fluorescent 1,N^6-etheno derivatives of SAM and SAH is described. The derivatization time was lowered to 4 h, and only one chromatographic system was used to separate SAM and SAH. Moreover, the use of an ion-pairing reagent in the mobile phase was avoided. Furthermore, a solid-phase extraction (SPE) was employed, resulting in an increase of the method's sensitivity. The present method was applied to plasma samples from apparently healthy subjects. The same plasma samples were analysed using liquid chromatography coupled with tandem mass spectrometry, as described by Struys et al. [4]. Comparison of the results obtained with both the methods allowed the study of the accuracy of the method here described.

2. Experimental

2.1. Sample collection

Overnight fasting blood samples were collected in a vacutainer tube containing EDTA cooled in ice. Samples were immediately centrifuged at 2000 *g* for 10 min, 4 °C. A 1 ml aliquot of the obtained plasma was mixed with 625 μl of 10% perchloric acid, vigorously mixed and stored at −20 °C until analysis.

2.2. Chemicals

SAM and SAH were obtained from Sigma (St. Louis, MO). 2-Chloroacetaldehyde (45% in water, used without further purification) and HPLC grade solvents were products of Merck (Darmstadt, Germany). Other reagents used for sample preparation were of analytical grade. SPE was performed using Oasis HLB (hydrophilic–lipophilic balance copolymer) cartridges (60 mg, 3 ml) obtained from Waters Assoc. (Waters Corporation, MA).

2.3. Sample preparation

Prior to analysis, samples were thawed and centrifuged at 2000 *g* for 10 min at 4 °C. The supernatant was collected and used for subsequent SPE. Oasis cartridges were activated with 1 ml of methanol and equilibrated with 1 ml of 10 mM SDS pH 2.10, adjusted with orthophosphoric acid, and prepared just prior to use. Samples (1 ml) were loaded on the top of the cartridges, which were then washed with 3 × 1 ml of methanol–water (50:50, v/v). SAM and SAH were eluted with 1 ml of 2.5% acetic acid in methanol. The eluates were collected and dried down under nitrogen atmosphere at 30 °C. The dried residues were resuspended in 100 μl of 0.2 M perchloric acid, mixed with 25 μl of chloroacetaldehyde and 12.5 μl of 3 M sodium acetate (the pH was checked and if not between 3.5 and 4.0,

was corrected by the addition of more 3 M sodium acetate), and incubated for 4 h at 40 °C, to obtain the corresponding 1,N^6-etheno derivatives.

2.4. Apparatus

HPLC analysis was carried out using a Shimadzu system (Kyoto, Japan) consisting of model LC-10AD pump and model RF-551 fluorescence detector. Samples were introduced via a Rheodyne 7125 injection valve (Rheodyne, Cocati, CA) fitted with a 65 μl sample loop. The chromatographic separation was carried out on a Waters Symmetry C_{18} (150 × 3.9 mm I.D., 5 μm particle size) analytical column. The column was maintained at 10 °C in a jacket connected to a circulating refrigerated water bath. The fluorescent SAM and SAH 1,N^6-etheno derivatives were monitored at an excitation and an emission wavelength of 270 and 410 nm, respectively. The signal output was acquired on a computer using dedicated software (MILLENNIUM32, Waters Assoc.).

2.5. Chromatographic conditions

The mobile phase used consisted of 0.1 M sodium acetate buffer, pH 4.5, containing 4.2% acetonitrile (v/v), filtered through a 0.45 μm HA filter (Millipore, Bedford, MA) and degassed just prior to use.

SAM and SAH derivatives were resolved by an isocratic elution with a flow rate of 1 ml/min during 7 min. Then the column was washed over a period of 6 min by a linear increase of the acetonitrile content of the mobile phase from 4.2 to 50%, in 1 min, at a flow rate of 1 ml/min. A return in 1 min to the initial conditions, followed by a stabilisation period of 5 min prior to the next injection was used to ensure the reproducibility of the retention times. This chromatographic run allowed the evaluation of SAH. However, SAM eluted as an impure peak, requiring further purification. For this purpose, the corresponding SAM fraction, in this chromatographic run, was collected, dried down under nitrogen atmosphere at 30 °C, redissolved in 140 μl of mobile phase and 65 μl was re-injected on the same HPLC system, under the chromatographic conditions described above. Peak-start and peak-end time of SAM were determined using an enriched pooled plasma sample at the level of 250 nM. At this level no major interferences were visible.

2.6. Linearity and quantification

Standard curves were prepared by spiking pooled plasma samples with 25, 50, 100 and 250 nM of SAM and SAH. The calibrators were then processed as described in Section 2.3. The peak-areas of each individual standard (corrected for the peak-areas of a blank pooled plasma sample) were submitted to linear regression analysis. The estimated slope was used to calculate the unknown concentration of SAM and SAH in plasma samples.

2.7. Applicability and comparability

In order to evaluate the applicability of the present method, SAM and SAH concentrations were determined in plasma samples from eight apparently healthy volunteers. The same plasma samples were also analysed by the method of Struys et al. [4], which uses liquid chromatography coupled with tandem mass spectrometry.

3. Results and discussion

3.1. Chromatographic profiles

The resolution of plasma SAM and SAH 1,N^6-etheno derivatives obtained in the first chromatographic run is shown in Fig. 1A. The identity of the peaks was performed either by comparison of authentic standard retention times or by re-chromatography after spiking the samples with appropriate standards. SAH elutes as a single and symmetrical peak at a retention time of 6.1 min. SAM elutes at a retention time of 2 min as an impure peak. The poor resolution invalidated the quantification purposes of the developed method. However, this problem was overcome by further purification of SAM by re-injection of the previously collected SAM fraction on the same HPLC

system. An SAM chromatographic peak free from the major previous co-eluting interferences was achieved with a retention time of 1.9 min. A representative chromatogram is shown in Fig. 1B. In the method described by Loehrer et al. [13–16], a good resolution of SAM from other polar compounds present in the plasma matrix was achieved by increasing its retention time through the addition of an ion-pairing reagent to the mobile phase, which may impair the reproducibility of the method. Furthermore, a second HPLC system, using a mobile phase ion-pairing free, was required to evaluate SAH.

3.2. SPE process

In order to evaluate the accuracy of the SPE process, and to assess the possible interference of the plasma matrix, a pooled plasma spiked with SAM (25 nM) and SAH (25 nM) was deproteinized with 10% perchloric acid solution. From the supernatant, 100 μl (fraction A) was saved for later derivatization, whereas 1000 μl (fraction B) was loaded into an activated Oasis cartridge and processed as described. The derivatization reaction on those two fractions and their HPLC analysis were then performed using the present method. The recovery of SAM and SAH was estimated using the respective area ratios (fraction B/fraction A), taking into account the concentration factor ($\times 10$) in fraction B. The recovery of SAM and SAH were $88 \pm 8\%$ (mean $\pm$ SD; $n = 4$) and $92 \pm 3\%$ (mean $\pm$ SD; $n = 4$), respectively, showing that the plasma matrix interference on the SPE process was negligible, and the accuracy of the SPE process was suitable.

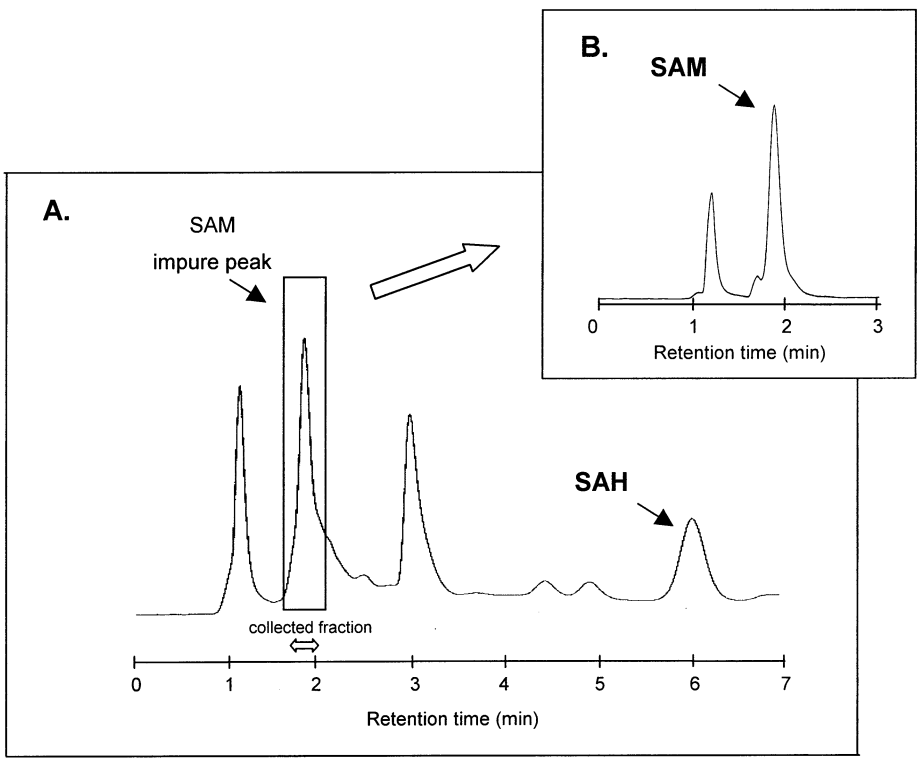

Fig. 1. Chromatographic profiles of a control plasma sample analysed according to the method described; SAH and SAM corresponding to a concentration of 26 and 56 nM, respectively. (A) Before SAM further purification, SAH as a well-resolved peak; (B) collected fraction, SAM was resolved as a pure peak.

3.3. Linearity and calibration

The method showed good linearity for the studied concentration range (25–250 nM) for SAM and SAH; the linear regression equations for SAM and SAH respectively, were: $Y = 3.28 \times 10^5 + 4.45 \times 10^3 \ X$ (intercept $SE = 6.07 \times 10^4$; slope $SE = 2.61 \times 10^2$) and $Y = 8.54 \times 10^5 + 2.72 \times 10^4 \ X$ (intercept $SE = 1.21 \times 10^5$; slope $SE = 9.87 \times 10^2$). The signal obtained for non-enriched calibrator was subtracted from the signal of the enriched calibrators. Calibration curves obtained with SAM and SAH standard solutions in 10% perchloric acid displayed, after linear regression analysis, a slightly different slope from those prepared in plasma (data not shown). This phenomenon is probably due to the presence of plasma compounds, which interfere with the derivatization reaction. To avoid this problem, quantification of plasma samples was performed using plasma-based calibrators.

3.4. Precision

The precision of the full method was assessed by quantification of SAM (74 ± 7.7 nM; mean $\pm$ SD) and SAH (43 ± 2.0 nM; mean $\pm$ SD) in five individual aliquots of the same plasma sample. The intra-assay coefficients of variation were 10.4 and 4.7% for SAM and SAH, respectively.

3.5. Sensitivity

The lower limit of detection (LLOD) of the method, defined as signal-to-noise ≥ 5, were 5.0 and 2.5 nM for SAM and SAH, respectively. These values compare favourably with those reported by Loehrer et al. (10 nM for both SAM and SAH) [16]. An improvement in LLOD was achieved due to the introduction of the SPE procedure.

3.6. Normal plasma SAM and SAH concentrations

Normal SAM and SAH concentrations were evaluated in plasma samples from eight apparently healthy volunteers. The values were 57 ± 12 and 28 ± 3 nM (mean $\pm$ SD) for SAM and SAH, respectively. These results are in agreement with those reported by Loehrer et al. (60 ± 20 and 24 ± 7 nM for SAM and SAH, respectively) [16].

3.7. Accuracy

In order to evaluate the comparability of the present method, the plasma samples were also analysed by the method of Struys et al. [4]. The achieved results were 63 ± 16 and 24 ± 5 nM for SAM and SAH, respectively, which were not significantly different (Student's t-test) than the results obtained by our method.

In conclusion, based on the HPLC method published by Loerher et al. [13–16] for the HPLC measurement of plasma SAM and *S*-adenosylhomocysteine as their fluorescent $1,N^6$-etheno derivatives, a simplified method is now reported. The improvements introduced in the present report were: (1) the derivatization time was decreased from 8 to 4 h; (2) an SPE was employed allowing an increase of the method's sensitivity; (3) finally, one chromatographic system was used for the detection of both SAM and SAH.

Our approach to the analysis of plasma SAM and SAH described in the present article requires technology currently available in most laboratories. In fact, this method only uses commercially available reagents and a standard HPLC apparatus equipped with a fluorescence detector. Such availability ensures a widespread use of the method contributing to a better knowledge of the role of the SAM/SAH ratio in crucial reactions in the organism.

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