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SHORT COMMUNICATION

Validated HPLC-Fl Method for the Analysis of S-Adenosylmethionine and S-Adenosylhomocysteine Biomarkers in Human Blood

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Abstract The natural methyl donor group, S-adenosylmethionine and its product, S-adenosylhomocysteine play an important role in many biochemical reactions involving transmethylation reactions. These compounds can be used as biomarkers in incipient diagnosis of various pathological disorders therefore the validation of a suitable method to routinely analysis of these compounds is very important. In this paper, a high performance liquid chromatrography method for S-adenosylmethionine and S-adenosylhomocysteine measurement as fluorescent 1,N^6-ethanoderivatives from biological samples was validated in terms of selectivity, linearity range of the response (R>0.9993), detection limit (9×10^{-9} and 4.4×10^{-9} molL^{-1}), the limit of quantitation (9.7×10^{-9} and 5.7×10^{-9}mol L^{-1}), precision, trueness and robustness. The method for quantification simultaneous of these compounds is rapid, sensitive and precise and appropriate for clinical analysis.

Keywords S-adenosylmethionine · S-adenosylhomocysteine · HPLC · Validation · Human blood · Biomarkers

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Introduction

The natural methyl donor group, SAM (S-adenosylmethionine) is an important physiologic compound playing an important role in many biochemical reactions involving enzymatic transmethylation [1, 2]. SAM forms SAH (S-adenosylhomocysteine) after the transfer of a methyl group from SAM to a methyl acceptor with subsequent conversion to homocysteine.

The concentrations levels of SAM and SAH, respectively, as well as their ratio are critical factors for metabolic processes, because these can be used as important biomarkers in incipient diagnosis of various pathological disorders [3–7].

Under physiologic conditions, SAH concentrations generally are several-fold lower than SAM concentrations and their measurement is time-consuming and difficult. The low concentration levels of SAM and SAH in body fluids (micromolar range in tissues and cells, nanomolar range in plasma) make necessary the development and validation of a proper rapid, sensitive and precise method for quantification simultaneous of these compounds, due to compounds significance for clinical analysis.

Various analytical methods have been developed for the analysis of SAM and SAH based on HPLC (high performance liquid chromatography). For compounds determination in matrices where SAM and SAH concentrations are at micromolar concentrations level, HPLC with ultraviolet detection is frequently used [8–10]. SAM and SAH can be separated by isocratic elution and determined by coulometric detection with detection limits in femtomolar range, but this method is not suitable in routine clinical analyses due to the relatively long elution times (40 min) [11]. HPLC-MS (high performance liquid chromatography coupled with tandem mass spectrometry) is a fast, precise, high selective and

sensitive analytical technique that allows precise and simultaneous quantification of SAM and SAH in tissue, plasma, serum and urine samples [12–16]. A drawback of these methods is the cost of analysis. A sensitive HPLC–FL method (HPLC with fluorimetric detection) based on the conversion of SAM and SAH to 1,N^6-ethanoderivatives by derivatization with chloroacetaldehyde and sodium acetate, that allow detections of SAM and SAH in the nanomolar range from plasma and cerebrospinal fluid, has been described [17–19].

Validation should be considered as a complement to the development of analytical methods providing evidence on the efficiency of developed methods. The validation is always required, whether national or international procedures standards apply. Also, validation is a continuous process; any change in the analytical system (another laboratory, another analyst) requires validation of the method in the new working conditions.

This paper aimed to demonstrate good performance characteristics of HPLC-FL method. The method validation in several terms (reproducibility, repeatability, trueness, etc.) was attempted in order to provide a fast and reliable analytical tool for screening routine SAM-SAH analysis [20, 21].

Experimental

Reagents and Materials

The SAM (Sigma, A7007) and SAH (Sigma, A-9384) stock solutions, $5.8\times10^{-3}\,\mathrm{mol\,L^{-1}}$ and $1.2\times10^{-3}\,\mathrm{mol\,L^{-1}}$, respectively, were prepared by water dissolving of the appropriate amount of substance. Stock samples were stored at dark and/or freeze between the experiments.

All the other reagents, methanol (ULC/MS-BIOSOLVE), KH_2PO_4 (Sigma, P-5379), heptanesulfonic acid (Sigma, H-2766), chloroacetaldehyde 45 % (Sigma, 10907), sodium acetate (Fluka, 71183), diethyl ether (Fluka, 31675), trichloroacetic acid, and H_3PO_4 were analytical pure or of chromatographic grade and they were used after filtration. The ultra pure water was obtained using a system for purification of water, Elix 3 (Millipore).

Instrumentation and Chromatographic Conditions

The chromatographic measurements were performed using two complete Shimadzu and Jasco HPLC system. UFLC Prominence Shimadzu (2D-HPLC) system was equipped with: five pumps LC-20AD, two column ovens CTO-20AC, a Kromasil, 100 10C18 10 μm 25×46 column, two degassers DGU-20A_5, a controller CBM-20A, a autosampler SIL-20A_{HT}, a fluorescence detector RF-10AXL and LC Solution software. HPLC Jasco system was equipped with: a Jasco PU-1580 Intelligent HPLC Pump, a Kromasil, 100 10C18 10 μm 25×46 column, a Jasco DG-2080-54 4-Line Degasser, a Jasco LG-1580-02 Ternary Gradient Unit and Borwin software. The used detector was Jasco FP-2020-Plus Intelligent Fluorescence Detector, set at appropriate excitation and emission wavelengths. The fluorescent SAM and SAH 1,N^6-ethanoderivatives were monitored at an excitation wavelength of λ_{ex}=270 and an emission wavelength λ_{em}=410 nm.

The mobile phase was sonicated in order to eliminate the dissolved air and then subjected to filtration using a Millipore PTFE 0.22 μm membrane. The samples were filtrated before injection using Chromafil PTFE, 0.2 μm, (Macherey-Nagel).

Sample Preparation

A small amount of fresh blood, 1 mL, was placed in an Eppendorf tube. The protein was precipitated by adding 40 % (w/v) trichloroacetic acid equal to 1:5 of blood volume. To remove the precipitated protein, the tubes were

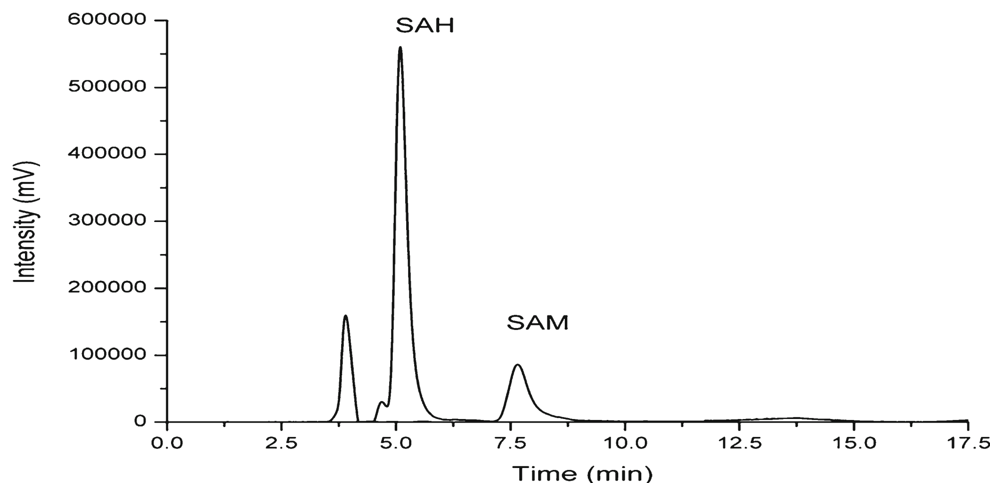

Fig. 1 SAM and SAH 1,N^6-ethanoderivatives chromatogram

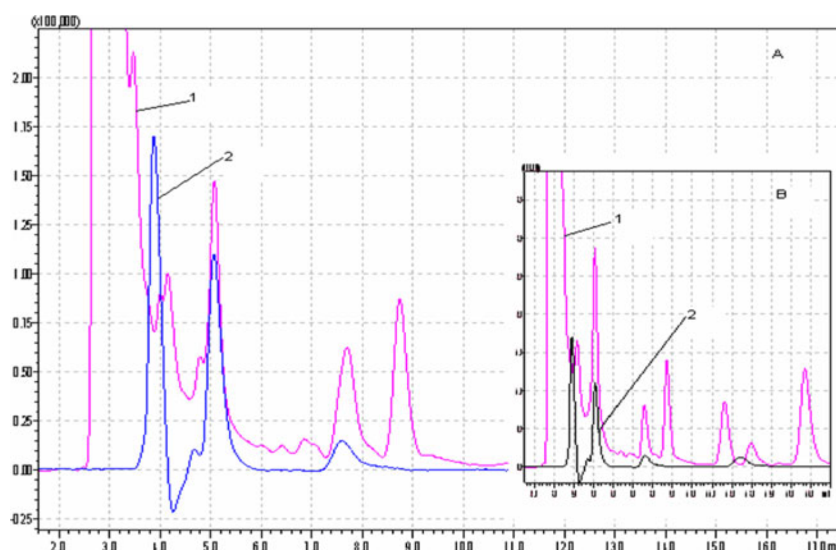

Fig. 2 Overlapped HPLC-FL chromatograms of blood sample (1) and mixture standards (2), for 70 years old volunteer (**a**), respectively 56 years old volunteer (**b**)

centrifuged at 25,000g for 10 min at 4^0C. The supernatant was then transferred to centrifugal filter units (Ultrafree-MC, 0.45 μm; Millipore) and an equal volume of diethyl ether was added (to extract lipids, to extract excess trichloroacetic acid). After that the supernatant containing tubes were centrifuged to separate the phases. The top layer was drawn off and discarded. When necessary, the samples were stored at 0–4^0C until derivatization.

Prior to analysis, the samples (mixture of SAM and SAH standards/blood) were subjected to pre-column derivatization using a derivatization reagent formed by chloroacetaldehyde (4.3×10^{-5} molL^{-1}) and sodium acetate (1.8×10^{-2} molL^{-1}), and incubated for 2 h at 60^0C, to obtain the corresponding 1, N^6-etheno derivatives.

Derivative samples (10 μL) were HPLC analysed immediately after derivatization. The column was equilibrated for 1 h before injections were started. A mobile phase of KH_2PO_4 (5×10^{-2}molL^{-1}) and heptanesulfonic acid (10^{-2} molL^{-1}) containing methanol 30 %, adjusted to pH 4.30 with phosphoric acid was used. For the SAM and SAH derivatives separation an isocratic elution was used, with a flow rate of 1 mLmin^{-1}, during 20 min. Then the column was washed over a period of 10 min with mobile phase using the same flow rate. After completion of series of analyses, the HPLC system was cleaned with water and methanol 50:50 (v/v) for 1 h. The experiments were performed at 25^0C.

Results and Discussion

A standardized method of SAM and SAH determination is not yet reported. The HPLC-FL method applied in the present study was previously developed [22] and we used for the validation of the HPLC-FL method the assessment of fluorescent 1,N^6-ethanoderivatives of SAM and SAH. The method was validated in terms of selectivity, linearity range of the response, detection limit (LoD) limit of quantitation (LoQ), precision, trueness and robustness.

To demonstrate selectivity of method the following experiments were performed:

– Overlapping the chromatogram of the fluorescent derivatives of standard compounds (SAM and SAH standard mixture) over the chromatogram of the derivatization reagent.

The chromatogram for the fluorescent 1,N^6-ethanoderivatives of SAM and SAH was registered and it was observed that the t_R (retention times) for SAM was 7.56± 0.07 min, while SAH retention time was 5.06±0.05 min and the peaks being properly resolved (Fig. 1).

In order to accurate discriminate between the retention times of SAM and SAH and the retention times of the derivatisation reagent, the chromatograms for the derivatisation reagent were registered and it was observed that the specific peaks for the derivatisation reagent are different from the peaks of SAM and SAH.

Table 1 Some performance characteristics of the proposed HPLC-FL method for SAM and SAH analysis

Compound	t_r (min)	The linear regression equations	Linearity range of response (mol L^{-1})	R	LoD (mol L^{-1})	LoQ (mol L^{-1})
SAM	7.56±0.07	$A = 5.8E + 12 \times CmolL^{-1} - 51067$	10^{-8}–5×10^{-7}	0.9993	9×10^{-9}	9.7×10^{-9}
SAH	5.06±0.05	$A = 1.9E + 13 \times CmolL^{-1} - 75205$	7.5×10^{-9}–2.5 10^{-7}	0.9998	4.4×10^{-9}	5.7×10^{-9}

Table 2 The experimental results of the injection repeatability and analytical repeatability of the proposed HPLC–FL method

Injection repeatability			Analysis repeatability		
Compound	Concentration (mol L^{-1})	CV	Compound	Concentration (mol L^{-1})	CV
SAM	2.5×10^{-7} 5×10^{-7}	0.63 % 0.83 %	SAM	10^{-7}	1.39 %
SAH	7.5×10^{-8} 10^{-7}	0.31 % 0.058 %	SAH	2.5×10^{-8}	0.98 %

– Calculating the resolution of retention times for the analytes of interest.

For estimate of R_s (resolution) were used retention times corresponding SAM and SAH and as noticed from the resolution values obtained (2.6 and 3.9), a confirmation of a properly separation of the peaks is provided. A resolution between two separated components accepted has to be minimum 1–1.5 [23].

– Overlapping the chromatogram corresponding to the standards fluorescent derivatives mixture (SAM and SAH) over the chromatogram of blood sample processed as described in the experimental section.

In order to accurate discriminate between the retention times of SAM and SAH and the retention times of the components from blood sample matrix, the chromatograms of the blood samples were registered in a first instance and it was noticed a clear difference between the specific peaks of the blood matrix and those of SAM and SAH derivatives (Fig. 2).

The calibration curves (A=f(c), A is peak area and c is compound concentration) were obtained from points resulting from the mean values of five measurements per point, corresponding to seven different concentrations, in the domain 10^{-8}–5×10^{-7} molL^{-1} (t_r=7.56±0.07 min) for SAM and respectively 7.5×10^{-9}–2.5×10^{-7} molL^{-1} for SAH. As observable from Table 1, the linearity range of the response proper for analysis of SAM in blood samples (SAM and SAH concentrations in plasma are in nanomolar range) for both compounds was of 1.5 decades, namely the correlation coefficients (R) were 0.9998 for SAH and 0.9993 for SAM.

Detection limit was calculated as 3 times the signal-to-noise ratio, and the limit of quantitation as 10 times the signal-to-noise ratio, respectively. In order to assess the noise contribution injection of mobile phase were performed (five replica), the peak area at the specific retention times being determined and further used to obtain the values corresponding to the detection limit and the limit of quantitation. The values obtained for SAM and SAH LoD and LoQ confirm as this method is able to be use for SAM and SAH analysis in blood samples (Table 1).

The precision of method was assessed by measuring the repeatability, the reproducibility and the intermediate precision of the method.

– The repeatability

For monitoring the injection repeatability, CV (coefficients of variation) for peak area and, respectively, retention time, were calculated for two samples (SAM and SAH mixture, 5 repetitive injections). It is recommended for the validation method as CVs to be≤1 % and for retention time, the values obtained are smaller than 1 % (Table 2).

The analysis repeatability of HPLC–FL method was determined by performing ten consecutive analysis runs for SAM and SAH, respectively, within one day. The obtained CV values are smaller than 1.5 % (Table 2).

– The reproducibility

To estimate the method reproducibility SAM and SAH assays were performed on five different days, during four months, five consecutive runs for each sample prepared everyday and the obtained CV values are≤5 % (Table 3).

– The intermediate precision

The intermediate precision of method HPLC–FL was determined by analyses of blood samples (64), using same method, in same laboratory but were used 2 different HPLC systems and the experiments were performed for several months. For experiments performed was used a HPLC Shimadzu system, each blood sample being five times injected, and a HPLC Jasco system, in this case each blood sample being three times analyzed. The variations between mean values SAM/SAH ratio obtained by two HPLC systems were monitored and CV value calculated (2.04 %) demonstrates that the method has a good intermediate precision.

Table 3 The experimental results demonstrating the reproducibility of the proposed HPLC–FL method

Compound	Concentration^n mean ± SD, (molL^{-1})	CV	Compound	Concentration^n mean ± SD, (molL^{-1})	CV
SAM	$2.49\times10^{-7}\pm10^{-8}$	4 %	SAH	$2.06\times10^{-7}\pm10^{-8}$	5 %

n average of 5 experiements

The method recovery was assessed using three blood samples fortified with standard SAM and SAH mixture, each sample being analyzed five times. The obtained values were in the range 89–111 % proving that for employed concentration level (5×10^{-8}–10^{-7} molL^{-1} for SAM and 2.5×10^{-8}–7.5×10^{-8} molL^{-1} for SAH, respectively) both the sampling and the HPLC-FL method were accurate enough to not introduce errors affecting significantly the analysis results.

To study the method robustness injections of SAM and SAH standard mixture were performed slightly changing the operational parameters of the HPLC, such are column temperature, controlled at 25^0C or at room temperature and pH of the mobile phase (4.29, 4.36, 4.39).

Obtained data proved that by performing these slight modifications of the operational parameters the changes occurring in the values of retention times for each standard are not significant, registered differences framing in the CVs values which still allows the compound identification using retention time criteria. Moreover, the modifications occurring in the peak area for a certain concentration (we used 5×10^{-8} molL^{-1} for SAM and SAH, respectively) are small, not affecting response linearity.

Conclusions

In this study, a HPLC method for SAM and SAH measurement as fluorescent 1,N^6-ethanoderivatives from biological samples was fully validated. The method was validated in terms of selectivity, linear range of the response, detection limit, limit of quantitation, precision (repeatability -injection repeatability and analysis repeatability-, reproducibility and intermediate precision of method), trueness and robustness.

The validated method is specific and selective (R_S between 2.6 and 4.6) with respect to compounds of interest, precise and robust presenting recovery from blood samples between 89 and 111 %, the detection limits (9×10^{-9} and 4.4×10^{-9} molL^{-1}, respectively) and the limits of quantitation (9.7×10^{-9} and 5.7×10^{-9} molL^{-1}, respectively) suitable for quantitative SAM and SAH measurement from biological samples (blood and tissues). The response proved to be linear (R>0.9993) and accurate (CVs for repeatability, reproducibility and intermediate precision of method <5 %). Considering all these results it has to be concluded that the method has an appropriate degree of robustness and simplicity to be used in determination of SAM and SAH in clinical studies.

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