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A Hydrophilic-interaction Chromatography Tandem Mass Spectrometry Method for Quantitation of Serum s-Adenosylmethionine in Patients Infected with Human Immunodeficiency Virus

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Abstract

Background—S-adenosylmethionine (SAM) has been suggested as a diagnostic test and surrogate marker for *Pneumocystis jirovecii* pneumonia (PCP) in HIV-positive patients. In this study, we report a robust hydrophilic-interaction liquid chromatography tandem mass spectrometry (LC-MS/MS) assay that can be used to quantitate serum SAM in clinical laboratories.

Methods—Proteins in serum samples were precipitated using trichloroacetic acid. The supernatant was separated after centrifugation. D₃-SAM was added as the internal standard. SAM and d₃-SAM were extracted using a mixed-mode cation exchange column. Extracts were dried under nitrogen and reconstituted in H₂O and acetonitrile (1:9, vol:vol). HPLC-tandem mass spectrometry analysis was performed with a silica column and multiple reaction monitoring for SAM and d₃-SAM.

Results—The limit of quantitation (LOQ) for SAM was 10 ng/mL. The assay was linear between 10–500 ng/mL. Intra-assay coefficient of variation (CV) was 8% and inter-assay CV was 17% at the LOQ. Turnaround time for each specimen was approximately 1 hour. Using this method, we found that serum SAM concentration was correlated with fasting status, especially methionine intake. We also measured acute and convalescent serum SAM levels of 8 HIV-positive patients with PCP and non-PCP pneumonia. SAM concentrations in convalescent samples were significantly increased compared to acute levels only in patients with PCP.

Conclusions—The LC-MS/MS method had sufficient analytical sensitivity for detecting low levels of SAM found in HIV-infected patients and can be used for quantitative measurements in a clinical laboratory. This method facilitates research and possible clinical application of SAM as a marker for PCP.

¹Nonstandard abbreviations HIV, Human Immunodeficiency Virus; SAM, S-adenosylmethionine; D₃-SAM, 2H₃-SAM; LC-MS/MS, liquid chromatography mass spectrometry; LOQ, limit of quantitation; CV, coefficient of variance; BAL, bronchoalveolar lavage; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid; BSA, bovine serum albumin.

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The incidence of *Pneumocystis jirovecii* pneumonia (PCP) remains high in individuals unaware of their HIV status and those failing to receive, adhere to, or respond to combination antiretroviral therapy and PCP prophylaxis[1]. Diagnosis of PCP is often based on empiric clinical and radiological findings [2]. Due to the nonspecific nature of these findings and the toxicities associated with treatment, a rapid, sensitive and specific laboratory method to diagnose PCP is needed. The traditional method used for this purpose (i.e., histological examination or immunofluorescence staining of induced sputum or bronchoalveolar lavage (BAL) fluid specimens[2]) relies heavily on morphology recognition and cannot distinguish dead pneumocystis cysts and trophic forms from living fungi. Thus, false positive results may result from patients recovering from previous PCP. For the same reason, these methods have limited value for monitoring response to PCP therapy.

S-adenosylmethionine (SAM) has been suggested as a diagnostic test for PCP in HIV-positive patients. S-adenosylmethionine is synthesized from methionine and ATP by SAM synthetase. It serves as a universal methyl donor in biometabolism [3]. Some studies indicated that pneumocystis must scavenge SAM from its hosts due to deficiency in SAM synthetase [4]. Two clinical studies reported that plasma SAM levels of patients with PCP were significantly lower than those of healthy controls and those with HIV and other pulmonary infections. SAM concentrations increased gradually in several PCP patients after 3–14 days of successful treatment [5,6]. Another study suggested pneumocystis encoded a functional SAM synthetase gene [7]. Conflicting literature reports warrant further investigation of the correlation between SAM and PCP.

SAM quantitation requires a sensitive method. Plasma or serum levels 60–160 nmol/L (24–64 ng/mL) have been reported for healthy people in several studies [5,8–10]; these levels are further decreased in PCP infected patients [5,6]. In the clinical studies cited [5,6], plasma SAM was measured using HPLC after derivatization with a fluorescent reagent [4]. This method has the advantage of high sensitivity, but requires an expensive derivatization reagent and fluorescence detectors are not routinely used or available in clinical laboratories. A sensitive coulometric electrochemical method was also reported but had the same disadvantage of not being amenable to routine clinical use [11]. Methods using reverse phase HPLC tandem mass spectrometry require ion-pairing reagents in order for the polar SAM molecule to be retained by C18 columns [9,10,12]. The presence of ion-pairing reagents can affect the performance of other LC assays. In contrast, hydrophilic-interaction chromatography is suitable for retaining highly polar analytes and can be used in clinical laboratories. In this study, we report a sensitive and convenient method for quantitation of SAM in a clinical laboratory based on hydrophilic-interaction chromatography- tandem mass spectrometry.

Serum SAM was first extracted using solid-phase extraction. One hundred microliters of trichloroacetic acid solution (10 g/dL) were added to 500 μ L of serum to precipitate proteins and disrupt SAM protein binding. The supernatant was separated by centrifugation at 2000g for 10 min. The internal standard d₃-SAM (CDN Isotopes) was added to 450 μ L of supernatant to a final concentration of 50 ng/mL. Oasis MCX mixed-mode cation exchange columns (Waters) were prepared by washing with 2 mL of methanol followed by 2 mL of H₂O. The supernatant from each sample was then slowly drawn through the column by vacuum. Columns were then washed with 2 mL of methanol. After the columns were dried under high vacuum for 5 minutes, SAM and d₃-SAM were eluted with 2 mL of a freshly prepared solution containing methanol and NH₃•H₂O (50 mL of NH₃•H₂O/L). The extracts were evaporated to dryness with a stream of nitrogen at 37 °C and reconstituted in 50 μ L of acetonitril and H₂O (9:1, vol:vol).

Ten microliters of reconstituted sample were injected by an autosampler into an HP 1200 HPLC (Agilent Technologies) interfaced with a 3200 Q TRAP® LC-MS/MS (Applied Biosystems).

Chromatographic separation was achieved on a 2.1mm ×50mm Atlantis HILIC silica column with a particle size of 3.0 μm (Waters). Samples were eluted at a flow rate of 500 μL/min with a mobile phase gradient from 1:9 (vol:vol) H₂O and acetonitrile to 100% H₂O in 4 min. Trifluoroacetic acid (TFA, 0.25 mL of TFA/L) was supplemented in the mobile phase for optimal peak shapes. Since TFA is known to suppress electrospray ionization signals of positively-charged analytes due to its ability to form gas-phase ion pairs with the analyte ions, we also added propionic acid (10 mL of propionic acid/L) in the mobile phase to compensate for ionization signal decrease caused by TFA [13]. SAM and d₃-SAM co-eluted at approximately 1.5 min. The mass spectrometry was operated in the positive mode. The source collision energy was set at 50 eV for the optimized transitions: m/z 399.0→250.0 for SAM; m/z 402.0→250.2 for d₃-SAM.

We found that SAM frequently adhered to the injection port and the chromatography tubing due to its very polar nature. This caused carryover between injections and imprecision. To address this issue, we first built a custom injector program in which the injection needle was washed in 100% H₂O for 4 times after drawing a sample. The needle was then washed in H₂O and acetonitrile (1:9, vol:vol) twice before injection. In addition, a wash run was programmed between every two sample injections: a blank water injection and a 3 min-perfusion with 100% H₂O were used to wash any residual SAM from the injection port and tubing. These measures efficiently removed carryover and improved precision. The LOQ, which was defined as the lowest SAM concentration at which the intra-assay CV was less than 20%, was determined to be 10 ng/mL.

Since SAM is present in all serum samples, we calibrated the assay using 8 spiked standards with bovine serum albumin (BSA) supplemented phosphate buffer (pH 7.4) as the matrix. We compared the above buffer with serum on LC-MS/MS and did not observe any significant matrix difference. The assay was linear between 10–500 ng/mL and was calibrated with the standards every time a new batch of serum samples was run.

Previous studies indicate that methionine administration caused rapid increase in SAM liver concentration in mice[14]. Since methionine is the precursor of SAM, we hypothesized that serum SAM levels would be correlated with fasting status and would increase after ingestion of methionine. We measured serum SAM concentrations of 5 healthy volunteers under fasting conditions and after a methionine-rich meal containing roasted peanuts. Fasting (baseline) SAM concentrations varied between individuals (mean ± SD, 96 ± 28 ng/mL). Two hours after the meal, SAM concentrations increased to different extents in all individuals (mean ± SD, 178 ± 56 ng/mL; mean increase ± SD, 81 ± 39 ng/mL) (Fig. 1). These results indicated that SAM levels are best interpreted with knowledge of fasting status and individualized baseline.

In a pilot study, we measured serum SAM concentrations of 8 patients with HIV infection and hospitalized with pneumonia. Among these patients, PCP was diagnosed in 4 by histological examination of Giemsa (Diff Quik) stained induced sputum or BAL fluid (“Pos” in Table 1) and was excluded in 4 patients who had other infections (“Neg”). Two serial samples were tested for each patient. The acute sample was drawn at time of acute pneumonia presentation and the convalescent sample was drawn 3–13 days after successful treatment. We also attempted to determine the patient’s fasting status at the time of blood draw. This information was not available for every sample. As shown in Table 1, convalescent SAM concentrations were 124–178% of acute levels in diagnosed PCP patients (mean increase 145%, 95%CI: 107–183%). SAM levels for patients without PCP did not demonstrate any significant increases (mean 98%, 95% CI: 81–115%) and were significantly different from the changes in SAM for patients with confirmed PCP (p=0.012).

We note that SAM levels in our study were higher than those reported before. In a recent study using HPLC with fluorescence derivatization to measure SAM, PCP diagnosed patients had SAM levels of <5–58 nM (<2–23 ng/mL, fasting status unknown); healthy subjects and patients with other infections had SAM levels of 67–249 nM (27–100 ng/mL, fasting status unknown) [6]. The difference between our results and the reported ones could be due to differences in extraction and detection methodology. SAM concentration has also been shown to be affected by other medications, such as vitamins, anti-depression drugs and levodopa [15–17]. These drugs, together with dietary intake of methionine, could have influenced SAM levels in our study. Future research is needed for further exploration of this issue.

In summary, we developed a robust and reproducible method for quantitation of SAM in serum of HIV-positive patients. This method can be implemented conveniently in a clinical laboratory. Using this method, we showed that serum SAM concentration varied between individuals and was affected by methionine intake. We also confirmed that SAM levels increased after successful PCP treatment. Although in our study acute SAM levels could not distinguish PCP positive patients from the ones without PCP, this should be further explored in a future study with knowledge of the fasting status. Regardless, serial fasting SAM levels may be useful for following up PCP therapy response.

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Fig. 1. Correlation between serum SAM concentration and fasting status

Serum SAM concentrations of 5 healthy volunteers under fasting condition and after a methionine-rich meal were plotted. Data points from the same individual are connected by a straight line. The non-fasting specimens were drawn 2 hours after the meal.

Table 1 Acute and convalescent serum SAM concentrations of 8 HIV-positive patients.

PCP status (by histological examination)	Acute		Convalescent		Change
	SAM (ng/mL)	Fasting status	SAM (ng/mL)	Fasting status	
Pos	51.1	n/a	63.6	fasting	124%
Pos	75.6	n/a	100	n/a	132%
Pos	142	fasting	208	n/a	146%
Pos	245	n/a	436	n/a	178%
Neg	98.7	fasting	84.8	n/a	86%
Neg	125	n/a	137	n/a	109%
Neg	197	n/a	183	n/a	93%
Neg	270	fasting	284	fasting	105%