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Development and validation of an LC-MS/MS assay for the quantification of the trans-methylation pathway intermediates S-adenosylmethionine and S-adenosylhomocysteine in human plasma

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ABSTRACT

Background: Although increased levels of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) have been implicated as markers for renal and vascular dysfunction, until now there have been no studies investigating their association with clinical post-transplant events such as organ rejection and immunosuppressant nephrotoxicity.
Methods: A newly developed and validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for the quantification of SAM and SAH in human EDTA plasma was used for a clinical proof-of-concept pilot study. Retrospective analysis was performed using samples from a longitudinal clinical study following *de novo* kidney transplant patients for the first year (n = 16).
Results: The ranges of reliable response were 8 to 1024 nmol/l for SAM and 16 to 1024 nmol/l for SAH. The inter-day accuracies were 96.7–103.9% and 97.9–99.3% for SAM and SAH, respectively. Inter-day imprecisions were 8.1–9.1% and 8.4–9.8%. The total assay run time was 5 min.
SAM and SAH concentrations were significantly elevated in renal transplant patients preceding documented acute rejection and nephrotoxicity events when compared to healthy controls (n = 8) as well as transplant patients void of allograft dysfunction (n = 8).
Conclusion: The LC-MS/MS assay will provide the basis for further large-scale clinical studies to explore these thiol metabolites as molecular markers for the management of renal transplant patients.

1. Introduction

Kidney transplantation is the preeminent treatment for patients with terminal kidney failure, both in terms of survival advantage and quality of life [1,2]. Although short-term allograft survival has significantly improved over the years, long-term graft survival past 5 y has remained largely unchanged [3,4]. Considerable advances have been made in the discovery and qualification of novel molecular markers that have shown promise as predictive markers of renal dysfunction and nephropathy, however, allograft biopsy in addition to monitoring changes in serum creatinine have remained the 'gold standard' for assessing renal function in clinical settings [2,5,6]. Nevertheless, the lack of sensitivity and specificity of serum creatinine and the risk of damaging the allograft during a biopsy have underlined the need for more sensitive and specific noninvasive diagnostic tools capable of monitoring and predicting kidney dysfunction in a clinical setting. While chronic allograft dysfunction is the primary cause of renal allograft loss after the first year, cardiovascular complications remain the foremost cause of death [1]. The increased prevalence of cardiovascular disease in transplant recipients is the result of multiple factors, and homocysteinemia was found in approximately 60% of renal transplant patients [7]. Unfortunately, due to large variation in endogenous homocysteine levels, its application as a molecular marker has often been unsuccessful [8,9]. As a result, primary focus has shifted towards methylation cycle intermediates, in particular trans-methylation pathway intermediates S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) Fig. 1 [8–11]. Methionine sulfur metabolism is thought to occur primarily via the trans-sulfuration pathway which results in the transfer of the sulfur from methionine, an essential amino acid that is primarily metabolized in the liver, to serine resulting in the formation of cysteine [12,13]. The first step in methionine metabolism is the formation of S-adenosylmethionine (SAM) in a reaction catalyzed by methionine adenyltransferase (MAT) [12]. Under normal conditions, most of the SAM generated in this process is used in trans-methylation reactions, whereby SAM acts as a universal methyl donor for a large variety of acceptor compounds by conversion into S-adenosylhomocysteine through the trans-methylation pathway [13]. This reaction is catalyzed by S-adenosylhomocysteine hydrolase and is reversible with the equilibrium favoring formation of SAH [12]. Several studies have shown evidence that elevated concentrations

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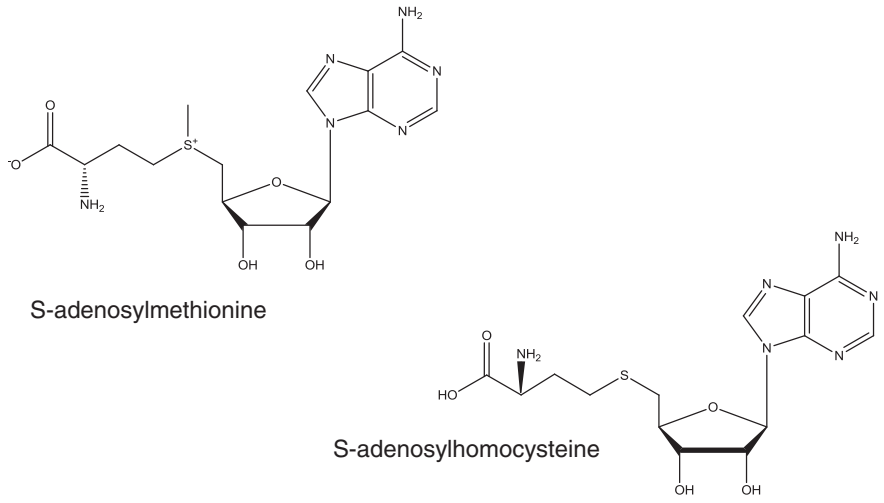

Fig. 1. Structures of SAM and SAH.

of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) can be used as sensitive indicators of vascular disease [8]. Moreover, decreases in the SAM/SAH ratio, which is also commonly referred to as "methylation potential" have been observed in patients with end-stage renal failure suggesting a link between disturbed trans-methylation reactions, vascular dysfunction and impaired renal function [10,11,14]. Although previous studies have identified both SAM and SAH as potential markers for cardiovascular and renal dysfunction, to date, no studies have been performed evaluating SAM and SAH concentrations and/or ratios in plasma from renal transplant patients as an early marker for acute rejection and nephrotoxicity. One of the major limitations has been the availability of reliable high-throughput assays that can be used for larger clinical trials and, later, potentially for clinical monitoring. While several quantitative assays have been developed to measure both metabolites in human plasma, utilizing coulometric electrochemical detection, liquid chromatography with ultraviolet detection (LC–UV), as well as liquid chromatography mass spectrometry (LC–MS), these assays involve tedious sample preparation, extended analysis times, and/or lack of proper bioanalytical method validation following current guidelines [14–18].

2. Materials and methods

2.1. Reagents and reference materials

Solvents and reagents (HPLC grade acetonitrile, water, formic acid, and ammonium formate) used for sample preparation and as mobile phases were from Fisher Scientific (Fair Lawn, NJ) and used without further purification. SAM, SAH and DL-homocysteine reference materials as well as dithiothreitol were from Sigma Aldrich (St. Louis, MO). SAH hydrolase was purchased from Abcam (Cambridge, MA). d5-Adenosine was from Cambridge Isotopes (Andover, MA), deuterated internal standard (d3-SAM) was purchased from C/D/N Isotopes (Pointe-Claire, QC, Canada), and deuterated internal standard (d5-SAH) was synthesized as previously described by Stabler et al. [18]. Six separate lots of human plasma were supplied by Bonfils Blood Center (Denver, CO). Six separate lots of human plasma with elevated triglyceride and bilirubin levels for matrix interference testing were supplied by Bioreclamation (Westbury, NY). The use of de-identified blood bank plasma for assay validation, calibration and quality control was considered exempt by the Colorado multi-institutional Review Board (COMIRB, Aurora, CO).

2.2. Sample extraction

Internal standard solution with d3-S-adenosylmethionine and d5-S-adenosylhomocysteine was prepared at a concentration of 5 μmol/l in 0.1% formic acid. Two hundred microliters of plasma sample was transferred into a microfuge tube. Each sample was spiked with 50 μl internal standard solution, vortexed for 5 min and incubated at 4 °C for 10 min. Five hundred and fifty microliters acetone stored at −20 °C was then added to each sample, vortexed for 10 min and incubated at 4 °C for an additional 10 min. Samples were then centrifuged for 10 min at 13400 ×*g* at 4 °C and 500 μl of the clear supernatant was transferred into an HPLC auto sampler vial.

2.3. LC-MS/MS assay for the quantification of SAM and SAH

Samples were analyzed using an Agilent 1200 series HPLC system consisting of a G1312 binary pump, a G1322A vacuum degasser, and a G1316A thermostated column compartment (Agilent Technologies, Palo Alto, CA) in combination with a Leap CTC PAL auto sampler (Carrboro, NC). The HPLC system was interfaced with an ABSciex 5000 triple quadrupole mass spectrometer (Foster City, CA) operating with an electrospray ionization source (ESI) using nitrogen (purity: 99.99%). Twenty microliters of the extracted sample were injected onto a 3.0 × 150 mm, 3.5 μm RP-Amide column, Supelco (St Louis, MO). The starting mobile phase consisted of 5% acetonitrile and 95% 10 mmol/l ammonium formate buffer (pH 3.4) with a flow of 0.6 ml/min for the first minute. After 1 min, the flow rate was increased to 0.8 ml/min and a gradient from 5% to 95% acetonitrile within 2.5 min was run. Acetonitrile was then held at 95% for 0.5 min. The column was re-equilibrated for 1 min to starting conditions. The mass spectrometer was run in the multiple reaction monitoring (MRM) mode with the interface heated to 500 °C. Nitrogen of >99.999% purity was used as collision activated dissociation (CAD) and curtain gas. The first quadrupole (Q1) was set to selectthe protonated molecular ion $[M + H]^+$ of each compound, SAM ($m/z = 399.0$), SAM-d3 ($m/z = 402.0$), SAH ($m/z = 385.1$) and SAH-d5 ($m/z = 390.0$). The declustering potential (DP) was 90 V and the entrance potential (EP) 10 V. Collision energy (CE) settings were 28 eV for SAH and SAH-d5 and 32 eV for SAM and SAM-d3. The second quadrupole (Q2) was used as collision chamber, and the third quadrupole (Q3) to select the characteristic product ions of SAM ($m/z = 250.1$ and $m/z = 136.2$), SAM-d3 ($m/z = 250.1$ and $m/z = 136.2$), SAH ($m/z = 136.2$), and SAH-d5 ($m/z = 137.2$).

2.4. Assay validation

The assay was validated following the FDA Guidelines on Bioanalytical Method Validation [19] as considered fit-for-purpose. Calibrators were prepared by spiking known concentrations of SAM and SAH into human plasma 1/5 diluted with PBS at 8 levels (8, 16, 32, 64, 128, 256, 512, and