Journal of Chromatography, 345 (1985) 150—156
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2768

Note

Determination of adenosine and S-adenosyl derivatives of sulfur amino acids in rat liver by high-performance liquid chromatography

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(First received April 10th, 1985; revised manuscript received June 17th, 1985)

The metabolism of methionine in rat liver is primarily dependent on its conversion to S-adenosylmethionine (SAM), the labile methyl group donor in a number of transmethylation reactions [1, 2]. The demethylated product of transmethylation, S-adenosylhomocysteine (SAH), an allosteric inhibitor of many SAM-dependent transmethylase enzymes [3, 4], is broken down into adenosine (Ado) and homocysteine by a reversible reaction. Consequently Ado and homocysteine concentrations directly influence SAH concentration and transmethylation [4]. Separation and quantitation of hepatic SAM, SAH and Ado are therefore necessary in many studies addressing methionine metabolism or transmethylation in liver.

Several methods for the determination of SAM, SAH and Ado using high-performance liquid chromatography (HPLC) have appeared in recent years [3, 5—9]. However, these require either lengthy sample preparation [5—7], derivatization [8] or gradient elution [3, 9] and only one [8] is designed to measure all three metabolites. We present an isocratic reversed-phase HPLC procedure for the determination of SAM, SAH and Ado in rat liver which was adapted from a method used to measure these metabolites in rat brain [10]. Rapid sample preparation using a Sep-Pak cartridge is followed by a single injection onto a C_{18} column with UV detection at 254 nm. The method may also be used to quantitate the concentration of hepatic S-adenosylethionine (SAE), produced from the carcinogenic methionine analogue, ethionine, when it is administered to rats [11].

0378-4347/85/$03.30

EXPERIMENTAL

Chemicals and reagents

SAM, SAH, SAE, Ado, protocatachuic acid (PCCA) and catechol-O-methyltransferase (COMT) were obtained from Sigma (St. Louis, MO, U.S.A.). SAM and SAE were purified by HPLC using the method described below. S-[Carboxyl-^{14}C]adenosylmethionine ([^{14}C]SAM) was obtained from Amersham (Arlington Heights, IL, U.S.A.) and [8-^{14}C]adenosine was obtained from New England Nuclear (Boston, MA, U.S.A.). 1-Octanesulfonic acid, sodium salt, was obtained from Aldrich (Milwaukee, WI, U.S.A.).

Synthesis of S-[carboxyl-^{14}C] adenosylhomocysteine ([^{14}C]SAH)

[^{14}C]SAH was synthesized using a modification of the procedure for radiochemical assay of SAM described by Yu [12]. [^{14}C]SAM was reacted with PCCA, a methyl-accepting catechol derivative, in the presence of COMT at pH 7.9. Tris buffer (200 m*M*), dithiothreitol (17 m*M*), magnesium chloride (4 m*M*), PCCA (1.2 m*M*), COMT (200 U) and [^{14}C]SAM (0.04—0.08 MBq) were combined in a final volume of 300 μl and incubated at 37°C for 60 min. A 300-μl volume of 2 *M* formate buffer, pH 3.5, was then added and protein was removed by centrifugation. [^{14}C]SAH was isolated by HPLC using the method described below. Radiochemical purity of greater than 95%, determined by HPLC, was obtained. This radionuclide was used in the determination of SAH recovery and stability, described below.

Sample preparation

Fresh rat liver was homogenized in 2 vols. of cold 0.4 *M* perchloric acid and centrifuged for 10 min at 9000 *g*. For recovery determinations and standard curve generation, known amounts of labeled or unlabeled SAM, SAH, SAE or Ado were added to the supernatant. The pH of 2.0 ml of the supernatant was adjusted to 6.5—7.5 using potassium hydroxide. After precipitation of the perchlorate salt was complete, 2.0 ml of the neutralized supernatant were applied to a C_{18} Sep-Pak cartridge (Waters Assoc., Milford, MA, U.S.A.) which had been charged with 1.0 ml of methanol and washed with 4.0 ml of water. The cartridge was then washed with 3.0 ml of water, which was discarded. SAM, SAE, SAH and Ado were eluted by applying 1.2 ml of 0.175 *M* acetic acid, followed by 3.0 ml of 25% methanol in 0.175 *M* acetic acid, to the cartridge. The eluates were pooled and 100 μl were used for the HPLC step.

High-performance liquid chromatography system

The stationary phase was μBondapak C_{18}, 5 μm particle size, packed in a stainless-steel column 300 × 3.9 mm (Waters Assoc.). The mobile phase was 5 m*M* octanesulfonic acid in 5% methanol, pH 4.0. The isocratic elution was carried out using a flow-rate of 2.8 ml/min. Each of the compounds was detected by monitoring the absorbance at 254 nm, since each contains the adenine ring structure. A full scale absorbance setting of 0.02 was used. The amount of each compound present in liver samples was determined by comparison of peak areas with the increases in peak area obtained by adding various amounts of each standard to perchloric acid supernatant before neutralization.

Recovery of added standards

The recoveries of SAM, SAH and Ado from rat liver were determined by adding labeled standard to 2.0-ml aliquots of perchloric acid supernatant and determining the total dpm present in the pooled eluate from the Sep-Pak cartridges. Radiochemical purity was then checked by HPLC to obtain estimates for any losses which may have occurred during sample preparation. Recovery of SAE was estimated by addition of known amounts of unlabeled compound to perchloric acid supernatant. By determining the amount of SAE present in the corresponding HPLC peak (from the absorbance of the eluate at 257 nm) and assuming complete stability throughout the procedure, the amount of SAE recovered from the Sep-Pak eluates was calculated.

Animals and diets

Ten weanling male Sprague—Dawley rats were housed individually in suspended wire mesh cages and fed a commercial ration ad libitum for fourteen days. Five rats were fasted for 8 h prior to the killing of all ten rats by decapitation. The average body weight (± S.E.) was 162 ± 2 g for the fasted group and 165 ± 2 g for the fed group. Livers were rapidly removed and prepared for SAM, SAH and Ado determination as described above.

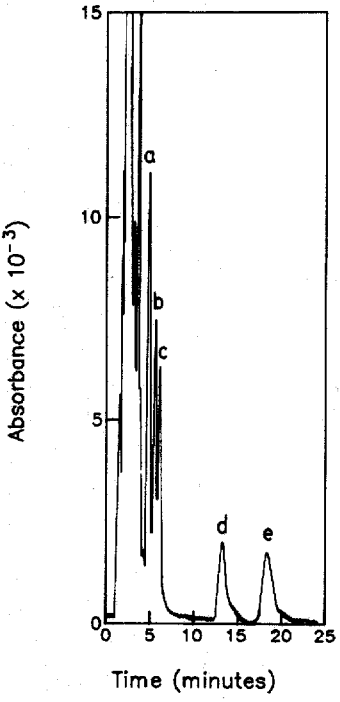

Fig. 1. Representative high-performance liquid chromatogram of a 100-μl injection of Sep-Pak eluate of rat liver supernatant to which had been added 100 nmol of S-adenosylethionine. The letters indicate the peaks corresponding to adenosine (a), an unknown compound (b), S-adenosylhomocysteine (c), S-adenosylmethionine (d), and S-adenosylethionine (e). Absorbance was monitored at 254 nm and the flow-rate was 2.8 ml/min.

RESULTS

HPLC elution profile

Fig. 1 shows a representative chromatogram of 100 μl of Sep-Pak eluate of a rat liver supernatant to which 100 nmol of SAE had been added. SAE was added since this compound is not normally found in tissues. The retention times were: Ado (peak a), 4.5 min; SAH (peak c), 5.75 min; SAM (peak d), 13.75 min; SAE (peak e), 19.0 min. Peak b corresponds to an unknown compound which could not be resolved from adenosine using the conditions of the original method described by Gharib et al. [10] for brain. This peak remained when neutralized liver supernatant was treated with adenosine deaminase, which destroyed the adenosine in the sample.

Recovery and stability of added standards

Ado and SAH were completely recovered and stable throughout the sample preparation and chromatography steps (Table I). SAM was also stable throughout the procedure but only 75% could be recovered. Making the assumption that SAE was also stable throughout the procedure, recovery was determined to be only 29%. As SAM and SAE are the first to elute from the Sep-Pak cartridge, the smaller recoveries for these compounds can be explained by loss during the washing of the cartridge with 3.0 ml of water. This step, however, is mandatory to ensure elimination of compounds which would otherwise interfere with the detection of Ado and SAH.

TABLE I

RECOVERY AND STABILITY OF STANDARDS ADDED TO RAT LIVER SUPERNATANT

The indicated standard was added to rat liver perchloric acid supernatant, which was prepared for HPLC by the method reported. The percentage of standard recovered in Sep—Pak eluate was determined by using labeled standards for adenosine, S-adenosylhomocysteine and S-adenosylmethionine. For S-adenosylethionine, it was determined by measuring the absorbance of the HPLC eluate and calculating the amount present in the Sep-Pak eluate, assuming 100% stability. Data are presented as means ± S.E.

Standard	*n*	Percentage of standard recovered in Sep-Pak eluate	Percentage radiochemical purity of Sep-Pak eluate (% stability)
Adenosine	4	99 ± 2	95 ± 2
S-Adenosylhomocysteine	4	98 ± 1	89 ± 1
S-Adenosylmethionine	4	75 ± 2	95 ± 1
S-Adenosylethionine	7	29 ± 3	–

Sensitivity and peak area response

This procedure will accurately detect concentrations of Ado and SAH as low as 5 nmol/g of liver and concentrations of SAM as low as 10 nmol/g of liver. When standards were added to perchloric acid supernatant, increases in peak

TABLE II

CONCENTRATIONS OF ADENOSINE, S-ADENOSYLHOMOCYSTEINE AND S-ADENOSYLMETHIONINE IN RAT LIVER

Data are presented as means ± S.E. of five rats. The diet was a commercial ration and was fed for fourteen days prior to killing.

Compound	Concentration (nmol/g)	
	Ad libitum fed	Fasted 8 h
Adenosine	36.5 ± 4.4	38.0 ± 3.1
S-Adenosylhomocysteine	17.9 ± 1.4	18.1 ± 1.1
S-Adenosylmethionine	75.6 ± 4.7	72.6 ± 6.3

areas were found to respond linearly ($r > 0.98$) up to 50 nmol/g of liver for Ado, 40 nmol/g of liver for SAH and 140 nmol/g liver for SAM, concentrations well above those we found in normal liver. Peak area responses were also linear ($r = 0.99$) for SAE between 150 and 1400 nmol/g of liver, the range of concentrations that have been found when rats are fed between 0.2 and 0.5% ethionine in the diet [11].

Concentrations of Ado, SAH and SAM in rat liver

The concentration of each metabolite in liver of rats fed a commercial ration is presented in Table II. Fasting the rats for 8 h prior to killing had no effect on these concentrations.

DISCUSSION

The method reported here provides a simplified procedure for the quantitation of Ado, SAH and SAM compared to the original method described by Gharib et al. [10]. It allows determination of all three compounds by making a single injection of a pooled Sep-Pak eluate onto the C_{18} column, whereas the original method required two injections for each sample.

In light of the fact that the original procedure was used to assay brain, several changes had to be introduced in order to permit successful analysis of liver tissue. We reduced the methanol concentration of the mobile phase from 10 to 5% and increased the pH from 3.5 to 4.0. We also discovered that when heptanesulfonic acid, the paired ion used in the original method, was employed to assay rat liver, the peaks corresponding to adenosine (peak a, Fig. 1) and an unidentified compound (peak b) could not be adequately resolved. The presence of the unidentified compound was demonstrated by treating neutralized liver supernatant with adenosine deaminase to destroy the adenosine peak. Substituting octanesulfonic acid as the paired ion permitted resolution of adenosine (peak a) and the unidentified compound (peak b).

The concentrations of SAM and SAH in rat liver as determined by our procedure are in good agreement with those reported by Finkelstein et al. [13], who used a radiochemical method [14]. They found that fasting for 24 h re-

sulted in a decrease in SAM to 55% of the control value and an increase in SAH to 193% of the control value for rats fed a stock diet [13]. These trends were not found when our rats were fasted for only 8 h (Table II). The SAM and SAH concentrations of our rats were both significantly higher than the values reported by Hoffman et al. [4], but their relative concentrations were comparable. The coefficients of variation for determination of SAM and SAH by our method were 14 and 18%, respectively. This represents a significant improvement in precision when compared to the coefficients of variation for the data reported by Finkelstein et al. [13] (14% and 54%, respectively) and Hoffman et al. [4] (29% and 75%, respectively). The hepatic adenosine concentration of our rats was found to be at least four times higher than previously published values [15].

In addition to Ado, SAH and SAM, SAE may also be detected and can be quantitated using our method. Despite the fact that recovery of SAE added to liver supernatant was found to be only 29%, the magnitude of SAE production by rats fed as little as 0.2% ethionine in the diet (230 nmol/g of liver) [11] permits successful quantitation of this metabolite.

This is, therefore, the first isocratic HPLC method which permits the simultaneous assay of Ado, SAH, SAM and SAE in rat liver. The possibility also exists that a non-physiological analogue of adenosine could be used as an internal standard to compensate for small differences in recoveries between samples during sample preparation. The use of such a compound, when found, would further improve the procedure reported here.

ACKNOWLEDGEMENTS

The authors wish to acknowledge M. Tullis for his contributions regarding the use of this procedure for SAE determination. This work was supported by USPHS Grant No. AM 32521, USDA Grant No. 82—CRCR-1-1153 and by the College of Agricultural and Life Sciences and the Graduate School of the University of Wisconsin-Madison.

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