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Note

Simple separation of adenine and adenosyl-sulfur compounds by high-performance liquid chromatography

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Much information has been accumulated concerning the role of S-adenosyl-L-methionine (SAM) in transmethylation, transsulfuration and aminopropylation reaction[1–3]. Its demethylated product, S-adenosyl-L-homocysteine (SAH), is one of the most potent inhibitors for various transmethylation reactions[4] and spermine synthetases[5]. These two compounds, SAM and SAH, are key intermediates in the regeneration of methionine methyl group[6] as well as in the transfer of the sulfur atom from methionine to cysteine[7]. 5′-Deoxy-5′-methylthioadenosine (MTA), a cleavage product of S-adenosyl-(5′)-3-methylthiopropylamine (decarboxy-SAM), which is the intermediate for polyamine synthesis, acts as a product inhibitor for the reaction[8,9], and inhibitor for some of transmethylases[10]. These two reaction products, SAH and MTA, subsequently give rise to the formation of adenine, a strong competitive inhibitor of MTA phosphorylase[11]. Therefore, the above mentioned inter-related metabolic pathway suggests that the simultaneous and quantitative determination of the metabolites in tissues should greatly enhance the study for their biological functions.

Numerous methods for determination of SAM metabolites have been described[11–26], including some that utilize high-performance liquid chromatography (HPLC)[11–16]. Although the reported HPLC methods are capable of resolving a few SAM metabolites, the simultaneous separation of various metabolites of SAM is impossible; poor resolution between adenine and MTA and/or MTA and SAH has been a drawback. In the present paper, we describe a simple improved method for simultaneous and quantitative measurement of adenine, MTA, SAH and decarboxy-SAM in addition to SAM.

MATERIALS AND METHODS

Apparatus

A Partisil-10/SCX (25 × 0.46 cm) I.D. column was obtained from Whatman. A solvent-delivery system, a Model 660 solvent programmer equipped with Model 45 single-piston pump and Model 6000A dual-piston pump, a Model U6K Universal Injector and a Model 440 dual absorbance detector were from Waters Assoc. The chromatographic runs were recorded on an Omniscribe recorder from Houston Instrument.

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Materials

Adenine, SAM and MTA were purchased from Sigma. SAH was from Calbiochem Decarboxy-SAM was generously provided by Dr. Vincenzo Zappia through Dr. Patrizia Galletti of the University of Naples (Italy). Acetonitrile was a HPLC grade preparation from Fisher Scientific. Other chemicals were analytical grade. Dowex 50W-X8 (200–400 mesh) was from Bio-Rad Labs. Male Sprague-Dawley rats (weighing *ca.* 200 g) were obtained from Charles River Breeding Labs.

Tissue extraction

All extraction steps were done at 0–4°C. After decapitation of rats, tissues were rapidly removed, rinsed in cold saline and blotted on filter paper. About 1 g of freshly excised tissue was homogenized with 1.5 *M* perchloric acid (1:4, w/v). After centrifugation, the deproteinized supernatant was chromatographed using a column of Dowex 50 (H^+) (resin bed, 4 × 0.5 cm) previously equilibrated with 0.1 *M* hydrochloric acid to remove contaminating compounds, and the desired metabolites of SAM were collected with 10 ml of 6 *M* hydrochloric acid. The 6 *M* hydrochloric acid eluate was mixed with 20 μl of thiodiglycol prior to evaporation and then dried under reduced pressure at 33–35°C with Buchi Rotovapor. Samples were routinely stored at −20°C

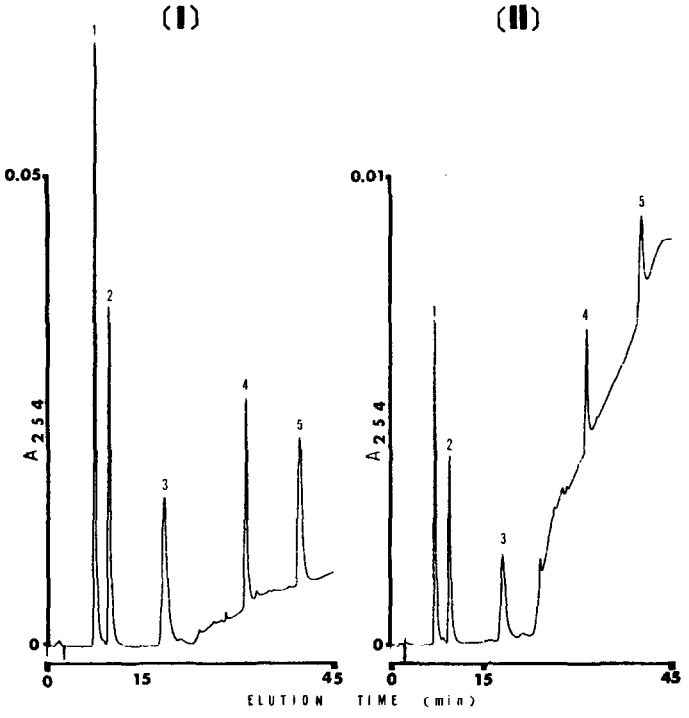

Fig. 1. Analytical HPLC elution patterns of adenine and adenosyl-sulfur compounds. The biphasic chromatographic elution was employed as described in Materials and Methods: [I], 1.25 nmoles of each compound with 0.1 a.u.f.s.; [II], 125 pmoles of each compound with 0.02 a.u.f.s. Peaks: 1 = MTA; 2 = adenine; 3 = SAH; 4 = SAM; 5 = decarboxy-SAM.

overnight. Just before HPLC analysis, this dried sample was redissolved in 1 ml of deionized water (final sample pH 3.0–3.4).

HPLC separation

The acid-soluble extracts of tissues were analyzed at room temperature for adenine and adenosyl-sulfur compounds using a Whatman Partisil-10 strong cation-exchange HPLC column. The flow-rate was 1.2 ml/min. Two elution buffers were utilized: buffer A, 15% (v/v) acetonitrile in 0.01 *M* ammonium formate, pH 2.6; buffer B, 15% (v/v) acetonitrile in 1.0 *M* ammonium formate, pH 4.3.

Prior to sample application, the column was equilibrated with buffer A. Following sample (10 μl) injection, the separation of SAM metabolites was effected by an isocratic elution with buffer A for initial 15 min followed by a linear 30-min gradient to 30% buffer B. There was a lag of *ca.* 7.5 min between the change to gradient elution buffer and the initial appearance of buffer B absorbance on the chromatogram (Fig. 1). The effluent was monitored by absorbance at 254 nm.

Calibration curves and recovery

SAM was purified by HPLC and found to be 84.2% pure using the molar absorbance as 15,000 $mole^{-1}$ cm^{-1} (ref. 2). Purities of other commercial standard compounds were analyzed individually by HPLC. Each compound exhibited more than 97% of UV-absorbing material as a single peak. All calibration curves were consistently linear over a range from 125 pmoles to 5 nmoles with less than 5% variation between identical samples (Fig. 2).

Recovery of each compound was determined as follows: 50 nmoles of each standard were added to a set of the original perchloric acid tissue homogenates, which served as the internal standards. Absorbance of each added standard peaks were then corrected by subtracting the absorbance due to the endogenous origin which was obtained from the another set of tissue preparations without added standards. The percentage recovery of added standards were then calculated on the basis of added amount of each compound. Usually 1/100 of the extract, which is equivalent to 500 pmoles of the added standard, was applied to HPLC.

Quantitation

The amount of each compound per gram of wet tissue was first estimated directly from the calibration curve based on the peak heights, and the values were corrected according to the recovery percent of each standard.

RESULTS AND DISCUSSION

Separation of MTA, adenine and SAH

Although HPLC method is frequently used to quantitate various nucleosides and their analogues, the available methods do not adequately resolve MTA, adenine and SAH, all of which are present in most biological samples. As an initial experiment, we used 0.01 *M* ammonium formate buffer, pH 3.3 (not containing acetonitrile) at a flow-rate of 2.5 ml/min as the mobile phase, and found that adenine, MTA and SAH essentially coeluted under these conditions (not shown). However, by decreasing the PH and flow-rate of the mobile phase to pH 2.6 and 1.2 ml/min, re-

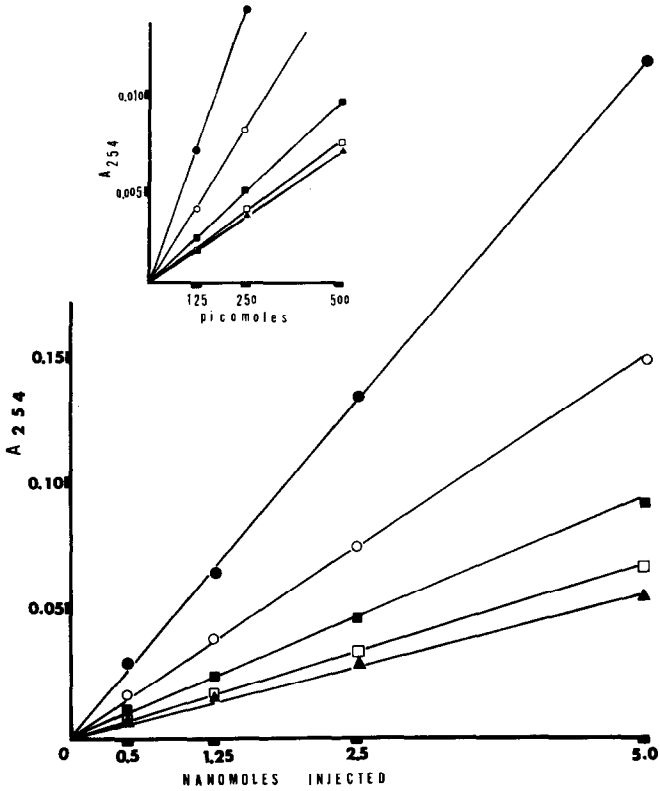

Fig. 2. Calibration curves of standard compounds. The chromatographic conditions were the same as Fig. 1. ● = MTA; ○ = adenine; ■ = SAM; □ = SAH; ▲ = decarboxy-Sam.

spectively, the SAH peak was clearly separated from those of adenine and MTA, although the latter two compounds were not effectively resolved (Fig. 3 [I]). However, on addition of acetonitrile to this low-pH mobile phase at 15% concentration, complete separation of all three peaks was achieved (Fig. 3 [II]). The use of this acetonitrile containing buffer, not only decreased the capacity factor (compare capacity factors, k', in Fig. 3 [I] and [II] for each compound)[27], but also increased the absorbance of SAH *ca.* 1.3-fold (A_{254} = 0.076 *vs.* 0.102). The apparent reason for the better resolution when acetonitrile is present in the elution buffer is that the retention times of the compounds on cation-exchange HPLC are proportionately decreased in a manner dependent on acetonitrile concentration due to the hydrophobicity of the solute[28].

Sensitivity of the improved HPLC method

Compared with the previously reported HPLC methods for the separation of SAM and its metabolites[12–16], the present method has the advantages of higher sensitivity and better resolution efficiency as illustrated in k' values in Table I and Fig. 1. We estimate that for each compound the limit of detection using this technique is somewhat less than 100 pmoles per 10-μl injection (Fig. 1 [II]). All the standard

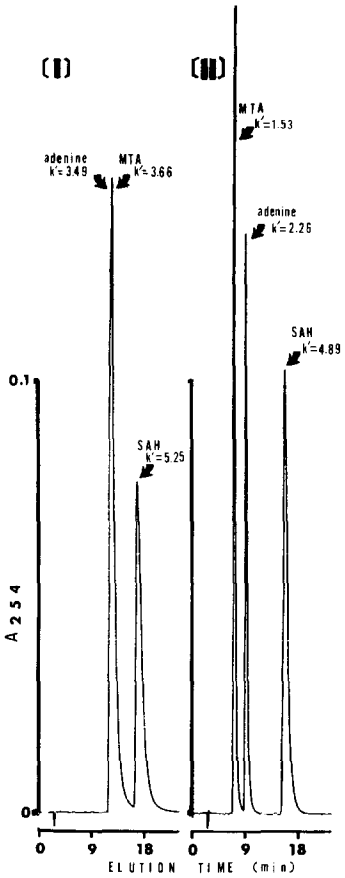

Fig. 3. Isocratic separation of MTA, adenine and SAH by HPLC. The standard mixture (10 μl) containing MTA (4 nmoles), adenine (4 nmoles) and SAH (8 nmoles) was applied on HPLC using a flow-rate of 1.2 ml/min at 850 p.s.i. and 0.2 a.u.f.s. at ambient temperature. The mobile phases applied were: [I], 10 m*M* ammonium formate buffer, pH 2.6; and [II], buffer A (a 5:1 mixture of the above ammonium formate buffer and acetonitrile).

TABLE I

CAPACITY FACTORS OF ADENINE AND ADENOSYL-SULFUR COMPOUNDS

Compounds	*Capacity factor (k')**
5'-Methylthioadenosine (MTA)	1.53 ± 0.02
Adenine	2.26 ± 0.03
S-Adenosyl-L-homocysteine (SAH)	4.89 ± 0.06
S-Adenosyl-L-methionine (SAM)	9.76 ± 0.05
S-Adenosyl-(5')-3-methylthiopropylamine (decarboxy-SAM)	12.37 ± 0.11

* The capacity factors were calculated according to the equation in ref. 27 from the retention time of distilled water, and indicate mean ± standard error of three independent determinations.

TABLE II

TISSUE CONTENTS OF ADENINE AND ADENOSYL-SULFUR COMPOUNDS IN NORMAL RATS

Compounds	*Concentration (nmoles/g wet weight)**		
	Liver	*Heart*	*Brain*
MTA	<0.05	ND**	ND
Adenine	32.8	98.2	110.9
SAH	18.0 (45.5)***	5.48	3.34
	(43.8)§	(3.9)§	(3.4)§
SAM	59.0 (66.3)***	27.1	20.6
	(57.5)§	(38.5)§	(25.4)§
	(72.7)§§	(65.2)§§	(27.6)§§
Decarboxy-SAM	1.98	ND	ND

* Values were corrected by recovery factors of each compounds as follows; 57.6% for MTA, 85.1% for adenine, 40% for SAH, 98.7% for SAM and 91.0% for decarboxy-SAM.

** Not detectable.

*** Ref. 15, Table 2.

§ Ref. 30, Table 2.

§§ Ref. 31, Table 1.

curves are linear for concentration up to 5 nmoles (Fig. 2). The sensitivity of the present method is comparable to that of the isotope-dilution technique[21,22].

Extraction of SAM and its metabolites from tissues

In establishing optimal conditions for the quantitative measurement of SAM and its metabolites in tissues, recovery of these compounds in the steps prior to the HPLC analysis is an extremely important factor for the accurate quantitation. Preliminary experiments using a Dowex 50 (H^+) column to remove undesirable compounds from the tissue extract by successive washings of the column with 0.1 *M* hydrochloric acid and 1.8 *M* hydrochloric acid[12] indicated considerable loss of MTA, adenine and SAH in the 1.8 *M* hydrochloric acid wash. Therefore, the column was washed with 50 ml of 0.1 *M* hydrochloric acid followed by only 15 ml of 1.0 *M* hydrochloric acid, and finally the compounds of interest eluted with 10 ml of 6 *M* hydrochloric acid. Although the 1.0 *M* hydrochloric acid wash still contained small amounts of MTA and adenine, massive loss of the compounds can be prevented using the washing procedure described. Thiodiglycol (20 μl) was added to the 6 *M* hydrochloric acid eluate before concentrating it, since the addition of this compound is known to suppress hydrolysis of SAH[22] which might be occurring during the subsequent steps. With these precautions, the recoveries of MTA and SAH were found to be 57.6% and 40%, respectively, while that of adenine was 85.1%. On the other hand, the recoveries of SAM (98.7%) and decarboxy-SAM (91.0%) were quite satisfactory. These recovery values were used to calculate the tissue concentrations of each compounds after HPLC analysis.

Application of the method to estimate the concentration of SAM and its derivatives in tissues

The present improved HPLC method was used for quantitative simultaneous

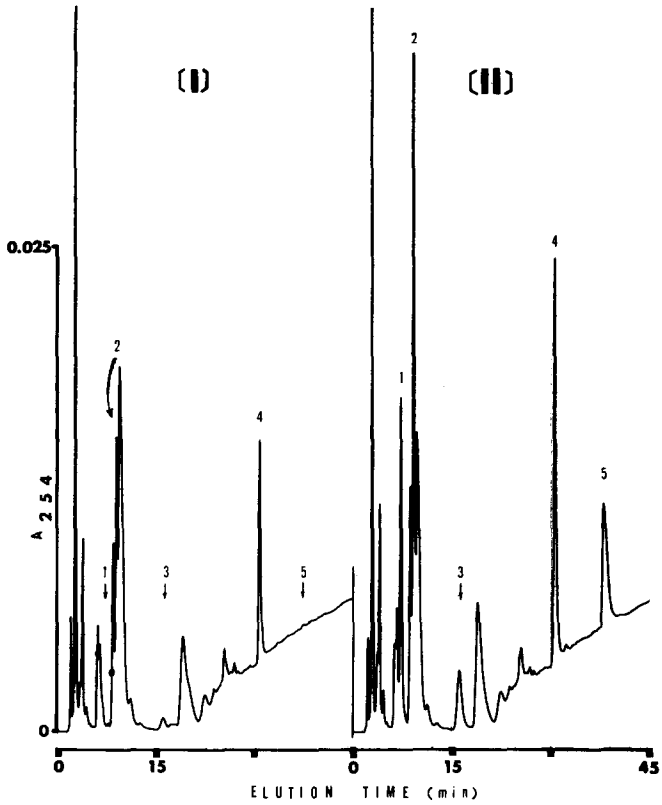

Fig. 4. Separation of adenine and adenosyl-sulfur compounds in rat liver. The chromatographic conditions were the same as Fig. 1 with sensitivity of 0.05 a.u.f.s.: [I], the extract equivalent to 8 mg of wet liver; [II], the above plus internal standards equivalent to 0.5 nmole of each compounds. Peak numbers as in Fig. 1.

estimation of adenine and adenosyl-sulfur compounds in rat tissues (Table II and Fig. 4). Although the data generally show slightly lower values than those reported by others[15,30,31], this is most likely due to higher efficiency of resolution with our method. The full separation of five compounds takes only 45 min.

For separation of one or two of these five compounds (SAM, SAH, adenine, MTA and decarboxy-SAM), the elution pattern can be adjusted in order to save time and elution buffer. For instance, the separation of SAM and decarboxy-SAM can be achieved by using a linear-gradient elution without the initial isocratic elution.

The first chromatographic method[20] ensuring reliable quantitation of SAM and SAH in tissues was based on the separation and purification by several chromatographic columns and subsequent estimation of the ribose moiety by the orcinol reaction. However, Eloranta[29] recently pointed out that even the improved method[15,17] was not suitable for the simultaneous analysis of SAM and SAH in routine analysis. Moreover, there had been no method available until now for the simultaneous quantitation of adenine and biologically important adenosyl-sulfur compounds.

We can conclude from the present study that, although various compounds which are present in the tissue samples prepared by Dowex 50 (H+) column interfere with HPLC separation of adenine and adenosyl-sulfur compounds, the described cation-exchange HPLC method is a highly sensitive and reproducible method for the simultaneous and quantitative determination of the major SAM metabolites.

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