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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF NATURAL ADENOSYL-SULPHUR COMPOUNDS

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SUMMARY

A rapid, sensitive and specific high-performance liquid chromatographic method for the simultaneous separation of natural adenosyl-sulphur compounds has been developed.

The compounds were separated by using the strong cation-exchange resin Partisil 10 SCX with isocratic elution. The adenosyl-compounds were monitored by an ultraviolet detector operating at 254 nm. Sensitivity was greater than 50 pmoles for all compounds tested and standard curves were found to be linear for concentrations of up to 50 nmoles. The method can be applied to biological samples for the estimation of S-adenosyl-L-methionine, S-adenosyl-L-homocysteine and S-adenosyl-(5')-3-methylthiopropylamine and for measurement of enzyme activities involving the above-mentioned compounds.

INTRODUCTION

S-Adenosyl-L-methionine (AdoMet) is the most widely recognized methyl donor in a variety of biological methyl-transfer reactions[1], S-adenosyl-L-homocysteine (AdoHcy) being its demethylated product[2]. Both compounds are key intermediates in the transfer of the sulphur atom from methionine to cysteine[3] as well as in the regeneration of the methionine methyl group in the sulphur-conservation pathway[4]. AdoMet also functions as regulator in a number of enzymatic reactions[5,6] that include 5'-methylthioadenosine (MTA) among the products[7].

Moreover, the sulphonium compound is a key intermediate in the biosynthesis of aliphatic polyamines: decarboxylation of AdoMet to S-adenosyl-(5')-3-methylthiopropylamine (decarboxy-AdoMet), followed by the transfer of propylamine moiety to putrescine and/or spermidine, represents the first step of the biosynthetic pathway[8]. Decarboxy-AdoMet has recently been reported to be a precursor of the new polyamines *sym*-nor-spermidine and *sym*-nor-spermine[9]. Some of the reported biological roles of AdoMet are summarized in Fig. 1; few other molecules are involved in so many different types of reaction within the cell.

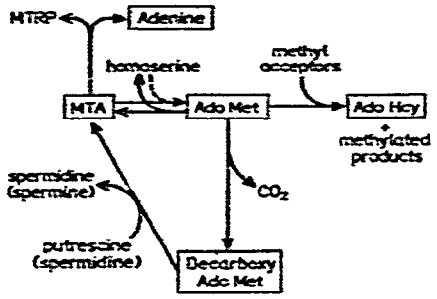

Fig. 1. The main metabolic pathways of S-adenosylmethionine in mammalian tissues. The molecules in the squares can be separated and analysed by the aid of the reported HPLC system. AdoMet = S-adenosyl-L-methionine; AdoHcy = S-adenosyl-L-homocysteine; decarboxy-AdoMet = S-adenosyl-(5′)-3-methylthiopropylamine; MTA = 5′-methylthioadenosine; MTRP = methylthioribose-1-phosphate.

The study of these intricate metabolic pathways is greatly enhanced by the possibility of a quantitative and simultaneous estimation of the involved adenosyl-sulphur compounds.

Methods for estimation of AdoMet, AdoHcy and their metabolic products have been described[10–15]. However, they are all quite laborious and generally permit the estimation of only single molecular species. Furthermore, despite the biological importance of decarboxy-AdoMet, few data are reported on the occurrence of this sulphonium compound in biological materials[16].

This paper reports a rapid method for the separation of the natural adenosyl-sulphur compounds listed in Fig. 1, using a high-performance liquid chromatographic (HPLC) system, which combines cation-exchange liquid chromatography under pressure and detection of peaks by UV absorbance. The method permits the quantitative estimation of AdoMet, AdoHcy and decarboxy-AdoMet in biological samples.

EXPERIMENTAL

Chemicals

Reagents were analytical grade and were always prepared in freshly glass-distilled water.

Since commercial AdoMet is contaminated by numerous impurities, the compound was routinely prepared from cultures of *Saccharomyces cerevisiae*[17] and isolated by ion-exchange chromatography[18]. Decarboxy-AdoMet was obtained by enzymatic decarboxylation of AdoMet[19]. The decarboxylated sulfonium compound was purified according to the procedure of Zappia *et al.*[20]. MTA was prepared by acid hydrolysis (pH 4.5, 100° for 30 min) of AdoMet[21]. The reaction has been monitored spectrophotometrically using adenosine deaminase from *Aspergillus oryzae*[22,23]. S-Adenosyl-(5′)-3-methylthiopropanol was prepared by partial deamination of decarboxy-AdoMet[24]. Adenine and AdoHcy were obtained from Sefochem, Fine Chemical Ltd., Israel.

All standard solutions were prepared in water at a concentration of 2 m*M*.

The chemical purity of the standards was checked by paper and thin-layer chromatography and high-voltage electrophoresis[2]. UV quenching at 254 nm, ninhydrin spray[25] and platinum iodide spray were used for the detection.

Enzymes

Non-specific adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) was purified 200 fold from *A. oryzae* powder (Sanzyme; Calbiochem, Los Angeles, Calif., U.S.A.) according to Kaplan[22] and Sharpless and Wolfenden[23].

AdoMet decarboxylase was purified from *E. coli* by the method of Wickner *et al.*[19], including the first ammonium sulphate precipitation.

Apparatus

A Model LC-65T Perkin-Elmer liquid chromatograph equipped with a Model LC-15 high-performance recording UV detector operating at a fixed wavelength of 254 nm was used. The column (25 cm × 4.6 mm I.D.) was prepacked with Partisil 10 SCX (Whatman). Integration was performed electronically using a Spectra-Physics Minigrator.

Operating conditions

Ammonium formate buffer, 0.5 *M*, pH 4.0, was used as eluent at room temperature. The flow-rate, 3.0 ml/min, produced a pressure drop of 3000 p.s.i.

Injection of sample was done via Model 70-10 sample injector valve and Model 70-10 loop filler port (Rheodyne). A Hamilton syringe (25–50 μl) was used to inject the sample volumes.

Quantitation of peaks

Concentrations were determined from calibration curves constructed by plotting the ratio of peak area measurement of sulphur compounds to internal standard (10 nmoles), over the concentration range.

S-Adenosyl-(5')-3-methylthiopropanol, a synthetic analogue of decarboxy-AdoMet, was used as internal standard.

Biological samples clean-up for HPLC analysis

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 through a Dowex-50 (H^+) column (resin bed 2 × 0.2 cm) previously equilibrated with 0.1 *M* HCl. Elution was carried out stepwise with 50 ml 0.1 *M* HCl and 20 ml 1.8 *M* HCl, to remove contaminating nucleotides and nucleosides, and the desired metabolites were collected with 6 *M* HCl. The 6 *M* acid eluate, containing AdoMet, AdoHcy, decarboxy-AdoMet and residual amounts of adenine and MTA, was concentrated under reduced pressure to 1 ml and the pH was adjusted to 4.0. Then 20 μl of this sample were analysed by HPLC. The recovery of AdoMet, AdoHcy and decarboxy-AdoMet is quantitative, while MTA and adenine are partly eluted with 1.8 *M* HCl. The internal standard, 0.5 μmol of S-adenosyl-(5')-3-methylthiopropanol, was added to the tissue during the homogenization with perchloric acid.

RESULTS

Separation of adenosyl-sulphur compounds

To check the reproducibility of the results, the stability of the instrument and column conditions, standard solutions of adenosyl-sulphur compounds were analysed several times. A representative chromatogram of these compounds is shown in Fig. 2 and the relative retention times are given in Table I. All the samples were eluted within 20 min of injection.

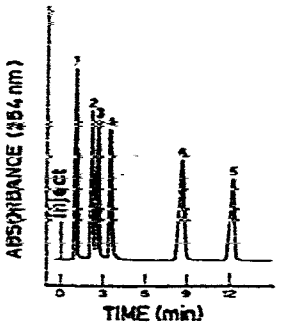

Fig. 2. HPLC separation of adenine and adenosyl-sulfur compounds. 1 = AdoHcy; 2 = adenine; 3 = MTA; 4 = AdoMet; 5 = decarboxy-AdoMet; 6 = S-adenosyl-(5')-3-methylthiopropanol (internal standard). The compounds were added in the amount of 10 nmoles each. Column, Partisil 10 SCX; temperature, ambient; sensitivity, 2.048 a.u.f.s.; eluent, ammonium formate, 0.5 *M*, pH 4.0; flow-rate, 3.0 ml/min.

TABLE I

RETENTION TIMES OF NATURAL ADENOSYL-SULPHUR COMPOUNDS

Operating conditions as in Fig. 2.

Compound	*Retention time (sec)*	*Coefficient of variation (%)*
Adenine	142	3
S-Adenosyl-L-homocysteine	92	2
S-Adenosyl-L-methionine	225	2
5'-Methylthioadenosine	170	3
5'-Methylthioinosine	82	2
S-Adenosyl-(5')-3-methylthiopropylamine	750	3.5
S-Adenosyl-(5')-3-methylthiopropanol (I.S.)	530	3.5

Sensitivity and reproducibility

The lower limit of detection for the tested compounds in our chromatographic system depends on the detector-sensitive noise level and column efficiency. Fig. 3b shows that in the system used, 50 pmoles of the above mentioned compounds are easily detectable. The peak shape remains constant up to 50 nmoles, as indicated in Fig. 3a.

Excellent reproducibility of retention times and peak areas was always obtained. The coefficients of variation of the retention times for five consecutive injections of standard solutions are reported in Table I.

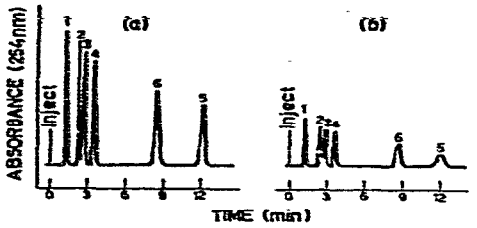

Fig. 3. Detection limits for HPLC separation of adenine and adenosyl-sulphur compounds. (a) $5 \cdot 10^{-8}$ moles of each standard adenosyl-sulphur compounds. Detector sensitivity, 2.048 a.u.f.s. (b) $5 \cdot 10^{-11}$ moles of each standard adenosyl-sulphur compounds. Detector sensitivity, 0.032 a.u.f.s. Operating conditions as in Fig. 2.

Linearity

Response curves were linear for all five tested compounds in amounts ranging from 5 to 50 nmoles (Fig. 4). The wide linear range for the different sulphur-containing compounds is more than adequate for their analysis in biological samples.

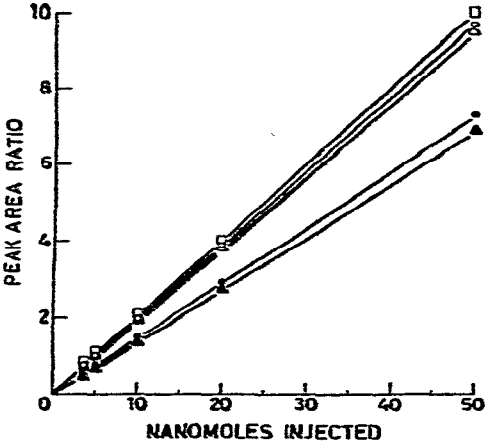

Fig. 4. Calibration curves of standard natural adenosyl-sulphur compounds. Operating conditions as in Fig. 2. □, Decarboxy-AdoMet; ○, AdoMet; △, MTA; ●, adenine; ▲, AdoAcy.

"Enzymatic peak shift"

The "enzymatic peak shift" was used to verify peak identities. This technique utilizes the specificity of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) from *Aspergillus oryzae*. Previous data indicate that this enzyme is very effective in deaminating adenosyl-5'-thioethers[26], whereas it is unable to convert the corresponding adenosyl-5'-sulphonium compounds[27]. Fig. 5 shows the effect of the deamination on the retention times of AdoMet, adenine and MTA. After the reaction the peak of MTA is substituted by a new peak, with a lower retention time, corresponding to MTI. The peaks of AdoMet and adenine were not altered by the reaction. The same procedure can also be applied in order to identify AdoHcy which can be quantitatively deaminated to S-inosyl-L-homocysteine, with a lower retention time.

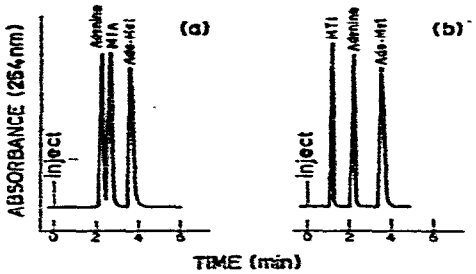

Fig. 5. Peak shift caused by reaction of non-specific adenosine deaminase on a standard solution of adenine, MTA and AdoMet. (a) Separation of a standard solution containing 10 nmoles each of adenine, MTA and AdoMet. (b) The same solution after reaction with 20 μg of non-specific adenosine deaminase, for 15 min at room temperature. 5'-Methylthioadenosine is quantitatively converted into 5'-methylthioinosine (MTI).

The "enzymatic peak shift" reaction can be extremely useful in clarifying or "unmasking" a chromatogram. When adenine is present in large amounts, it may hide the presence of small amounts of MTA which has a retention time close to that of adenine. With the aid of adenosine deaminase, MTA is quantitatively converted in to MTI which differs significantly from adenine in retention time (Table I).

Separation of adenosyl-sulphur compounds from rat liver

The adenosyl-sulphur compounds were analysed from 1 g of freshly excised rat liver using the sample clean-up procedures previously described. Samples equivalent to 20 mg of tissue were injected for each HPLC analysis. Fig. 6 shows a chromatogram from such an analysis.

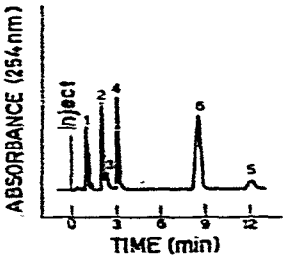

Fig. 6. Separation of adenosyl-sulphur compounds in rat liver extract. Sample of 20 μl equivalent to 20 mg of fresh tissue was injected into the column. The sample clean-up procedure is described in the text. Operating conditions as in Fig. 2.

DISCUSSION

This method offers several advantages over previous ones[10–15]. A rapid and excellent separation of the compounds can be achieved at room temperature with high sensitivity, selectivity and efficiency. The results are quantitative and minimal sample preparation is required.

The sensitivity of analysis permits accurate detection of 50 pmoles of each compound in the applied volume.

Since an isocratic elution is used no re-equilibration time is required between the analyses. The "enzymatic peak shift" represents a useful tool for the positive identification of the investigated thioethers.

The procedure has also been employed in our laboratories for the assay of enzymatic reactions where AdoMet and its related compounds are involved as substrates or products, *i.e.* AdoMet lyase (EC 3.3.1.–), MTA phosphorilase (EC 2.4.2.1)[28] and AdoMet decarboxylase (EC 4.1.1.50).

HPLC can be an excellent tool in the study of the overall metabolism of AdoMet in cell extracts as well as for checking the chemical purity of adenosyl-sulphur compounds. It appears to be less expensive and less time-consuming than the standard methods described.

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