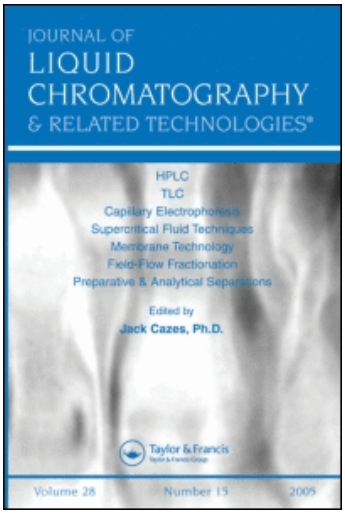

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF S-ADENOSYLMETHIONINE AND S-ADENOSYLHOMOCYSTEINE IN RAT LIVER

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ABSTRACT

A sensitive and precise high performance liquid chromatographic method for the simultaneous quantitation of S-adenosylmethionine (SAMe) and S-adenosylhomocysteine (SAH) in rat liver is described.

Liver is extracted by homogenization in 1.5 M PCA. SAMe and SAH are purified from contaminating substances by chromatography on Dowex 50 W x 2 and analyzed by HPLC on conventional styrene-type anion exchanger. Analytical chromatography, performed under isocratic elution conditions, is achieved in 18 min. SAH is determined as such while SAMe as adenine after hydrolysis at 60° C, under alkaline conditions. The sensitivity of the analytical procedure allows the determination of SAMe and SAH in less than 200 mg of liver sample.

INTRODUCTION

The quantitative determination of S-adenosylmethionine and S-adenosylhomocysteine in biological samples represents a somewhat complex analytical problem. Several procedures have been reported for its resolution, but remarkable experimental difficulties had to be overcome.

The radioenzymatic determination of SAMe (2), even if sensitive and specific, proved to be unsatisfactory owing to its laboriousness; moreover, the method does not allow the estimation of the demethylated derivative of SAMe, which is metabolically important as well.

The simultaneous analysis of the two metabolites in tissue samples has then been tackled by using ion-exchange chromatography (3-6). Shapiro and Ehninger (3) and Salvatore et al. (4) have thus described a chromatographic procedure involving a strong cationic resin. The procedure requires two chromatographic processes, the isolation of the two metabolites by precipitation with phosphotungstate and finally the alkaline hydrolysis of SAMe. The method needs about 10 g of tissue. Furthermore, owing to the use of a strong acidic eluent (6N H_2SO_4) and to the operational complexity, considerable losses of the two metabolites are often observed; thus, the use of an isotopic dilution technique is necessary.

A significant improvement in the analyses of SAMe and of its demethylated derivative has been achieved using high resolution liquid chromatography (5). This procedure is simple and rapid enough but, as the liver extracts are directly adsorbed on the analytical column, possible interferences due to uv-absorbing substances with the elution bands of SAMe and SAH, are likely to occur.

More recently, a chromatographic method using phosphocellulose beds has been proposed (6). After extract adsorption and washing of the column to remove the interfering materials, SAMe and SAH were eluted stepwise by using increasing concentrations of HCl. However, in this case also, the spectrophotometric determination of SAMe and SAH may be impaired by the co-elution of contaminating substances.

On performing a research dealing with the effects of SAMe administration during severe liver cell injury, a new method for the assay of rat liver SAMe and SAH has been devised. The analytical procedure, that is reported in this paper, involves the PCA extraction of rat liver samples, the clean-up of the extracts by Dowex 50W-X2 chromatography and the final simultaneous determination by high performance liquid chromatography of SAH and SAMe (as adenine), after the alkaline hydrolysis of SAMe.

MATERIALS AND METHODS

Chemicals and Chromatographic Packings

S-adenosylmethionine was obtained from BioResearch Co. (Liscate, Milan, Italy); S-adenosylhomocysteine, 5'-deoxy-5'-methylthioadenosine (5'-MTA) and adenine (Ade) were obtained from Sigma Chemical Co. (St.Louis, Mo., USA); 2-amino-2-methyl-1-propanol (MAP) was obtained from E. Merck (Darmstadt, West Germany). Dowex 50W-X2 (100-200 mesh) and Aminex A-14 (20 $\pm$ 3 μ) were obtained from Biorad Labs. (Richmond, USA). Other chemicals were obtained from commercial sources.

Extraction and Purification of SAMe and SAH

All steps prior to chromatography were carried out at 0-4°C. Male Sprague-Dawley rats of 150-180 g body weight were sacrified by decapitation, the livers quickly removed, and 1-1.5 g of tissue portions were homogenized in 4-6 ml of 1.5 M ice-cold PCA. After centrifugation for 10 min at 15,000 x g, the supernatant was withdrawn while the precipitate was resuspended in 5 ml of 1.5 M PCA and again centrifuged. The second supernatant was combined with the first and the solution obtained was three-fold diluted with bidistilled water. The diluted solution was adsorbed into a 15 cm x 1.3 cm i.d. column packed up to 2 cm with Dowex 50W-X2,

H^+ form, equilibrated with 0.5 M PCA. The column was first washed with 50 ml of 0.5 M PCA at 90-100 ml/h, in order to elute the uv-absorbing contaminating substances (nucleotides, nucleosides and bases), and then washed with 10 ml of 10^{-2}N HCl. SAMe and SAH were eluted with 35 ml of 0.5 M NH_4OH (the first 5 ml were discarded). The ammonia eluate was heated to 60°C for 10 min and then concentrated to dryness under vacuum at 60°C on a rotary evaporator. The residue was taken up in 2 ml of 0.1 M 2-amino-2-methyl-1-propanol, 0.07 M NaCl, 2% propanol pH 9.35 buffer (Buffer A). Aliquots of this solution were used for the HPLC analyses.

High Performance Liquid Chromatography

The analytical chromatography was performed by using an LC 20 Pye Unicam liquid chromatograph equipped with an UV detector operating at 254 nm, with a cell of 1 mm diameter and 10 mm of path length (8 µl capacity). The detector output, linear within the range 0.010-1.28 a.u.f.s., was connected to a 10 mV chart recorder, with a chart speed of 12 cm/h.

Chromatography was carried out by using Aminex A-14, Cl^- form, as stationary phase. The resin, processed as described elsewhere (7) was packed in 0.3 M HCl up to 25 cm in a glass column (30 cm x 0.4 cm i.d.) thermostated at 50° C. Prior to chromatography the column was equilibrated with Buffer A for 15 min. After adsorption of the sample (0.2-0.5 ml), isocratic elution was carried out at a flow rate of 75 ml/h with Buffer A. Usually several samples were chromatographed in succession; after 20-30 analyses the column was regenerated by washing with 0.3 M HCl for 10 min. After equilibration with Buffer A for 15 min, the column was again ready for new analyses.

In some experiments, performed in order to identify the products obtained from the alkaline hydrolysis of SAMe, the column equilibration and elution were carried out with 0.1 M MAP, 0.1 M NaCl, 1% propanol, pH 10.30 buffer (Buffer B). This elution system allowed the separation of 5'-MTA, SAH and Ade.

Chromatographic Quantitation

The calibration curves, obtained by correlating the heights of the chromatographic peaks with the nanomoles of the corresponding substances, were determined by introducing into the chromatograph known amounts of synthetic SAMe and SAH treated as those of the tissue extracts. The starting concentrations of SAMe and SAH solutions were checked by using an ε = 14,700 at 256 nm in 6 N HCl. The quantitation of sample peaks was carried out by using the calibration curves previously drawn.

Recovery Studies

The efficiency of SAMe and SAH recoveries were established by adding known amounts of the two synthetic metabolites to liver homogenates. Two identical aliquots of the same rat liver (1 g each) were homogenized in PCA. After addition of synthetic SAMe and SAH to one of the homogenates, the extracts were treated as described above.

The recovery was determined by comparing the amounts added with the increase of the content of the two metabolites of the spiked sample, above those of the original sample.

RESULTS AND DISCUSSION

Extraction and Purification of SAMe and SAH

One of the main difficulties for the dosage of SAMe and SAH arises from their different chemical stability which is strongly dependent upon pH and temperature conditions. SAMe, which has a

sulfonic group, is sufficiently stable under acidic conditions, while SAH, which has a thioether bond, is unstable in concentrated solutions of strong mineral acids. Moreover, SAMe is almost unstable in neutral or alkaline conditions, giving place to different degradation products with respect to the pH value of the solution (8). On the contrary, SAH is stable in dilute alkaline solutions. The assay procedure here reported has been selected in order to avoid these analytical difficulties. The extraction of SAMe and SAH from rat liver using ice cold 1.5 M PCA is efficient and does not result in a significant hydrolysis of the thioether bond of SAH. The next purification step, which according to our experience is necessary to obtain reliable results, is carried out at conditions which yield a satisfactory recovery of the two metabolites. The solution used to elute the compounds investigated from the cationic exchanger was chosen in order to obtain pH conditions to allow the complete hydrolysis of SAMe to adenine and pentosylmethionine during drying at 60° (8). Under these conditions, SAMe (as adenine) and SAH are obtained from the biological sample in a stable form, suitable for their simultaneous separation by HPLC at isocratic elution conditions.

Chromatographic Resolution

Figure 1 shows the elution chromatogram of a standard mixture of SAH and SAMe processed as described. The peaks having t_R= 10 min and t_R= 13 min are due to 16 nmoles of SAMe (as adenine) and 10 nmoles of SAH. The unique uv-absorbing product of SAMe degradation is adenine; 5'-deoxy-5'-methyl-thioadenosine, which rises from the hydrolysis of SAMe under mild-acid or neutral conditions, is completely absent. This is evident from Figure 2, which shows the elution chromatogram of synthetic 5'-MTA, SAH and Ade (curve A)

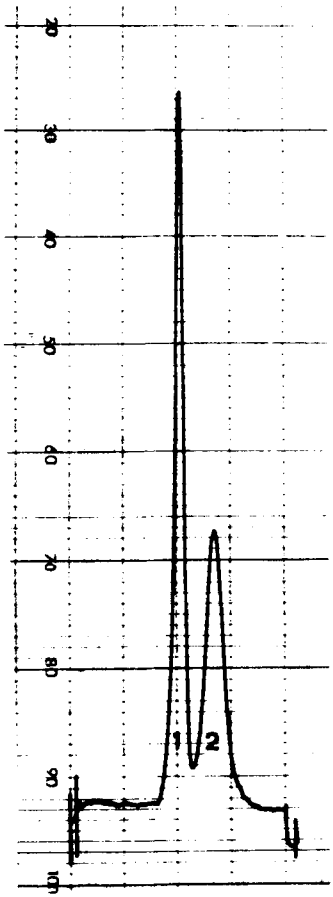

Figure 1. Chromatogram of pure SAMe (16 nmoles) and pure SAH (10 nmoles) treated as described in the text. The column was eluted isocratically with Buffer A. Flow rate : 75 ml/h. Absorbance unit full scale : 0.640 O.D. at 254 nm.
The peaks correspond to : 1, SAMe, as adenine; 2, SAH.

and the chromatographic profile of synthetic SAMe and SAH hydrolyzed with 0.5 M ammonia solution at 60° (curve B). The chromatographic separations are carried out by using Buffer B as eluent. No

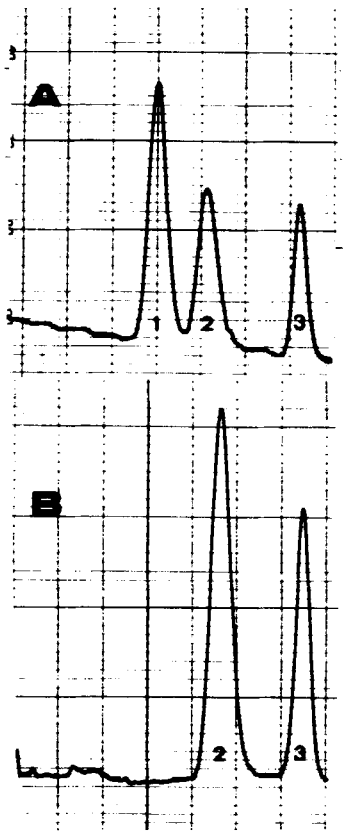

Figure 2. (A) : Chromatogram of pure 5'-MTA (20 nmoles), pure SAH (10 nmoles) and pure Ade (7 nmoles). The column was eluted isocratically with Buffer B. Flow rate : 75 ml/h. Absorbance unit full scale : 0.640 O.D. at 254 nm. The peaks correspond to : 1, 5'-MTA; 2, SAH; 3, Ade. (B) Chromatogram of the compounds obtained after the alkaline hydrolysis of SAH and SAMe. The hydrolysis, carried out in 0.5 M ammonia at 60°, lasted 10 min. The rotary evaporated derivatives were taken up in Buffer B. Aliquots corrisponding to 15 nmoles of SAMe and 25 nmoles of SAH were chromatographed under the experimental conditions described in (A) above.
The peaks correspond to : 1, SAH; 2, Ade.

trace of 5'-MTA is detectable in curve B. On the other hand, it is worthy to point out that the chromatography of SAH after its treatment with 0.5 M ammonia at 60° (chromatogram not reported here) shows only one peak at the same t_R as the one observed for the native SAH.

These conditions are of paramount importance for the assay reliability, because they prove that SAH is not destroyed during the hydrolysis step, and that SAMe is quantitatively converted to adenine.

The correlation between scalar amounts of SAH and SAMe and heights of the corresponding chromatographic peaks is reported in Figure 3. These two parameters show a linear relationship in the concentration range of 2-40 nmoles.

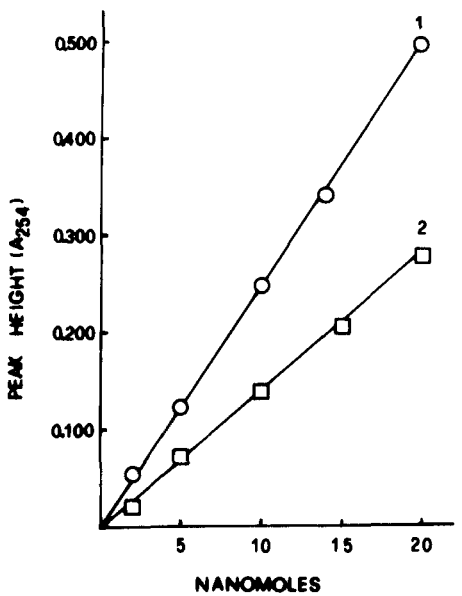

Figure 3. Linearity between peak heights and nmoles of SAMe : 1 and SAH:2.

Analysis of SAMe and SAH of the Rat Liver Pool

A typical chromatogram relative to an analysis of a rat liver extract, corresponding to 200 mg of wet tissue, is reported in Figure 4. Apart from the extreme left-hand portion, the inspection of the chromatogram shows two peaks, which according to their re-

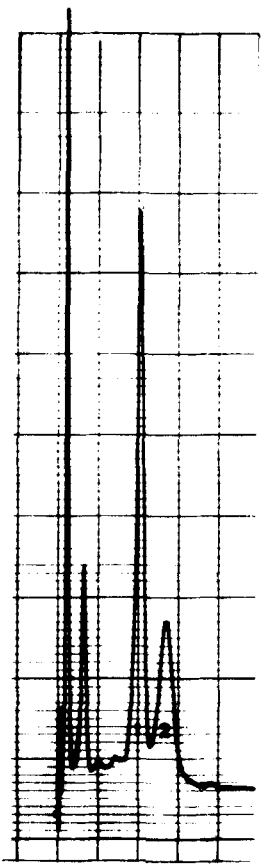

Figure 4. Chromatogram of a PCA liver extract processed as described in the text. The sample passed through the column correspond to 0.200 g of fresh tissue. The column was eluted isocratically with Buffer A under the experimental conditions described in Figure 1. The peaks correspond to : 1, SAMe, as adenine; 2, SAH.

tention times might be identified as Ade and SAH, respectively. Furthermore, the identity of these peaks has been confirmed by a new chromatography of the same liver extract previously added with known amounts of SAH and Ade. In this experiment the synthetic compounds co-elute with the peaks previously ascribed to adenine and SAH and the respective bandwidths of these peaks are coincident with those obtained from the chromatography of the standard mixture. As it has been reported (9) that the retention time and constancy of bandwidths are useful parameters for the identification of eluted substances, the peaks of interest have been identified as Ade and SAH, respectively. The chemical nature of the substance eluted as small peaks not interfering with the elution curves of SAH and adenine has not been investigated.

The results reported above exclude the presence of contaminating products in the elution peaks of adenine and SAH and demonstrate the efficiency both of the HPLC system and the preliminary purification step.

The recoveries of the two metabolites, determined as described under Methods, are 90 $\pm$ 3.7% and 85 $\pm$ 3.4%, for SAH and SAMe respectively.

The quantitative results of the analyses of the rat liver pool of SAMe and SAH are reported in Table 1.

CONCLUSIONS

High performance liquid chromatography provides a fast and specific method for the determination of several classes of metabolites. Nevertheless, its use for the quantitation of SAMe and SAH is restricted due to the presence of interfering substances

TABLE 1

SAH and SAMe Content of Rat Liver
nmoles/g

Rat	SAH	SAMe
A	31.0 ± 1.1	58.1 ± 2.8
B	29.2 ± 0.9	55.7 ± 2.5

The values are the mean of five determinations (± SD). Rats A and B were both sacrified after 15 h starvation.

in the biological samples and to the physico-chemical properties of the two metabolites.

The rationale behind the procedure described in this paper is that, after preliminary clean-up, SAMe is quantitatively broken down to adenine under alkaline conditions. In this way adenine and SAH can be simultaneously determined by HPLC at pH conditions which allow a suitable positive interaction of the two components to the anionic exchanger. The pH, ionic strength and propanol concentration of the mobile phase are selected to allow an isocratic separation of adenine and SAH in a short time (18 min). The problem of interfering substances present in the PCA extract of liver is worked out by direct treatment of extracts on Dowex 50W-X2. This preliminary fractionation procedure does not involve any laborious or time-consuming manipulation of the samples, and several extracts can be processed in 80-90 min by using a series of small chromatographic columns.

Therefore, the easy preparation of the sample, the fast and reproducible HPLC analysis, and the sensitivity of the present

method, allow a favourable comparison with previously described column chromatographic methods (3-6).

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1. Abbreviations used : SAMe, S-adenosylmethionine; SAH, S-adenosylhomocysteine; 5'-MTA, 5'-deoxy-5'-methyl-thioadenosine; Ade, adenine; MAP, 2-amino-2-methyl-1-propanol; HPLC, high performance liquid chromatography; PCA, perchloric acid.

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