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SPECIES AND TISSUE DIFFERENCES IN THE CATABOLISM OF S-ADENOSYL-L-HOMOCYSTEINE: A QUANTITATIVE, CHROMATOGRAPHIC STUDY

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SUMMARY

Using a rapid, quantitative high pressure liquid chromatographic (HPLC) procedure for the separation of S-adenosyl-L-homocysteine (AdoHcy) and its purinic metabolites in rat and mouse brain, kidney and liver, we found that uric acid is the principal catabolic product of AdoHcy metabolism in the liver, but that none forms in brain. Rat kidney formed about 10 times as much uric acid and half as much hypoxanthine as did mouse kidney. The HPLC procedure has been adapted to assay AdoHcy hydrolase activity which was found to be lowest in the brain and highest in the liver.

The ability of S-adenosyl-L-homocysteine (AdoHcy) to inhibit the activity of histamine-N-methyltransferase (1,2), catechol-O-methyltransferase (3), indolethylamine-N-methyltransferase (4), tRNA methyltransferase (5,6) and phenylethanolamine-N-methyltransferase (7) has led to much interest in the role of AdoHcy as a regulator of methylation processes. The enzyme responsible for AdoHcy hydrolysis (AdoHcy hydrolase, E.C. 3.3.1.1.) (8) may thus be crucial in the regulation of tissue AdoHcy levels (8-11), and hence of cellular transmethylation. In vitro AdoHcy hydrolase is inhibited by the end products of the hydrolytic reaction homocysteine (Hcy) and adenosine (Ado). Ado is further metabolized to inosine (Ino), hypoxanthine (Hyp), xanthine (Xan) and, in liver, to uric acid (12).

Previously described methods for the separation of AdoHcy from its purinic metabolites are rather time consuming as they require column (9,13, 14), paper (8,10,12,13) or thin layer (15) chromatographic procedures followed by an independent quantitative determination of each separated metabolite. The most rapid separation takes 2.5 h using low voltage paper electrophoresis (16). The development of high pressure liquid chromatography (HPLC) has greatly decreased the time necessary for separation and analysis of many substances. Indeed, an HPLC method requiring less than 30 min for the separation and quantitation of several purines has recently been described (17). We report a rapid and facile method, also using reverse phase HPLC, for the separation and quantitation of nanogram amounts of AdoHcy and its purinic metabolites and requiring only 6-8 min for each sample. The method has been applied to the quantitation of the purinic metabolites formed by incubating AdoHcy with soluble extracts of rat and mouse liver, kidney and brain tissue and for an assay of AdoHcy hydrolase.

MATERIALS AND METHODS

Apparatus. A μBondapak C_{18} (a non-polar material consisting of a monomolecular layer of octadecyltrichlorosilane) column (30 cm x 0.4 cm I. D. for reverse phase chromatography was obtained from Waters Associates, Inc. Milford, MA). A solvent delivery system equipped with dual piston pump (model 6000 A), a universal liquid chromatography injector (model U6K) and an absorbance detector (model 440) were also from Waters Associates. The chromatographic runs were recorded on an Ominiscribe recorder (Texas Instruments, Austin, TX). In some cases, fractions (0.12 ml) from chromatographic runs were collected using an LKB fraction collector (model 7000, LKB Instruments, Chicago, IL).

Reagents. Adenosine, inosine, hypoxanthine, xanthine, uric acid, S-adenosyl-L-homocysteine, 6-methylamino-9-ribofuranoside (MAPR) and adenosine deaminase (adenosine aminohydrolase, E.C. 3.5.4.4, Type I from calf intestinal mucosa) were obtained from Sigma Chemical (St. Louis, MO). D,L-homocysteine (free base) was from ICN Nutritional Biochemicals (Cleveland, OH) and [8-^{14}C] adenosine (sp. act. 52 mCi/mmol, from New England Nuclear (Boston, MA). Radioactive [8-^{14}C] AdoHcy was prepared from Hcy and [8-^{14}C] adenosine in the presence of the adenosine deaminase inhibitor MAPR (11) using a dialyzed high speed supernatant (100,000 g x 35 min) from rat brain as a source of AdoHcy hydrolase. After a 2 h incubation at 37° C the reaction was stopped by addition of 4 N perchloric acid. The sample was then neutralized by addition of 1 N KOH and filtered (0.45 μMetricel filter). The filtered solution was injected onto a μBondapak C_{18} column and the AdoHcy fraction collected (see Chromatographic separation for details). The radioactive AdoHcy was placed on a Dowex 50 column [H^+] equilibrated with 1 N HCl, after which the column was eluted with water followed by 3 N NH_4OH (14). The NH_4OH fraction contains AdoHcy and this step was performed to free AdoHcy from the HPLC buffers. Finally, the AdoHcy fraction was lyophilized and its specific activity adjusted to 0.27 mCi/mmol by dilution with non-radioactive AdoHcy.

Chromatographic separation. Varying aliquots of a standard solution containing AdoHcy, Ado, Ino, Hyp, Xan and uric acid (45 μg/ml of each) were applied to a μBondapak C_{18} column via the injector septum at room temperature using a 100 μl syringe (Precision Sampling Corp., Baton Rouge, LA) at a constant flow rate (buffer 1) of 2 ml/min. Buffer 1 was 0.04 M dibasic ammonium phosphate (5%, v/v methanol, pH 8.0) and buffer 2 was 0.04 M monobasic ammonium phosphate (20%, v/v methanol, pH 4.7). There was a lag of 8.4 ml between buffer changes and their appearances on the spectrophotometer tracing. In initial experiments elution with buffer 2 was begun 1.25 min (2.5 ml) after sample injection. Subsequently we found that sample injection, 0.5 min after beginning elution of the column with buffer 2, reduced the time necessary for separation of each sample (see Figures 1 and 3 for detailed chronology of elution). Both buffers and all samples were filtered (0.45 μMetricel filter) prior to use. In addition, buffers were degassed *in vacuo* before use.

Animals, tissue preparation and enzyme assay. Adult male Sprague-Dawley rats (120-200 g) and Swiss-Webster mice (25-30 g) (Spartan Research Animals, Haslett, MI) were sacrificed by decapitation, after which brain, kidney and liver were rapidly excised, rinsed with buffer (0.05 M potassium phosphate, pH 7.4), blotted and weighed in tared beakers containing buffer. Tissues were homogenized in 5 vol buffer, centrifuged at 100,000 g x 35 min and the supernatants dialyzed against three changes of buffer (0.005 M potassium phosphate, pH 7.4). The AdoHcy hydrolase reaction mixture contained 50 nmol [8-^{14}C] AdoHcy (119 μM), 0.1 ml dialyzed supernatant and 0.051 units of adenosine deaminase in phosphate buffer (0.05 M, pH 7.4) (total vol.: 0.31 ml). Blanks con-

tained heated supernatant (5 min, 95° C). The reaction mixtures were incubated at 37° C for 15 min after which the reaction was stopped by addition of 15 µl of 4 N perchloric acid. Samples were than neutralized with 60 µl of 1 N KOH, diluted with water (final vol.: 0.54 ml), and filtered. An aliquot of 90 µl was injected onto the Bondapak column 30 sec after changing from elution with buffer 1 to elution with buffer 2 (see Figure 3 for detailed chronology of elution).

Quantation of AdoHcy and Metabolites

Absorbance. Standard curves of AdoHcy and its purinic metabolites were constructed by comparing known amounts of standards to the maximum peak height (A_{254}) produced by each compound. These data were then analyzed by linear regression analysis. For quantitation of enzyme reaction mixtures, the change in peak height absorbance of samples, compared to blanks, was determined followed by comparison of these values with those of standards run the same day.

Radiometric. Column eluates were collected (0.12 ml fractions) directly into scintillation vials and, after addition of 2 ml PCS (a tissue solubilizer-scintillation mixture, Searle Analytic, Arlington Heights, IL), the samples were counted. Counting efficiency was determined using the channels ratio method. The total radioactivity in each sample peak was compared to its blank and this difference used to determine the amount of AdoHcy remaining or the amount of metabolite formed by calculation from the specific activity of [8-^{14}C] AdoHcy (0.27 mCi/mmol).

RESULTS AND DISCUSSION

Figure 1 represents a typical reverse phase HPLC separation of a standard mixture of AdoHcy and its purinic metabolites. The compounds, in their order of elution, are uric acid, Xan, Hyp, Ino, AdoHcy and Ado. Buffer 1, alone, resolved all the above compounds, however, the retention volumes of Ino, AdoHcy and Ado were increased and their respective peaks became quite braod, making peak height measurements (A_{254}) inaccurate. Buffer 2, on the other hand, cleanly resolved Ino, AdoHcy and Ado with a much higher peak absorbance than buffer 1, but Xan and Hyp were not resolved and appeared as one peak. The combination of buffers 1 and 2, however, provided a rapid separation paired with the maximum peak heights attainable for each individual compound. The order of elution of uric acid, Xan and Hyp is different than previously reported (17) in that Hyp eluted before Xan, however, the elution sequence shown in Figure 1 is as would be predicted from the pK_a values of the individual compounds (5.75, 7.7 and 8.8, respectively). That solvent affinity of the various compounds also plays a role in this separation was indicated by the poor resolution of uric acid, Xan and Hyp seen after decreasing the polarity (methanol concentration increased from 5% to 20%) of the mobile phase (buffer 1). The pK_a values of Hyp and Ino are quite close (8.8 and 8.85, respectively) and separation of these compounds is effected by decreasing the pH of the mobile phase from 8.0 to 4.7 at a point precisely between the retention volumes of Hyp and Ino. Ino is less polar at pH 4.7 and is consequently retained longer on the stationary phase of the column. Addition of the amino acid substituent (Hcy) to Ado results in increased polarity of the resultant compound (AdoHcy) compared to Ado as evidenced by their elution order (AdoHcy followed by Ado) (Fig. 1). Ado eluted last in this series which generally agrees with another HPLC study (17) in which, of 23 related compounds tested, only one, cAMP, eluted after Ado. Solvent affinity also plays a role in the separation of Ino, AdoHcy and Ado as evidenced by the fact that reducing the methanol concentration of buffer 2 (from 20% to 5%) markedly extended the retention time of these compounds (especially AdoHcy and Ado) although their elution order remained unchanged (Schatz and Sellinger, unpublished work).

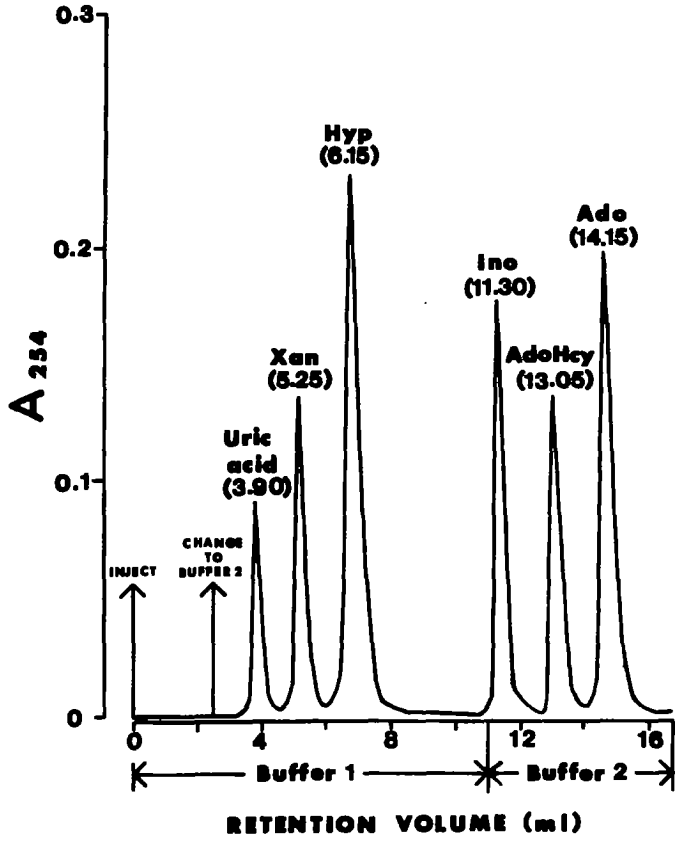

FIG. 1

Reverse phase HPLC separation of a standard mixture of AdoHcy and its purinic metabolites. A 75 µl aliquot of a standard mixture containing 3.375 µg each of AdoHcy, Ado, Ino, Hyp, Xan and uric acid was injected onto a µBondapak C_{18} column at a flow rate of 2ml/min. Buffer 1 was 5% (v/v) methanol in 0.04 M dibasic ammonium phosphate (pH 8.0; pressure, 2500 psi) and buffer 2 was 20% (v/v) methanol in 0.04 monobasic ammonium phosphate (pH 4.7; pressure, 3000 psi).

The linearity of the spectrophotometric response over a 40-fold range of increasing amounts of standard (Fig. 2) demonstrates the reliability and reproducibility of absorption measurements for the quantitative determination of AdoHcy and of its purinic metabolites. The sensitivity of this method allowed detection of amounts as low as 180 ng. Absorption measurements of lesser amounts of standards were complicated by the fact that the baseline increased

slightly with buffer 2 and this increase became greatly amplified at low detector sensitivity settings, making true peak height determination difficult and of doubtful accuracy.

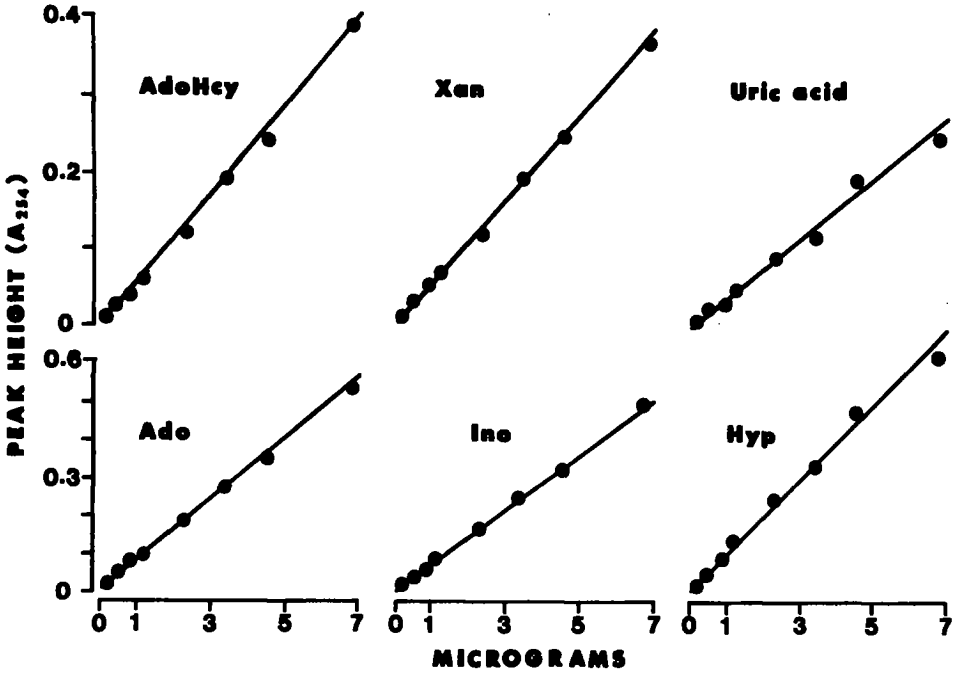

FIG. 2

Standard curves of a mixture of AdoHcy and its purinic metabolites. Aliquots, in triplicate (variation less than 5%), of a standard mixture containing 45 μg/ml each of AdoHcy, Ado, Ino, Hyp, Xan and uric acid were injected onto a μBondapak column at a flow rate of 2 ml/min. Time of sample injection and buffer change were as in Fig. 1. Peak height values were determined by multiplying the maximum absorbance (A_{254}) by the appropriate detector sensitivity setting. The correlation coefficient (r) was at least 0.99 for all compounds. Also, a test of the hypothesis (r=0) was rejected in all cases.

Figure 3 is an elution profile of an AdoHcy hydrolase reaction mixture using rat brain as the source of enzyme. In all tissues examined (Table 1 and Fig. 3), retention volumes of peaks of radioactivity corresponded exactly to previously determined retention volumes of "cold" standards. Recoveries from the Bondapak column were from 96-100% and AdoHcy or its purinic metabolites accounted for all of the radioactivity present in the injected sample. Table 1 shows that hydrolysis of AdoHcy proceeded most rapidly in liver followed by kidney and brain, respectively. Generally, little interspecies difference

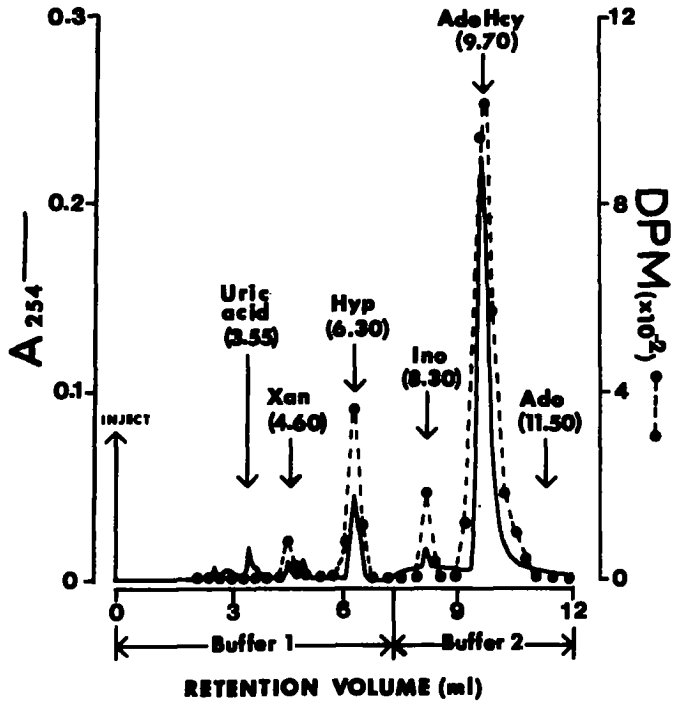

FIG. 3

Reverse phase HPLC of AdoHcy and its purinic metabolites in rat brain. A 90 µl aliquot of an AdoHcy hydrolase reaction mixture was injected onto a µBondapak column at a flow rate of 2 ml/min, 30 sec after changing to buffer 2. Buffers 1 and 2 are described in Methods. Solid line denotes absorbance (A_{254}) and dotted line denotes DPMs. Fractions (0.12 ml) were collected directly into scintillation vials and counted after addition of 2 ml of scintillant. Arrows and numbers in parentheses indicate retention volumes of a standard solution containing AdoHcy and its purinic metabolites. Note that retention volumes are different in Fig. 1 owing to the different lag time between changing of buffers and injection of sample.

(Table 1) existed in the extent of AdoHcy hydrolysis for a given tissue, however, hydrolysis was somewhat greater in rat brain than in mouse brain. In both rat and mouse brain Ino and Hyp represented most of the AdoHcy hydrolytic products, with very little Xan and no uric acid being detected. In rat and mouse liver, 83% and 91% respectively, of the AdoHcy was converted to uric acid. In rat kidney, the distribution of AdoHcy hydrolytic products was found to be relatively even between uric acid (31%), Xan (27%), Hyp (26%) and Ino (15.5%) whereas, in mouse kidney, most of the AdoHcy was converted to Ino (26%) and Hyp (54.5%), 16.5% to xanthine and less than 4% to uric acid.

The formation of Hyp in all tissues tested (Table 1) confirms the presence of inosine-guanosine phosphorylase (E.C. 2.4.2.1; 2.4.2.15) while the formation of Xan or uric acid implies that of xanthine oxidase (E.C. 1.2.3.2). As the AdoHcy incubations were conducted in the presence of an excess of

TABLE 1

Conversion of AdoHcy to purinic metabolites in rat and mouse tissues

Tissue	nanomoles[a]. Uric acid	Xan	Hyp	Ino	AdoHcy
Rat brain	0	1.9	7.5	2.6	37.9
Mouse brain	0	0.6	3.9	3.4	42.1
Rat liver	41.7	0	0	3.4	4.8
Mouse liver	45.3	1.4	0.4	0.5	2.3
Rat kidney	8.3	7.1	6.8	4.1	23.6
Mouse kidney	0.8	4.7	15.5	7.3	21.5

[a]. The data show the distribution of AdoHcy and its purinic metabolites after reaction (15 min) of 50 nmol AdoHcy [8-^{14}C] with 0.1 ml dialyzed high-speed supernatant (see Methods) in the presence of excess intestinal adenosine deaminase. Values were calculated as described in Methods. Recoveries from the Bondapak column varied from 96-100% and the values are corrected accordingly.

intestinal adenosine deaminase, no direct conclusions can be made concerning the presence of this enzyme, however, the presence of adenosine deaminase has been demonstrated in the brain, kidney and liver of the rat and the mouse (18). Preliminary experiments in our laboratory (Schatz and Sellinger, unpublished work) have demonstrated the feasibility of using the HPLC separation procedure for the separate assay of adenosine deaminase, purine-nucleoside phosphorylase (18,20) and xanthine oxidase (21), by using the appropriate substrate and by meeting the different pH and cofactor requirements for each enzyme. Table 2 shows the usefulness of the described HPLC method of separation and quantitation as an assay for AdoHcy hydrolase. As may be seen data obtained by absorbance or radiometric measurements were in close agreement. Comparison of the AdoHcy hydrolase activity with literature values is complicated by the use of different buffers, pH and assay conditions; however, the present values are in the same range as previously reported values (9,14). Further, comparison of AdoHcy hydrolysis between tissues (Table 2) showed hydrolysis to be fastest in liver followed by kidney and brain as has been reported (9,14). Values for AdoHcy hydrolase in liver are (Table 2) probably an underestimate as most of the substrate (AdoHcy) was consumed during incubation (Table 1). Indeed, further experiments were conducted using less liver enzyme and AdoHcy hydrolase activity was found to be 12.9 and 16.1 μmol AdoHcy hydrolyzed/g tissue/15 min in mouse and rat liver, respectively (see Table 2, footnote b).

TABLE 2

Comparison of AdoHcy hydrolase activity in 3 tissues of rat and mouse

Tissue	AdoHcy hydrolase[a]. Absorbance	DPM
Rat brain	0.68	0.72
Mouse brain	0.40	0.45
Rat liver[b].	2.74	2.71
Mouse liver[b].	2.83	2.87
Rat kidney	1.47	1.58
Mouse kidney	1.73	1.72

[a]. AdoHcy reaction mixtures were as described (Methods or Table 1). Enzyme activity (μmol AdoHcy hydrolyzed/g tissue/15 min) was calculated as AdoHcy disappearance on the basis of peak height (A_{254}) or total DPM in the AdoHcy peak. Absorbance measurements represent averages of triplicate samples, whereas DPM measurements represent averages of duplicate samples (variation less than 5%).

[b]. The liver values were determined under conditions of excess substrate consumption, and are thus an underestimate. In the presence of 0.01 ml instead of 0.1 ml (see Methods) enzyme, AdoHcy hydrolase activity was 12.9 and 16.1 μmol AdoHcy hydrolyzed/g tissue/15 min in mouse and rat liver, respectively, using peak height (A_{254}) measurements to determine activity.

In conclusion, a reverse phase HPLC method is described for the separation of AdoHcy and its purinic metabolites. This method may be used for the routine determination of the activity of AdoHcy hydrolase. An added advantage is that radioactive AdoHcy is not necessarily required.

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