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Two-dimensional high-performance liquid chromatographic method for assaying S-adenosyl-L-methionine and its related metabolites in tissues

Morteza P. Hamedani, Klára Valkó☆, Xiaofei Qi, Kevin J. Welham and William A. Gibbons*

University–Industry Centre for Pharmaceutical Research, Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX (UK)

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ABSTRACT

S-Adenosyl-L-methionine (SAM) is a methyl-donor compound which is actively involved in a variety of biochemical reactions. An assay has been developed permitting the quantitative measurement of SAM and its related metabolites (S-adenosylhomocysteine, decarboxylated SAM, methylthioadenosine, adenosine and adenine) in liver and cell cultures. As gradient reversed-phase chromatographic or cation-exchange chromatographic methods often resulted in overlapping peaks, a two-dimensional high-performance liquid chromatographic (HPLC) procedure was developed involving gradient reversed-phase chromatographic separation followed by ion-exchange chromatography. After precipitating large molecules in the sample by perchloric acid, gel permeation was carried out on a Sephadex G 25 column to separate small water-soluble metabolites from proteins and membrane fragments. The freeze-dried sample was injected onto an ODS column and a 0–10% acetonitrile gradient in 10 m*M* ammonium formate buffer (pH 2.9) (20 min, linear) was applied. The relevant fractions were collected and injected onto a cation-exchange column (Partisil SCX, 10 μm, 250 mm × 4.6 mm I.D.). Elution and quantification were carried out using ammonium formate buffers of various concentration (15–400 m*M*), pH 2.9. The detector response (254 nm) as a function of concentration was linear over the concentration range 30–500 pmol. The detection limits of the compounds after the two-dimensional chromatographic procedure ranged from 10 to 60 pmol and the recovery was higher than 70%. The reproducibility of the results obtained from given samples was within 9–22% for rat liver and 6–24% for mast cells.

INTRODUCTION

S-Adenosyl-L-methionine (SAM) is an endogenous compound that is heavily involved in a variety of biochemical reactions of cellular functions [1], such as methylation of lipids, proteins, RNA and DNA, transsulphuration and polyamine biosynthesis [2–4]. Immunological effects [5] and cellular differentiation [6,7] can be triggered by perturbation of the SAM metabolic pathways. SAM has also been shown to play an important role in the biochemistry connected with schizophrenia and depression [8,9] and has therefore been the target for several assays in body fluids and tissues [10–16]. The role of SAM and its related metabolites in methylation processes and polyamine synthesis is shown in Fig. 1. The aim of this study was to develop an assay to monitor the levels of the demethylated product of SAM, S-adenosylhomocysteine (SAH), the decarboxylated derivative of SAM (dc-SAM), the end-

* Corresponding author.
☆ On leave from Central Research Institute for Chemistry, Hungarian Academy of Sciences, P.O.B. 17, Budapest H-1525, Hungary.

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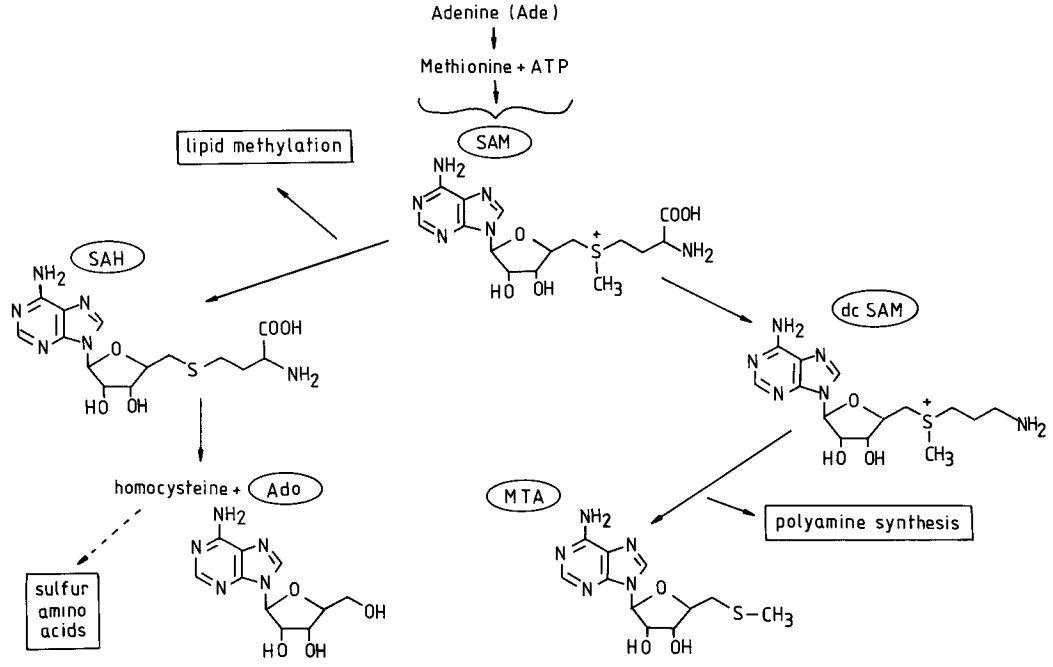

Fig. 1. Structures of the compounds investigated and their relation to other biochemically important reactions.

products that resulted from the spermidine and spermine synthesis, methylthioadenosine (MTA), adenosine (Ado) and adenine (Ade).

All metabolites are small polar molecules, which makes their isolation and determination difficult. Reversed-phase chromatography using only aqueous mobile phases has already been applied for assaying these compounds [14]. To increase the retention time of the positively charged SAM, reversed-phase ion-pair chromatographic methods have been suggested using heptane- or octanesulphonic acids in the acidic mobile phase [17–20]. The drawback of the relatively short retention times is manifested in the difficulties in the separation of SAM and its related metabolites from other polar endogenous compounds [21]. The application of cation-exchange chromatography has already been published [22,23] and, as expected, SAM had a much longer retention time, being a cation at every mobile phase pH; but SAH, MTA and adenosine showed relatively short retention times. Most of the publications utilized only precipitation with perchloric acid (PCA) and centrifugation for sample preparation. The detection of the compounds was carried out by ultraviolet absorbance at 254–267 nm, which provided acceptable detection limits.

Since non-homogenous peaks for SAM and each of its metabolites were observed in one-dimensional chromatography, a two–dimensional HPLC assay was developed involving consecutive use of reversed-phase and cation-exchange separations, and thus increasing the selectivity of the assay. A gel-permeation chromatographic sample preparation method was introduced to enhance the column lifetime in this study and to provide a preliminary separation of small metabolites from proteins.

EXPERIMENTAL

Chemicals

The SAM, SAH, MTA, Ade and Ado standard compounds were purchased from Sigma (St. Louis, MO, USA). The ds-SAM sample was kindly provided by Professor J. Wagner (Strasbourg Research Center, Strasbourg, France). The standard SAM obtained from Sigma con-

tained 78–81% SAM, 15% MTA, 3% SAH and 1% Ade. The purity of SAM was always taken into account in the calculations. The compounds were dissolved in distillled water at 1 mg/ml and kept in the freezer below 0°C. While SAM 100% decomposes at room temperature and pH 6–7, the aqueous solution of all compounds proved to be stable [24] for more than thirty days when stored below 0°C.

Apparatus

The HPLC measurements were carried out using a Gilson HPLC system (Gilson, Villier le Bel, France) equipped with an M 303 pump and an M 116 variable-wavelength UV detector (Gilson, Beltline, Middletown, PA, USA). Data acquisition and handling were carried out by using Gilson 714 software running on an IBM AT personal computer interfaced through Gilson GSIOC. The samples were injected through a Rheodyne (Cotati, CA, USA) M 7010 injection valve with a 100-μl sample loop. The mobile phase was pumped at a flow-rate of 1.00 ml/min.

Gel-permeation chromatography was carried out using a Watson-Merlow M5029 peristaltic pump (Watson-Merlow, Cornwall, UK) and LKB M2238 UvicordsII UV detector (LKB, Bromma, Sweden).

Sample preparation

Male albino rats (230 ± 25 g) were starved for 24 h before the experiments, then sacrificed with diethyl ether (5–10 min in a gas chamber). The fresh rat livers were immersed in normal saline solution, blotted with filter paper, divided into three pieces, frozen in liquid nitrogen, weighed (approximately 2–3 g), homogenized (Polytron PTA 10-35, Kinematica, Kriens/Luzern, Switzerland) and dissolved in 12 ml of ice-cold 1.5 *M* PCA (approximately 4 ml/g of liver). The mixture was homogenized and centrifuged at 1000 *g* for 10 min at 4°C (Beckman L8-M, Beckman Instruments, Fullerton, CA, USA). The pH of the supernatant was adjusted to 4 with 1.5 *M* KOH. After centrifuging, 4 ml of the supernatant were fractionated using a Sephadex G 25 fine glass column (Pharmacia LKB, Uppsala, Sweden) with dimensions of 350 mm × 16 mm I.D. The mobile phase, which was 10 m*M* ammonium formate (pH 5.5), was pumped through the column at a flow-rate of 1.7 ml/min and the detection carried out at 276 nm. The fraction of the small molecules (between 62 and 85 min) was collected and freeze-dried. Gel-permeation chromatography was carried out at 4°C.

The concentrations of SAM and its related metabolites were also investigated in cultured mast cells (RBL-2H3). After homogenization of the cells, the macromolecules were precipitated using 1.5 *M* PCA solution. After 15 min sonication, the sample was kept in a cold cabinet at 4°C for 30 min. Then the pH of the sample was adjusted to 4–5 with 1.5 *M* KOH solution. The sample was kept at 4°C for another 30 min to complete precipitation and then centrifuged for 15 min at 9000 *g* (Biofuge A, Heraeus Sepathech, Germany). The supernatant was freeze-dried and later subjected to the HPLC measurements.

Reversed-phase chromatography

The freeze-dried samples were dissolved in 1 ml of 10 m*M* ammonium formate buffer and injected onto the reversed-phase column (HiChrom ODS2-2597, 250 mm × 4.6 mm I.D., HiChrom, Reading, UK). The mobile phases were: (A) 10 m*M* ammonium formate pH 2.9 (analytical grade, Pharmacos, Southend-on-Sea, UK); (B) acetonitrile (HPLC grade, Rathburn, Walkerburn, UK). A 20-min gradient from 0% B to 10% B followed by 90% A and 10% B for 20 min was applied. The flow-rate was 1.00 ml/min. The detection of the peaks was carried out at 254 nm (0.05 AUFS). The chromatogram was recorded for 40 min, and fractions corresponding to the standard retention times of SAM, SAH, MTA, Ado, dc-SAM and Ade were collected and freeze-dried immediately.

Cation-exchange chromatography

The cation-exchange chromatographic measurements of the fractions obtained from reversed-phase chromatography were carried out on a HiChrom P10 SCX-2846 250 mm × 4.6 mm I.D. column. The mobile phase flow-rate was

1.00 ml/min, detection was at 254 nm (0.05 AUFS). The mobile phases for each reversed-phase fraction contained various concentrations of ammonium formate buffer, *e.g.* adenosine and SAH fractions required 20% acetonitrile in 12 m*M* ammonium formate (pH 2.9), SAM, MTA and adenine fractions required 250 m*M* ammonium formate (pH 3.7) and the mobile phase contained 20% acetonitrile in 250 m*M* ammonium formate (pH 2.9) for the measurements of dc-SAM.

Calibration study, detection limits and recovery

The linearity of the calibration curves was checked for triplicate injections of each compound. Five different concentrations in the range 20–500 pmol were used for calibration.

The detection limit of each component was determined by injecting decreasing amounts until the signal-to-noise ratio reached the value of 5:1. The detection limit was calculated as the amount of injected compound which produced the above signal-to-noise ratio. The recovery was studied using known amounts of standard compounds injected onto the reversed-phase column followed by injection onto the cation-exchange column. The recovery after both HPLC procedures was calculated by injecting 0.7, 1.4 and 2.1 nmol of MTA, 1.78, 3.56 and 5.34 nmol of SAH and 1.43, 2.86 and 4.29 nmol of SAM. The recovery from tissue samples was studied by standard addition methods.

RESULTS AND DISCUSSION

A typical gel-permeation chromatogram is presented in Fig. 2. The exclusion volume of the column was 25 ml, and the total permeation volume was 86 ml. Fig. 3 shows the reversed-phase chromatogram obtained by injecting the standard compounds. The double peak for SAM (peak 1) is due to the separation of the *R,S* and *S,S* diastereomers. Figs. 4 and 5 show typical reversed-phase chromatograms obtained from liver and mast cell samples. Five fractions were collected from 6 to 8.5 min for SAM, 8.5 to 10 min for dc-SAM, 11 to 13 min for Ade, 15 to 17 min for SAH and Ado, and 28 to 31 min for MTA. Fig. 6 shows typical chromatograms obtained on cation-exchange columns from each fraction. It can be seen from Figs. 4 and 6 that the two types of chromatography greatly enhance the selectivity and accuracy of the method. Thus a peak which seemed to be a single compound in reversed-phase chromatography very often produced three or more peaks when subjected to cation-exchange chromatography.

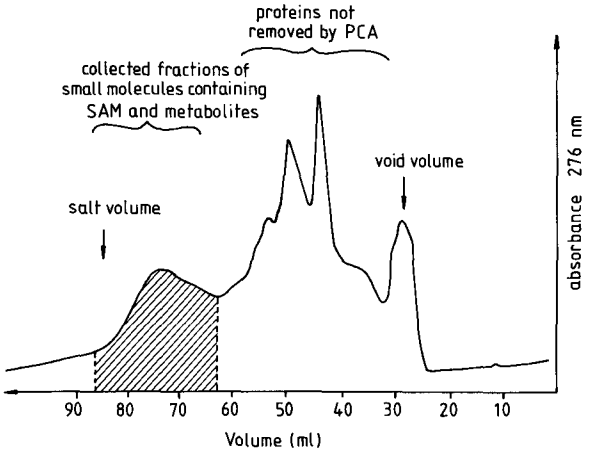

Fig. 2. Typical gel-permeation chromatogram of a liver sample. Column: Sephadex G 25 fine glass, 350 mm × 16 mm I.D., mobile phase: 10 m*M* ammonium formate pH 5.5; flow-rate: 1.7 ml/min; detection: 276 nm.

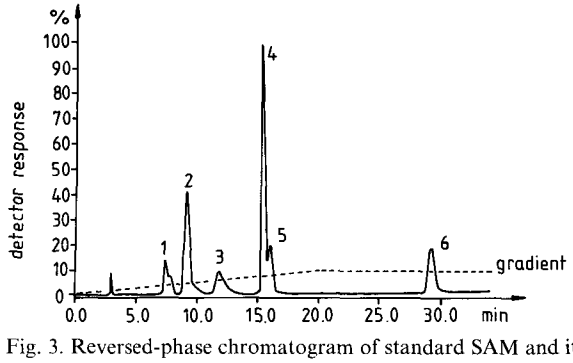

Fig. 3. Reversed-phase chromatogram of standard SAM and its related metabolites. Column: HiChrom ODS2, 10 μm, 250 mm × 4.6 mm I.D.; mobile phase: (A) 10 m*M* ammonium formate; (B) acetonitrile; linear gradient from 0% B to 10% B in 20 min; detection: 254 nm (0.05 AUFS). Peaks: 1 = SAM; 2 = dc-SAM; 3 = Ade; 4 = SAH; 5 = Ado; 6 = MTA.

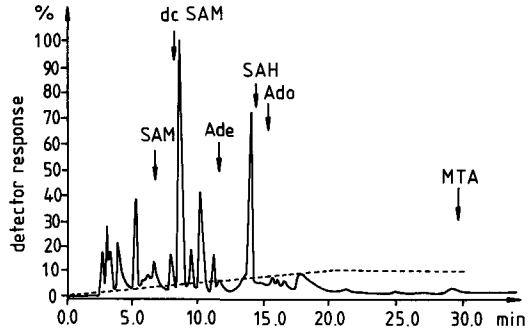

Fig. 4. Typical reversed-phase chromatogram obtained from rat liver. Chromatographic conditions are the same as described in Fig. 3.

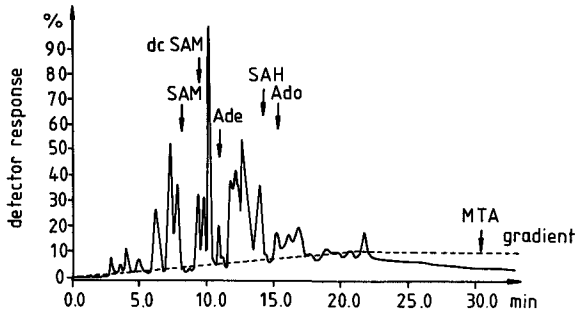

Fig. 5. Typical reversed-phase chromatogram obtained from mast cells. Chromatographic conditions are the same as described in Fig. 3.

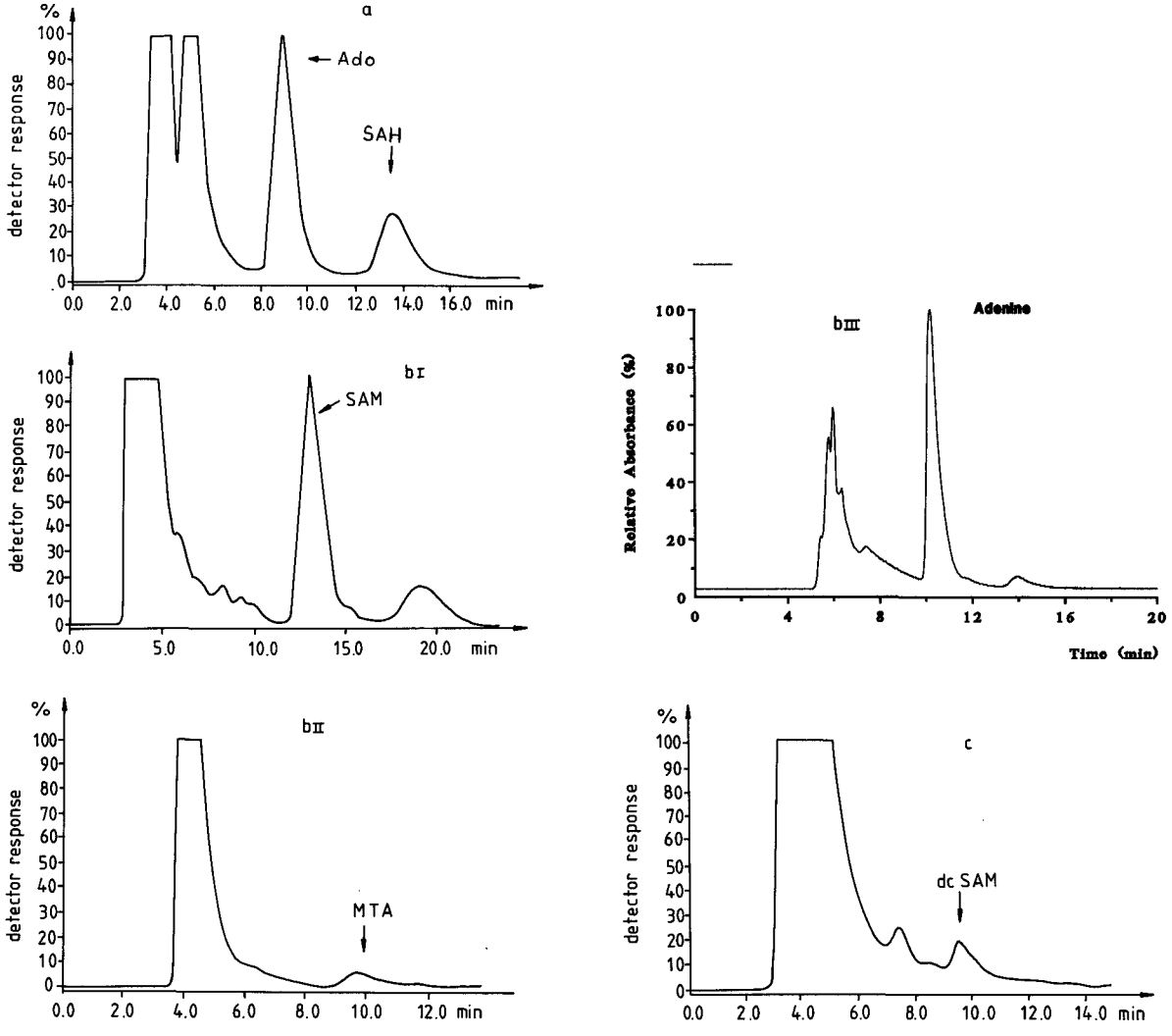

Fig. 6. Typical SCX chromatogram of the reversed-phase fractions collected by injecting liver sample. Column: Partisil-SCX (Hi-Chrom), 10 μm, 250 mm × 4.6 mm I.D.; mobile phase: (a) 12 mM ammonium formate buffer pH 2.9–20% acetonitrile; (b) 250 mM ammonium formate buffer pH 3.7; I, II, and III denote the collected fractions of SAM, MTA and Ade, respectively; (c) 250 mM ammonium formate buffer pH 2.9–20% (v/v) acetonitrile; flow-rate: 0.6 ml/min.

TABLE I

RETENTION DATA, DETECTION LIMITS AND CALIBRATION PARAMETERS OF THE COMPOUNDS INVESTIGATED

Compound	Retention time (min)		Detection limit (pmol)	Slope	Intercept	r	n^a	s^b	Relative error (%)
	Reversed-phase	Cation exchange							
SAM	7–8	13	50	444 ± 4	1945	0.982	21	18379	2.8–8.3
SAH	15–16	14	12	877 ± 12	−6378	0.997	18	13366	0.9–8
MTA	29–30	10	60	748 ± 23	21892	0.988	15	15336	4.8–8
Ade	10–12	10	10	482 ± 4	−1029	0.999	21	2410	1.1–6
Ado	15–16	9	14	489 ± 2	1182	0.999	21	1911	1.0–5.7
dc.SAM	9–10	10	50	280 ± 5	−2988	0.996	15	1746	2.2–6.6

[a] n = the number of data points.
[b] s = the standard error of the estimate.

Table I contains the retention time values of the compounds both in reversed-phase and cation-exchange chromatography, their detection limits and the parameters of the calibration curves obtained in cation-exchange chromatography. The recovery from the two HPLC methods (six experiments) was 94 ± 9.5% for SAM, 92 ± 9.0% for SAH and 83 ± 7.8% for MTA. The average recovery (five experiments) for all procedures including the sample preparation was determined by the standard addition method and was found to be 68% for SAM, 55% for SAH, 68% for MTA, 99.2% for dc-SAM, 103% for Ado and 103% for Ade. Thus the two-dimensional chromatographic assay of the adenosyl derivatives permitted accurate detection of 0.050 nmol of SAM, MTA and dc-SAM and 0.015 nmol of SAH, Ade and Ado. This permitted the quantification of a sample equivalent to 0.020 g of liver tissue for SAM, SAH, Ade and Ado, 0.250 g of liver tissues for MTA and dc-SAM, and $7 \cdot 10^5$ mast cells (RBL-2H3) for SAM. The within-day precision of the method was 9.6% for SAM, 8.4% for SAH and 7.8% for MTA. The day-to-day variation was less than 10% for each compound.

Tables II and III contain the results obtained

TABLE II

CONCENTRATIONS OF SAM AND ITS RELATED METABOLITES IN RAT LIVER

Compound	Concentration (nmol/g)	
	One rat[a]	Average of four rats[b]
SAM	28.2 ± 2.0	35.5 ± 7.3
SAH	8.3 ± 0.8	10.2 ± 2.3
MTA	0.2 ± 0.03	0.3 ± 0.05
Ado	43.8 ± 6.3	43.2 ± 4.0
Ade	48.6 ± 2.7	49.3 ± 4.3
dc-SAM	3.5 ± 0.8	4.0 ± 0.5

[a] Data are the average values obtained from three measured data from one rat liver divided into three parts.
[b] Data are the average from the measured values of four livers (*i.e.* the number of measured peak-area values = 12).

TABLE III

CONCENTRATIONS OF SAM AND ITS RELATED METABOLITES IN FOUR DIFFERENT BATCHES OF MAST CELLS

Compound	Concentration (ng/10^6 cells)	Standard deviation (%)
SAM	36.9 ± 4.4	12.8
SAH	13.4 ± 1.1	6
MTA	Not determined	
Ado	17.8 ± 6.2	24
Ade	16.2 ± 4.2	21
dc-SAM	Not determined	

TABLE IV

CONCENTRATIONS OF SAM AND ITS RELATED METABOLISM IN RAT LIVER OBTAINED FROM THE LITERATURE

Ref.	Method	Concentration (nmol/g)				
		SAM	SAH	MTA	Ade	dc-SAM
25[a]	Ion-pair	72	18	–	38	–
26[b]	Ion-pair	33.0	56	2.9	–	–
11	Cation-exchange	66.5	45.8	9.0	13.0	1.7
24	Cation-exchange	66.3	45.5	–	–	–
14	Reversed-phase	63.4	25.3	–	–	–
22	Partisil	59.0	18.0	0.05	32.8	–

[a] The data were obtained from rats starved for 24 h.
[b] The data were obtained from rats statved for 8 h.

from rat liver and mast cells, respectively. Table IV shows the concentration values of SAM and its related metabolites in rat liver published in the literature. Comparison of the data shown in Tables II and IV shows that, as expected, the concentrations obtained by the two-dimensional chromatographic method are lower. This can be either because of the longer (24 h) starvation of rats, or because of the higher selectivity of our method. As reported by Fell *et al.* [25] starvation can cause a decrease of the concentration of SAM and increase of SAH. As our rats were starved for 24 h, our concentration levels of SAM are in good agreement with the published results of Wagner *et al.* [26]. The hepatic concentrations of SAH and MTA were thus found to be lower than those previously reported.

In conclusion, two-dimensional HPLC has proved to be a suitable, selective and accurate assay for SAM and its related metabolites in different tissues and cultured cells, and has suggested that assay procedures using single HPLC methods may give erroneous results.

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