Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 4/5, pp. 361–366, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

A comparative study of the reversed-phase HPLC retention behaviour of *S*-adenosyl-L-methionine and its related metabolites on Hypersil ODS and Supelcosil™ LC-ABZ stationary phases

KLÁRA VALKÓ,*† MORTEZA P. HAMEDANI,† TRACY L. ASCAH‡ and WILLIAM A. GIBBONS†

† *Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK*
‡ *Supelco, Inc., Supelco Park, Bellefonte, PA 16823-0048, USA*

Abstract: *S*-Adenosyl-L-methionine (SAM) and its metabolites *S*-adenosyl-L-homocysteine (SAH) and methylthioadenosine (MTA) are endogenous compounds that are heavily involved in a variety of biochemical processes, and have therefore been the target for several assays in body fluids and tissues. Reversed-phase chromatographic behaviour of SAM and its metabolites has been studied by using Supelcosil™ LC-ABZ column, specially designed for analysis of acidic, basic, zwitterionic and neutral compounds, and on a Hypersil ODS column as a function of mobile phase pH. The retentions of the compounds, expressed by the capacity ratio (k'), are measured on both column with mobile phases comprised of 10% acetonitrile and 10 mM ammonium formate buffer with pH values ranging from 2 to 9. Higher selectivity is observed on Supelcosil LC-ABZ within pH range 4–6. Different retention properties are observed at very low pH and seemed as if the Supelcosil LC-ABZ column reduced the effect of the mobile phase pH by about 1 pH unit. Whilst the Supelcosil column can be recommended for the routine analysis of SAM and its related metabolites in biological fluids by using mobile phase pH 5, the Hypersil ODS column may be suggested for use with mobile phase pH values of 3–4.

Keywords: *S-Adenosyl-L-methionine and related metabolites; reversed-phase HPLC; dependence of the RP-HPLC retention on pH.*

Introduction

S-Adenosyl-L-methionine (SAM) is an endogenous compound that is heavily involved in a variety of biochemical reactions of cellular functions, such as methylation of lipids, proteins, RNA and DNA, trans-sulphuration and polyamine biosynthesis [1–4]. Immunological effects [5] and cellular differentiation [6–7] can be triggered by the perturbation of the SAM metabolic pathways. SAM also has 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 [10–13].

SAM and its metabolites *S*-adenosyl-L-homocysteine (SAH) and methyl-thioadenosine (MTA) are relatively polar compounds and as a consequence they display relatively short retention times under reversed-phase chromatographic conditions even if very low concentrations of organic modifier are used [14–15]. To increase the retention time of the positively charged SAM, reversed-phase ion-pair chromatographic methods have been suggested using heptane or octane sulphonic acids in the acidic mobile phase [16–19]. 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 [20]. The application of cation-exchange chromatography already has been published [21–22] and, as expected, SAM had a much longer retention time, being a cation at every mobile phase pH; but SAH, MTA and adenosine (Ado) showed relatively short retention times. All of these separations were carried out on a silica-based HPLC stationary phase. HPLC on silica is limited by the poor chromatography of silanophillic compounds. Symptoms of silanol

* Author to whom correspondence should be addressed.

interaction between a sample and the silica support are peak asymmetry, retention drift and irreproducible separations. The Supelcosil™ LC-ABZ column was designed to be suitable for analyses of acidic, basic, zwitterionic, and neutral compounds, without the use of silanol-suppressing mobile phase conditions and additives.

The comparative study of the retention behaviour of SAM, SAH, MTA, Ado (adenosine) and iso-propyl-deoxyuridine (IPDU) as a function of mobile phase pH is presented here obtained using Supelcosil LC-ABZ and Hypersil ODS stationary phases.

Experimental

The SAM, SAH, MTA, and Ado standard compounds were purchased from Sigma (Sigma Chemical Co., St Louis, CA, USA). The potential internal standard for the biological assay of the investigated compounds, iso-propyldeoxyuridine (IPDU), was a gift from L. Ötvös (Central Research Institute for Chemistry, Hungarian Academy of Sciences, Budapest, Hungary). IPDU is a registered anti-herpes agent in Hungary; it is available in high purity and is a very stable compound. The investigated compounds were dissolved in distilled water at 1 mg ml^{-1} concentration and kept in the freezer until required for use.

The HPLC measurements were carried out using Gilson HPLC system (Gilson, Villiers le Bel, France) equipped with a M 303 pump and a Holochrom variable wavelength UV detector. Detection was performed at 254 nm. A Hewlett–Packard M3396 Series II (Hewlett–Packard Co., Avendale, PA, USA) recorder–integrator was used to measure retention times. The samples were injected by means of a Rheodyne M7125 (Rheodyne, Cotati, CA, USA) injection valve equipped with a 20 µl sample loop. The mobile phase was pumped at a flow rate of 1.00 ml min^{-1}.

The mobile phases always contained 10% (v/v) acetonitrile (HPLC grade, obtained from Rathburn Chemical Ltd, Walkerburn, UK) and 10% (v/v) stock solution of 100 mM ammonium formate (Analytical grade, Pharmacos Ltd, Southend-on-Sea, UK) (pH 6.4). The mixture of the two solutions was diluted by double distilled water up to 95%. The pH* of the mobile phase was adjusted with concentrated formic acid (BDH Chemicals Ltd, Poole, UK) for lower than pH 6.4 and concentrated ammonia solution (BDH Chemicals Ltd, Poole, UK) for higher pH values. The mobile phase solutions were then diluted up to 100% volume by water and the final mobile phase pH* value was determined. The pH* values of the mobile phases used in the experiments are listed in Tables 1 and 2. The same solution of the mobile phase was used for the two columns. The Hypersil ODS 5 µm stationary phase (Shandon Scientific Ltd, Runcorn, UK) was packed into a 4.0 × 150 mm column (Chromlab, Budapest, Hungary). The Supelcosil LC-ABZ (5 µm) column (150 × 4.6 mm) was a kind gift from Supelco, Inc. (Bellefonte, PA, USA).

Table 1
The capacity ratios (k') of SAM, SAH, MTA, Ado and IPDU obtained by mobile phases containing 10% acetonitrile and 10 mM ammonium formate with various pH on Supelcosil LC-ABZ column

	Capacity ratio (k')				
pH*	SAM	SAH	MTA	Ado	IPDU
2.20	0.083	0.300	1.083	0.350	3.917
2.50	0.180	0.467	1.233	0.633	4.250
3.00	0.048	0.700	2.083	0.683	5.017
3.46	0.033	0.750	3.367	0.917	5.133
3.90	0.083	0.750	4.950	1.083	4.617
4.55	0.333	0.833	6.250	1.333	4.850
4.96	0.250	0.833	6.367	1.367	4.867
5.50	0.183	0.950	6.083	1.350	4.717
5.90	0.250	0.750	5.833	1.300	4.533
6.82	0.233	0.750	6.333	1.333	4.750
7.52	0.917	0.833	6.833	1.433	5.000
8.02	0.750	0.867	7.000	1.500	5.333
8.50	0.817	0.833	6.333	1.417	4.750

Table 2
The capacity ratios of SAM, SAH, MTA, Ado and IPDU obtained by mobile phases containing 10% acetonitrile and 10 mM ammonium formate with various pH* on Hypersil ODS column

	Capacity ratio (k')				
pH*	SAM	SAH	MTA	Ado	IPDU
2.20	0.167	0.333	2.333	0.583	3.125
2.50	0.150	0.333	3.333	0.333	3.917
3.00	0.250	0.417	3.667	0.483	3.833
3.46	0.302	0.450	4.750	0.467	4.250
3.90	0.208	0.333	5.250	0.683	4.167
4.55	0.333	0.350	4.917	0.667	4.000
4.96	0.333	0.333	5.167	0.667	3.958
5.50	0.417	0.417	5.083	0.667	3.917
5.90	0.417	0.417	4.483	0.517	3.500
6.82	0.333	0.333	5.500	0.667	4.167
7.52	0.350	0.367	5.250	0.667	4.000
8.02	0.333	0.383	5.667	0.833	4.333
8.50	0.383	0.417	5.167	0.667	3.833

The retention time measurements were repeated three times consecutively and the average was taken into account in the calculations of the capacity ratio (k'). The first solvent peak was regarded to be the dead time.

Results and Discussion

The chemical structure of the investigated compounds are shown in Fig. 1. As it can be seen IPDU can be considered to be a neutral compound since protonation or dissociation would not be expected within the investigated pH range. SAM has a positive charge at all pH values and possibly SAH as well due to the amino group in the amino acid side chain. The pK_a values for the —COOH groups in methionine and homocysteine are 2.28 and 2.22, respectively, and for the NH_2 groups the pK_a values are 9.21 and 10.86, respectively. MTA and Ado can be regarded to be very weak bases and thus can be protonated only at low pH (below 4). The pK_a value for the 6-NH_2 group in adenine is 4.14.

The k' values for the five compounds, SAM, SAH, MTA, Ado and IPDU, obtained at various pH* values on Supelcosil LC-ABZ column and Hypersil ODS column are summarized in Tables 1 and 2, respectively. In general, reversed-phase retention is independent of the mobile phase pH for neutral compounds. When the pH change causes dissociation or protonation of the sample molecule and, thus changing its hydrophobicity, it is manifested in the reversed-phase retention as well. In the present case the mobile phase pH can effect the protonation of MTA, SAH, SAM and Ado, but also it can affect the protonation of the functional groups on the Supelcosil LC-ABZ stationary phase. Therefore, the change in retention as a function of mobile phase pH* can be caused as a sum of the two effects. Figure 2 shows the capacity ratios of the five model compounds as a function of mobile phase pH on Supelcosil LC-ABZ. As a comparison, Fig. 3 shows the same plot obtained on Hypersil ODS. The largest change in retention values were observed for MTA on Supelcosil LC-ABZ column. The capacity ratio of IPDU (which may be re-

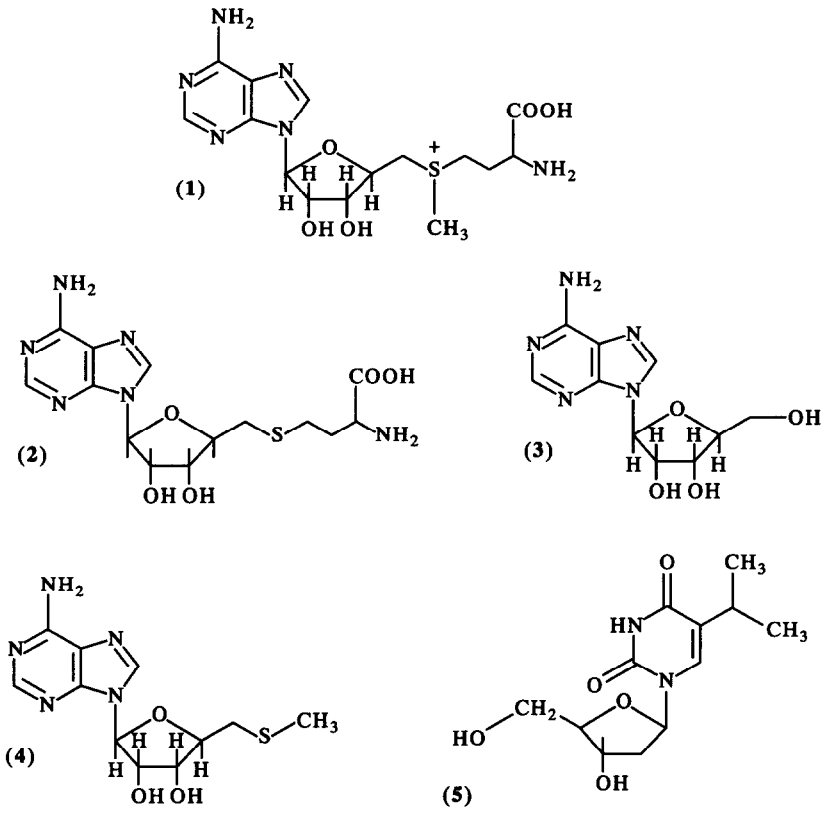

Figure 1
The chemical structures of the investigated compounds. 1, *S*-adenosylmethionine (SAM); 2, *S*-adenosylhomocysteine (SAH); 3, adenosine (Ado); 4, methylthioadenosine (MTA); 5, iso-propyl-deoxyuridine (IPDU).

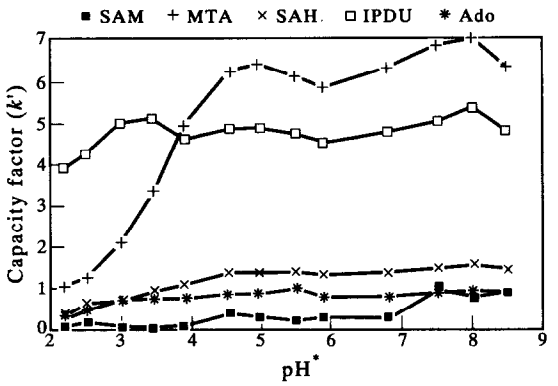

Figure 2
The plot of the capacity ratios (k') as a function of mobile phase pH* measured on Supelcosil LC-ABZ column.

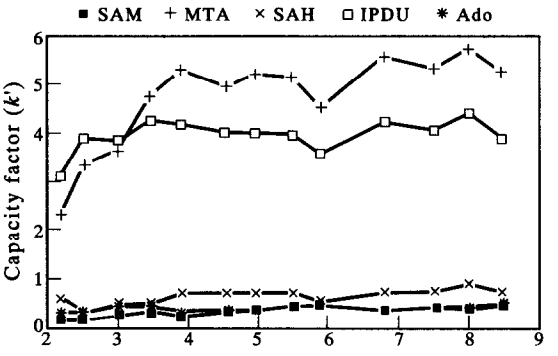

Figure 3
The plot of the capacity ratios (k') as a function of the mobile phase pH* measured on Hypersil ODS column.

garded to be a neutral compound) remained around 4 and 5 within the investigated pH* range of the mobile phase. As a consequence the retention order of IPDU and MTA changed when the pH* was lower than 4 on both stationary phases. Very short retention times were measured on both stationary phases for SAM as it has a positive charge at any pH value, and the capacity ratio of SAM did not change significantly. From the data presented in Figs 2 and 3 it can be concluded, that the Supelcosil LC-ABZ stationary phase showed similar retention properties as a function of pH as the Hypersil stationary phase in the case of neutral (IPDU) and positively charged (SAM) molecules. However, the retention change due to protonation of MTA was more significant, and it occurred at higher pH value on Supelcosil LC-ABZ column than on Hypersil ODS.

As the retention times and even the retention order of the compounds could be changed by the variation of mobile phase pH*, it provides another possibility for optimization of the separation of the investigated biochemically important molecules. As Figs 2 and 3 suggest the separation of Ado, SAH and SAM proved to be the most difficult. The selectivity factors ($\alpha = k'_1/k'_2$) for SAM and SAH in relation to Ado were plotted against the mobile phase pH* obtained on the two investigated stationary phases and are shown in Figs 4 and 5. Higher selectivity and therefore better separation could be observed on Supelcosil LC-ABZ within the range pH 4–6. If the separation factor of MTA and IPDU is considered as well, the optimum separation can be achieved between pH 5 and 6. The application lower than pH 4 can be suggested on Hypersil ODS stationary phase, but then the separation of IPDU and MTA becomes the most critical point. The chromatogram obtained under the optimum separation conditions is shown in Fig. 6. Figure 7 shows the chromatogram obtained

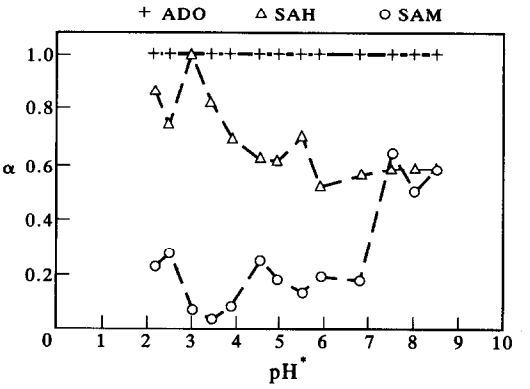

Figure 4
The plot of separation factors ($\alpha = k'_1/k'_2$) of SAH and SAM in comparison to Ado as a function of mobile phase pH* obtained on Supelcosil LC-ABZ column.

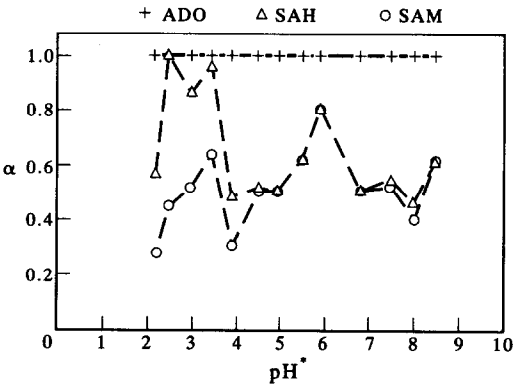

Figure 5
The plot of separation factor ($\alpha = k'_1/k'_2$) of SAH and SAM in comparison to Ado as a function of mobile phase pH* obtained on Hypersil ODS stationary phase.

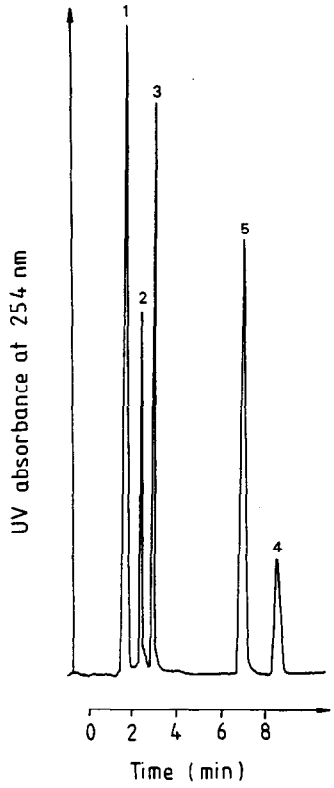

Figure 6
The chromatogram of the investigated compounds obtained on Supelcosil LC-ABZ column. Mobile phase: 10% acetonitrile, 10 mM ammonium formate buffer, pH* = 5.5; flow rate: 1.00 ml min^{-1}; detection wavelength 254 nm. 1, SAM; 2, SAH; 3, Ado; 4, IPDU; and 5, MTA.

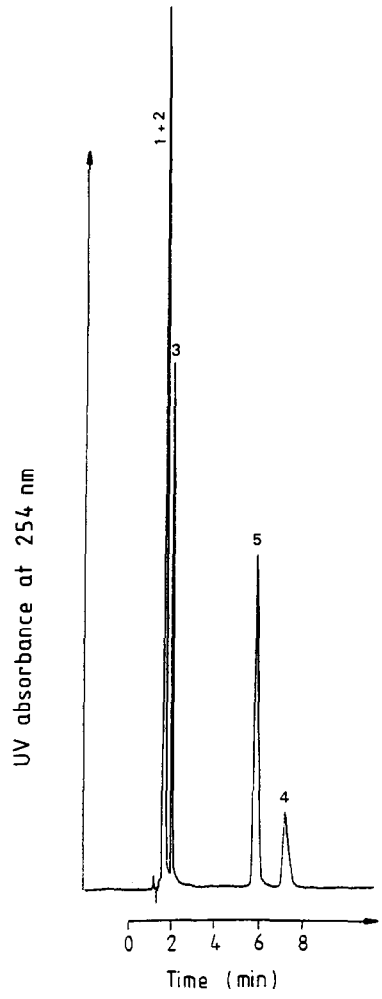

Figure 7
The chromatogram of the investigated compounds obtained on Hypersil ODS column. For conditions and key see Fig. 6.

under the same conditions on Hypersil ODS column.

In conclusion, the Supelcosil LC-ABZ column showed slightly different retention properties for the investigated polar charged compounds as a function of mobile phase pH than the Hypersil ODS column. The Supelcosil LC-ABZ column is recommended for the routine analysis of SAM and its related metabolites in biological samples by using mobile phase pH 5. The Hypersil ODS column can be suggested for the separation of the investigated compounds at pH 3–4.

Acknowledgements — K. Valkó's contribution to the work was supported by a Maplethorpe Fellowship, that is greatly acknowledged.

References

[1] F. Salvatore, E. Borek, V. Zappia, H.G. Williams-Ashman and F. Schlenk, *Biochemistry of Adenosylmethionine.* Columbia University Press, New York (1977).

[2] D.R. Hoffman, D.W. Marion, W.E. Cornatzer and J.A. Duerre, *J. Biol. Chem.* **255**, 10822–10827 (1980).

[3] R.K. Chawla, H.L. Bonkosky and J.T. Galambos, *Drugs* **40**, (Suppl. 3), 98–110 (1990).

[4] H. Hibasami, R.T. Borchardt, S.Y. Chen, J.K. Coward and A.E. Pegg, *Biochem. J.* **187**, 419–428 (1980).

[5] P.K. Chiang, *Science* **211**, 1164–1166 (1981).

[6] P.A. Crooks, R.N. Dreyer and J.K. Coward, *Biochemistry* **18**, 2601–2609 (1979).

[7] T.P. Zimmerman, M. Iannone and G. Wolberg, *J. Biol. Chem.* **259**, 1122–1126 (1984).

[8] T. Bottiglieri, M.W.P. Carney, J. Edeh, M. Laundry, R. Martin, E.H. Reynolds, C. Thomas and B.K. Toone, in *Biochemistry of S-Adenosyl Methionine and Related Compounds* (E. Usdm and R.H. Brochardt, Eds), pp. 327–338. Macmillan, London (1982).

[9] C. Fazio, V. Andreoli and A. Agnoli, *IRCS Medical Science — Nervous System* **2**, 1015–1028 (1974).

[10] D.O. Adams and S.F. Young, *Proc. Natl. Acad. Sci.* **76**, 170–174 (1979).

[11] G.A. Miura, J.R. Santangelo, R.K. Gordon and P.K. Chiang, *Anal. Biochem.* **141**, 161–167 (1984).

[12] D. Fell, L.E. Banjamin and R.D. Steele, *J. Chromatogr.* **345**, 150–156 (1985).
[13] H. Gourdeau, R. Lavoie, J.H. Grose and L. Bélanger, *Anal. Biochem.* **158**, 64–67 (1986).
[14] T. Bottiglieri, *Biomed. Chromatogr.* **4**, 239–241 (1990).
[15] A.M. Mollay, D.G. Weir, G. Kennedy, S. Kennedy and J.M. Scott, *Biomed. Chromatogr.* **4**, 257–260 (1990).
[16] J. Wagner, C. Danzin and P. Mamont, *J. Chromatogr.* **227**, 349–368 (1982).
[17] J. Wagner, N. Claverie and C. Danzin, *Anal. Biochem.* **140**, 108–116 (1984).
[18] J. Wagner, C. Danzin, S. Hout-Olivier, N. Claverie and M.G. Plafreyman, *J. Chromatogr.* **290**, 247–262 (1984).
[19] S.H. Zeisel, T. Zola, K.-A. DaCosta and E.A. Pomfret, *Biochem. J.* **259**, 725–729 (1989).
[20] B. Guattari, *J. Chromatogr.* **567**, 254–260 (1991).
[21] B.-G. Chun, W.K. Paik and S. Kim, *J. Chromatogr.* **264**, 321–328 (1983).
[22] V. Zappia, P. Galletti, M. Porcelli, C. Manna and F.D. Ragione, *J. Chromatogr.* **189**, 399–405 (1980).

[*Received for review 5 March 1992; revised manuscript received 10 August 1992*]