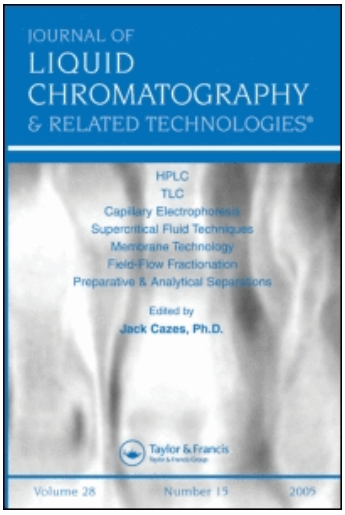

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Alonso, J. , Nogúes, M. V. and Cuchillo, C. M.(1985) 'Separation of RNA Derivatives by High-Performance Anion-Exchange Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 8: 2, 299 — 313

To link to this Article: DOI: 10.1080/01483918508067079

URL: http://dx.doi.org/10.1080/01483918508067079

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(2), 299-313 (1985)

SEPARATION OF RNA DERIVATIVES BY HIGH-PERFORMANCE ANION-EXCHANGE LIQUID CHROMATOGRAPHY

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ABSTRACT

In this paper the separation of different mixtures of RNA derivatives by high-performance anion-exchange liquid chromatography is presented. By using this method different mixtures of nucleosides, 3'- and 5'- nucleotides and 2':3'-cyclic nucleotides can be separated by isocratic elution at room temperature and in a short analysis time. The separation was optimized by properly adjusting the ionic strength and pH of the mobile phase. The pH is a critical factor in the order of elution and in the resolution capacity whereas the ionic strength affects primarily, the retention time. The separation method was applied to the analysis of RNAase A substrates and ligands.

 0148-3919/85/0802-0299$3.50/0

INTRODUCTION

Several authors have applied the HPLC technique to the separation of complex mixtures of RNA derivatives (1-3). Most of the chromatographic systems described use either reverse or ion exchange as stationary phases and the elution is carried out either with phosphate buffers (4-6) or with very acidic (pH 2.8 or lower) solutions. These working conditions are not convenient when dealing with problems relevant to the mechanism of action of bovine pancreatic RNAase A. On the one hand, phosphate is a powerful competitive inhibitor of the enzyme (7) and thus it is important to avoid phosphate buffers. On the other hand, the use of very low pH values shortens considerably the life of the column (8). Finally, with regard to the study of the specificity of the different sub-sites of the enzyme, currently investigated in our laboratory (9-13) it is important to be able to separate mixtures of nucleosides, 2'-, 3'- and 2':3'-cyclic mononucleotides as well as some nucleotides analogues such as halogenated purine nucleotides and N^1-oxide purine derivatives.

For all these reasons a detailed study of the separation conditions of nucleosides and 3'- and 5'-nucleotides was undertaken. The best conditions found were then applied to the separation of different derivatives of RNA, i.e. N^1-oxide, cyclic and halogenated nucleosides and nucleotides. These experiments were carried out with an anion-exchange column (Nucleosil 10SB) using a mild solvent system (ammonium acetate) that does not interfere with later enzymatic studies.

MATERIALS AND METHODS

Materials: Adenosine (Ado), Guanosine (Guo), Cytidine (Cyd), Uridine (Urd), Adenosine 3'-phosphate (3'-AMP), Guanosine 3'-phos-

phate (3'-GMP), Cytidine 3'-phosphate (3'-CMP), Uridine 3'-phosphate (3'-UMP), Adenosine 5'-phosphate (5'-AMP), Guanosine 5'-phosphate (5'-GMP), Adenosine 2':3'-phosphate (2':3'-AMP), Guanosine 2':3'-phosphate (2':3'-GMP), Cytidine 2':3'-phosphate (2':3' CMP), Uridine 2':3'-phosphate (2':3'-UMP), Adenosine N^1-oxide (N^1-oxide Ado), Adenosine N^1-oxide 5'-phosphate (N^1-oxide AMP) Cytidine 2'-phosphate (2'-CMP) were products of Sigma Chemical Company (Saint Louis, MO, USA). Cytidine 5'-phosphate (5'-CMP) Uridine 5'-phosphate (5'-UMP), Adenosine 2'-phosphate (2'-AMP) 8-Bromoadenosine (8-BrAdo), were obtained from Cambrian Chemicals (Croydon, UK). 8-Bromoadenosine 5'-phosphate (8-BrAMP) was synthesized by direct bromination of 5'-AMP by the procedure of Ikehara et al. (14). Analytical grade 2-propanol, sodium acetate and ammonium sulphate were from Merck (Darmstadt, GFR), ammonia and acetic acid HPLC grade were obtained from Scharlau (FEROSA, Barcelona, Spain).

Anion-exchange column Nucleosil 10SB (300x8x4 mm) and precolumn stationary phase Vydac-301SB were purchased from Macherey & Nagel (Düren, GFR).

TLC cellulose plates (without fluorescent indicator) 20x20 cm, 0.1mm layer thickness, were obtained from Merck.

Apparatus: All experiments were carried out with a modular HPLC Waters (Waters Associates, Milford, MA, USA) consisting of pump Model 6000A controlled by the Automated Gradient Controller Model 680. Samples were detected by monitoring the effluent at 254nm with an Absorbance Detector Model 441 and a Microprocessor Data Module Model M730 and a Universal Liquid Chromatograph Injector Model U6K. Injections were made with a Hamilton precision syringe Model 802RNE (Reno, NV, USA). An oven Eldex CX4-2 and a Temperature Control Unit Model III were used in the studies on the temperature dependence.

Procedures: The effects of pH and ionic strength on the chromatographic properties of RNA derivatives were studied by using as

mobile phase solutions of ammonium acetate of 0.2, 0.3, 0.4, 0.5 and 0.6M at pH values of 4, 5 and 7.5.

Twice distilled water was filtered through a 0.22μm Millipore membrane filter (Millipore, Madrid, Spain) and degassed under vacuum for 20 minutes. The adjusted solutions were degassed under vacuum for 10 minutes before use.

25 or 50 μl of the different mixtures (0.5 - 1 mg/ml of each component) dissolved in ammonium acetate with the same pH and ionic strength as those of the eluent were loaded onto the previously equilibrated column. Before injection all samples were centrifuged for 5 minutes in an Eppendorf 3414 centrifuge (Eppendorf Geratebau, Hamburg, GFR) to remove any particulate material.

Sample separations were performed isocratically at room temperature and a flow rate of 1 ml/min. Routinely, the column was run at a pressure between 12400-13800 kPa (1800-2200 psi). At the end of each day, the column and the chromatographic system were washed with twice-distilled water for 30 minutes to remove salts.

The identification of the different peaks was carried out by means of TLC on cellulose plates using saturated $(NH_4)_2SO_4$: 1M sodium acetate:2-propanol (80:18:2 by vol) as solvent system (15). An Uvatom 70 dual-wavelength UV lamp (Atom, Barcelona, Spain) was used to locate the spots on the chromatograms.

RESULTS AND DISCUSSION

The separation of nucleosides and 3'- and 5'-nucleotides was optimized by studying the separation conditions in relation to ionic strength and pH. At all pH values studied the retention time of nucleosides was independent of ionic strength. In general, purine nucleosides are more retarded than pyrimidine ones, the re-

tention being stronger in both cases at high pH values (Figs. 1, 2 and 3). At pH 4 the order of elution was Cyd, Urd, Ado, Guo. At pH 5 the Urd + Cyd and Ado + Guo pairs were eluted together and at pH 7.5 the order was Urd, Cyd, Ado, Guo. Probably this could be explained on the basis of two factors: a) the pK_{a2} values of the amino groups in the bases (16) and b) some unspecific interactions between purine or pyrimidine rings and the stationary phase, as the retention time is not affected by changes in the ionic strength. This phenomenon is more important for purine nucleosides as they show longer retention times than the pyrimidine ones.

At pH 7.5 an interesting effect in the elution of nucleosides was observed. At low ionic strength values all nucleosides were eluted before the 3'- and 5'-nucleotides, but at higher ionic strength values purine nucleosides were more retarded than 3'- and 5'-pyrimidine nucleotides (Fig. 1a). Again, this is probably due to the effect of unspecific interactions between purine nucleosides and the stationary phase, its intensity being independent on the ionic strength, whereas the interaction of 3'- and 5'-nucleotides is due, primarily, to the effect between the phosphate group and the positive groups on the matrix. As shown in Fig. 1b the separation of 9 fractions out of 12 standard compounds was achieved in a reasonable time of 40 minutes using 0.2M ammonium acetate as mobile phase. The order of elution was as follows: pyrimidine nucleosides, purine nucleosides, 5'-pyrimidine nucleotides, 3'-pyrimidine nucleotides, 5'-purine nucleotides and 3'-purine nucleotides.

At pH 5 the best separation was obtained with 0.4M ammonium acetate but only 6 fractions out of 12 compounds were completely separated in 25 minutes (Fig. 2b). Each peak contained two compounds related both by the phosphate position and the kind of base (pyrimidine or purine). The order of elution was the same as that found at pH 7.5.

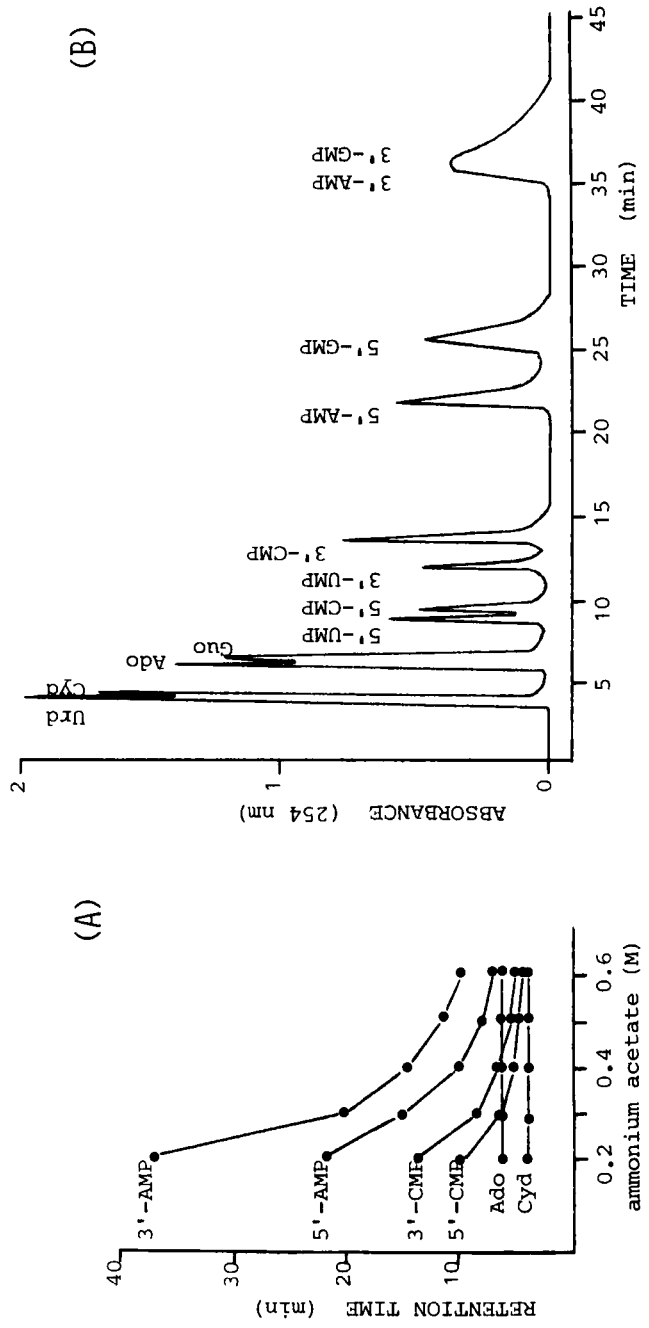

FIGURE 1.- (a) Variation of retention time with concentration of eluent for some nucleosides and nucleotides at pH 7.5. (b) Separation of nucleosides, 3'- and 5'-nucleotides by isocratic elution with 0.2M ammonium acetate, pH 7.5. For other conditions see text.

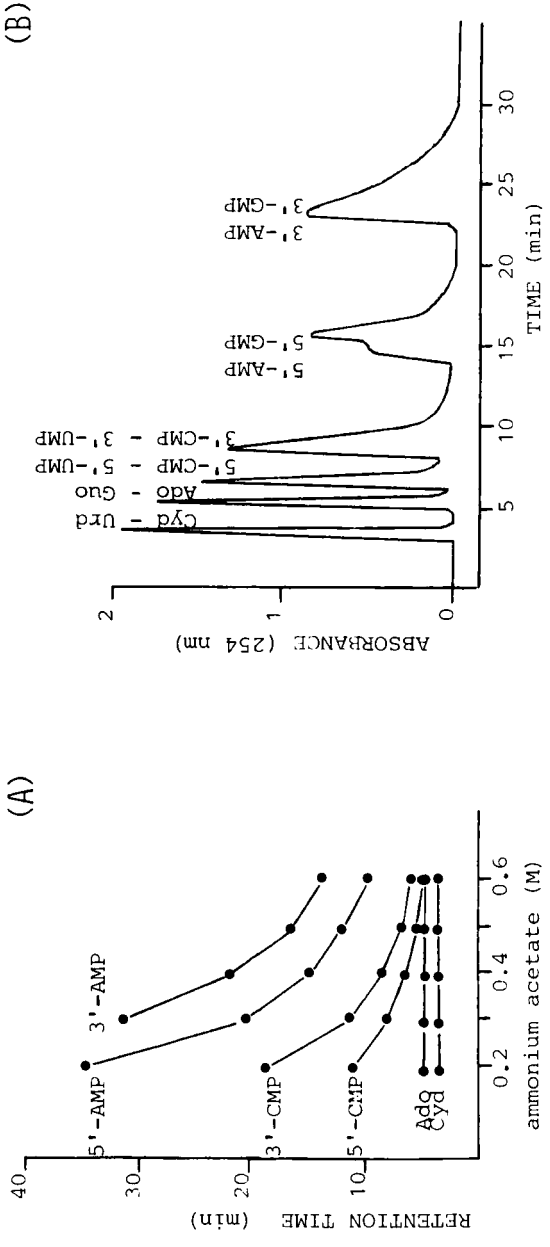

FIGURE 2.- (a) Variation of retention time with concentration of eluent for some nucleosides and nucleotides at pH 5. (b) Separation of nucleosides, 3'- and 5'-nucleotides by isocratic elution with 0.4M ammonium acetate, pH 5. For other conditions see text.

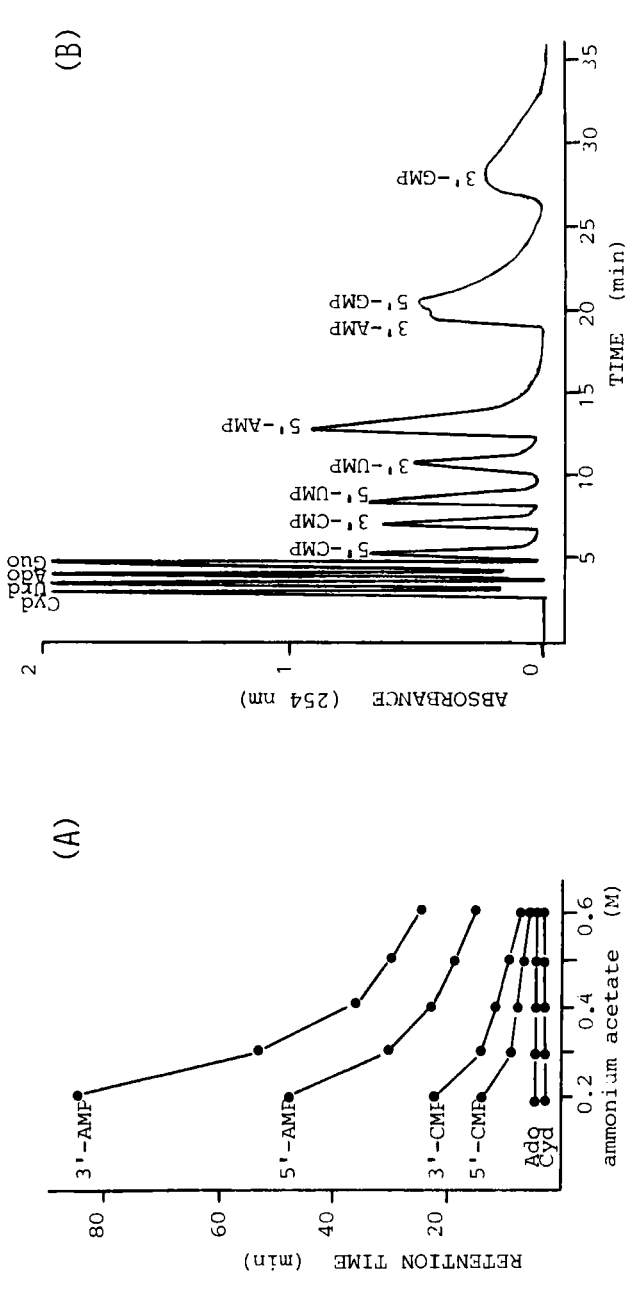

FIGURE 3.- (a) Variation of retention time with concentration of eluent for some nucleosides and nucleotides at pH 4. (b) Separation of nucleosides, 3'- and 5'-nucleotides by isocratic elution with 0.6M ammonium acetate, pH 4. For other conditions see text.

The most complete separation was obtained at pH 4 and ionic strength of 0.6. The separation of 11 peaks out of 12 components was achieved in less than 30 minutes. Only the mixture of 3'-AMP and 5'-GMP was not resolved (Fig. 3b). By decreasing the ionic strength the resolution was not improved and the chromatography time increased. At this pH it can be noticed the effect of the amount of sample on the retention time. By increasing the quantity loaded onto the column, the retention time decreased slightly (Table 1). This effect is more apparent with the compounds with longer retention times but the resolution is not affected.

The order of elution of 3'- and 5'- nucleotides is highly pH-dependent. Both the amino group and the secondary phosphate pK_a values are higher for the 5'-nucleotides than for the 3'-nucleotides (16, 17). For this reason the separation between the 3'- and 5'- isomers was always observed. The ionic strength modifies the retention time, the lower the ionic strength the higher the retention time for all compounds. This effect is more noticeable with the most retarded compounds. At low ionic strengths a widening of the peaks is observed and the resolution is highly affected. Only at pH 7.5 the best separation was obtained at an ionic strength of 0.2 (Fig. 1b). The retention times showed little variation between experiments carried out under the same experimental conditions and they never affected the elution patterns.

Neither the resolution nor the retention time showed any dependence on the temperature (15-55°C) and changing in the flow rate (between 0.5 and 2 ml/min) did not affect the resolution.

The above chromatographic procedures were also applied to the separation of RNA derivatives used in the study of the mechanism of action of RNAase A. A reasonable good separation of a mixture of the 2':3'-cyclic nucleotides was achieved in 25 minutes with the previously described column using as eluent 0.6M ammonium acetate, pH 4 (Fig. 4).

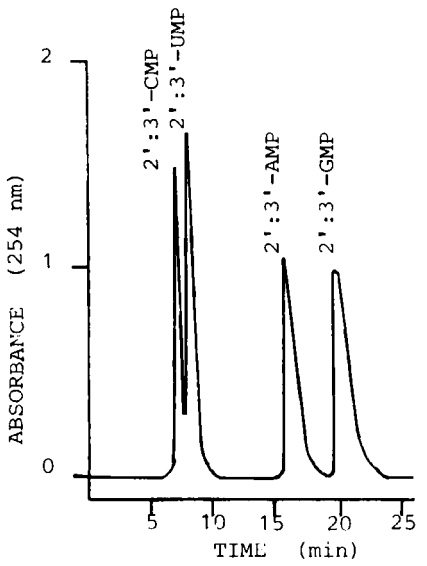

FIGURE 4.- Elution profile of a mixture of 2':3'-cyclic nucleotides with 0.4M ammonium acetate, pH 4 as eluent. For other conditions see text.

TABLE 1.- Variation of the Retention Time with the Amount of Sample Loaded onto the Column (pH 4).

SAMPLE	RETENTION TIME (minutes)			
	0.3M		0.6M	
	A	B	A	B
Cyd	3.0	3.0	2.9	2.9
Ado	4.0	4.0	4.4	4.4
5'-CMP	5.5	5.4	9.0	8.5
3'-CMP	7.2	6.9	14.1	12.9
5'-AMP	13.1	12.4	30.6	28.4
3'-AMP	20.3	18.5	53.0	48.5

A. Sample contained 0.02mg of each compound in a final volume of 25µl.
B. Sample contained 0.04mg of each compound in a final volume of 50µl.

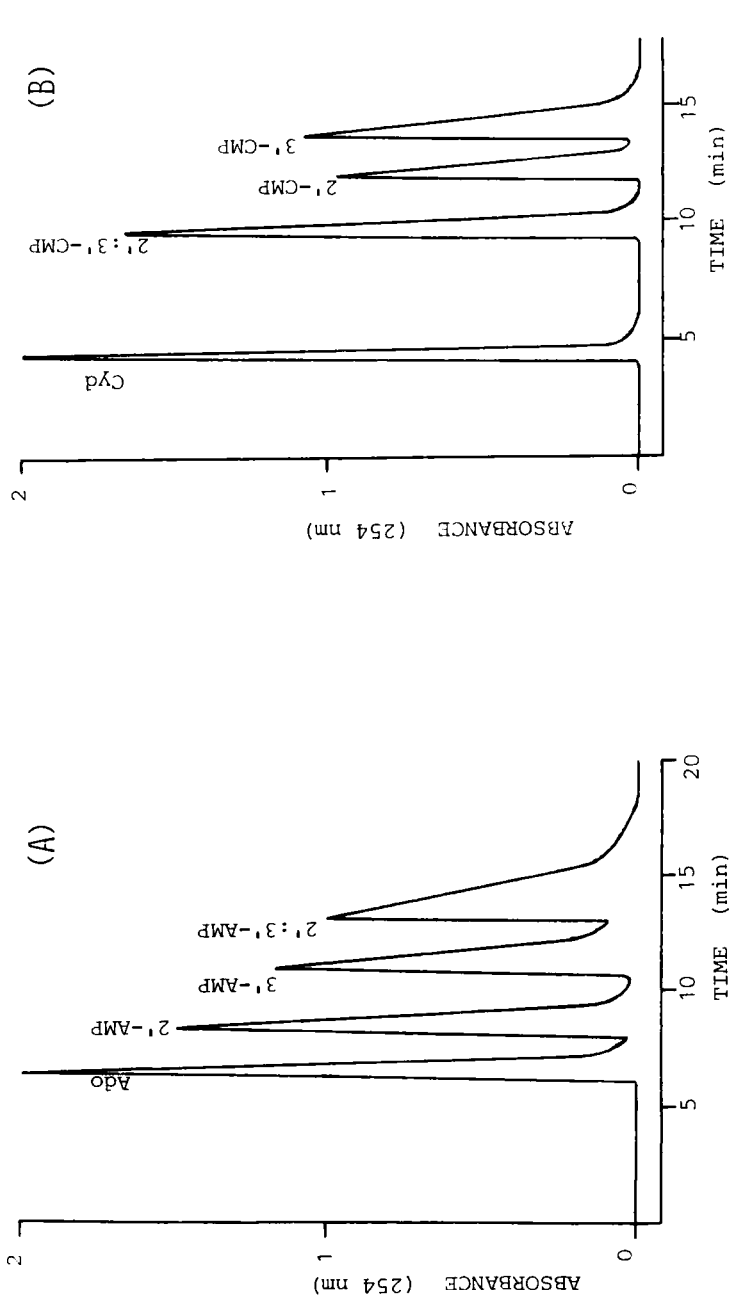

FIGURE 5.- (a) Separation of a mixture of Ado, 2'-AMP, 3'-AMP and 2':3'-AMP by isocratic elution with 0.5M ammonium acetate, pH 7.5. (b) Elution profile of a mixture of Cyd, 2'-CMP, 3'-CMP and 2':3'-CMP by isocratic elution with 0.2M ammonium acetate, pH 7.5. For other conditions see text.

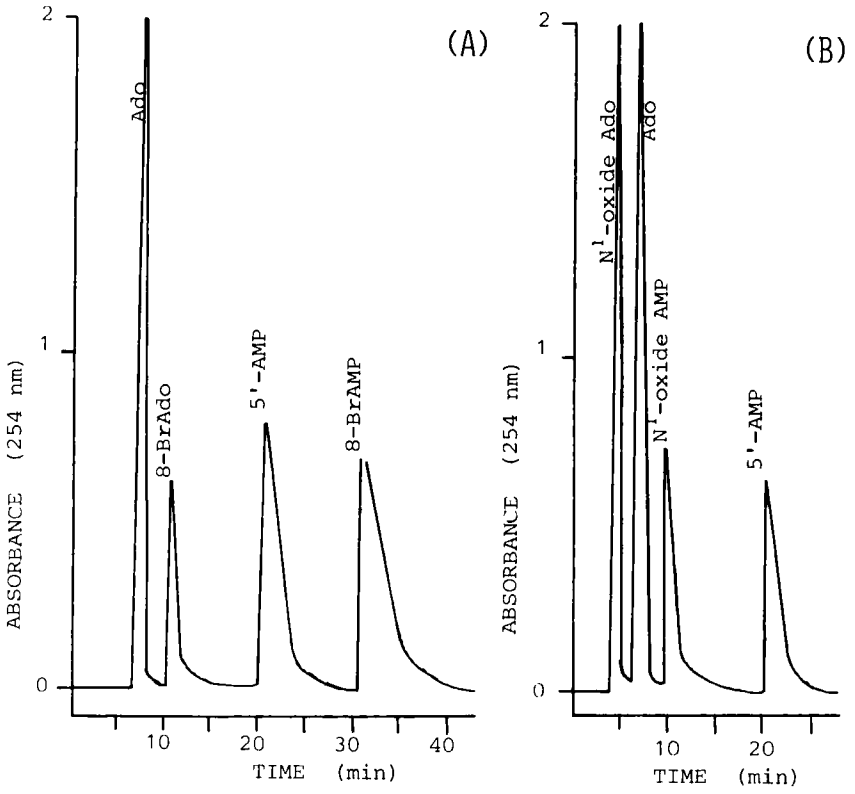

FIGURE 6.-Isocratic separation with 0.2M ammonium acetate, pH 7.5 (a) Ado, 8-BrAdo, 5'-AMP and 8-BrAMP and (b) Ado, N^1-oxide Ado, 5'-AMP and N^1-oxide AMP. For other conditions see text.

Fig. 5 shows a good separation of the hydrolytic products of 2':3'-AMP and 2':3'-CMP which was achieved using ammonium acetate pH 7.5 in 15 minutes, similar results were found with 2':3'-GMP and 2':3'-UMP although in these cases the 2'-isomers were ommitted (results not shown). The ionic strength is critical to obtain a good separation; at 0.5M ammonium acetate the order of elution was: nucleosides, 2'-nucleotides, 3'-nucleotides and 2':3'-cyclic nucleotides. At this ionic strength the complete separati-

on of purine derivatives was achieved (Fig. 5a). Under conditions of low ionic strength the highest separation efficiency was obtained with the pyrimidine derivatives. It is interesting to note that at 0.2 ionic strength the cyclic nucleotides were less retarded than both the 2'- and 3'-nucleotides, the order of elution being: nucleosides, 2':3'-cyclic nucleotides, 2'-nucleotides and 3'-nucleotides (Fig. 5b).

By using 0.2M ammonium acetate, pH 7.5 it was possible to separate a mixture of the 8-Bromo and N^1-oxide derivatives of 5'-AMP as well as their degradation products. The elution was achieved in 35 and 22 minutes respectively and the order of elution can be seen in Fig. 6. An increase in the ionic strength lowers the resolution and at the same time there is a change in the order of elution: the 8-BrAdo was more retarded than the 5'-AMP, the 8-Br AMP was the slowest fraction and the N^1-oxide AMP was eluted before the Ado fraction.

ACKNOWLEDGMENTS

This work was supported by grant n°1716/82 from the "Comisión Asesora de Investigación Científica y Técnica" of the Ministerio de Educación y Ciencia, Spain and by the Fundació M.F. de Roviralta, Barcelona, Spain.

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