Journal of Chromatography, 326 (1985) 281–291
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 593

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOTIDES AND OLIGONUCLEOTIDES

B. ALLINQUANT*, C. MUSENGER and E. SCHULLER
Laboratoire de Neuro-Immunologie, INSERMU 134, Hôpital de la Salpêtrière, 75651 Paris Cedex 13 (France)

SUMMARY

Nucleosides, nucleoside monophosphates (5′-, 3′- and 2′-isomers) and cyclic nucleotides (2′→3′- and 3′→5′-) were separated by ion-pair reversed-phase high-performance liquid chromatography on a Radial-Pak C_{18} column (5 μm particle size). In this way, isomeric-sequence oligonucleotides, such as CpU and UpC, CpA and ApC, UpG and GpU and GpA and ApG, may easily be separated. By decreasing the amount of counter-ion, larger molecules may be chromatographed with shorter retention times. This technique may be useful in the identification and quantitation of enzyme reactions of nucleic acid catabolism and in the analysis of specific cleavages of oligonucleotides used as substrates by different endonucleases.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been widely applied to the separation of nucleobases, nucleosides and nucleotides. The two most commonly employed separation techniques involve either ion-exchange[1–5] or reversed-phase chromatography[6–14].

In reverseed-phase HPLC, compounds are retained on columns according to their hydrophobicity. This mode of chromatography has been used for the separation of oligodeoxyribonucleotides from their phosphorylated derivatives[15,16] and for the separation of sequence-isomeric pyrimidine oligodeoxyribonucleotides[17]. Larger molecules have also been resolved by reversed-phase HPLC[18,19]. In this technique, the addition to the mobile phase of an ion-pairing agent with a hydrophobic moiety that could adsorb on to the stationary phase and an ionic group that could pair with the negatively charged nucleotide resulted in the greater retention of nucleotides on reversed-phase columns[20,21]. Ion-pair chromatography combines the advantages of reversed-phase and ion-exchange chromatography. It has been applied to the separation of ribonucleoside mono-, di- and triphosphates[22–26], of oligonucleotides[27] and of nucleic acids[28,29].

We report here an ion-pair reversed-phase HPLC method that can separate bases, nucleosides, nucleotides (5′-, 3′- and 2′-isomers) and cyclic nucleotides (2′→3′- and 3′→5′-). Using the same programme, isomeric-sequence oligonucleotides such as

0021-9673/85/$03.30

CpU and UpC, CpA and ApC, UpG and GpU and GpA and ApG may be easily separated. By decreasing the amount of counter-ion, larger molecules may be chromatographed with shorter retention times.

This technique may be suitable for the study of enzyme reactions of nucleic acid catabolism. It can be also useful in the analysis of specific cleavages of oligonucleotides by different endonucleases.

EXPERIMENTAL

Chemicals

All nucleobases, nucleosides, nucleotides, oligonucleotides and polynucleotides were obtained from Sigma (St. Louis, MO, U.S.A.) and PL-Biochemicals (Milwaukee, WI, U.S.A.).Tetrabutylammonium phosphate (Pic A reagent) used for ion pairing was purchased from Waters Assoc. (Milford, MA, U.S.A.). The water used for the buffer was purified in a Milli-Q system to give a resistivity of 18 MΩ/cm (Millipore, Bedford, MA, U.S.A.). Acetonitrile was of HPLC grade (Carlo Erba, Milan, Italy).

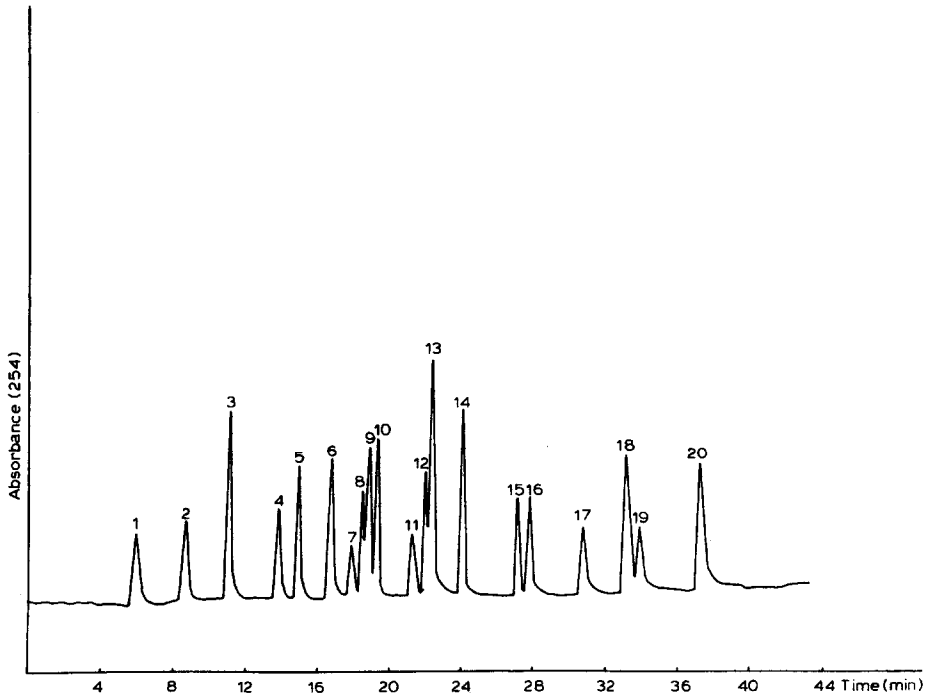

Fig. 1. Elution profile of a test mixture of 5'-, 3'-, 2'-mono-, 5'-di- and 5'-triphophates, as detected at 254 nm; 0.05 a.u.f.s. (separation conditions are described under Experimental). Retention times in minutes are as follows: (1) 5'-CMP, 5.62; (2) 5'-UMP, 8.41; (3) 5'-GMP, 10.87; (4) 2'-CMP, 13.60; (5) 3'-CMP, 14.76; (6) 5'-AMP, 16.54; (7) CDP, 17.68; (8) 3'-GMP, 18.16; (9) 3'-UMP, 18.66; (10) 2'-UMP, 19.13; (11) UDP, 21.06; (12) GDP, 21.84; (13) 2'-GMP, 22.19; (14) 3'-AMP, 23.88; (15) 2'- AMP, 26.97; (16) ADP, 27.64; (17) CTP, 30.59; (18) GTP, 33.02; (19) UTP, 33.78; (20) ATP, 37.19.

Instrumentation

HPLC was performed on a Waters Assoc. instrument, equipped with two solvent delivery systems (Model M45 and Model 6000A), a Model 720 solvent programmer, a Model 440 UV-absorbance detector operating at 254 and 280 nm, a Model U6K sample injector and a Model 730 integrator.

The analytical reversed-phase column used was a Radial-Pak C_{18} (100 × 8 mm I.D., 5 μm particle size) in a Waters Assoc. radial compression module (RCM-100).

Chromatographic conditions

A gradient elution system was used, consisting of two mobile phases. Mobile phase A was 0.02 mol/l monopotassium phosphate buffer (pH 6.5), containing 5 m*M* Pic A reagent. Mobile phase B was a 1:1 (v/v) mixture of mobile phase A and acetonitrile. Before use, the phosphate buffer and acetonitrile were filtered through a Millipore membrane filter [Type HA for phosphate buffer (pore size 0.45 μm) and type FH for acetonitrile (pore size 0.5 μm)].

Elutions were first performed with mobile phase A (isocratic conditions). After

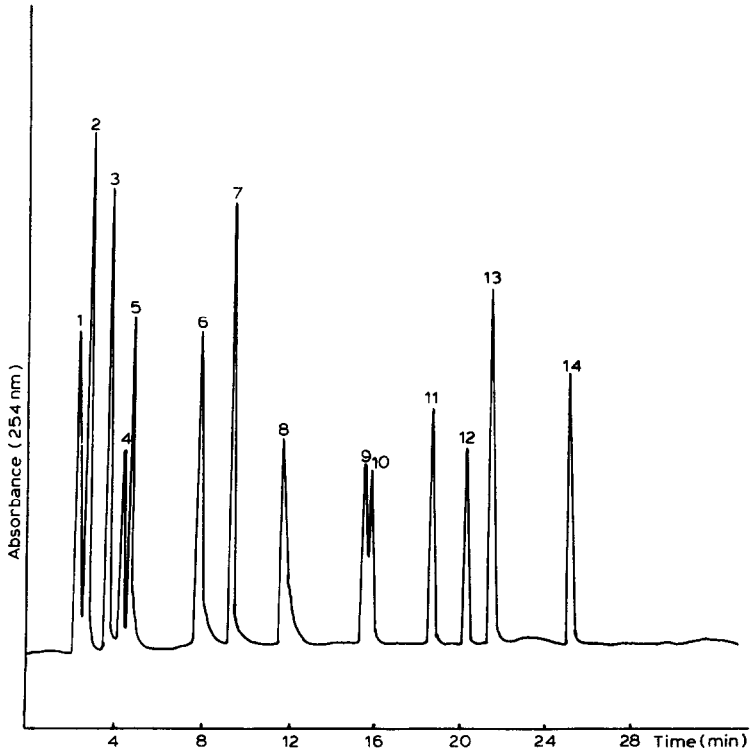

Fig. 2. Elution profile of a test mixture of bases, nucleosides and cyclic nucleotides, as detected at 254 nm; 0.05 a.u.f.s. (separation conditions are described under Experimental,). Retention times in minutes are as follows: (1) cytosine, 1.83; (2) uracil, 2.33; (3) hypoxanthine, 3.30; (4) xanthine, 3.95; (5) uridine, 4.36; (6) 2′→3′-cCMP, 7.54; (7) 2′→3′-cUMP, 9.12; (8) adenosine, 11.45; (9) 2′→3′-cGMP, 15.16 (10) 3′→5′-cCMP, 15.42; (11) 3′→5′-cUMP, 18.28; (12) 3′→5′-cGMP, 19.90; (13) 2′→3′-cAMP, 21.08; (14) 3′→5′-cAMP, 24.70.

3 min, a linear gradient (solvent programme No. 6) was started from the initial conditions, leading in 45 min to the final conditions, consisting of eluent A–eluent B (1:1). At this time, automatic resetting to the initial conditions occurred, followed by a waiting time of 15 min before the next injection. The solvent flow-rate was kept constant at 2.0 ml/min.

RESULTS AND DISCUSSION

Chromatography

Fig. 1 illustrates a typical analysis of a standard mixture containing 5'-, 3'-, 2'-mono-, 5'-di- and 5'-triphosphates. Cytidine nucleotides, particularly 5'-CMP, which is slightly negatively charged, eluted first and adenine nucleotides last. With the exception of adenine nucleotides, the order of retention times of the nucleotides follows the predicted order for an anion-exchange column[1–3]. Strong retention of adenine nucleotides is probably the result of hydrophobic interaction with the stationary reversed phase. Fig. 2 shows the separation of bases, nucleosides, 2'→3'-cyclic and 3'→5'-Cyclic nucleotides. 2'→3'-Cyclic nucleotides were eluted before their 3'→5'-cyclic isomers.

Using the same programme, di- and trinucleotides may be easily resolved in 30 min. Dinucleotide 3'→5'-monophosphates such as CpU and UpC, CpA and ApC, UpG and GpU and GpA and ApG may be easily separated (Fig. 3). Their order of

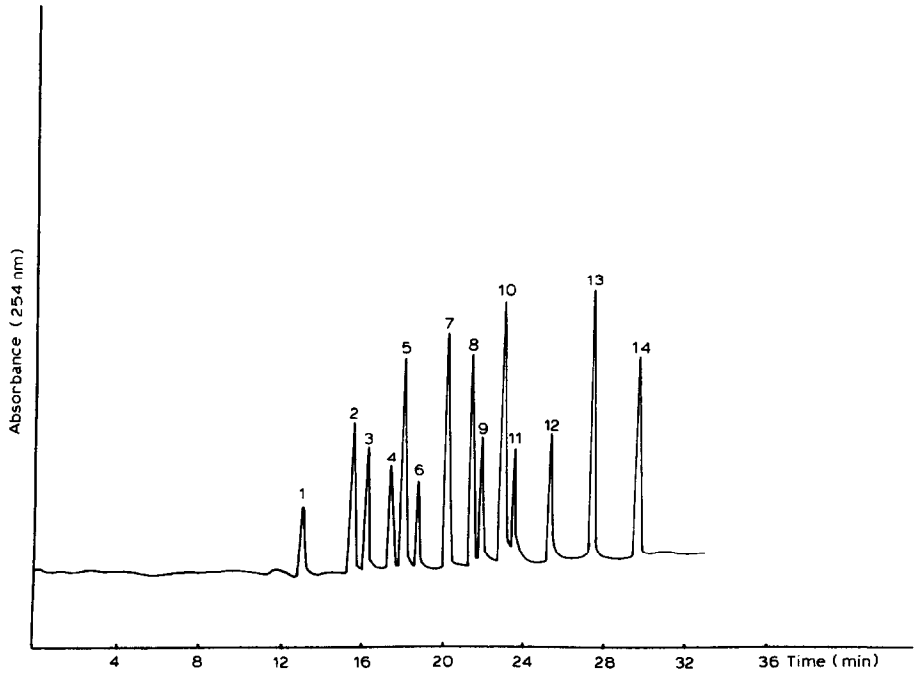

Fig. 3. Chromatogram of oligonucleotides, as detected at 254 nm; 0.05 a.u.f.s. Retention times in minutes are as follows: (1) CpC, 13.08; (2) CpU, 15.6; (3) UpC, 16.25; (4) GpC, 17.72; (5) UpG, 18,14, (6) CpA, 18.76; (7) GpU, 20.30; (8) ApC, 21.56; (9) GpA, 22.04, (10) ApG, 23.12; (11) ApU, 23.66; (12) ApA, 25.48; (13) GpApC, 27.65; (14) ApUpG, 29.90. Conditions as described under Experimental.

retention times follows the predicted order for the 5'-nucleotides. The nature of the second nucleotide determines secondly the order of elution.

The gradient conditions used give the best separation with reasonable retention times and with a very slight baseline drift. By increasing the gradient slope with a shorter time programme, the chromatographic separation may be altered and overlapping occurs.

The mono-, di- and trinucleotides can be quantitated with a detection limit of 20 pmol, the response of the UV detector at 254 nm being linear from 20 to 1500 pmoles by measurement of the peak heights or the peak areas. For the mononucleoside triphosphates the detection limit is 50 pmoles, the response of the detector being linear from 50 to 1500 pmole by measurement of the peak areas. The relative standard deviation of the peak areas for ten successive injections of 1 nmole is 0.6%.

Fig. 4 shows that the retention times of the different compounds depend strongly on the amount of tetrabutylammonium in the concentration range studied (0.2–5 m*M*).

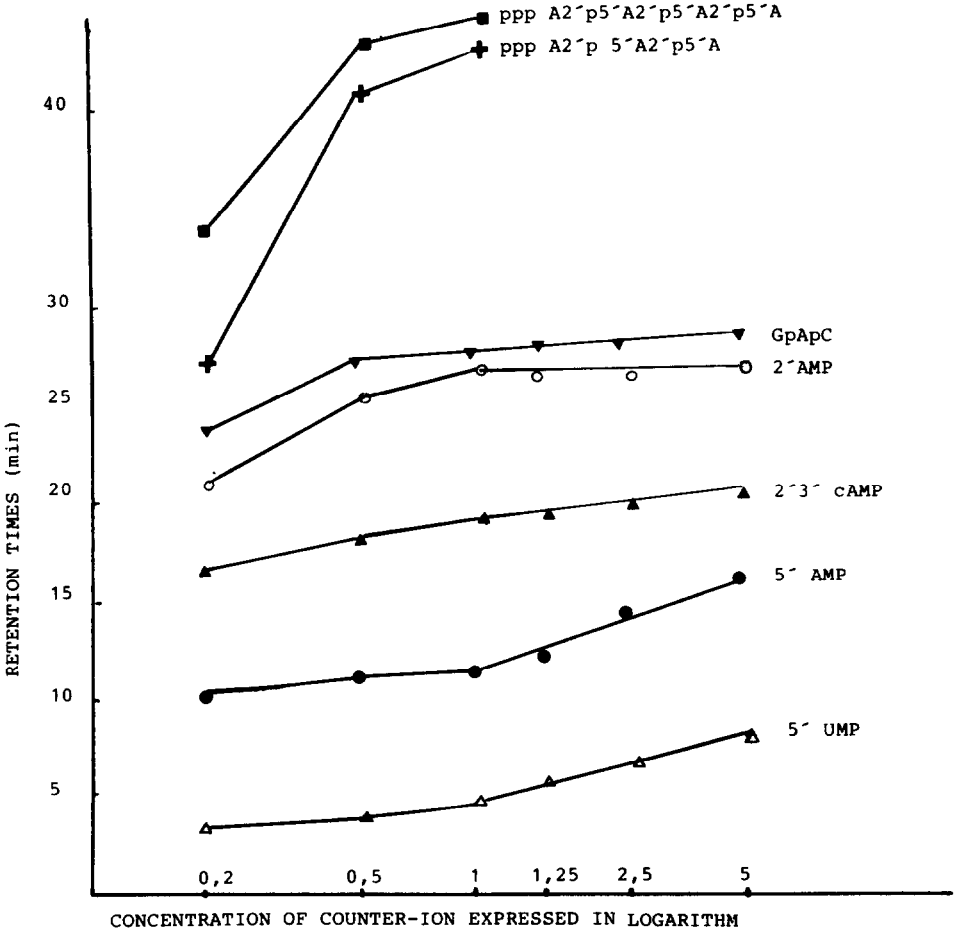

Fig. 4. Relationship between the retention times and the logarithm of the counter-ion concentrations (m*M*) of the mobile phases.

All chromatograms were obtained under the same gradient conditions as described under Experimental. From 0.2 to 1 m*M*, inhibitors of protein synthesis found in interferon-treated cells (trimer 5'-triphosphate pppA2'p5'A2'p5'A and tetramer pppA2'p5'A2'p5'A2'p5'A) may be chromatographed in our system. Similarly, Wagner *et al.*[30] observed that the concentration of the counter-ion determines the retention times of amines in ion-pair reversed-phase HPLC. Without any counter-ion and with the same pH of the mobile phase, polynucleotides may be separated according to their hydrophobicity, similarly to the method described by Garcia and Liautard[19] (Fig. 5).

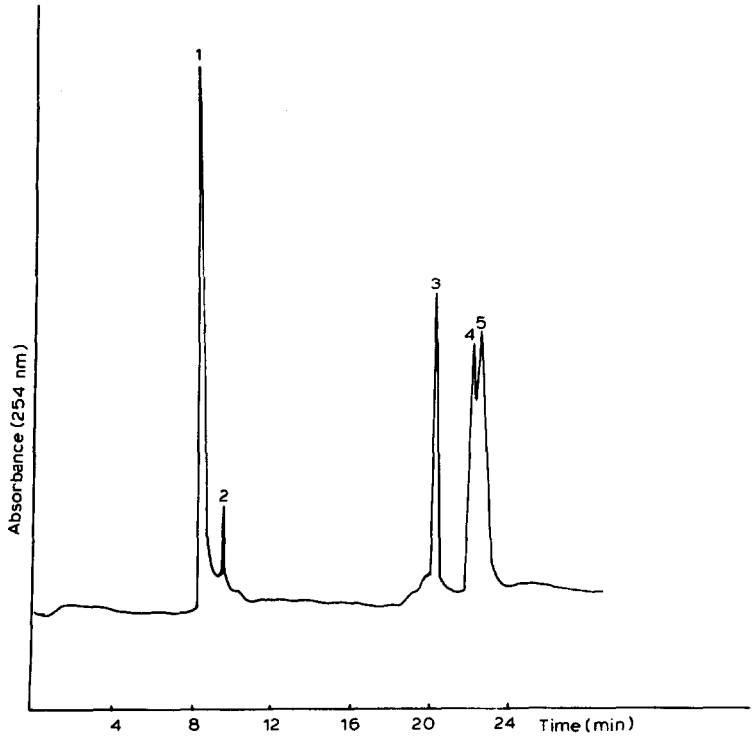

Fig. 5. Chromatogram of oligo- and polynucleotides. Chromatographic conditions are as described, except no counter-ion was added. Peaks: (1) pppA2'p5'A2'p5'A; (2) pppA2'p5'A2'p5'A2'p5'A; (3) Poly(U); (4) Poly(A-U); (5) Poly(A).

After use, the column is washed with water–acetonitrile (1:1, v/v) for 45 min at a flow-rate of 2.0 ml/min. Methanol is never used for washing. The Radial-Pak cartridges are known to have shorter lifetimes than steel columns. Under these chromatographic conditions, the lifetime of the column is about 130 runs.

APPLICATIONS

This chromatographic separation may be applied to the study of nucleic acid catabolism.

Study of enzymes of nucleic acid catabolism

Different types of enzymes may be involved, as follows.

2′→3′-cyclic phosphodiesterase. This enzyme (E.C. 3.1.4.37), which catalyses the hydrolysis of several 2′→3′-cyclic nucleoside monophosphates to their respective 2′-nucleoside monophosphates, has a very high activity in the central nervous system, particularly in myelin[31–34].

5′- and 2′-nucleotidases. Although not specific for myelin, 5′-nucleotidase is a myelin-associated enzyme[35], increasing during development[36]. Recently, a 2′-nucleotidase has been found in mammalian brain[37].

(2′,5′)-Oligoisoadenylate synthetase. This enzyme synthesizes a series of (2′,5′)-linked oligoadenylates from ATP in the presence of double-stranded RNA. The level of this enzyme is elevated 10- to 1000-fold on interferon treatment of various cells in tissue cultures[38]. The activity of this enzyme may be monitored by using our programme without any counter ion (Fig. 5).

By using our HPLC technique, the increase in enzymatic products may be followed and quantified in parallel with a decrease in substrate (Figs. 6 and 7).

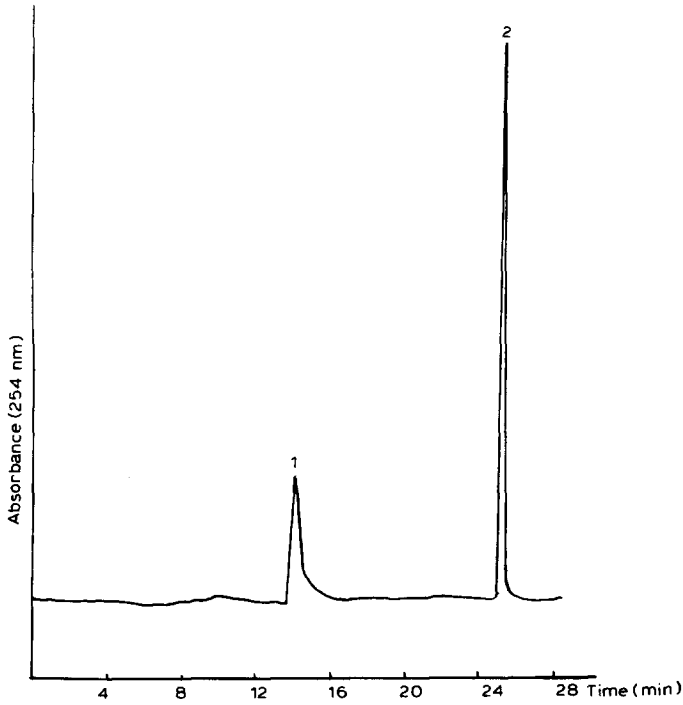

Fig. 6. Product and substrate of 2′→3′-cyclic phosphodiesterase from rat brain. Enzyme reaction was carried out with 20 µl of 30 µ*M* 2′→3′-cAMP in a total volume of 130 µl. After 1 h at 37°C, reaction was stopped by heating for 2 min at 100°C and then chilling. After centrifugation, 50 µl, diluted 1:100, was injected. Chromatographic conditions as described under Experimental. Peaks: (1) 2′→3′-cAMP; (2) 2′-AMP.

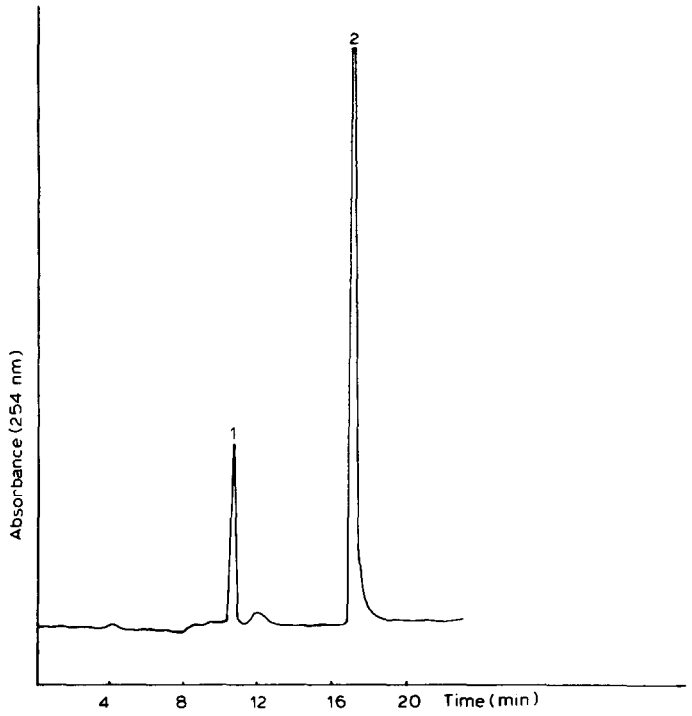

Fig. 7. Product and substrate of 5'-nucleotidase enzyme reaction as described[36]. After 1 h at 37°C, the reaction was stopped by heating. Chromatographic conditions as described under Experimental. Peaks: (1) adenosine; (2) 5'-AMP.

Analysis of specific cleavages of oligonucleotides used as substrates by different endonucleases

Most ribonucleases (which act as endonucleases) in human cerebrospinal fluid split ribonucleic acids after pyrimidine sequences and particularly after cytidylic residues, producing oligonucleotides ending in a 2'→3'-cyclic phosphate, as demonstrated in Figs. 8 and 9. In this way, such products may be purified. Their length may be determined as already described by Crowther *et al.*[27]. When each of the substrates CpA, CpC, CpG and CpU was incubated with the same enzyme for a longer time, only CpA was cleaved, suggesting that such endonucleases preferentially cleave between cytidylic and adenylic residues (Fig. 10).

CONCLUSION

With this reversed-phase system, oligonucleotides of various lengths may be separated by varying the counter-ion concentrations. Without any counter ion, polynucleotides may be eluted. With a 5 m*M* concentration of counter ion, maximal resolutions of isomeric nucleotides occurred with reasonable retention times.

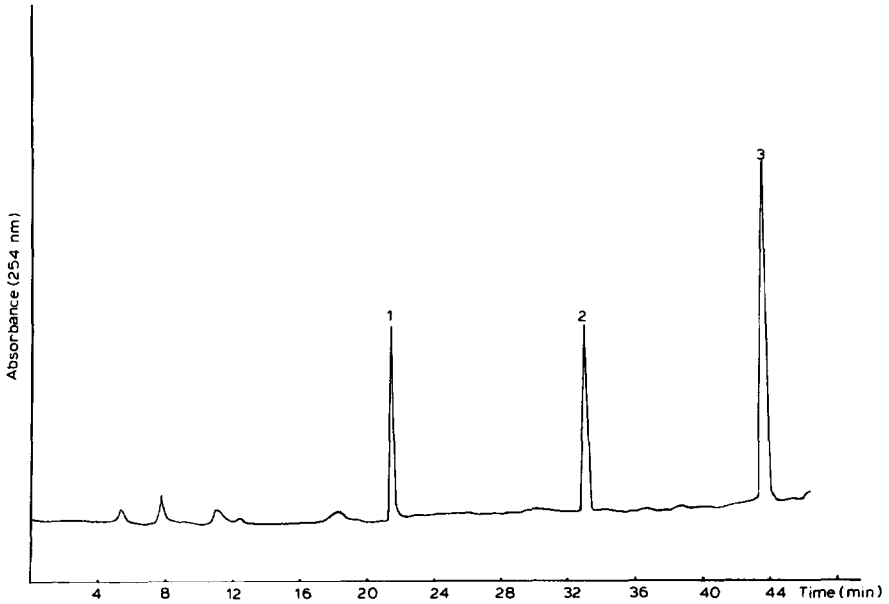

Fig. 8. Oligonucleotides produced by cerebrospinal fluid ribonuclease activity with a poly(C) substrate (*ca.* 650 nucleotides). Enzyme reaction was carried out with 15 μl of cerebrospinal fluid, 15 μl of 9 μmol/ml poly(C) under the conditions described in ref. 39. After 30 min at 37°C, reaction was stopped by heating. Chromatographic conditions as described under Experimental. Each peak represents oligocytidylates (< 10 nucleotides) ended by a 2′→3′-cyclic phosphate. Elution of larger oligocytidylates is performed by decreasing counter-ion concentrations. Without any counter-ion (conditions as in Fig. 5) poly(C) eluted at 17.58 min.

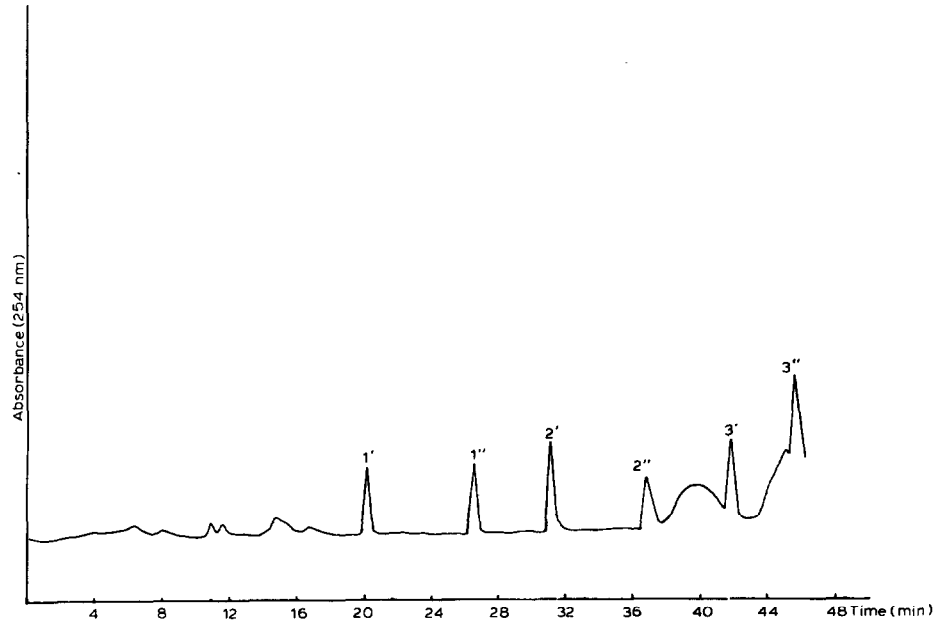

Fig. 9. Oligonucleotides produced in Fig. 8 after action of 0.1 *N* $HClO_4$ for 5 min. The supernatant injected in Fig. 8 was treated with 0.1 *N* $HClO_4$ to split terminal 2′→3′-cyclic phosphate in 2′-phosphate- and 3′-phosphate-isomers. In this way, each component in Fig. 8 yields two peaks.

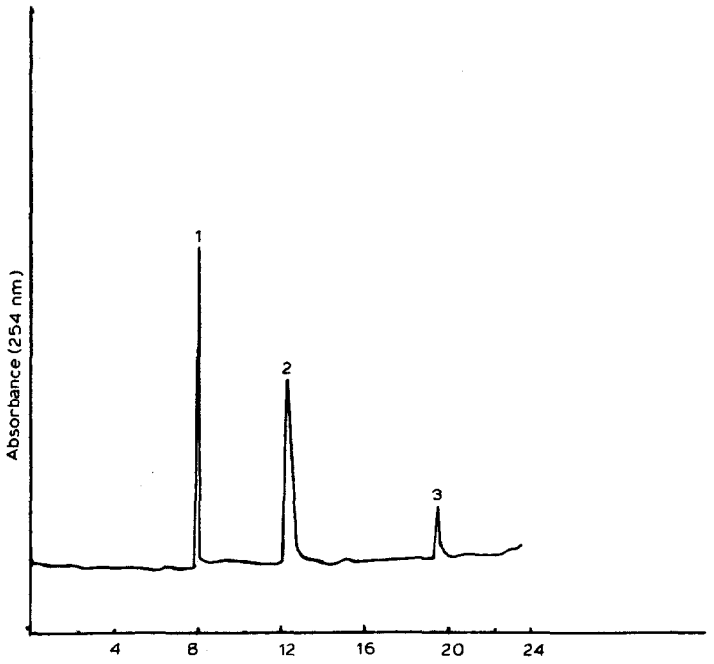

Fig. 10. Product and substrate of cerebrospinal fluid ribonuclease activity on CpA after 16 h at 37°C. Chromatographic conditions as described under Experimental. Peaks: (1) 2′→3′-cyclic CMP; (2) adenosine; (3) residual substrate CpA.

ACKNOWLEDGEMENTS

This work was supported by grants or supplies from the Institut National de la Santé et de la Recherche Médicale (INSERM), Faculté de Médecine Pierre et Marie Curie (Paris VI) and Association pour la Recherche sur la Sclérose en Plaques (ARSEP), and we thank all of them.

REFERENCES

1 A. Floridi, C. A. Palmerini and C. Fini, *J. Chromatogr.*, 138 (1977) 203.
2 B. Bakay, E. Nissinen and L. Sweetman, *Anal. Biochem.*, 86 (1978) 65.
3 A. A. de Abreu, J. M. van Baal, J. A. J. M. Bakkeren, C. H. M. M. de Bruyn and E. D. A. M. Schretlen, *J. Chromatogr.*, 227 (1982) 45.
4 K. P. Naikwadi, S. Rokushika and H. Hatano, *J. Chromatogr.*, 280 (1983) 261.
5 P. D. Reiss, P. F. Zuurendonk and R. L. Veech, *Anal. Biochem.*, 140 (1984) 162.
6 P. R. Brown, S. Bobick and F. L. Hanley, *J. Chromatogr.*, 99 (1976) 587.
7 A. M. Krstulovic, P. R. Brown and D. M. Rosie, *Anal. Chem.*, 49 (1977) 2237.
8 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 143 (1977) 383.
9 C. W. Gehrke, K. C. Kuo, G. E. Davis, R. D. Suits, T. P. Waalkes and E. Borek, *J. Chromatogr.*, 150 (1978) 455.
10 P. R. Brown and A. M. Krstulovic, *Anal. Biochem.*, 99 (1979) 1.
11 R. J. Simmonds and R. A. Harkness, *J. Chromatogr.*, 226 (1981) 369.
12 R. A. Hartwick, S. P. Assenza and P. R. Brown, *J. Chromatogr.*, 186 (1979) 647.
13 G. A. Taylor, P. J. Dady and K. R. Harrap, *J. Chromatogr.*, 183 (1980) 421.

14 R. A. de Abreu, J. M. van Baal, C. H. M. M. de Bruyn, J. A. J. M. Bakkeren and E. D. A. M. Schretlen, *J. Chromatogr.*, 229 (1982) 67.
15 M. F. Aukaty, S. N. Bubenschikova, V. K. Kagrammanova and L. A. Baratova, *J. Chromatogr.*, 137 (1977) 351.
16 A. M. Delort, A. Derbyshire, A. M. Duploa, A. Guy, D. Molko and R. Teoule, *J. Chromatogr.*, 283 (1984) 462.
17 H. Schott, H. D. Meyer and E. Bayer, *J. Chromatogr.*, 280 (1983) 297.
18 M. H. Simonian and M. W. Capp, *J. Chromatogr.*, 266 (1983) 351.
19 S. Garcia and J. P. Liautard, *J. Chromatogr. Sci.*, 21 (1983) 398.
20 M. Zakaria and P. R. Brown, *J. Chromatogr.*, 226 (1981) 267.
21 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, p. 454.
22 N. E. Hoffman and J. C. Liao, *Anal. Chem.*, 49 (1977) 2231.
23 E. Juengling and H. Kammermeier, *Anal. Biochem.*, 102 (1980) 358.
24 F. K. Chow and E. Grushka, *J. Chromatogr.*, 185 (1980) 361.
25 J. H. Knox and J. Jurand, *J. Chromatogr.*, 203 (1981) 85.
26 J. L.-S. Au, M. G. Wientjes, C. M. Luccioni and Y. M. Rustum, *J. Chromatogr.*, 228 (1982) 245.
27 J. B. Crowther, R. Jones and R. A. Hartwick, *J. Chromatogr.*, 217 (1981) 479.
28 P. N. Nguyen, J. L. Bradley and P. M. McGuire, *J. Chromatogr.*, 236 (1982) 508.
29 R. Bischoff and L. W. McLaughlin, *J. Chromatogr.*, 296 (1984) 329.
30 J. Wagner, C. Danzin and P. Mamont, *J. Chromatogr.*, 227 (1982) 349.
31 G. I. Drummond, N. T. Iyer and J. Reith, *J. Biol. Chem.*, 227 (1962) 3535.
32 T. Kurihara and Y. Tsukada, *J. Neurochem.*, 15 (1968) 827.
33 T. Kurihara, J. L. Nussbaum and M. Mandel, *J. Neurochem.*, 17 (1970) 993.
34 T. Kurihara, S. Kawakami, K. Uehi and Y. Takahashi, *J. Neurochem.*, 22 (1974) 1143.
35 W. Cammer, S. R. Sirota, T. R. Zimmerman and W. T. Norton, *J. Neurochem.*, 35 (1980) 367.
36 J. M. Bourre, C. Chanez, O. Dumont and M. A. Flexor, *J. Neurochem.*, 38 (1982) 643.
37 S. Nakamura, S. Yamao, J. Iho and M. Kameyana, *Biochim. Biophys. Acta*, 568 (1979) 30.
38 I. Krishnan and C. Baglione, *Nature (London)*, 285 (1980b) 485.
39 B. Allinquant, C. Musenger and E. Schuller, *Acta Neurol. Scand.*, 70 (1984) 12.