Journal of Chromatography, 516 (1990) 456–460
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 528

Note

Reversed-phase high-performance liquid chromatographic study of the formation of complexes of nucleotides and oligonucleotides with Lu(III)

ALEXANDER SIGALOV
U.S.S.R. Research Centre for Preventive Medicine, Petroverigsky Street 10, 101953 Moscow (U.S.S.R.)
(First received February 28th, 1989; revised manuscript received May 1st, 1990)

High-performance liquid chromatography (HPLC) is widely used in the analysis of nucleoside–nucleotide mixtures and in the isolation of synthetic oligonucleotides[1–3]. As shown previously[4,5], the presence of Mg(II) ions in the mobile phase greatly affects the retention times of the nucleotides, and in some instances allows the separation time to be considerably reduced and the process to be simplified.

This paper reports investigations on the reversed-phase HPLC of deoxynucleosides, deoxynucleotides and oligodeoxynucleotides in standard systems of solvents in the presence of lutetium chloride ($LuCl_3$). This lanthanide has the smallest radius among the rare earth elements and so possesses the maximum ability to form complexes with different n- and π-donors.

EXPERIMENTAL

Materials and apparatus

Deoxynucleosides and deoxynucleotides were obtained from SKTB BAV (Novosibirsk, U.S.S.R.). [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham (Amersham, U. K.). UV spectra were recorded on a Specord M40 spectrophotometer.

HPLC procedures

Reversed-phase HPLC was carried out at 25°C on a Gilson liquid chromatograph equipped with a UV detector (254 nm). For the separation of the deoxynucleosides and deoxynucleotides, a column (250 × 4 mm I.D.) with LiChrosorb RP-18 (LKB, Bromma, Sweden), particle size 10 μm, as the stationary phase was used; the mobile phases were (A) 0.05 M KH_2PO_4 (or 0.05 M $HCOONH_4$)–methanol (89:11, v/v), pH 4.2, and (B) 0.05 M CH_3COONH_4–methanol (89:11, v/v), pH 6.2, with or without 80 μM $LuCl_3$. The flow-rate was 2 ml/min. For the analysis of a crude 20-mer oligodeoxynucleotide and a slab gel-purified 20-mer oligodeoxynucleotide, a column (250 × 4 mm I.D.) with LiChrosorb RP-8 (LKB), particle size 10 μm was used; the mobile phases were 0.05 M CH_3COONH_4 (pH 6.2), with or without 80 μM $LuCl_3$,

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with a stepped acetonitrile gradient from 0 to 60% (0–8 min, 0–8%; 8–10 min, 8%; 10–15 min, 8–15%; 15–20 min, 15%; 20–25 min, 15–30%; 25–30 min, 30%; 30–35 min, 30–60%; and 35–40 min, 60% acetonitrile). The flow-rate was 1 ml/min. The capacity factor, k', is defined by $k' = (V_R - V_0)/V_0$, where V_R is the retention volume of the substance of interest and V_0 that of an unretarded substance (usually the solvent).

Oligonucleotide synthesis

The oligodeoxynucleotides CCAGCCCTAGGGATTGAG (I), CTTATGTG-CACGATGCACCT (II) and AACGAGGGTACCAACGGCTA (III) were synthesized by a solid-phase phosphoramidate method according to a previously described procedure[6]. Aminopropyl-CPG-550Å (Fluka, Buchs, Switzerland) was used as a polymeric carrier. As soon as the synthesis and last detritylation had been accomplished, the P-methoxy protecting groups were removed by the treatment with thiophenol–dioxane–triethylamine (1:3:1, v/v/v) at 20°C for 45 min. Subsequently, the carrier was washed with ethanol and diethyl ether and dried. The splitting of the oligodeoxynucleotides from the carrier was performed with 25% ammonia solution at 20°C for 3 h; thereafter the carrier was filtered off and the solution sealed in an ampoule and maintained for 20 h at 60°C. The crude mixture was separated by preparative 20% polyacrylamide slab gel electrophoresis under denaturated conditions. The zone containing the desired product was cut off and the transfer of oligodeoxynucleotide was performed on DEAE-cellulose by electroelution. The oligodeoxynucleotide was then eluted with a minimum volume 1.5 *M* aqueous lithium perchlorate solution for 10 h at 37°C, precipitated with 2% lithium perchlorate in acetone, washed with acetone and ethanol–water (80:20) and dried.

Analysis of a crude 20-mer oligodeoxynucleotide

The crude 20-mer oligodeoxynucleotide (II) was analysed by reversed-phase HPLC under the above conditions. The fractions were collected, evaporated, dissolved in water and precipitated with 2% lithium perchlorate in acetone by the described technique. All fractions were radiolabelled using T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol) in a standard reaction mixture[7]. An aliquot of each sample was loaded onto 20% polyacrylamide slab gel. After electrophoresis, the gel was fixed and then dried. Autoradiography was performed at −70°C using an intensifying screen for 1 h.

RESULTS AND DISCUSSION

The results (Table I) showed that in buffer A at pH 4.2 in the presence of 80 μM $LuCl_3$ the capacity factors (k') for both the deoxynucleosides and deoxynucleotides decrease. This phenomenon may be explained by the formation of Lu(III) complexes with the heterocyclic bases, which leads to some increase in hydrophilicity. The latter was observed for protein molecules during the isolation of α-interferon by HPLC when $LaCl_3$ was added to the mobile phase[8]. Similar retention behaviour was also observed for the deoxynucleosides on applying buffer B (pH 6.2) (Table I).

The effect of Lu(III) is much more noticeable with deoxynucleotides when buffer B is used. The capacity factors for these compounds increase considerably, prob-

TABLE I

CAPACITY FACTORS OF DEOXYNUCLEOSIDES AND DEOXYNUCLEOTIDES IN REVERSED-PHASE HPLC WITH (k'_{Lu}) AND WITHOUT (k') 80 μM $LuCl_3$

Compound	*Buffer A (pH 4.2)*[a]			*Buffer B (pH 6.2)*[a]		
	k'	k'_{Lu}	k'_{Lu}/k'	k'	k'_{Lu}	k'_{Lu}/k'
dA	11.53	7.73	0.67	13.84	11.65	0.84
dG	3.42	2.41	0.70	3.89	3.42	0.88
dC	1.03	0.77	0.75	1.26	1.14	0.90
dT	3.95	2.87	0.73	4.28	3.77	0.88
dCMP	0.40	0.39	0.98	0.19	1.25	6.58
dGMP	0.95	0.86	0.91	0.63	4.47	7.10
dAMP	2.03	1.45	0.71	1.42	11.33	7.98
dADP	0.83	0.63	0.76	0.74	6.68	9.02
dATP	0.53	0.39	0.74	0.40	4.62	11.55

[a] See *HPLC procedures*.

ably owing to the formation of Lu(III)–deprotonated phosphate group complexes. The existence of such complexes is also confirmed by the increase in the capacity factors for deoxynucleotide as the number of phosphate groups increases. Thus, the k'_{Lu}/k' ratio is 11.55, 9.02 and 7.98 for dATP, dADP and dAMP, respectively, and the retention order is dAMP $<$ dADP $<$ dATP (Table I). The retention order for these deoxynucleotides with a mobile phase not containing Lu(III) is dATP $<$ dADP $<$ dAMP. There is evidence[4,5] that Mg(II) ions form strong complexes with deprotonated phosphate groups when Mg(II) salts are added to the mobile phase at a concentration $8.1 \cdot 10^{-4}$ M, which results in a decrease in the capacity factors of nucleotides in anion-exchange and reversed-phase HPLC. The reasons for the decrease are not clear. In this work, probably the formation of a strong complex of Lu(III) with the deprotonated phosphate groups and the bases results in phosphate charge neutralization and a decrease in hydrophilicity. Correspondingly, an increase in the retention indices of the deoxynucleotides is observed in reversed-phase HPLC with the addition of $8.0 \cdot 10^{-5}$ M Lu(III) to the mobile phase.

The chromatographic behaviour of the oligodeoxynucleotides in the presence of Lu(III) was studied by using a slab gel-purified 20-mer oligodeoxynucleotide (I). The capacity factor appears to increase in the presence of 80 μM $LuCl_3$ and the ratio k'_{Lu}/k' is 1.47. The analysis of the crude mixture obtained by the synthesis of the oligodeoxynucleotide II under the same conditions of separation indicates that the presence of 80 μM $LuCl_3$ induces a change in the general separation pattern and an improvement in the purification of the desired product (Fig. 1, top). The k'_{Lu}/k' ratio is 1.81–2.10 (depending on the gradient profile of acetonitrile) for the zone containing the principle amount of the desired oligodeoxynucleotide (up to 90%, determined by slab-gel electrophoresis of the radiolabelled collected fractions and subsequent autoradiography). In addition, the application of the above buffer containing 80 μM $LuCl_3$ permits the flow-rate to be increased from 1 to 2 ml/min without worsening the separation pattern (data not shown).

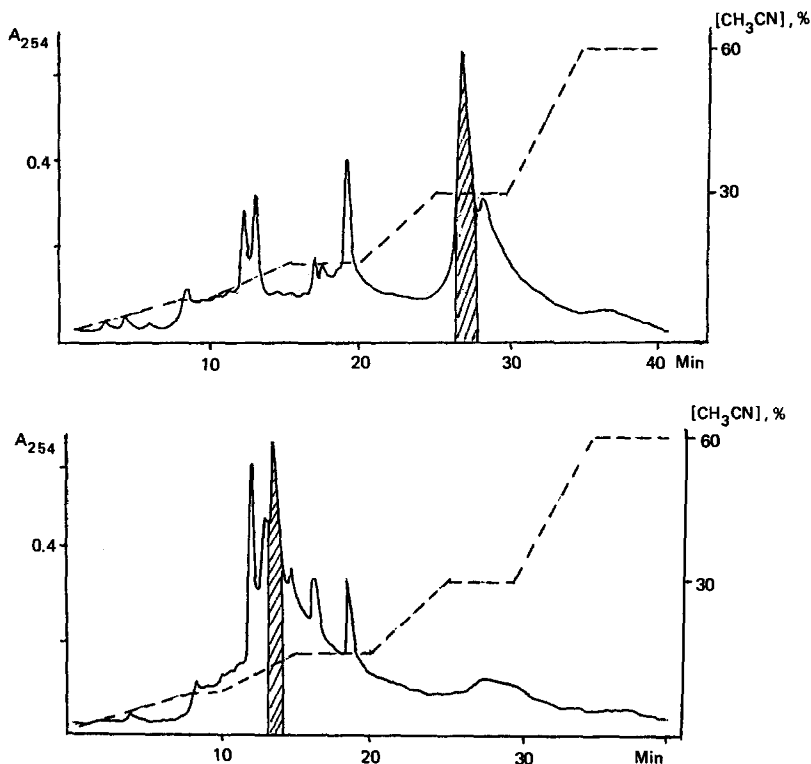

Fig. 1. Reversed-phase HPLC of crude 20-base oligodeoxynucleotide CTTATGTGCACGATGCACCT. Column, LiChrosorb RP-8 (250 × 4 mm I.D.). Mobile phase: gradient of acetonitrile concentration (broken line) in 0.05 *M* ammonium acetate (pH 6.2), (top) with and (bottom) without 80 μM $LuCl_3$. Flow-rate, 1 ml/min. The shaded area is that containing the major amount (up to 90%) of the desired product.

Hence the addition of 80 μM $LuCl_3$ to the mobile phases usually used in reversed-phase HPLC leads to a pronounced increase in the capacity factors for compounds with deprotonated phosphate groups (deoxynucleotides and oligodeoxynucleotides).

The UV spectra of the oligodeoxynucleotide III at various concentrations were measured to investigate the mechanism of the interaction of Lu(III) with oligodeoxynucleotides in aqueous solutions. Fig. 2 illustrates that the change in the molar absorption coefficient is 12–17% at λ_{max} = 260.4 nm, whereas the $LuCl_3$-to-phosphate molar ratio ranges from 0.7 to 7.0. This effect may be associated with a disturbance of the "stacking" interaction between the heterocyclic oligodeoxynucleotide bases caused by the formation of the Lu(III) complexes with both the phosphate groups and the heterocyclic bases. A change in the oligodeoxynucleotide structure seems to be another reason for the increase in the capacity factors of the oligodeoxynucleotides in the presence of 80 μM $LuCl_3$.

An interesting analogy is observed by comparison of the above results with

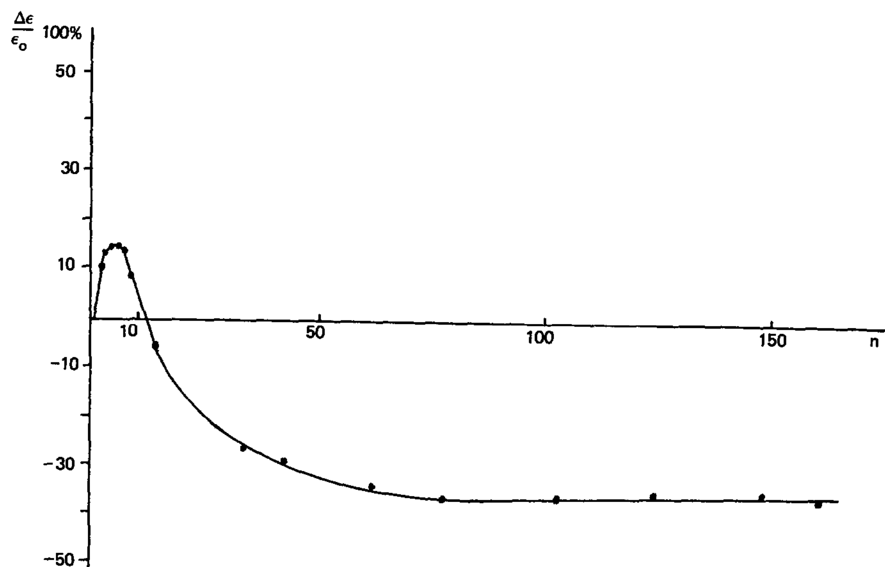

Fig. 2. Relationship between $\Delta\varepsilon/\varepsilon_0$ [ε_0 = molar absorption coefficient at λ_{max} = 260.4 nm of the initial oligodeoxynucleotide (III)] and the molar ratio of $LuCl_3$ to phosphate groups of oligodeoxynucleotide.

those from a study of the fluorescence of Tb(III) complexes with nucleic acids[9]. The Tb(III) fluorescence appeared to increase considerably from 0.4–0.6 with increase in the Tb(III)-to-phosphate group molar ratio up to n = 2.8 in the presence of a single-stranded RNA ($rC_{75}G_{75}$). Unfortunately, the Tb(III) fluorescence at $n > 2.8$ was not investigated, and therefore we cannot compare the titration curve in our study with the corresponding[9] characteristic over the entire range of n values.

In conclusion, the results presented here show that the formation of strong Lu(III) complexes with deoxynucleotides and oligodeoxynucleotides in aqueous solutions results in alterations of both the optical characteristics and the chromatographic behaviour of these compounds.

ACKNOWLEDGEMENTS

The author is grateful to N. Petcheritsa and V. Pedchenko for assistance.

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