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Note

An improved separation of diastereomers of nucleoside phosphorothioates using reversed-phase high-performance liquid chromatography

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In the past decade, high-performance liquid chromatography (HPLC) on both ion-exchange and reversed-phase columns has been used to separate the diastereomers of nucleoside phosphorothioates. Although ion exchangers were frequently used in earlier work, *e.g.*, ref. 1, it has recently become apparent that in many cases reversed-phase HPLC is both more effective and more straightforward, *e.g.*, refs. 2–4).

An outstanding unresolved problem is the separation of the diastereomers of nucleoside thiotriphosphates, which has not been achieved either by ion-exchange or reversed-phase HPLC. In addition, although the separation of the diastereomers of ADP (α-*S*) and ATP (α-*S*) on reversed-phase columns is easily performed on an analytical scale, the complete resolution of the corresponding guanosine nucleotides is much more critically dependent on such factors as the age of the column and the performance of the system in general. This is due largely to the fact that in the buffers normally used for these separations, such as ammonium phosphate, the retention times for the guanosine nucleotides are very short. We report here that these retention times can be influenced dramatically by taking advantage of ion-pair effects, and that a combination of this and complexation with divalent metal ions leads to significant improvements in the solution of both these problems.

MATERIALS AND METHODS

A solvent delivery system and UV detector (Latek, Heidelberg, F.R.G.) were used for HPLC separations. Data were analyzed using a C-R1A data processor (Shimadzu, Japan). Two different reversed-phase column materials were used. For routine separations, C_{18} ODS-Hypersil (5 μm) from Shandon Southern Products (Runcorn, U.K.) was used. For the resolution of the diastereomers of ATP (β-*S*), C_{18}-Ro Sil (4–5 μm) from RSL (Alltech Europe, Gent, Belgium) was found to be preferable. The columns (250 × 4.6 mm) were eluted isocratically with the buffers described in the legends to the figures and tables. For C_{18} (Shandon), a flow-rate of 2 ml/min was used; for C_{18}-Ro, 1 ml/min was employed due to the higher pressure needed for this material.

Nucleoside thiophosphates were prepared as previously described[5–7]. All other regents were commercially available.

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RESULTS AND DISCUSSION

In the course of seeking a method for the analytical separation of the isomers of ATP(β-*S*), it seemed to us that the behaviour of nucleotides on reversed-phase columns should be strongly influenced by formation of nucleotide–divalent metal ion complexes. When 20 m*M* Mg^{2+} was included in the elution buffer, it was found that diphosphates and triphosphates showed considerably longer retention times, although monophosphates were not significantly affected. This is presumably due to reduction of the net charge on the complexes with Mg^{2+}. In addition, a partial separation of the diastereomers of ATP(β-*S*) was also obtained. In order to avoid possible competition for the Mg^{2+} by phosphate in the buffer which was normally used, this was replaced by Tris–HCl buffers of various concentrations and pH. This led to a further increase in the retention time of nucleoside diphosphates and triphosphates, and this is of particular value for the guanosine derivatives, since their short retention times lead to poor separations (Table I). The effect of Tris is presumably due to formation of ion-pairs between the nucleotide and the Tris cation, thus increasing the hydrophobic nature of the complex. This idea is supported by the observation that the order of elution of polyphosphates of a particular nucleoside is now reversed. Using phosphate buffer, the retention time increases in the sequence ATP, ADP, AMP. In Tris–HCl buffer at sufficiently high concentration, AMP is eluted first, followed by ADP and then ATP.

The best separations of the thiophosphate diastereomers were obtained using Tris buffer and $MgCl_2$, and it is seen from Table II and Fig. 1 that the separation of the diastereomers of GDP(α-*S*) and GTP(α-*S*) is now complete, in contrast to the situation in the standard ammonium phosphate buffer (Table I). In addition, a partial separation of the diastereomers of ATP(β-*S*) could now be obtained (see Table II and Fig. 2). This separation was almost complete using the C_{18},Ro Sil material. Although a similar separation could not be achieved for the diastereomers of GTP(β-*S*), the pure isomers could be distinguished on the basis of slightly different retention times (Table II).

TABLE I

RETENTION TIMES OF GUANOSINE NUCLEOTIDES ON A C_{18}-ODS (SHANDON) COLUMN (250 × 4.6 mm)

Buffer: 50 m*M* potassium phosphate, pH 6.0. Flow-rate: 2 ml/min.

Nucleotide	*Retention time (min)*
GTP	1.23
GDP	1.34
GMP	1.76
(S_p)-GTP(α-*S*)	1.48
(R_p)-GTP(α-*S*)	2.01
(S_p)-GTP(β-*S*)	1.27
(R_p)-GTP(β-*S*)	1.27
(S_p)-GDP(α-*S*)	1.68
(R_p)-GDP(α-*S*)	2.19
GDP-(β-*S*)	1.31

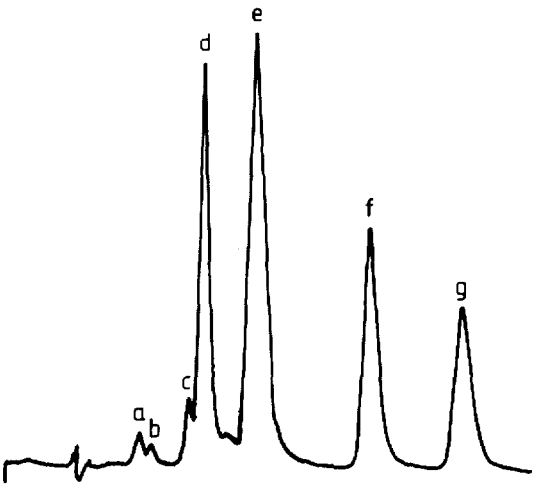

Fig. 1. Separation of guanosine nucleotides on C_{18} (Ro Sil). Conditions as in Table II. Peaks a = GTP; b = GDP(β-*S*); c = (R_p)-GTP(β-*S*); d = (S_p)-GDP(α-*S*); e = (S_p)-GTP(α-*S*); f = (R_p)-GDP(α-*S*); g = (R_p)-GTP(α-*S*).

TABLE II

RETENTION TIMES OF GUANOSINE AND ADENOSINE NUCLEOTIDES ON C_{18} (Ro Sil) AND C_{18}-ODS (SHANDON) COLUMNS (250 × 4.6 mm)

Buffers: 300 m*M* Tris–HCl, pH 8.0, 20 m*M* $MgCl_2$. Flow-rates: C_{18} (Ro Sil), 1 ml/min; C_{18}-ODS (Shandon), 2 ml/min.

Nucleotide	*Retention time (min)*	
	C_{18}-Ro Sil	*C_{18} Shandon*
GTP	3.67	2.79
(S_p)-GTP(α-*S*)	6.85	3.78
(R_p)-GTP(α-*S*)	12.64	7.35
(R_p)-GTP(β-*S*)	6.38*	
(S_p)-GTP(β-*S*)	6.59*	
(S_p)-GDP(α-*S*)	5.5	2.86
(R_p-GDP(α-*S*)	10.09	5.6
GDP(β-*S*)	4.06	2.13
ATP	7.45	5.93
ADP	6.82	5.02
AMP	6.15	3.41
(S_p)-ATP(α-*S*)	17.53	
(R_p)-ATP(α-*S*)	34.8	
(S_p)-ATP(β-*S*)	10.55	5.6
(R_p)-ATP(β-*S*)	11.58	6.19
ATP(γ-*S*)	7.73	
(S_p)-ADP(α-*S*)	13.37	
(R_p)-ADP(α-*S*)	26.1	
ADP(β-*S*)	7.91	4.23
AMP(*S*)	7.83	

* In these separations, the buffer employed was: 300 m*M* Tris–HCl, pH 6.2 with 20 m*M* $MgCl_2$.

Fig. 2. Separation of adenosine nucleotides on C_{18} (Ro Sil). Conditions as in Table II. a = AMP; b = ADP; c = ATP; d = AMP(S), ADP(β-S), ATP(γ-S); e = (S_p)-ATP(β-S); f = (R_p)-ATP(β-S); g = (S_p)-ADP(α-S); h = (S_p)-ATP(α-S); i = (R_p)-ADP(α-S); j = (R_p)-ATP(α-S).

The results reported here indicate that the use of ion-exchange HPLC for the separation of nucleoside thiophosphates is no longer necessary. This is of significance, since ion-exchange HPLC is considerably more troublesome than reversed-phase HPLC, and the separations achieved are in all cases inferior to those reported here. Ion-exchange resins are in our experience considerbly more sensitive and of much shorter useful lifetime than the reversed-phase materials. In addition, high concentrations of corrosive salts which can cause problems with the solvent delivery system are not needed for reversed-phase HPLC. A further advantage of the method described here is that, for a particular separation, retention times can easily be modified by slightly changing the conditions. For example, the slowly eluted nucleotides in Table I can be eluted more rapidly by inclusion of low concentrations of methanol in the buffer. It also seems likely that retention times and separations can be modified drastically by use of other cations to produce the ion-pairs, and possibly by using other multivalent metal ions. A systematic investigation of these effects could lead to complete separation of the isomers of ATP(β-S) and GTP(β-S).

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