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ION-PAIR CHROMATOGRAPHY OF NUCLEOTIDES

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SUMMARY

Some of the variables that affect the separation of the 5′-mono, di- and triphosphate ribonucleotides by ion-pair reversed-phase chromatography (IPC) were investigated. The variables included the concentration of the pairing agent, chain-length of the alkyl group of the quaternary amine, and the pH of the mobile phase.

The tetrabutylammonium ion (TBA) was found to be the most effective for retarding the elution of the majority of the nucleotides, especially the triphosphates. No pairing agents had any significant effect on the cytidine nucleotides; conversely, with the thymidines, the retention times of all of their nucleotides were greatly increased by the presence of TBA.

At both pH 3.0 and 5.7, only the ion-paired thymidine nucleotides showed a marked increase in retention time and only those of cytosine showed no significant change in retention behavior. At pH 5.7, all ion-paired adenosine nucleotides showed large increases in retention times, as did the triphosphate nucleotides of inosine and guanosine.

At pH 5.7, the elution order of all ion-paired nucleotides follow the structure retention rules proposed by Brown and Grushka for nucleosides and their bases, but the same pattern of behavior was not observed at pH 3.0. Also, the elution order observed for all compounds at pH 5.7 was mono < di < tri, the order found for anion exchange. However, at pH 3.0 a few diphosphates were eluted before their respective monophosphates.

From these data, it is evident that the structure of the base and the type and position of substituent groups, as well as the charge on the phosphate group or groups, is involved in the ion-pairing reaction.

INTRODUCTION

Traditionally, nucleotides have been chromatographed by ion exchange[1–4], but ion-exchange chromatography has the disadvantages of requiring long equilibration times in gradient programming and lack of reproducibility. To alleviate these shortcomings, the separation of nucleotides by reversed-phase (RP) chromatography has been investigated[5–9]. The 5′-mono-, di- and triphosphate nucleotides are ionic mole-

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cules in the pH range 2–8, the operating range of silica-based columns. Due to their ionic character, the nucleotides are not well retained by the non-polar stationary phase. By the selection of a low-pH mobile phase, the secondary ionization of the phosphate moiety may be suppressed and improved reversed-phase separations of the nucleotides obtained[10,11]. However, the dissociation of the phosphate group makes the nucleotides excellent candidates for ion-pair chromatography (IPC). Numerous reports have been published on the RP-IPC of the nucleotides[12–22]. Many are concerned only with separation of the adenine nucleotides[12–14], other papers reported on the monoribonucleotides[15,16], while in still others, the mono-, di- and triphosphates of adenine, cytosine, guanine and uracil were studied[17,22]. However, there has not been a comprehensive investigation of the variables affecting IPC of the major purine and pyrimidine nucleotides, including those of hypoxanthine and thymine. In this paper the effects of concentration and alkyl chain length of the quaternary amine pairing agents and the pH of the mobile phase on the retention of the major, naturally-occurring purine and pyrimidine mono-, di- and triphosphate nucleotides are presented.

EXPERIMENTAL

Instrumentation

A Waters liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), equipped with an M6000A pump and a Model 440 dual-wavelength detector, monitoring the effluent at 254 and 280 nm were used. Retention times were recorded by the Perkin-Elmer Sigma 10 data station (Perkin-Elmer, Norwalk, CT, U.S.A.) and a dual-pen strip chart recorder (Omniscribe Houston Instruments, Austin, TX, U.S.A.). The column was a Whatman Partisil 10/25 ODS-3 (Whatman, Clifton, NJ, U.S.A). To provide longer column lifetimes, a guard column (5 cm × 4.6 mm), tap-packed with pellicular reversed-phase material (Co-Pell, Whatman) was used.

Chemicals and preparation of solutions

Potassium dihydrogen phosphate, HPLC grade, was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The mobile phase consisted of 0.02 *M* potassium dihydrogen phosphate plus one of the following pairing agents; tetramethylammonium hydroxide pentahydrate (TMA), tetraethylammonium hydroxide (TEA) (Aldrich, Milwaukee, WI, U.S.A.) and tetrabutylammonium phosphate (TBA) (Eastman Kodak, Rochester, NY, U.S.A.). The pH of the mobile phase was adjusted with either phosphoric acid or potassium hydroxide and filtered through 0.45-μm filters, Type HA (Millipore, Milford, MA, U.S.A.). The following nucleotide standards were purchased from Sigma (St. Louis, MO, U.S.A.): the 5′-mono, di- and triphosphate nucleotides of adenine (Ade), guanine (Guo), hypoxanthine (Hyp), cytosine (Cyt), uracil (Ura) and thymine (Thy) (Table I). The structures of the base in each nucleotide and their acid dissociation constants are shown in Fig. 1. The nucleotides were prepared as millimolar solutions in doubly-distilled, deionized water.

Procedures

Prior to use, the column was allowed to equilibrate overnight with the mobile phase used overnight at a flow-rate of 0.3 ml/min. All separations were isocratic with

TABLE I

LIST OF ABBREVIATIONS OF BASES, NUCLEOSIDES AND NUCLEOTIDES*

Ade	=	adenine
Ado	=	adenosine
AMP	=	adenosine 5'-monophosphate
ADP	=	adenosine 5'-diphosphate
ATP	=	adenosine 5'-triphosphate
Cyt	=	cytosine
Cyd	=	cytidine
CMP	=	cytidine 5'-monophosphate
CDP	=	cytidine 5'-diphosphate
CTP	=	cytidine 5'-triphosphate
Gua	=	guanine monophosphate
Guo	=	guanosine
GMP	=	guanosine 5'-monophosphate
GDP	=	guanosine 5'-diphosphate
GTP	=	guanosine 5'-triphosphate
Hyp	=	hypoxanthine
Ino	=	inosine
IMP	=	inosine 5'-monophosphate
IDP	=	inosine 5'-diphosphate
ITP	=	inosine 5'-triphosphate
Thy	=	thymine
Thd	=	thymidine
TMP	=	thymidine 5'-monophosphate
TDP	=	thymidine 5'-diphosphate
TTP	=	thymidine 5'-triphosphate
Ura	=	uracil
Urd	=	uridine
UMP	=	uridine 5'-monophosphate
UDP	=	uridine 5'-diphosphate
UTP	=	uridine 5'-triphosphate

* All nucleotides except the thymidines used in this study are the ribonucleotides. The thymine nucleoside and nucleotides are the deoxyribo analogue.

a 0.02 *M* phosphate buffer plus the desired concentration of pairing agent, (pH adjusted) as eluent. A flow-rate of 1.5 ml/min was used and all experiments were carried out at ambient temperature. The value of t_0 of the system was determined by the injection of 5 μl of a 3 *M* potassium chloride solution with doubly distilled, deionized water as the mobile phase. For 10 replicate injections, the t_0 value was 2.17 min.

RESULTS

Effects of concentration and chainlength of the pairing agent

In order to optimize an ion-pairing separation, the concentration and alkyl chainlength of the pairing agent may be varied. For each pairing agent used in this

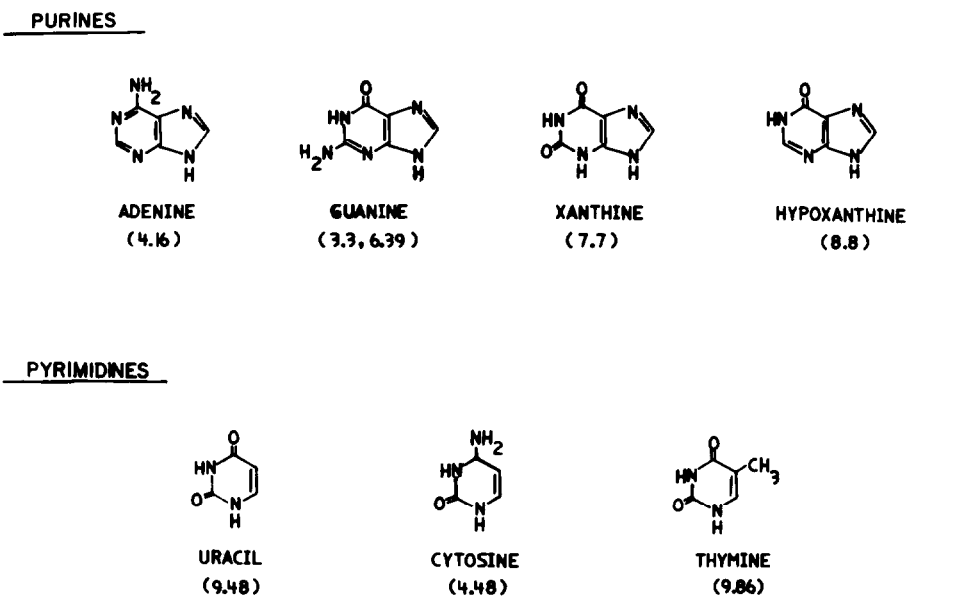

Fig. 1. Structure and acid dissociation constants of the purine and pyrimidine bases. pK_a values from D. O. Jordan, *The Chemistry of Nucleic Acids,* Butterworths & Co., Ltd., London, 1960, p. 137.

investigation, concentration studies were performed at pH 3.0 and 5.7. Although concentrations of 10^{-6} *M* through 10^{-2} *M* were examined, no changes in retention were found with 10^{-6} *M* and 10^{-5} *M* TMA. With TEA, some changes in k' values were found at 10^{-4} *M*. There were also changes at 10^{-5} *M* concentration, but there was no variation in retention at 10^{-6} *M*. With TBA, changes in the retention behavior were noted with concentrations as low as 10^{-6} *M* (Table III). The k' values for all the mono- and diphosphate nucleotides, except for the cytosine and adenine nucleotides, increased. At the higher concentration of TBA, there was a marked increase in k' for all triphosphates, especially for ITP, UTP and TTP. Therefore, proper selection of concentration is a critical factor in controlling retention times.

Although the pairing agent most commonly used for the separation of nucleotides is TBA[12,14,17,20,21], we investigated the effect of the length of the alkyl chain of the pairing agent on the retention behavior of the nucleotides. We found that the retention of the cytidine and guanosine nucleotides was not affected by the presence of TMA or TEA. Thus, only the k' values of the inosine, adenosine, uridine and thymidine nucleotides are shown in Table II. With both TMA and TEA, the k' values of the monophosphate nucleotides decreased. With the diphosphates, anomolous behavior was observed; the k' values increased with the TMA, but with TEA, k' values increased with IDP, decreased slightly with TDP, and hardly changed with ADP and UDP. With the triphosphates, in the presence of TEA, the predicted behavior was observed; the k' values increased. Thus, in comparing the effects of the 10^{-4} *M* TMA with those of 10^{-5} *M* TEA, there was no difference in the retention of the monphosphate nucleotides, but with the diphosphates, the k' values of all but the IDP were smaller with the longer-chain reagent, TEA.

TABLE II

EFFECT OF ALKYL CHAINLENGTH OF THE PAIRING AGENT ON CAPACITY FACTORS

Mobile phase = pairing agent + 0.02 *M* KH_2PO_4 (pH 3.0). Retention times used to determine *k'* values are averages of 3 analyses. Value of t_0 was 2.17 min.

Compound	*k'*			
	No. pairing agent	*TMA (10^{-4} M)*	*TEA (10^{-5} M)*	*TBA (10^{-5} M)*
Purines				
IMP	1.85	1.46	1.58	2.41
IDP	0.96	2.08	3.97	2.94
ITP	2.63	–	3.71	9.86
AMP	1.60	1.47	1.47	1.77
ADP	0.80	1.06	0.78	1.44
ATP	1.59	–	1.81	4.04
Pyrimidines				
UMP	0.75	0.63	0.65	1.80
UDP	0.39	1.03	0.41	2.24
UTP	2.12	–	2.39	6.24
TMP	3.83	2.58	2.86	7.01
TDP	1.87	2.59	1.52	8.70
TTP	4.74	–	5.20	20.77

Since the retention of all nucleotides was much greater with TBA than with either TEA or TMP, our results support the results of Walseth *et al.*[16], for the ribonucleoside monphosphates in which better separation and longer retention was reported when TBA at lower concentrations was used than with TMA or TEA at higher concentrations. Therefore, the pairing agent with the longer chainlength, TBA, is more effective in the separation of the nucleotides. In addition, since less TBA is required, it is comparatively less expensive.

The effects of pH in the presence of 10^{-5} *M* TBA were examined, and it was found that the *k'* values of GTP and all the adenosine and cytidine nucleotides were higher at pH 5.7 than at pH 3.0 (Table IV). A possible explanation of this retention behavior is that at pH 3.0, the bases in these nucleotides have a positive charge on the amine functional group. If the negatively charged phosphate group pairs with the positively charged ion-pairing reagent, there will then be a net positive charge on the nucleotide molecule. Hence, elution will be accelerated, especially if some of the cationic pairing agent is adsorbed on the stationary phase. Therefore, the decrease in retention will be due to repulsion of the positively charged ion pair by the positively charged stationary phase; *i.e.* an ion-exclusion effect predominates.

With the guanosine nucleotides, anomolous behavior was observed. Although the *k'* of GTP was larger at pH 5.7 than at 3.0, the *k'* values of GMP and GDP were about the same at pH 3.0 and pH 5.7. However, on comparing the retention behavior

TABLE III

EFFECT OF CONCENTRATION OF TBA ON CAPACITY FACTORS

Mobile phase = 0.02 *M* KH_2PO_4 + TBA, adjusted to pH 3.0. Retention times used to determine k' values are averages of 3 analyses. The t_0 value used to determine the k' values was 2.17 min.

Compound	k'		
	No TBA	10^{-6} *M*	10^{-5} *M*
Purines			
IMP	1.85	2.24	2.41
IDP	0.96	1.67	2.94
ITP	2.63	2.61	9.86
GMP	1.90	2.12	2.42
GDP	1.14	1.64	3.16
GTP	3.19	2.55	3.32
AMP	1.60	1.96	1.77
ADP	0.80	1.34	1.44
ATP	1.59	1.95	4.04
Pyrimidines			
CMP	0.47	0.45	0.48
CDP	0.23	0.42	0.40
CTP	0.42	0.63	1.08
UMP	0.75	1.06	1.80
UDP	0.39	0.82	2.24
UTP	2.12	1.53	6.24
TMP	3.83	4.85	7.01
TDP	1.87	3.52	8.70
TTP	4.74	4.73	20.77

of the guanosine nucleotides in the presence and the absence of TBA at pH 3.0, it was evident that the presence of TBA increased the retention times of GMP and GDP but had no effect on GTP. Thus ion pairing of TBA with GMP and GDP will occur at a pH of either 3.0 or 5.7 but ion pairing of the GTP occurs only at the higher pH. The zwitterionic GTP appears to ion-pair inter- or intramolecularly, whereas the GMP and the GDP form ion pairs only with an added pairing agent.

The trend we observed for the effects of pH on the ion pairing of the adenosine nucleotides and of GTP was opposite to that found by Ingebretsen *et al.*[14] and Knox and Jurand[19], who worked at higher pH values. In the ion-pairing systems used by both of these groups, decreases in the capacity factors were observed on increasing the pH from 4 to 6. Knox and Jurand[19] suggested that quadrupolar ion-pairs formed between the nucleotides and their zwitterionic pairing agents.

With the inosine nucleotides, a dichotomy was also found. The k' values of ITP decreased, but the k' values of IMP and IDP increased on increasing the pH from 3.0 to 5.7. Ion pairing of all the inosine nucleotides appeared to take place as

TABLE IV

EFFECT OF pH OF THE MOBILE PHASE ON CAPACITY FACTORS

Mobile phase = 0.02 *M* KH_2PO_4. Retention times used to determine the *k'* values are averages of 3 analyses. The t_0 values used to determine *k'* was 2.17 min.

Compound	*k'*		
	No TBA pH 3.0	*pH 3.0*	*pH 5.7*
Purines			
IMP	1.85	2.41	2.94
IDP	0.96	2.94	3.57
ITP	2.63	9.86	6.51
GMP	1.90	2.42	2.47
GDP	1.14	3.16	3.06
GTP	3.19	3.32	6.02
AMP	1.60	1.77	8.14
ADP	0.80	1.44	10.99
ATP	1.59	4.04	22.05
Pyrimidines			
CMP	0.47	0.48	0.73
CDP	0.23	0.40	0.92
CTP	0.42	1.08	1.77
UMP	0.75	1.80	0.81
UDP	0.39	2.24	0.80
UTP	2.12	6.24	1.54
TMP	3.83	7.01	5.63
TDP	1.87	8.70	6.96
TTP	4.74	20.67	12.91

the retention values increased significantly in the presence of TBA. Unlike those bases which have an amine group (*i.e.* adenine, guanine and cytosine), the bases uracil, thymine, and hypoxanthine are not protonated at either pH 3 or 5.7. Thus, the major factor in ion pairing should be the dissociation of the phosphate moiety. Since secondary as well as primary dissociation of the phosphate group occurs at pH 5.7, the molecules have a greater negative density at pH 5.7 than 3.0. Hence, it was postulated that ion pairing should be more effective for these 3 groups of nucleotides at pH 5.7 and longer retention times would be observed at that pH. However, with the exception of IMP and IDP, this was not the case; shorter retention of the thymidine and uracil nucleotides and ITP occurred at pH 5.7. Gelijkens and De Leenheer[23] also observed a decrease in the capacity factor of UMP with increasing pH of the mobile phase. Similiarly, Walseth *et al.*[16] reported the optimum pH for the separation of the ribonucleotide monophosphoates was pH 2.5. Therefore, it appears that the base is involved in the ion-pairing process. These data support studies done with nucleotides and metal cations that indicate that the cations are coordinated not only by the phosphate groups but also by various functional groups on the base[24–28].

Structural effects

At pH values greater than 1 and less than 5, the negative charge on the nucleotides is directly proportional to the number of phosphate groups. Thus, the negative charge density increases with an increasing number of phosphate groups, and the ribophosphates, with the greatest charge density, should ion-pair more effectively than the mono- and diphosphates. As is shown in Table IV at pH 5.7, the general elution order found, which was mono- < di- < triphosphates, follows the predicted trend. At pH 3.0, this elution order was also observed for the inosine, guanosine, uridine, and thymidine nucleotides. Although this elution order is that expected for ion-exchange chromatography[4,30,31], the retention order can also be explained on the basis of the solvophobic theory for a reversed-phase system. Since a monophosphate nucleotide molecule will pair with one molecule of TBA, the diphosphate with two, and the triphosphate with three, we would expect the triphosphate moiety to be much more solvophobic than its mono- or diphosphate counterpart.

At pH 3.0, the elution order of the cytidine and adenine nucleotides is di-, mono- and triphosphates (Table IV), which is the elution order found with most nucleotides at pH 3.0 when no ion-pairing reagent is present.

Substituent groups and positions

Brown and Grushka[24] postulated some structure–retention relationship rules for a selected group of nucleic acid components. They predicted that the group and position of the substituent would affect the capacity factors with the order of the effects being OH < H < NH < NHR. The presence of a methyl group greatly increases the capacity factor, and in the purines the 6-position is more important in controlling retention than the 2-position. If the phosphate group of nucleotides is effectively "ion paired", then the retention behavior in each group (mono-, di-, and triphosphates) should be controlled by the base structure, and the elution order should be the same as the corresponding nucleosides. At pH 5.7, our data followed this trend to a large extent (Table IV). Since the adenosine nucleotides are the only nucleotides that have an amine group in the 6-position, they are expected to be eluted last and, in fact, they are. At a pH of 3.0, no consistent behavior was observed and the rules of Brown and Grushka[24] were not obeyed. For the pyrimidine nucleotides the predicted trend would be Urd < Cyd < Thd. However, as has been noted in all separations involving cytosine compounds[25,26], the cytidine nucleotides had minimal retention, and the presence of the pairing agent had little effect on the k' value. The thymidine nucleotides has very large k' values compared to the k' values of the cytidine and uridine nucleotides, both at pH 3.0 and pH 5.7 (Table V). In fact, at pH 3.0 the thymidine nucleotides are retained longer than any other comparable groups, and at pH 5.7, they are retained longer than any group, except the adenine nucleotides. These large k' values could be due to either the presence of the 2'-deoxyribosyl group, which, as we have noted previously, increases retention over a comparable ribosyl moiety, or to the presence of the methyl group, which also increases retention[29]. It is postulated that the methyl group plays a greater role in increasing k' values, because of possible interaction of the methyl group with the hydrophobic moiety of the ion-pair reagent, thus creating a complex with a conformation that has a larger hydrophobic surface. Such a solvophobic complex would indeed have a longer retention time in a reversed-phase system. Therefore, these data support those

TABLE V

COMPARISON OF CAPACITY FACTORS WITH AND WITHOUT 10^{-5} *M* TBA, pH 3.0

Compound	k'_0	k'_{TBA}	k'_{TBA}/k'_0
Purines			
IMP	1.85	2.41	1.30
IDP	0.96	2.94	3.06
ITP	2.63	9.86	3.75
GMP	1.90	2.42	1.27
GDP	1.14	3.16	2.77
GTP	3.19	3.32	1.01
AMP	1.60	1.77	1.10
ADP	0.80	1.44	1.80
ATP	1.59	4.04	2.54
Pyrimidines			
CMP	0.47	0.48	1.02
CDP	0.23	0.40	1.73
CTP	0.42	1.08	2.57
UMP	0.75	1.80	2.40
UDP	0.39	2.24	2.49
UTP	2.12	6.24	2.94
TMPK	3.83	7.01	1.83
TDP	1.87	8.70	4.65
TTP	4.74	20.77	4.38

obtained with metallonucleotide complexes[24–28], *i.e.* with specific cations, the base is involved in complex formation along with the ionized phosphate group.

CONCLUSIONS

An investigation of the effects of concentration and alkyl chainlength of ion-pairing reagents as well as effects of pH of the mobile phase on the retention behavior of nucleotides is described. Tetrabutylammonium phosphate (TBA) was the most effective pairing agent. For ion pairing to occur, the pH of the mobile phase must be greater than the acid dissociation constants, especially those of adenine, guanine, and cytosine. At pH of 5.7, the elution order of mono- < di- < triphosphate nucleotides is that predicted in ion exchange. In addition, the elution order of each type of nucleotide (as catagorized by its base) is that predicted by the rules proposed by Brown and Grushka; thus indicating that only the phosphate moiety is involved in the ion pairing process. However, at pH 3.0 the elution order of the adenosine and cytidine nucleotides is that observed when a reversed-phase system at a pH of 3.0 is used without a pairing agent: di- < mono- < triphosphates. The cytosine compounds, which have minimal retention in most ion-exchange and reversed-phase systems, also display minimal retention under the ion-pairing conditions investigated. The greatest increase in retention was observed with the adenosine nucleotides at pH

5.7 and with the thymidine nucleotides at pH 3.0. The data obtained also support conclusions reached with metallonucleotide complexes, *i.e.* under certain conditions specific cations complex not only with the ionized phosphate group of the nucleotide but also involve the base structure.

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