THE RAPID SEPARATION AND ISOLATION OF MONO- AND OLIGO-NUCLEOTIDES BY HIGH SPEED LIQUID CHROMATOGRAPHY: AN ION EXCHANGE, REVERSED-PHASE SYSTEM.

R. A. Holton, D. M. Spatz, E. E. van Tamelen, and W. Wierenga†

Department of Chemistry
Stanford University
Stanford, California 94305

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SUMMARY

A new chromatographic material has been developed which has a combination of reversed-phase and ion exchange characteristics. In conjunction with high speed liquid chromatography, it provides a convenient method for the analysis and purification of nucleotides and their derivatives.

INTRODUCTION

A variety of chromatographic methods and procedures have been devised and employed to separate and purify nucleic acid derivatives including partition, adsorption, exclusion, and ion exchange chromatography. The recent commercial introduction of high pressure liquid chromatographic instruments has enhanced the role of ion exchange chromatography for the rapid identification particularly of nucleosides, nucleotides and small oligonucleotides.[1a,b,2]

As an adjunct to a synthetic program in nucleotide chemistry in our laboratories, we desired a chromatographic method with the best blend of qualitative and quantitative analytical capabilities as well as good preparative characteristics. Inherent in the many considerations are particularly those found wanting in the current methods employed, including rapid, reproducible analysis, ease of isolation and low cost. In this regard we have explored and developed a combination ion exchange and reversed-phase chromatographic (RPC) system, a method which incorporates several of the basic chromatographic phenomena. The capabilities of an RPC system in the area of

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RNA fractionation are indicated in the pioneering work of Kelmers, Novelli and coworkers.[3] We would like to describe our efforts in the development and utilization of an RPC system aimed initially at the mononucleotide level of nucleic acid chemistry which incorporates the beneficial features of earlier RPC systems and eliminates some problematic aspects.[4]

MATERIALS AND METHODS

The unmodified mononucleotides were purchased either from Nutritional Biochemicals, Cleveland, Ohio, or Sigma Chemical Company, St. Louis, Missouri. Oligomers of thymidylic acid were gifts from The Upjohn Company, Kalamazoo, Michigan. The remainder of the exemplary compounds included in the tables were prepared by classical methods of synthesis described in the nucleotide literature.[5]

The ion-exchange, reversed-phase chromatographic support (RPC-TMSN) was prepared in the following manner. A suspension of 500 g of silica gel H (E. Merck AG, 5-25 μ) in 1.5 l of toluene was treated with 50 g of trimethylsilyl chloride (TMS-Cl) and 50 g of hexamethyldisilazane (HMDS). For RPC-TPSN an equivalent amount of triphenylsilyl chloride (TPS-Cl) was employed with only a catalytic amount of HMDS. The solution was refluxed for two days during which an equivalent amount of TMS-Cl and HMDS was added at intervals to ensure complete silylation. The silylated silica was filtered, washed with chloroform and methanol with subsequent drying for ten hours at 100° and 1 Torr. To 500 g of the silylated silica was added a solution of 25 ml (5% V/W)* of trimethyl, C_8-C_{10} alkyl ammonium chloride (Adogen 464, Ashland Chemicals, Columbus, Ohio) in 300 ml of chloroform. Vigorous agitation with a mechanical stirrer in the hood at room temperature served to remove most of the chloroform over a 1-2 day period. Complete evaporation of the chloroform was accomplished at 0.01 Torr and 25°.

* This affords a maximum theoretical ion exchange capacity of 0.3 milliquivalents/gram of absorbent.

A Waters Model ALC 202 liquid chromatograph instrument with differential U.V. (monitored at 254 nm) and refractometer detectors and an M-6000 dual reciprocating piston pump was employed. The columns utilized for analytical purposes were 3/8" (O.D.)x 2' stainless steel, end-fitted with 5 μ porous frits (Waters Associates,Milford, Massachusetts). The columns were dry-packed with the RPC absorbents primarily by vertical tapping with minimal application of a vibrator. Extensive vibration during column packing leads to unacceptably high pressures at undesirable flow rates. Approximately 17 g of RPC-TMSN fills a 3/8" x 2' column (dry weight). The columns were conditioned prior to use by elution with 0.1 M ammonium acetate at pH 5.25 for several hours or until the UV-recorder baseline was stable.

All tabulated data was obtained at pH 5.25. The aqueous buffers were adjusted to pH 5.25 by the addition of acetic acid to the desired molar solution of ammonium acetate. Preparation of the buffered eluents in this manner results in the buffer designated 0.1 M NH_4OAc being 0.13 M in acetate and the 0.5 M NH_4OAc being 0.67 M in acetate.

RESULTS

A variety of ribo- and deoxyribonucleotides and derivatives thereof were examined on RPC-TMSN; the results are given in Tables 1 and 2 at two eluent concentrations. Analogously, several oligonucleotides in the uridine and thymidine series were scrutinized and are listed in Table 3.

The effect of a lower pH on retention times was determined on several representative examples. A solution of 0.1 M ammonium acetate, adjusted to pH 4.0 by addition of trifluoroacetic acid (6 ml/l), was arbitrarily selected for several purine and pyrimidine nucleotides. Preliminary data indicate the expected general reduction in retention times. For example, Ap has a retention time of 30 minutes at 0.1 M NH_4OAc, pH 4.0, 40% less than at pH 5.25. Similarly, pU and cyclo-pU have retention times of 8 and 7 minutes respectively at 0.1 M, pH 4.0. They begin to become distinguishable upon co-injection at

TABLE 1

RETENTION TIMES OF URIDINE MONONUCLEOTIDES AND DERIVATIVES[a,b]

	0.1 M NH_4OAc	0.5 M NH_4OAc
U	6	6
U-iso	12	12
Tr-U		15
pU	13	8
Up(3' & 2')	17	13
pU-iso		27
Ac-Up-2'Ac	21	
U-cyclo p	9	
Cyclo-pU	13	
MepU	8	
β-CEpU	22	
MepU-iso	28	10

TABLE 2

RETENTION TIMES OF ADENOSINE AND THYMIDINE NUCLEOTIDES AND DERIVATIVES[a,b]

	0.1 M NH_4OAc	0.5 M NH_4OAc
A	12	12
A-iso		18
pA	29	13
Ap(3'&2')	50	21
MepA	24	12
T	8	
Tr-T		14
pT	20	12
pT-Ac		18

a) Abbreviations: iso=2',3'-O-isopropylidene; β-CE= β-cyanoethyl

b) All values were determined at room temperature with flow rates of 4.0 ml/min. generating pressures of 2500-3000 psi.

0.05 M NH_4OAc, pH 4.0, where they have retention times of 11 and 9 minutes respectively. Adjustment to lower pH values should enhance the separation.

TABLE 3.

RETENTION TIMES OF OLIGOMERS OF URIDINE AND THYMIDINE PHOSPHATES[a,b]

	0.1 M NH_4OAc	0.5 M NH_4OAc	1 M NH_4OAc
UpU	13	8	7
UpU-iso		22	
U-5'-ppU-iso		17	
d(TpT)	28	13	
d(pTpT)	85	15	11
$d(pTpT)_2$			33
$d(pTpT)_3$			55

a) Abbreviations: iso=2',3'-O-isopropylidene;

b) All values were determined at room temperature with flow rates of 4.0 ml/min. generating pressures of 2500-3000 psi.

Application of RPC-TMSN to triphosphates of nucleosides is typified by the determination of a retention time of 27 minutes for ATP at 1.0 M NH_4OAc, pH 5.25.

An illustrative example of the preparative capability of this system is the ready separation of 50 mg of an equimolar mixture of pU and pU-iso(triethylammonium salts) in a single injection on a 3/8" x 2' column of RPC-TMSN with 0.25 M ammonium acetate eluent.

DISCUSSION

The choice of silica as the inert support of the RPC system described in this communication is the result of several requirements. The inert support had to be compatible with high pressures and flow rates, reasonably inexpensive for purposes of preparative applications, and available in a form suitable for coating with an appropriate quaternary amine. Silica is available in a wide variety of particle sizes with reproducible sizing and can be

functionalized by silylation, the resultant Si-O-Si bonds being thermally and hydrolytically stable. Treatment of the silylated silica with the quaternary amine, trimethyl-C_8-C_{10} alkyl ammonium chloride (Adogen 464) produces a stable chromatographic material. We have found insignificant bleeding of the alkyl ammonium salt employing acetate buffer eluents. Indeed, column longevity has reached six months of nearly daily usage without regeneration. The reproducibility of retention times from column to column has been less than $\pm$ 10% error and less than $\pm$ 5% error for the same column over an extended time period.

Although the pH span of ammonium acetate buffers is somewhat limited, the ammonium acetate can be easily and quickly removed from collected samples by reduced pressure at room temperature. An added advantage over phosphate buffers is its relative resistence to fungal growth, a characteristic of this RPC-adsorbent also.

The data in Tables 1 and 2 are organized in order of increasing substitution of the phosphate grouping. Several trends are apparent within a nucleic acid series. A significant reduction in retention time is noted in proceeding from pU to the methyl diester, 5'-MepU, suggesting that ionic interactions dominate the forces governing their relative retention. Substituting the larger alkyl group, β-cyanoethyl, for methyl highlights the strong hydrophobic interactions available to appropriate substrates. This is further exemplified in uridine monophosphate by the more than tripling of retention time in proceeding from pU to the 2',3'-O-isopropylidene derivative of pU. Esterification of a sugar hydroxyl as an acetate ester has a similar effect although less pronounced.

Another obvious and useful trend is evident in comparing several different nucleotides. For example, the retention time series pA>pT>pU arises from the data in Tables 1 and 2.

In an attempt to extend the versatility of RPC-TMSN, several representative dinucleotides and larger oligomers were examined as denoted by Table 3.

Cursory inspection reveals the expected propensity for longer retention times upon substitution of the hydrophobic protecting group, isopropylidene. Also of interest is the parallel of increasing retention with increasing oligomer length, $(pT)_n$.

Initial investigations with RPC-TPSN, where the silica has been silylated with triphenylsilyl chloride (TPS-Cl) instead of TMS-Cl, revealed a general decrease of retention times relative to RPC-TMSN. For example, pU, pU-iso, and pTpT have retention times of 5, 12, and 11 minutes respectively at 0.5 M NH_4OAc, pH 5.25. The latter example favorably indicates extension to higher oligomers at eluent ionic strenghts of half that employed for RPC-TMSN for similar retention.

These preliminary investigations reveal the rapid analysis and resolution of mono- and oligonucleotides characteristics of this high speed RPC-ion exchange system at the nanomole level without necessitating gradient elution. The excellent preparative capabilities of RPC-TMSN constitutes an additional desirable characteristic and, coupled with economic considerations, represents a distinct advantage over pellicular anion exchangers.

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