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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF OLIGONUCLEOTIDES AND OTHER NUCLEIC ACID CONSTITUENTS ON MULTIFUNCTIONAL STATIONARY PHASES

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SUMMARY

A new class of stationary phases has been created for high-performance liquid chromatography bonding various proportions of ionic and hydrophobic ligands to the surface of silica supports. The ratio of hydrophobic to ionic character can be reproducibly controlled by varying the volume ratio of monochlorosilane reagents in the bonding mixture. In this study, both octyl- and 3-chloropropyldimethylmonochlorosilane ligands were bonded, after which the chloro groups were converted into quaternary amines by nucleophilic substitution with benzyldimethylamine. The resulting hydrophobic ion exchangers proved to be well suited for the separation of the oligonucleotides up to about 20 in chain length, improved separations being observed in comparison to existing ion-exchange or reversed-phase separations alone. Selectivities of these mixed-mode packings towards polynucleotides were found to be comparable with those observed for RPC-5 and similar resins.

INTRODUCTION

The synthesis of a new class of hydrophobic ion-exchange stationary phases was undertaken in order to improve the separation of oligonucleotides and other biopolymers, as well as for the simultaneous separation of mixtures of neutral and ionic solutes. These reversed-phase ion-exchange stationary phases simulate in many regards separations obtained by using ion-paring reagents[1–3], but without the need of a secondary equilibrium to maintain weak levels of ion-exchange properties superimposed upon reversed-phase characteristics. These new multifunctional stationary phases are synthesized by bonding various proportions of dissimilar ligands to the silica support, thereby creating a phase with significant ion-exchange and hydrophobic properties[7]. The balance of these properties is controlled during the bonding step by varying the mole ratio of chlorosilane bonding reagents. A variety of phases can be produced in this manner, including cationic, anionic or zwitterionic reversed-phase packings. In addition, the polarity of the phase can be controlled by the addition of hydroxyl groups through various substitution reactions.

The control of stationary-phase ionic and hydrophobic character is critical in the separation of larger biopolymers, such as the oligonucleotides and proteins. These

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highly functionalized species are often charged at neutral pH. Short oligonucleotides and proteins have been chromatographed on reversed-phase materials, but since the retention of short oligomers increases with molecular weight, it is often found that even moderately long proteins and biopolymers are too strongly retained on simple reversed phases[4,5]. Furthermore, if the mobile phase is increased in strength with a higher concentration of organic modifier, solubility problems are encountered. Ion exchangers can be employed[6], but selectivity can often be lacking.

The hydrophobic ion exchangers described herein seem to be well suited for separations of ionic biopolymers, since a complex blend of hydrophobic hydrophilic and ionic properties can be imparted to the stationary phase. In addition, they are well suited to the simultaneous separation of neutral and ionic solutes of lower molecular weight[4], in a manner analogous to ion pairing[8]. These multifunctional phases behave in many regards like the RPC-5 and Adogen 464 coated Kel-F beads which are so successful in oligonucleotide separations[9,10]. The efficiencies and stabilities of bonded silicas are much higher though, since no coating, either dynamic or static, is involved.

The synthesis and characterization of hydrophobic anion exchangers is described, made by bonding monofunctional silane to silica support materials. The application of these phases to the purification of synthetic oligonucleotide fragments is also presented, and the retention characteristics of the mixed-mode packings are discussed.

EXPERIMENTAL

Chromatography

The high-performance liquid chromatographic (HPLC) system consisted of two Waters Model 6000 high-performance solvent pumps a Model 660 gradient solvent programmer, and a Model 440 detector (Waters Assoc. Milford, MA, U.S.A.). Injections were made via a 20-μl fixed-loop Rheodyne 7125 injector (Rheodyne, Cotati, CA, U.S.A.). Data were recorded and integrated using a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Columns were slurry packed with equipment and by procedures described elsewhere[7]. The proportion of water to 2-propanol used in the slurry solvent equalled the expected ratio of ion exchanger to reversed phase found on the support material.

All chromatographic solvents were HPLC grade and purchased from Burdick E Jackson (Muskegon, MI, U.S.A.). Buffers and mobile phase salts were of reagent grade and purchased from Fisher Scientific, (Pittsburgh, PA, U.S.A.). All solvents were filtered and then degassed with helium before use.

Biochemicals used as chromatographic standards were purchased from Sigma (St. Louis, MO, U.S.A.) except several longer oligomers, which were synthetic preparations provided by Vega Biochemicals (Tuscon, AZ, U.S.A.). Standard procedures for the base hydrolysis of the polyribonucleotides were followed for 50% bond cleavage.

Bonding

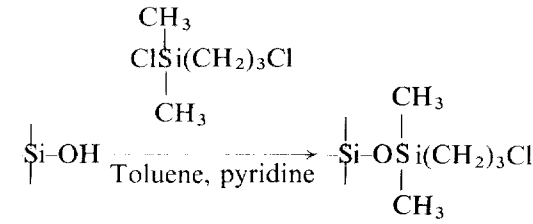
Throughout the study, a single batch of Whatman Partisil (Clifton, NJ, U.S.A.) 5-μm silica (Lot 10592) with a specific surface area of 427 m^2/g was used.

All monochlorosilane reagents are available from either Silar (Scotia, NY, U.S.A.) or Petrarch Systems (Bristol, PA, U.S.A.). Following bonding of the mixed silanes according to published procedures[7] the phases were capped with trimethylchlorosilane to reduce solute interactions with surface silanols. The bonded materials were then exhaustively washed with methanol, acetone, and finally water before they were functionalized according to the scheme outlined below. The mixed-mode materials were then washed, dried and packed.

RESULTS AND DISCUSSION

Synthesis

The blended stationary phase ligands were synthesized in a two-step procedure. Two monochlorosilane reagents with different terminal functionalities were first bonded to the silica support in the usual manner, by using toluene as the solvent and pyridine as an acid scavenger. The functionality of one of the bonded ligands was then activated by a second reaction step, which varied with the nature of the functionality and the desired ligand. In this case, commercially available octyldimethylchlorosilane and 3-chloropropyldimethylchlorosilane were added in the desired mole ratios to the toluene during the initial bonding. Iodide, which is a better leaving group than chloride, was substituted for chloride by refluxing the bonded silica in methyl ethyl ketone (MEK) and sodium iodide overnight. Iodine was then exchanged with a benzyldimethylamine (BDMA) group[11] in a simple substitution reaction. The quaternary amine formed produced an anion-exchange ligand with significant hydrophobic qualities due to its aromatic character. A summary of the reaction sequence is shown in eqns. 1–3.



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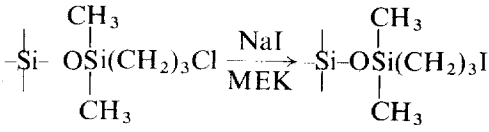

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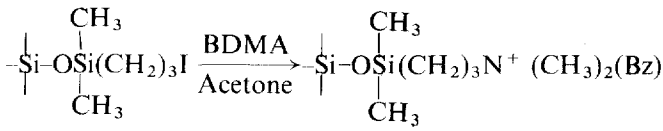

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Elemental analysis was used throughout the reactions as a confirmatory test of the progress of the bonding. For the amine functionalities however, it was found that percent nitrogen values were of limited diagnostic use, since at the low levels present

TABLE I

VARIATION OF CARBON CONTENT, LIGAND COVERAGE AND EXCHANGE CAPACITY WITH MOLE RATIO OF CHLOROSILANE REAGENTS

Carbon content is shown to increase with increasing amount of reversed-phase ligands on the support. Furthermore, it increases upon functionalization with BDMA, due to the increased molecular weight of the ligand. Coverage was determined by using an average of molecular weights for the ligands and a specific surface area of 427 m^2/g.

Mole ratio of chlorosilane reagents	*Percentage of carbon*		*Coverage**	Exchange capacity**
	Before functionalization	*After functionalization*		
100% N^+	6.60	8.09	1.27	1.13
50:50 N^+ C_8	8.00	9.53	1.53	0.610
20:80 N^+–C_8	9.70	9.79	2.20	0.136
100% C_8	13.27	–	3.20	–

* μmoles m^{-2}.
** mequiv. g^{-1}.

it was not possible to obtain values with more than 1 or 2 significant figures. The percent carbon loading was used to determine ligand coverage, and this, along with titration data, was sufficient to indicate surface concentrations. A summary of these coverages is presented in Table I. Further evidence of reaction efficiency was found by monitoring the loss in the chloro-ligand surface concentration on the silica surface upon conversion into quaternary amine. The chloro-ligand was quantitated by acid hydrolysis of the bonded silica, followed by gas chromatography–mass spectrometry of the hexane extract[18]. Conversion of the chloro ligand into the amine was found to be *ca.* 90%.

Since the mole ratio of the monochlorosilanes is the only means used to control the relative ligand concentrations in this particular bonding approach, it was essential to confirm that the bonding step reflected the mole ratios of chlorosilanes in the reaction flask. Fig. 1 shows the results of direct titration experiments on a series of blended phases over the range of 0% N^+–100% C_8 to 100% N^+ – 0% C_8, where N^+ is quaternary amine silane ligand. Excellent linearity and correlation between bonding mole ratios and exchange capacity was observed. Small absolute differences were observed, but these were reproducible, and had no effect upon the overall synthetic approach.

The bonded ligands were chosen so as to have similar rate constants during the initial bonding step. It is not necessary that these rate constants be identical, but only that they be reproducible. However, the rate constants must be similar in magnitude, otherwise it is experimentally difficult to control the exact ligand ratios at extremes of concentration.

Silica drying

During the primary bonding step, it is essential that no moisture be present on the silica surface. Although polymerization cannot occur with the monofunctional reagents used, diminished surface coverage and other problems may result if surface moisture is not removed from the silica prior to bonding. It was found that simple

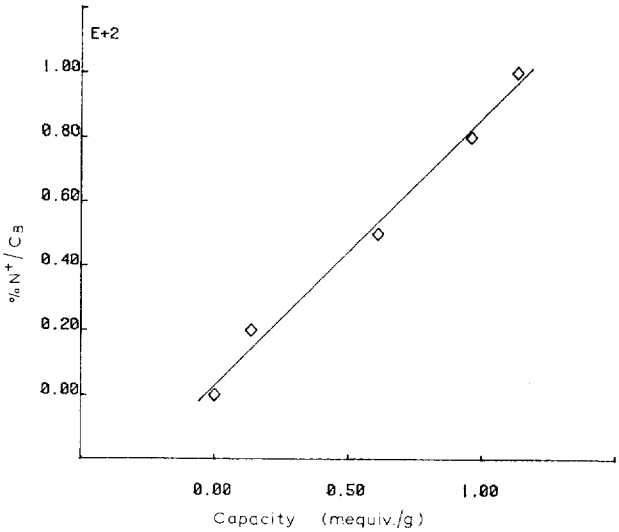


Fig. 1. Mixed-mode ligand coverage-exchange capacity relationship. Exchange capacity is observed to increase linearly as the mole ratio of propylchlorosilane (later functionalized to N^+) to octylsilane is increased. Direct titrations could be made with the mixed-mode materials in the –OH form.

microwave drying equalled or exceeded the results obtained from the use of a vacuum oven normally used for drying. A comparison between the two methods is presented in Fig. 2, where the same mass loss for the silica was achieved in 25 min by using the microwave oven, as opposed to 18 h at 170°C in the vacuum oven. No differences in silica reactivity, ligand bonded concentrations or surface properties between the two methods could be ascertained. Since no differences between the final products from silica batches dried by either method could be determined, the microwave technique was preferred for its simplicity and speed.

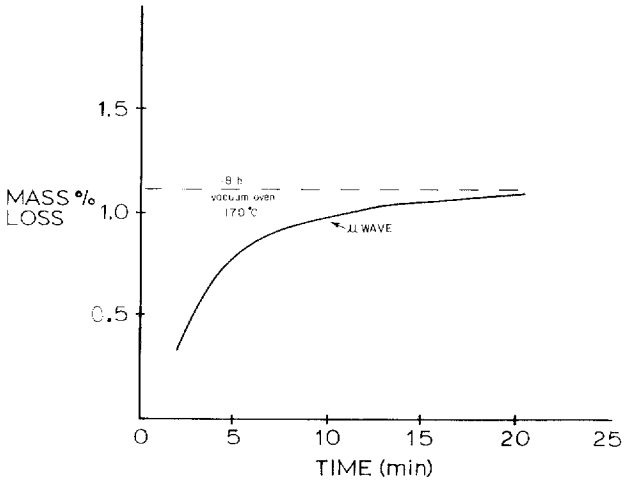
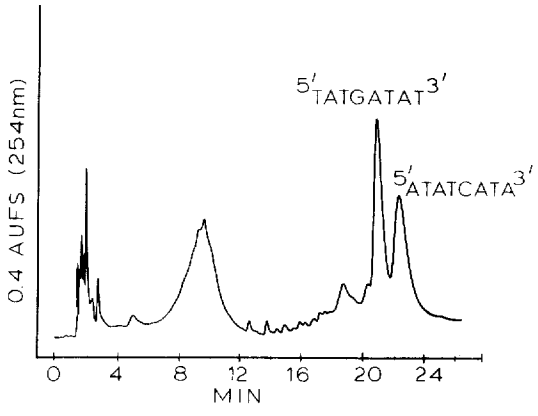


Fig. 2. Silica was dried prior to initial bonding by placing the material in a microwave oven at high power for 20 min. The results obtained (determined by weight loss) were similar to those for silica dried in a vacuum oven for 18 h.

Separation of the oligonucleotides

Due to the recent interest in genetic engineering, much effort has been expended on the development of efficient means for the synthesis of pure oligonucleotides with well-defined base sequences. In each of the popular techniques of synthesis, phosphodiester[12] or phosphotriester[13], via either batch or solid-phase synthesis, one of the most difficult steps is the efficient fractionation of the synthetic intermediates. These oligonucleotide building blocks, typically of chain length less than 20, have traditionally been separated on RPC-5 resins, or their various analogues[9,10,14]. These surface-coated polymers can fractionate relatively high-molecular-weight oligomers without requiring harsh mobile phases. RPC-5 phases have significant hydrophobic and cationic properties, but since they are dynamically coated with an alkylamine, their stabilities and applicabilities are limited.

Since the nucleotide biopolymers are highly charged (each internuclear bond produces a negatively charged phosphate linkage), bonded-phase ion-exchange chromatography is found to separate oligomers according to chain length. Typically, high salt concentrations are necessary to elute larger oligomers, and this complicates their analysis and purification. Thus by "diluting" the quaternary amine ligand concentration with reversed phase ligands, oligomers are eluted at a lower ionic strength while complete surface coverage is maintained. A further advantage of multifunctional phases is that oligonucleotides are separated according to chain length and base composition. In contrast, any differentiation among oligomers of equal chain length but dissimilar base composition in "pure" ion-exchange chromatography must



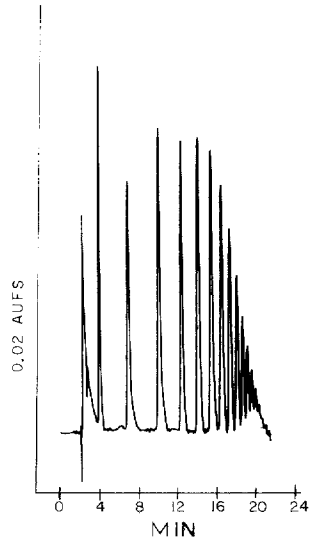


Fig. 3. A gradient separation of complementary octameric oligonucleotides on a 20% $N^+ - 80\%$ C_8 column. The low strength buffer was 10% in acetonitrile and 0.01 *M* in KH_2PO_4, and the high strength buffer was 40% acetonitrile, 0.10 *M* KH_2PO_4. A linear gradient from low to high strength buffer was achieved in 20 min.

Fig. 4. A gradient separation of poly-U base hydrolysate on a 20% $N^+ - 80\%$ C_8 column. The low strength buffer was 10% in methanol, 0.1 *M* in ammonium sulphate; while the higher strength buffer was 35% methanol and 1 *M* ammonium sulphate. The linear gradient from low to high strength buffer was achieved in 40 min.

be attributed to residual interactions with either the support material or the organosilane ligand. These multifunctional supports take advantage of these multiple modes of retention while they are specific and selective toward oligonucleotides of various chainlengths and base compositions.

Fig. 3 shows the separation of two complementary octameric oligonucleotides. Here, by using a simple phosphate buffer–acetonitrile gradient, separation of oligonucleotides of different composition but of equal length was achieved on the hydrophobic ion exchanger.

Fig. 4 shows the separation of the first sixteen fragments of polyuradinilic acid (poly-U), obtained from base hydrolysis of longer parent chains, by using the same column with adjusted mobile phase conditions. Excellent selectivities were also observed for the homologues of the oligonucleotides with other base monomers, and for mixed-base oligonucleotides. A linear gradient of methanol and ammonium sulphate was used to optimize the separation of the shorter chains, although it is possible to optimize the separation shown in Fig. 4 for longer chains as well.

The selectivity of the mixed-mode phases is contrasted with that of simple reversed phases in Fig. 5, where the separation of the same poly-U mixture is analysed on a C_8 packing under similar mobile phase conditions. Although poly-U has been found to undergo fractionation on reversed-phase materials[19], no separation occurs

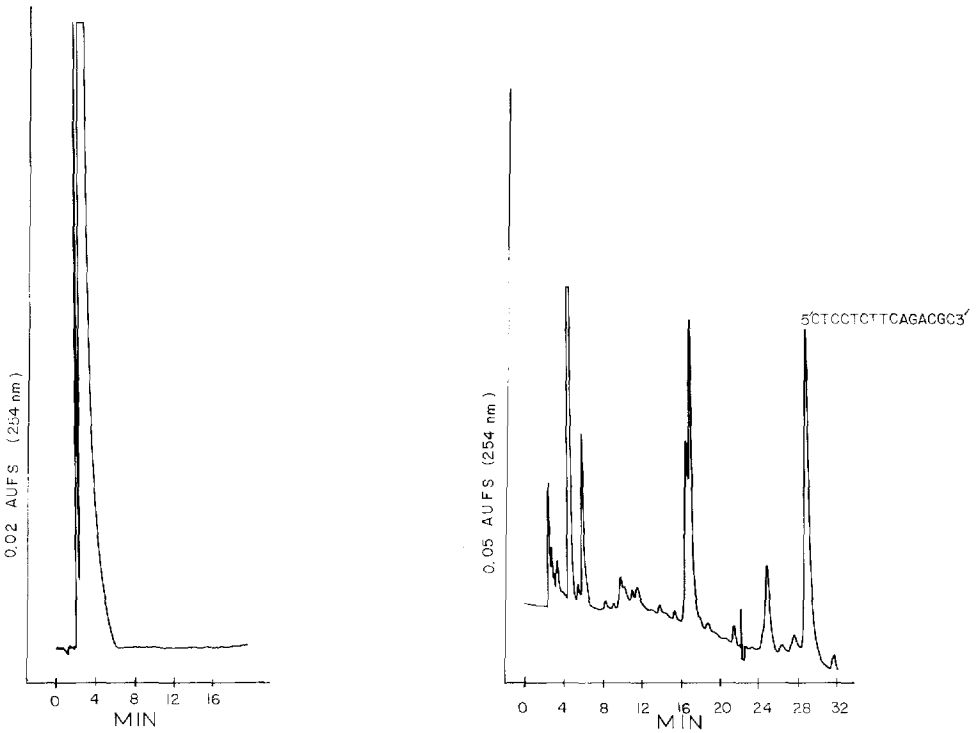


Fig. 5. Separation of the poly-U base hydrolysate on a capped Whatman 5-μm ODS column. Mobile phase conditions were the same as in Fig. 4.

Fig. 6. Separation of a synthetic mixture of reactants in the synthesis of a pentadecamer. Column and mobile phase conditions were the same as in Fig. 4.

when mobile phase conditions are used which are designed to elute progressively longer chains. The reversed-phase column (which had a high carbon loading) was found to be unsuited for the progressive separation of polymers relative to the mixed-mode packing; but was found to be very suitable for the analysis of short oligomers as well as for final purification of larger oligomers that had been previously purified by anion exchange. A simple strong anion exchanger was likewise unable to reproduce the separation shown in Fig. 4 unless extreme mobile phase conditions and temperatures were employed.

It is interesting to note that gradient elution can be effectively used with the mixed-mode packings, in contrast to either ion-pairing techniques or RPC-5 and similar surface coated packings. One of the major advantages of chemically bonding the ligands to the surface, rather than relying upon external equilibria for the creation of the stationary phase properties, is the stability of the bonded phases to mobile phase and temperature changes.

Fig. 6 shows the separation of a pentadecameric oligonucleotide of the sequence CTCCTCTTCAGACGC from its dimer building blocks. Excellent selectivities for the chain and high column efficiencies were observed. This was a particularly difficult separation, since minor amounts of other chain sequences and partially synthesized chains were present. This separation was achieved on a 20:80 N^+–C_8 packing, with a mild methanol–water mobile phase.

The ability to change the stationary rather than the mobile phase becomes more important as the molecular weight of the solute increases. Solubility of the biopolymers at high concentrations of organic compounds sets an upper limit on the stationary phase hydrophobicity that can be tolerated. The multifunctional approach to bonding allows substitution of ionic for hydrophobic groups as the solubility limit of the mobile phase is approached. In addition, the composition of the stationary phase can be incrementally altered to suit the separation required.

Chain length determination

The identification of collected oligonucleotides can present a problem as their length and complexity increase. While an absolute sequencing technique can be used, such as that of Maxam and Gilbert[15] or Sanger and Coulson[16], less time-consuming methods, such as simple chain length and base composition determination, can often confirm the presence or absence of the desired sequence during the various purification steps. It has previously been shown that the chain length, base composition, and leading and trailing nucleotide identies can easily be determined by the simultaneous analysis of the nucleosides and mononucleotides resulting from the hydrolysis of the oligonucleotide with phosphodiesterase I or II[17]. A simple method of analysing nucleosides and mononucleotides is HPLC using ion-pairing techniques[1]. However, the multifunctional stationary phases can also be used, and more efficient separations can be accomplished in less time.

Fig. 7 shows the separation of the major deoxyribonucleosides and their mononucleotides on a mixed-mode column of 50:50 N^+-C_8 ligand composition. Total separation time was *ca.* 9 min, *versus* 68 min when tetrabutylammonium phosphate is used as the mobile phase. The use of tailor-made phases for specific separations can increase the speed and efficiency of analysis of classes of compounds, especially when their routine analysis justifies the effort of producing the phase.

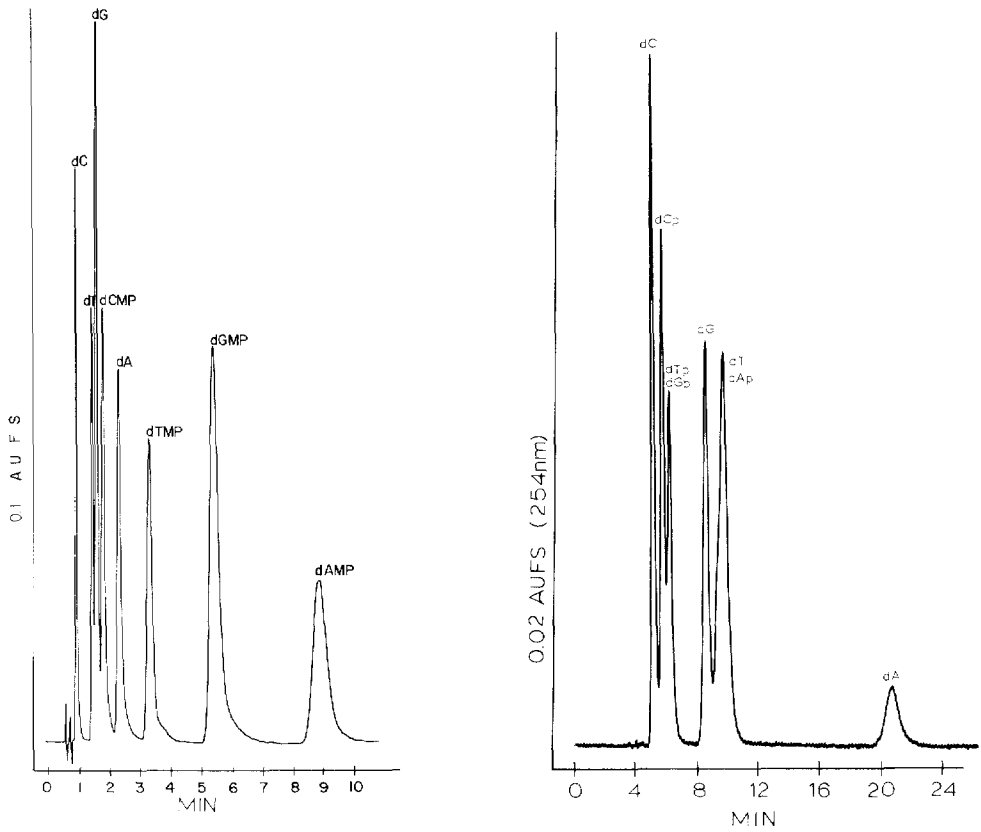


Fig. 7. Separation of the deoxynucleosides and their mononucleotides, on a multifunctional stationary phase of 80% $N^+ - 20\%$ C_8 packed into a 10-cm column. For the isocratic separation 10% methanol, 0.1 *M* KH_2PO_4 (pH 4.8) was used as the mobile phase at a flow-rate of 1.8 ml/min.

Fig. 8. Separation of deoxynucleotides and mononucleosides in a "mixed-bed" column containing a 50:50 weight ratio of Whatman anion exchanger and Whatmen C_8 10-μm material. Note that neither dTp/dGp nor dT/dAp are separated. Mobile phase conditions similar to those in Fig. 6, except flow-rate was 1 ml/min.

Comparison with mixed-bed columns

For lower-molecular-weight solutes, an alternative to the synthesis of multifunctional ion-exchange reversed-phase packings might be to mix physically ion-exchange and reversed-phase particles, and pack them in the same column. This approach was examined with SAX and C_8 packings, as shown in Fig. 8. A ratio of 50:50 was found to produce the best resolution of the nucleotides and nucleosides. However, even this best separation must be considered as inadequate when compared with the results of Fig. 7. The mixed-bed approach gave generally inferior results when compared with the multifunctional bonded phases, and cannot be used at all for high-molecular-weight solutes. With very hydrophobic solutes, the reversed-phase surface will act as a nearly irreversible adsorbent for the protein or oligomer. It appears from the data obtained thus far, that a retention mechanism different from that of simple additivity of ionic and hydrophobic particles results from the bonded

mixed ligands. This seems reasonable in light of the close proximity of the ligands and the complex interaction of Donnan and solvophobic forces involved. Further studies are underway to elucidate the mechanisms of separation on multifunctional phases.

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