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Comparison of Retention and Binding Behavior of dUTP and Biotin-Conjugated dUTP Using an Immobilized Silver Ion Chromatography Support

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ABSTRACT

A gel filtration chromatographic packing was modified to contain immobilized silver ions in order to study the retention and binding behavior of biotin-labeled b-dUTP versus dUTP. The immobilized silver column retains unlabeled dUTP (with the retention time depending on sodium chloride concentration in the mobile phase), but no affinity binding is evident with dUTP. In the absence of sodium chloride, dUTP was seen to have a retention time of 66 minutes using a 10.3-mL immobilized silver column, while b-dUTP is fully bound to the immobilized silver column. Approximately 90% of b-dUTP is recovered when b-dUTP is applied to the immobilized silver column using 10^{-3} M PBS and eluted using 0.2 M NaCl in the mobile phase. These results demonstrate the potential for using silver ions in immobilized soft metal affinity chromatography (ISMAC) in order to selectively target biotin labeled molecules. An analysis of the data yielding mathematical models with specific focus on the interaction between chloride and silver ions is provided in order to guide method development for other biotin-labeled oligonucleotides.

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INTRODUCTION

Immobilized metal affinity chromatography (IMAC) is a highly versatile method whose selectivity can be varied by a judicious choice of ligand and metal ion as well as by varying the modes of elution. Porath introduced the concept of metal chelate chromatography (1) which was later renamed immobilized metal affinity chromatography (2). This method was developed primarily for protein fractionation on the basis of the relative content of surface accessible imidazole, tryptophan, and cysteine residues as well as terminal amino groups, but in recent years there has been increased interest in separating nucleotides (3).

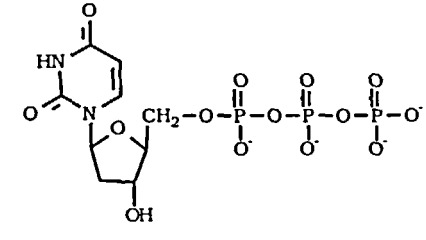
IMAC columns are generally packed with derivatives of a hydrophilic crosslinked biopolymer. Some of the insoluble matrix supports used include cellulose, polystyrene gels, polyacrylamide gels, porous glass, and agarose. The most commonly used metal ions are Zn(II), Ni(II), Co(II), and Cu(II) which are classified as borderline Lewis acids by Pearson (4). In this work Ag(I) has been the ligand of choice since soft acids have an affinity for soft bases such as thioethers (4). The purpose of this work is to extend the application of IMAC to the separation of biotin-labeled biomolecules by what we term immobilized soft metal affinity chromatography (ISMAC) using the soft metal ion Ag(I).

The thioether group on biotin is a particularly useful target ligand since it is a nonradioactive marker molecule used as a probe for the specific detection of target nucleic acid sequences (5). Biotin is currently widely used in DNA/RNA (6) detection due to the extremely high binding constants of biotin–streptavidin complexes. However, the downside of this technique is the difficulty in reversing the complex under reasonably mild conditions. Recovery of the biotin-labeled oligonucleotide molecule is crucial since oligonucleotides need to be released for subsequent use in recombinant DNA methods. Our studies are aimed at developing a useful, selective method employing ISMAC for recovering biotin-labeled oligonucleotides. Some of the intended applications are: 1) the isolation of full length oligonucleotides from failure sequences that result during automated solid support synthesis; 2) study of DNA/protein complexes; and 3) the isolation of PCR products resulting from incorporation of biotinylated primers.

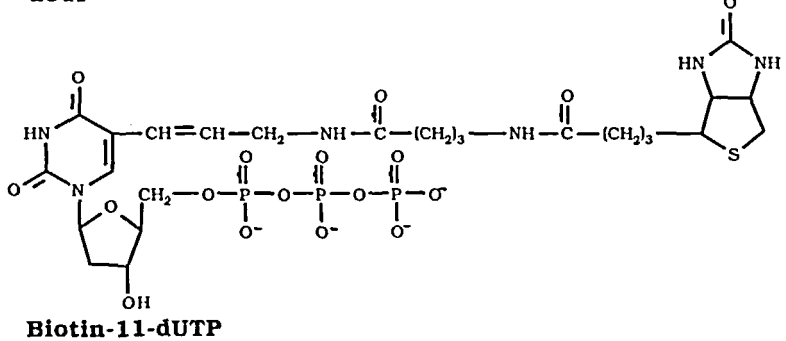
IMAC has been used to separate biotinylated proteins and nucleic acids. RecA-coated biotinylated probes have been used to recover plasmid DNA bearing a specific nucleotide sequence. Recovery of the DNA was accomplished by adding avidin to the reaction mixture followed by chromatography using a cupric iminodiacetic acid. However, in this case, complex-

ation of the copper ion with avidin, not biotin, resulted in retention of the conjugate on the column (7).

As a first step in developing a technique for selective recovery of biotin-labeled oligonucleotides, we chose to study the simple oligonucleotide dUTP with and without a biotin label (Fig. 1). One of our primary concerns was whether the highly charged phosphate groups would interact with the immobilized silver ions, thus causing loss of selectivity. Previous studies of ISMAC centered on amino acids and biotin-labeled BSA (8, 9) showed that chloride ions can play a competitive role for silver ion sites. Our studies with dUTP were aimed at manipulating sodium chloride concentrations in the mobile phase in order to moderate ion–ion interactions as well as in providing a very mild eluent for recovery of b-dUTP. Prior to the development of the immobilization method employing thiourea which is also used in this work, immobilized silver ions were easily removed from the stationary phase by low concentrations of phosphate and chloride salts (8). Thiourea-immobilized silver ions allow for phosphate and chloride salts to be used in the sample and mobile phases and, most impor-



dUTP



Biotin-11-dUTP

FIG. 1 Structures of dUTP and b-11-dUTP.

tantly, provide a valuable tool for eluting bound species since the interactions of chloride and silver ions are still quite strong.

Another focus of this study is on the reuse and long-term behavior of the immobilized silver column. In order to detect changes in column behavior as well as to aid chromatographers who wish to scale-up this technique, we employed a low pressure, low-end preparative scale (i.e., 1 cm I.D. × 13 cm; 10.27 mL) column in this work. While providing peaks of lower quality, the results provided here give a more representative picture of the behavior of a useful system when using oligonucelotides in recombinant DNA methods.

EXPERIMENTAL

Reagents

All chemicals used were of reagent or better grade. Henceforth either the reagent names or the nicknames will be used in this paper.

Equipment

A low pressure chromatography system (Bio-Rad Econo System, Bio-Rad Labs, Richmond, CA) was used in this work. The setup was designed to minimize tubing length in order to reduce dead volume and loss of pressure. Individual equipment in the chromatography system included: 1) a 1 cm I.D. × 20 cm long borosilicate columns with polymeric bed support; 2) a dual wavelength (254/280 nm) flow through a UV detector for monitoring of effluents. The optics module comes with a mercury lamp (with phosphor screen) and a 2-mm path length flow cell. Noise = 2.0×10^{-4} AU maximum from peak to peak (flowing liquid); 3) biocompatible silicone tubing (0.8 mm I.D.) for plumbing the system except for the peristaltic pump where Pharmed tubing (0.8 mm I.D.) was used; and 4) a two-channel, bi-directional, variable speed peristaltic pump programmable for fraction collections, fraction size, void volume, and total run volume. Degassing of the mobile phase was performed by vacuum degassing using an Ultrasound bath Model Branson 2200 (Baxter Scientific Product, McGraw Park, IL).

In addition to the chromatography system, a silver electrode Model 94-16 (Orion Research, Los Angeles, CA) was used to measure the amount of silver immobilized onto the Biogel P200 packing (Bio-Rad Laboratories, Hercules, CA). Mobile phase buffer pH was determined using a Corning multipurpose combination glass electrode and a pH meter (Fisher Scientific, Los Angeles, CA).

Preparation of Control and Silver Ion Modified Gels

A measured quantity of the Biogel P200 was placed in a 250-mL Erlenmeyer flask with an excess quantity of D.I. water, preferably in the ratio of 30 mL water per gram of dry gel in order to hydrate the gel. The Biogel series of resins offers a wide fractionation range. Biogel P200 has a fractionation range of 30,000 to 200,000 Daltons. This gel was selected in order to eliminate size exclusion effects so that oligonucleotides of up to 50 mers could be studied. The system was kept at room temperature for 24 hours, by which time the gel has swollen to a volume of 26.7 mL per gram of dry gel (8). The gel is thoroughly washed with D.I. water and set aside for reaction with glutaraldehyde (Sigma Chemical Co., St. Louis, MO) that has been reacted as follows in order to allow the glutaraldehyde to polymerize (9). The glutaraldehyde is polymerized at 70°C for 36 hours in a water bath. The reaction is given ample time for completion, and the unreacted glutaraldehyde remaining in the flask is washed at least six times with D.I. water.

Thoroughly washed hydrated gel is reacted with 25 wt% polymerized glutaraldehyde in the ratio of 35 mL of glutaraldehyde per gram of dry gel. The system is kept in a water bath equipped with a shaker at 50°C for 24 hours. The amido group on the gel reacts with the double bond of the glutaraldehyde. Hydrogen ion is evolved in the process (9).

Having covalently attached polymerized glutaraldehyde on the gel, reaction with thiourea (J.T. Baker Chemical Co.) provides the method for immobilizing silver, presumably by soft acid–base interactions with the sulfur atom of thiourea. After making sure that the glutaraldehyde-activated gel is washed thoroughly and is free of unreacted glutaraldehyde, 1.0 M thiourea is added to the system in the ratio of 25 mL per gram of dry gel. The reaction is allowed to be carried out for 24 hours at 50°C in a water bath with a wrist action shaker. After the reaction is complete, excess thiourea is decanted and the thiourea-activated gel is thoroughly washed with D.I. water.

The metal-free gel that we have developed is used as the control gel. A control column is prepared using this Biogel P200/Glut./Thio. gel. The control is compared to the gel activated with Ag(I) ions in order to measure the amount of retention of dUTP (Boehringer Mannheim, Corp.) and b-dUTP (Sigma Chemical Co., St. Louis, MO) on the immobilized silver gel. Henceforth in this paper, the Biogel P200/Glut./Thio. activated gel will be called the "control gel" and the corresponding column will be known as the "control column" or simply "control."

Having prepared the control gel, silver is immobilized on the gel. A 1.0 N $AgNO_3$ solution is added in the ratio of 0.5 mL per mL of swollen gel.

The reaction time is 24 hours at room temperature, at the end of which excess $AgNO_3$ solution is pipetted out and the gel is washed thoroughly with D.I. water. It is difficult to remove all of the excess $AgNO_3$ solution by batch washing, hence when the column is loaded with this gel the column is washed with D.I. water for 5 hours at a flow rate of 0.35 mL/min. This ensures that no free silver ions remain trapped in the gel. The amount of silver loaded was determined to be 0.02 mmol Ag(I) per mL of gel. The column was packed to a height of 13 cm for a packing volume of 10.27 mL, and therefore the silver ion loading on the column is 0.205 mmol. García et al. (9) showed that the immobilization of the Ag(I) ions is stable and that no leakage occurs even upon application of high sodium chloride concentrations in different buffers. This Biogel P200/Glut./Thio./Ag(I) gel will henceforth be known as the "immobilized silver gel" and the column as the "immobilized silver column."

Preparation of Mobile Phase Buffers

Six different buffer concentrations (10^{-5} M PBS, 10^{-4} M PBS, 10^{-3} M PBS, 10^{-2} M PBS, 10^{-1} M PBS, and 0.2 M PBS) were prepared. Phosphate buffer was prepared by mixing 152.5 mL of 0.2 M $NaH_2PO_4 \cdot H_2O$ and 97.5 mL of 0.2 M $Na_2HPO_4 \cdot 7H_2O$ and diluting the mixture to 500 mL with D.I. water. Sodium chloride was added in concentrations of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, and 0.2 M to prepare the corresponding saline buffers. All buffers were degassed before use, using an ultrasound bath and a vacuum pump.

Chromatography Conditions

The column was always equilibrated for at least 2 hours with the appropriate mobile phase buffer before a sample was run. The column was equilibrated for 30 minutes between runs before running the next sample. The columns were stored in D.I. water when not in use. It is interesting to note that possibly due to the antibacterial nature of Ag(I) ions, no bacterial growth or degradation of performance was noted on the immobilized silver column even after several months of storage. Nearly all chromatography experiments were run at the conditions given in Table 1. Any exceptions or additions to these conditions are noted in the discussion section wherever necessary.

Column Inter- and Intravoid Volume Determinations

The void fraction for unactivated Biogel P200 was determined by a batch technique, while the control and silver gel void volumes were mea-

TABLE 1
Column Conditions for the Experiments in this Work

Parameter	Value
Bed volume	0.79 cm^2 × 13 cm = 10.27 mL
Phosphate buffer concentration	0.05 M
Flow rate	0.35 mL/min
Sample volume	106 μL
Sample concentration	10 μM
UV range	0.02 AUFS
UV wavelength	280 nm

sured using a column technique. The immobilized silver column void volume was determined by chromatography with sodium benzoate. The batch and column techniques are compared in order to establish the elution volume for species that do not bind to the immobilized silver column and are not excluded from the intraparticle volume.

The batch technique used for measuring Biogel P200 inter- and intraparticle void volume ($V_0 + V_p$) consisted of weighing the dry gel and transfering the gel to three Erlenmeyer flasks where 12.000 mL of D.I. water was accurately added to each flask. The gel was allowed to swell for 48 hours, after which a 0.1 M solution of a solute (sodium benzoate or NaCl) was added to each flask. The flask was rotated gently for a period of 24 hours, and then aliquots of the supernatant were removed for analysis. Sodium benzoate concentrations were measured from the UV absorbance at 265 nm. Chloride concentrations were measured by titration with a silver nitrate stock solution. The total void fraction was calculated by assuming that any difference between the volume permeated by the solute and the total volume in the flask was equal to the apparent volume of the hydrated solid gel. The total void fraction could then by determined as

$$\epsilon_0 = 1 - \frac{(V_{gel} - V_{supernatant}) - (N_{solute}/C_{solute})}{V_{gel}}$$

The average value for the total void fraction determined in this fashion was 0.957. The total void fraction was also calculated as

$$\epsilon_0 = \frac{V_{gel} - (V_{gel} + V_{supernatant} - V_{water})}{V_{gel}}$$

The total void fraction calculated using this equation was 0.964. The data taken are summarized in Table 2.

TABLE 2
Determination of Void Fraction of Biogel P200 Using a Batch Method

Flask	Mass of gel (g)	V_t (mL)	Amount of 0.1 M solute added (mL)	Supernatant + V_t (mL)	Final concentration of solute (mmol/mL)	Void fraction, ϵ
A	0.400	10.673	0.1992[a]	14.377	0.01434	0.955
					0.01442	0.947
					0.01433	0.956
B	0.400	10.673	0.1992[b]	14.377	0.01436	0.954
					0.01412	0.975

[a] Sodium benzoate.
[b] NaCl.

Experiments were also run to determine the void volume of both control and immobilized silver columns. Sodium benzoate was chosen as the solute since it is well within the exclusion limit of Biogel P200 and it is known not to interact with the control gel (8). There may be some ionic interactions with the immobilized silver column. The results of these experiments were compared to the batch experiments described above to explore this issue.

The columns were first washed with D.I. water and then equilibrated until a stable baseline was achieved. The buffer and columns were prepared as discussed above. Run conditions were also as given above. Sodium benzoate was prepared in 0.05 M phosphate buffer. A 106-μL sample of 2.5×10^{-5} M sodium benzoate solution was then injected into the column and the experiment was run for 60 minutes. The results are shown in Fig. 2.

It should be noted that we also observed that the control column void volume varied depending on the flow rate. Also note that based on the batch experiments, a retention time of about 28 minutes was expected for the control column. We interpret the unusually low retention time for the control gel as due to instability of the gel. Low void volumes indicate that sections of the column have effectively been reduced due to gel compression in the control column. Gel collapse was also observed visually, especially at the top of the control column. This behavior was not found for the immobilized silver column, presumably due to structural reinforcement by the immobilized silver ions.

While the expected retention time is 28 minutes based on the batch experiments with the control gel, the immobilized silver column gave a

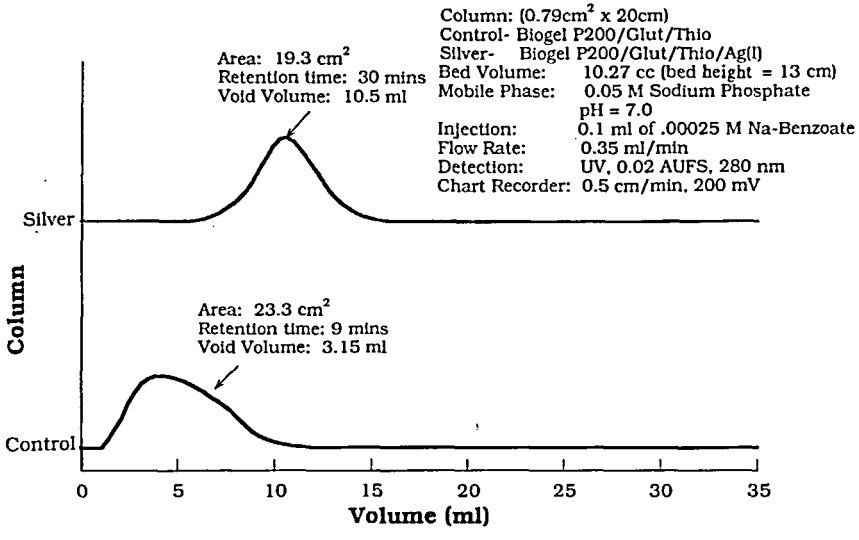


FIG. 2 Sodium benzoate injection in order to determine control and immobilized silver column void volumes.

retention time of 30 minutes. A retention time of 28 minutes was found for dUTP using the immobilized silver column when the mobile phase contained 0.1 M NaCl. The 2-minutes discrepancy is believed to be due to weak, nonspecific interactions which can be eliminated using moderate concentrations of NaCl in the mobile phase.

RESULTS AND DISCUSSION

Control Column Results with dUTP

Four different salt concentrations ranging over four orders of magnitude were investigated in order to study the effect of salt on the retention of dUTP. The experiments were run as described above. Plots and results are shown in Fig. 3. As the sodium chloride concentration is increased from no salt to 0.1 M NaCl, the area of the peaks increases with a maximum at 0.01 M PBS and then decreases as the salt concentration is increased further. The retention times are comparable to the sodium benzoate experiments described above, thereby leading to the conclusion that dUTP does not interact with the control column.

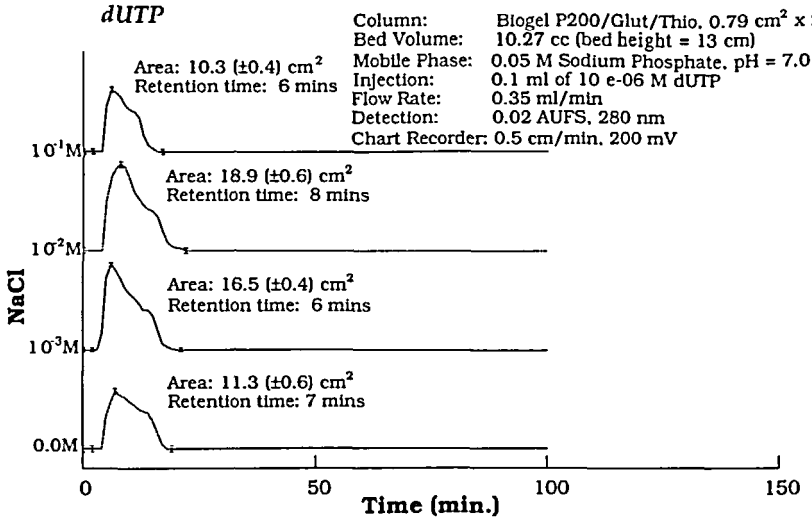


FIG. 3 Effect of varying the mobile phase salt concentration on the retention and peak area of dUTP on the control column. The area given for each chromatogram is the average and standard deviation for three separate chromatograms.

Immobilized Silver Column Results with dUTP

The results from chromatography of dUTP using the immobilized silver column is given in Fig. 4. They show the same trend as was seen on the control column except for the fact that with no saline in the buffer, dUTP shows a higher retention time and also considerable peak spreading. The retention times of the peaks above 10^{-3} M are also roughly the same. Small differences can be attributed to packing changes and flow characteristics during the runs. Nevertheless, the trend of an increase in the area of the peaks up to 0.01 M PBS and then a decrease on a further increase of salt concentration is the same. Except for the 0.05 M PB (no salt) run, the retention time for the other salt concentration runs is between 28 and 30 minutes. It was shown above that for sodium benzoate the retention time is 28 minutes. The increased retention time when no saline is present may be attributable to ion–ion interactions of dUTP with immobilized Ag(I) ions.

Experiments were performed with lower salt concentrations (10^{-5} and 10^{-4} M NaCl) in order to better understand the effect of chloride ions. These results are shown in Fig. 5. These runs show a decrease in retention time and also peak spreading as the salt concentration is increased from

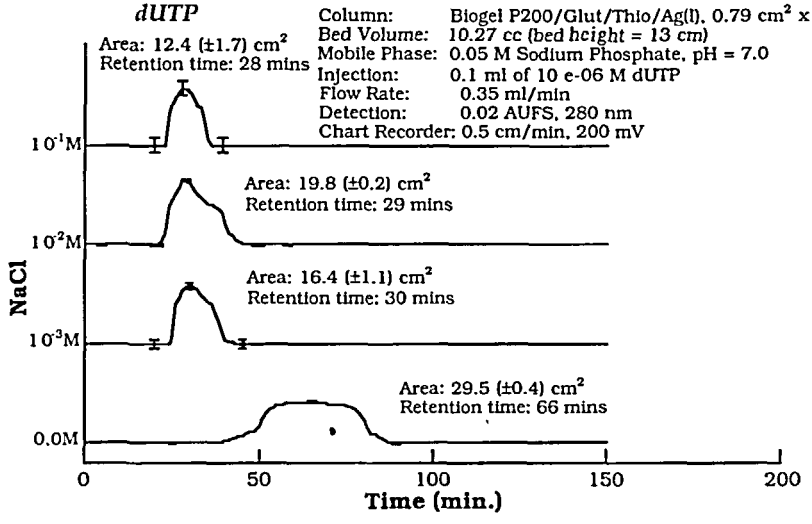


FIG. 4 Effect of varying the salt concentration in the mobile phase on the retention and peak area of dUTP on the immobilized silver column. The area given for each chromatogram is the average and standard deviation for three separate chromatograms.

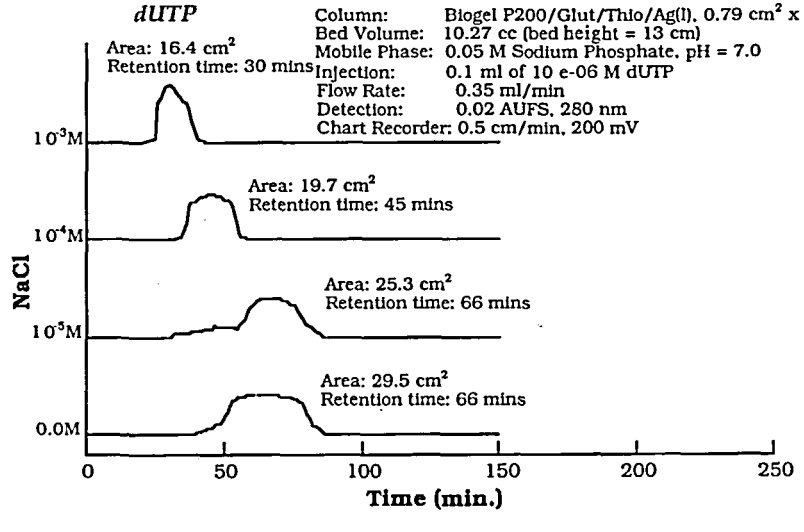


FIG. 5 Effect of varying the salt concentration in the mobile phase on the retention time and peak area for dUTP on the immobilized silver column.

no salt to 10^{-3} M NaCl. The decrease in retention time can be explained as a decrease in ion–ion interactions between dUTP and silver ions. Chloride masks the immobilized silver ion sites and prevents dUTP from interacting with the silver ions. At 10^{-2} and 10^{-1} M NaCl the ion–ion interactions between dUTP and the silver ions on the column become sufficiently suppressed so that the retention time tends toward a constant value. This suggests that at 10^{-3} M chloride ion concentration or higher, dUTP interaction with the silver ions can be virtually eliminated. Peak spreading might be the result of the complex nature of interactions between the antichaotropic solvent and the solute (10, 11).

Control Column Results with b-dUTP

Chromatograms for b-dUTP interactions with the control column are shown in Fig. 6. The antichaotropic effect of the salt is clearly visible here too, but it is more prominent here than it was with the dUTP runs. It is reasonable to expect stronger interactions because of the presence of biotin and C_{11} hydrophobic groups in the solute. Retention times are more or less the same, and slight variations may be due to packing and flow characteristics. As expected, peak area increases with increasing salt

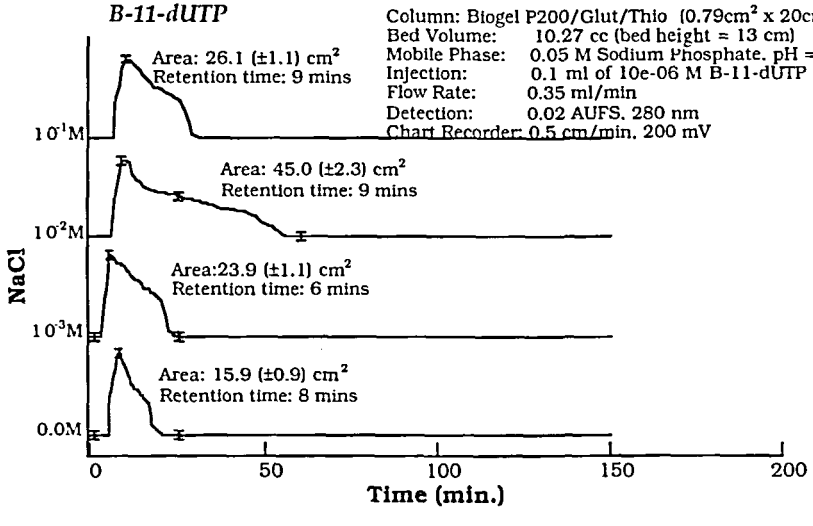


FIG. 6 Effect of varying the salt concentration in the mobile phase on the retention and peak area of b-dUTP on the control column. The area given for each chromatogram contains the average and standard deviation for three separate chromatograms.

concentration, but the peak for the 10^{-3} M PBS run has a considerable shoulder, due likely to the antichaotropic nature of the phosphate buffer.

Retention times are more or less the same as expected, and in conjunction with the sodium benzoate and dUTP experiments, it can be concluded that no retention or affinity interactions take place on the column.

Immobilized Silver Column Results with b-dUTP

Experimental results for the application of b-dUTP to the immobilized silver column are given in Fig. 7. It can be seen from the data and plots that if no saline is passed through the column, 100% of the b-dUTP injected is retained on the immobilized silver column. However, we did not determine the capacity of the column with no sodium chloride in the mobile phase since b-dUTP is rather expensive. The retention percentage decreases as salt concentration is increased to 0.1 M PBS. The column capacity decreases as salt concentration is increased. It is also noticed that the retention time for 0.01 M PBS is slightly higher than for others. The antichaotropic effect of the salt is also seen although it is less obvious here than it was with the control run because of the affinity binding of the

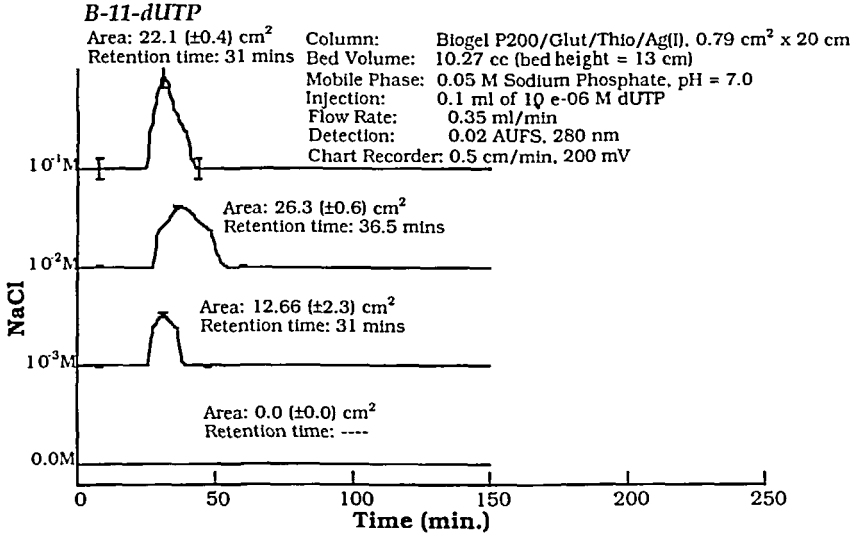


FIG. 7 Effect of varying the salt concentration in the mobile phase on the retention and peak area for b-dUTP on the immobilized silver column. The area given for each chromatogram is the average and standard deviation for three separate chromatograms.

solute to silver ions. Affinity binding dominates over all other nonspecific interactions.

It was seen that dUTP is strongly retained on the immobilized silver column due to ionic interactions when there is no NaCl in the mobile phase buffer. Also, the addition of small amounts of NaCl lowers the dUTP retention time. However, b-dUTP is bound to the immobilized silver column and is not eluted until a much higher concentration of saline is introduced into the mobile phase, as will be shown in the next section.

Recovery of b-dUTP from the Immobilized Silver Column

Based on observations that the mobile phase chloride ion concentration lowers the amount of affinity binding of b-dUTP, experiments were carried out in order to determine the extent to which bound b-dUTP can be recovered from the immobilized silver column. Experiments were carried out on the immobilized silver column where the b-dUTP sample was applied to the column using 10^{-3} M PBS buffer and eluted with 0.2 M PBS. After zeroing the baseline when the buffer was changed and comparing the effect of the buffer change on the detector using the control column, the amount of b-dUTP recovered was determined to be 90% (11). This result indicates that the soft acceptor/donor affinity binding can be reversed using the relatively mild and, certainly inexpensive, buffer concentration swing method.

Mathematical Models for Interpreting Immobilized Silver Column Data

dUTP Model for the Effect of Chloride Ions

The effect of chloride ions on the chromatography of dUTP on the immobilized silver column is interpreted based on the work of Noctor, Wainer, and Hage (12) who derived equations to predict competitive displacement at a single site and when solute binds to a secondary site unaffected by the competing agent. They showed that if a single site is involved in binding the solute (dUTP) as well as the competitive agent (Cl^-), the capacity factor is determined by the following equation:

$$\frac{1}{k'_{\mathrm{dUTP}}} = \left(\frac{V_0 K_{\mathrm{d}}}{K_{\mathrm{I}}[\mathrm{Ag_T}]}\right)[\mathrm{Cl}^-] + \frac{V_0 K_{\mathrm{I}}}{[\mathrm{Ag_T}]}$$

where the equilibrium constants are based on the following reversible reactions:

$$\mathrm{Ag^+—dUTP} \xleftrightarrow{K_{\mathrm{d}}} \mathrm{dUTP} + \mathrm{Ag^+}$$

$$Ag^+—Cl^- \overset{K_I}{\longleftrightarrow} Cl^- + Ag^+$$

and the capacity factor is determined using the void fraction obtained from the batch measurement for Bio Gel P-200. However, the data obtained in our system exhibited deviation from the simple site competition relationship in a manner similar to the systems investigated by Noctor and colleagues. In order to rationalize the deviation, they proposed that the solute binds to sites on the affinity packing where the competitor has no interaction with the solid phase. Noctor and coworkers (12) postulated that the above equation be modified to the following form:

$$\frac{1}{k'_{dUTP} - X} = \left(\frac{V_0 K_d}{K_I[Ag_T]}\right)[Cl^-] + \frac{V_0 K_I}{[Ag_T]}$$

where X is an adjustable parameter obtained by fitting the data. This approach can also account for the tendency of dUTP to bind to sites on the immobilized silver columns which would likely be less attractive to chloride ions since the interactions between silver and chloride ions are quite strong in aqueous solution. Fitting the data for chloride concentrations between 0 and 10^{-2} M provided a straight line ($r^2 = 0.9986$) using the values of $X = 0.03$, $K_d = 3.7 \times 10^{-6}$ and $K_I = 1.05 \times 10^{-4}$. It should be noted that since the retention volume at a chloride concentration of 0.1 M was equal to the void volume, this datum point could not be used in the regression because the inverse of the retention factor is infinite. Also, the value of K_I is highly dependent on the selected value of X for the best fit, especially for X values greater than 0.025. K_d also depends on X, but to a far lesser extent. This is due again to the use of data at higher chloride concentrations where the capacity factor is close to zero. Based on these observations, we recommend that chromatographers use the values $K_d = 5 \times 10^{-6}$ and $K_I = 10^{-3}$–10^{-4} in order to estimate the effect of chloride ions at other conditions.

b-dUTP Model for the Effect of Chloride Ions

In order to predict how the affinity binding of b-dUTP is affected by the chloride concentration in the mobile phase, we used the strategy of starting from a discrete stage model of the continuous packed column. Various discrete stage models have been used to rationalize chromatography peformance such as the number of theoretical plates and the height equivalent of a theoretical plate (H.E.T.P.). From the discrete stage model, we then used the relationship between chloride ion concentration and the fraction of b-dUTP bound as the basis for obtaining a structure for correlating the data and obtaining a predictive empirical equation.

For the discrete stage model it is assumed that the rate of change of b-dUTP in the immobilized silver gel phase is equal to a second-order, irreversible reaction dictated by the concentration of immobilized silver ions and the concentration of b-dUTP in the liquid phase. Thus the species balance for b-dUTP in the nth stage is given by

$$Q(C_{n-1} - C_n) = \epsilon V_n \frac{dC_n}{dt} + (1 - \epsilon)V_n \frac{dq_n}{dt}$$

where

$$dq_n/dt = kC_n[\mathrm{Ag}^+]$$

We further assumed that the concentration of free silver ions is lowered solely by the presence of chloride ions in the mobile phase since the amount of b-dUTP injected into the column is quite small. Based on a simple mass action expression, this suggests that

$$[\mathrm{Ag}^+] = \frac{[\mathrm{Ag_T}]}{1 + \dfrac{[\mathrm{Cl}^-]}{K_1}}$$

so that when the chloride concentration is zero, all of the immobilized silver ion sites are available for affinity binding to b-dUTP. Using the boundary and initial conditions which simulate the injection of a perfect pulse of b-dUTP at a concentration of C_f and solving the above equation using the Laplace transforms provides the following equation for the fraction bound:

$$\frac{C_n}{C_f} = \frac{\left(\dfrac{Qt}{\epsilon V_n}\right)^{n-1} \exp\left\{-\left(1 + \left(\dfrac{1 - \epsilon}{\epsilon}\right)\left(\dfrac{K_1[\mathrm{Ag_T}]}{K_1 + [\mathrm{Cl}^-]}\right) kt\right)\right\}}{(n - 1)!}$$

with the initial and boundary conditions:

$$@\ t = 0;\ C_n = 0$$

$$@\ t > 0;\ C_0 = C_f\delta(t)$$

Because this equation contains several adjustable parameters such as the number of stages (n), the reaction constant (k), and the equilibrium constant (K_1), rather that attach a specific meaning to these parameters it is more useful to use the discrete stage analysis in order to rationalize an empirical model. The equation above suggests that the affinity binding data for b-dUTP can be fitted by finding the best linear fit to a plot of $\ln(C_n/C_f)$ vs $1/(a + [\mathrm{Cl}^-])$ where a is an adjustable parameter. After fitting

the data, the best least-squares fit yielded $r^2 = 0.9355$ where the parameters and the empirical equation for predicting the effect of chloride on the fraction of b-dUTP bound was determined to be as follows:

$$\ln\left(\frac{C_n}{C_f}\right) = -1.772 + \frac{3.83 \times 10^{-3}}{2.25 \times 10^{-3} + [Cl^-]}$$

CONCLUSIONS

Biotin-labeled dUTP can be bound to immobilized silver ions under conditions where unlabeled dUTP is only retained, and not bound, on an immobilized silver column. Chloride ions can moderate ion–ion interactions between immobilized silver ions and dUTP as well as compete for affinity binding sites with b-dUTP. The retention time of dUTP can be shortened by adding sodium chloride, and at rather low salt concentrations (0.001 M NaCl and higher) the retention time of dUTP shows that there is no interaction with the column. At 0.001 M NaCl, about half of the b-dUTP applied to the column is bound to the column while all of the applied dUTP exits the column. About 90% of the bound b-dUTP can be then recovered merely by adding 0.2 M sodium chloride to the mobile phase. Mathematical models are provided to describe the competitive effect of chloride ions with chromatography of dUTP and b-dUTP on the immobilized silver column. Reproducible results with the immobilized silver column shows that addition of sodium chloride and various mobile phase conditions do not degrade column performance. These results suggest that immobilized silver ions could be useful in separating biotin-labeled oligonucleotides from their unlabeled counterparts.

SYMBOLS

$[Ag_T]$	total initial concentration of immobilized silver sites
C_f	feed concentration of b-dUTP
C_n	concentration of b-dUTP leaving the nth stage
$[Cl^-]$	equilibrium concentration of chloride ions
K_d	dissociation constant of dUTP-immobilized silver complex
k'_{dUTP}	capacity factor for the retention of dUTP
K_I	dissociation constant of the inhibitor, chloride-immobilized silver complex
V_e	volume required to elute a sample
V_{gel}	total volume of swollen gel ($V_s + V_0 + V_p$)
V_0	interparticle void volume of the column (mL)

V_p	internal pore volume of the gel (mL)
V_s	solid volume of the gel (mL)
$V_{supernatant}$	volume of supernatant above the gel after swelling and settling
V_t	total volume of the column
X	adjustable parameter
ϵ_0	total void fraction of the gel ($[V_0 + V_p]/V_t$)
ϵ	void fraction of the column (V_0/V_t)

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