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Note

Preparative separation of nucleotides by high-performance-ion exchange liquid chromatography using a volatile buffer system

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Purification of purine and pyrimidine nucleotides is often required prior to use in biological studies. Many excellent procedures for separations of nucleotides by either reversed-phase[1–4] or ion-exchange[5–9] chromatography exist but few systems allow easy removal of the eluent buffer. The most common procedure employing a neutral volatile buffer which uses a triethylammonium bicarbonate (TEAB) gradient system with DEAE cellulose was developed by Porath[10] and popularized by Smith and Khorana[11]. Other anion-exchange procedures used a Dowex 1 resin eluted with a stepwise gradient of buffers containing hydrochloric acid, formate or acetate[12]. Recently, high-performance liquid chromatographic (HPLC) procedures for nucleotide separations have been developed using strong anion-exchange resins eluted with volatile acidic buffers, such as hydrochloric acid or trifluoroacetic acid[13]. Linz[14] has reported nucleotide separations on a strong anion-exchange resin eluted with ammonium carbonate. Mahoney and Yount have purified micromolar quantities of nucleotides using reversed-phase HPLC with a TEAB–ethanol solvent system[15]. Alpert and Regnier[16] have reported the resolution of 5′-mononucleotides on a weak anion-exchange resin eluted with potassium phosphate (pH 3.0).

We have developed procedures for the purification of nucleoside mono-, di- and triphosphates on a high-performance weak anion-exchange support based on the use of the neutral volatile buffer TEAB for elution as described previously for DEAE cellulose[10,11]. The use of a neutral buffer system avoids hydrolysis of samples which occurs when acidic buffers are used for elution. Also, TEAB is easily removed by lyophilization. Separations were performed on both SynChroprep AX300 and SynChropak AX300 supports. Both isocratic and gradient elutions were used to effect separations of nucleotides.

MATERIALS AND METHODS

All nucleotides were purchased from Sigma. Triethylamine (TEA) was a product of Mallinckrodt and was distilled prior to use. All solutions were prepared in water filtered through a Millipore Milli Q water purification system.

TEAB was prepared by bubbling carbon dioxide through a 2 *M* TEA solution in a gas washing bottle at 4°C until no further change in pH was observed (pH 7.6–7.8). The actual concentration of the TEAB buffer was then determined by titration with a standardized hydrochloric acid solution.

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TEAB was removed from column effluents by lyophilization after shell freezing.

Separations were performed with a Glenco HPLC apparatus fitted with a 250 × 10 mm SynChroprep AX300 column or a 250 × 4.6 mm SynChropak AX300 column (products of Syn Chrom). Effluents were monitored at 254 nm or 280 nm using a Glenco 5680 UV detector. Flow-rates of 1.5–2.0 ml/min were maintained on the SynChroprep column with no detectable back pressure. All separations were done at room temperature.

To insure equilibration of the column, 200–300 ml of the initial elution buffer were pumped through the system prior to use. The column was washed with water at the end of each day. After extensive use the column was regenerated by thorough washing with water, 1.0 *M* acetic acid and then water again. The same SynChroprep column was used for many separations over several months with no signs of deterioration, requiring only occasional regeneration.

For routine analysis, 100 μl of a 1 m*M* neutralized nucleotide solution were injected. UV-absorbing fractions were collected and lyophilized. Freeze dried samples were dissolved in water and rechromatographed to determine purity and percentage recovery.

RESULTS AND DISCUSSION

Initial efforts in our laboratory on nucleotide separations were done with a SynChroprep AX300 analytical column. Both the SynChroprep and SynChropak supports are made of silica with a bonded polymeric layer of amine[16]. The SynChropak AX300 support has a particle size of 6.5 μm whereas the particle size of the

TABLE I

ISOCRATIC RETENTION TIMES OF COMMON NUCLEOTIDES*

Nucleotide	*Retention time*** *(min)*	*TEAB concentration (M)*
AMP	10.2	0.1
ADP	18.2	0.1
ATP	15.0	0.5
GMP	11.4	0.1
GDP	20.8	0.1
GTP	23.4	0.5
CMP	10.5	0.1
CDP	14.0	0.2
CTP	22.6	0.4
UMP	10.0	0.1
UDP	12.9	0.2
UTP	18.2	0.4
IMP	13.9	0.2
cAMP	9.6	0.1

* Flow-rate = 2 ml/min.

** Retention times are averages of 3–5 injections.

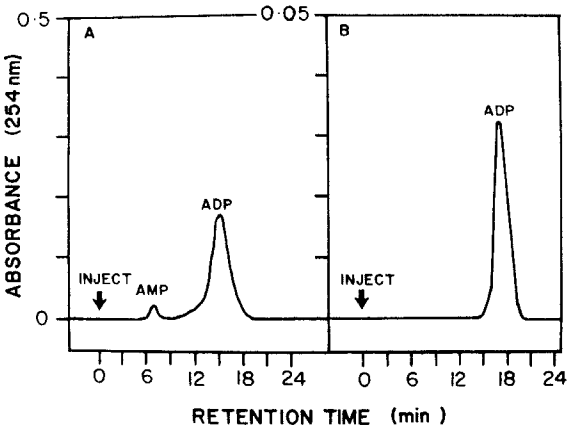

Fig. 1. Purification of ADP. Separations were performed on a 250 × 10 mm SynChroprep AX300 column eluted isocratically with 0.1 *M* TEAB at a flow-rate of 2 ml/min. (A) A volume of 100 μl of a 1 m*M* neutralized ADP solution was injected. The ADP peak was collected and lyophilized. (B) A volume of 1.0 ml of water was added to the lyophilized ADP sample. A volume of 100 μl of this solution was injected onto the column and was eluted under the same conditions as in A.

SynChroprep AX300 support is 30 μm. Resolution of nucleotides on the SynChropak AX300 support was somewhat better than that observed using the SynChroprep support. The smaller particle size and associated higher resolution of the SynChropak support make it very suitable for analytical work. However, due to higher operating pressures, bubble formation from the bicarbonate buffer on the low pressure side of the column made peak determination more difficult with the small diameter analytical SynChropak column. The use of a high-pressure detector and/or a large bore, short column (*e.g.* 50 × 10 mm) would eliminate this problem. The high capacity of the SynChroprep AX300 column combined with the ability to satisfactorily resolve nucleotides makes it useful in the preparative purification of nucleotides and was, therefore, our choice for the further studies described below.

Isocratic elution of nucleotides with TEAB affords several advantages over gradient elution. Resolution of individual nucleotides was found to be better using isocratic elution and equilibration times between runs was significantly reduced. Isocratic elution times for a number of common purine and pyrimidine nucleotides using the 250 × 10 mm SynChroprep AX300 column are shown in Table I. Based on these data, concentrations of TEAB can be chosen to afford maximum separation of mono- and dinucleotide pairs as well as mixtures of trinucleotides. Separation of adenine and guanine mononucleotides was accomplished by isocratic elution with 0.05 *M* TEAB (data not shown).

Fig. 1 shows the results of the isocratic elution of ADP. A 7% contaminant that was chromatographically identical to AMP was present in the commercial sample (Fig. 1A); no ATP was detected when the elution buffer was changed to 0.5 *M* TEAB. The effluent corresponding to the ADP peak was collected, lyophilized and resuspended in water. Upon rechromatography, no AMP was detected and 90% of the ADP was recovered (Fig. 1B). Recovery of an AMP sample under the same conditions was greater than 90% while 85% of a chromatographed ATP sample was

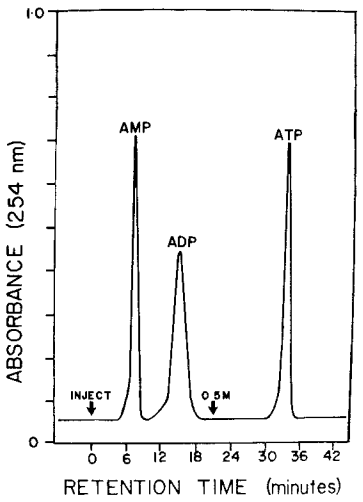

Fig. 2. Resolution of a mixture of adenine mono-, di- and trinucleotides. A volume of 100 μl of a neutralized solution containing 1 m*M* each AMP, ADP and ATP was injected onto a 250 × 10 mm SynChroprep AX300 column equilibrated with 0.1 *M* TEAB. AMP and ADP were eluted with 0.1 *M* TEAB. After the peak corresponding to ADP had been completely eluted, the buffer was changed to 0.5 *M* TEAB to effect elution of ATP. The flow-rate was 2 ml/min.

recovered using 0.5 *M* TEAB as elution buffer, with no AMP or ADP contaminants detected in purified samples. We were unable to obtain GTP free of GDP by this technique; the lyophilization step led to generation of a 6% GDP contaminant (this was approximately one half the level of GDP in the commercial sample). Improved procedures for removal of the buffer, such as flash evaporation, should allow quantitative recovery of GTP. We have used the isolated GTP fractions containing TEAB in enzyme reactions. No problems associated with the buffer could be detected thus indicating that for many applications removal of TEAB would not be required. The neutral pH of this buffer will allow direct use of column effluents more often than when more acidic conditions are used in HPLC separations.

The combined use of short gradients and isocratic elution at carefully chosen concentrations of elution buffer should allow separation of a wide variety of nucleotide mixtures. Fig. 2 shows the results of the separation of an equimolar mixture of adenine mono-, di- and trinucleotides. The column was equilibrated with 0.1 *M* TEAB and this buffer was used to elute AMP and ADP. ATP was eluted by changing the buffer to 0.5 *M* TEAB (ATP could be eluted with 0.1 *M* TEAB; however, the retention time was in excess of 90 min). The corresponding guanine nucleotides were completely resolved under the same conditions (retention times: GMP, 12 min; GDP, 19.2 min; GTP, 46.2 min). To determine the utility of the SynChroprep AX300 column in purifying preparative amounts of nucleotides, 55 mg of commercial ATP which had impurities of AMP and ADP was applied to the column and eluted isocratically with 0.5 *M* TEAB. The entire sample was retained by the column. Impurities of AMP and ADP were not resolved as separate peaks under these conditions, however, judicious sample collection resulted in nearly quantitative recovery of ATP which, upon reinjection, was free of any mono- or dinucleotides.

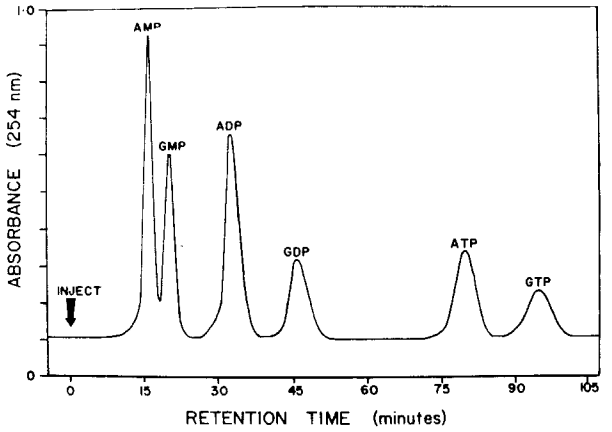

Fig. 3. Gradient elution of an equimolar mixture of adenine and guanine mono-. di- and trinucleotides. A volume of 100 μl of a solution containing 1 m*M* each AMP, ADP, ATP, GMP, GDP and GTP was injected onto a 250 × 10 mm SynChroprep AX300 column equilibrated with 0.1 *M* TEAB. Nucleotides were eluted with a 150 ml continuous concave gradient from 0.1 *M* to 0.5 *M* TEAB at a flow-rate of 1.5 ml/min.

To evaluate the resolving ability of the SynChroprep AX300 support an equimolar solution of adenine and guanine mono-, di- and trinucleotides was chromatographed. The results are shown in Fig. 3. The column was eluted with a 150-ml continuous concave gradient from 0.1 *M* to 0.5 *M* TEAB. AMP and GMP were not completely resolved in this buffer system; however, complete separation of these nucleotides can be achieved using 0.05 *M* TEAB as elution buffer. Adjusting the gradient to afford complete separation of mononucleotides resulted in extensive peak broadening of other components of the mixture. Complete resolution of all these peaks might be accomplished by a combination of an initial step gradient followed by the concave gradient.

The SynChroprep AX300 column exhibited high capacity (55 mg of ATP was completely retained) and is able to satisfactorily resolve nucleotide mixtures. Though resolution of nucleotides on the SynChropak AX300 column was better than on the SynChroprep column, the higher capacity of the latter column makes it more useful for preparative work. Additionally, the SynChroprep support can be dry packed and is considerably less expensive than the SynChropak support. Use of the SynChroprep AX300 column affords the preparative purification and separation of nucleotides in a simple, cost effective manner.

The procedures described here allow the rapid and quantitative purification of nucleotides using HPLC and a neutral volatile buffer system. TEAB is easily removed from column effluents by lyophilization and, because it is a neutral buffer, problems with hydrolysis associated with acidic elution systems are avoided. Careful choice of the concentration of TEAB will allow the resolution of mixtures of nucleotides. The methods reported here should have applications in the purification and separation of synthetic and radiolabelled nucleotides.

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REFERENCES

1 S. P. Assenza, P. R. Brown and A. P. Goldberg, *J. Chromatogr.*, 277 (1983) 305–307.
2 M. Ehrlich and K. Ehrlich, *Science,* 216 (1979) 319.
3 A. Wakizaka, K. Kurosaka and E. Okuhara, *J. Chromatogr.*, 162 (1979) 319–326.
4 T. F. Walseth, G. Graff, M. C. Moos and N. D. Goldberg, *Anal. Biochem.*, 107 (1980) 240–245.
5 B. Burnette, C. R. McFarland and P. Batra, *J. Chromatogr.*, 277 (1983) 137–144.
6 C. Garrett and D. V. Santi, *Anal. Biochem.*, 99 (1979) 268–273.
7 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651–662.
8 D.-S. Hsu and S. S. Chen, *J. Chromatogr.*, 192 (1980) 193–198.
9 K. Watanabe, W.-S. Chow and G. P. Royer, *Anal. Biochem.*, 127 (1982) 155–158.
10 J. Porath, *Nature (London)*, 175 (1955) 478.
11 M. Smith and H. G. Khorana, in S. P. Colowick and N. O. Kaplan (Editors), *Methods in Enzymology,* Academic Press, New York, 1963, Vol. 6, pp. 645–669.
12 W. G. Cohn, in S. P. Colowick and N. O. Kaplan (Editors), *Methods in Enzymology,* Academic Press, New York, 1957, Vol. 3, pp. 724–743.
13 J. T. Axelson, J. W. Bodley and F. Walseth, *Anal. Biochem.*, 116 (1981) 357–360.
14 U. Linz, *J. Chromatogr.*, 260 (1983) 161–163.
15 C. W. Mahoney and R. G. Yount, *Anal. Biochem.*, 138 (1984) 246–251.
16 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375–392.