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IMPROVED SEPARATION OF NUCLEOSIDES, NUCLEOTIDES, AND AMINOACYL tRNA ON A STRONG ANION-EXCHANGE RESIN

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SUMMARY

Nucleosides and nucleotides can be separated on a polystyrene–divinylbenzene anion-exchange resin, Chromex DA-X8-11 at 50° using isocratic elution with 1.0 M ammonium carbonate pH 8.8 or 0.05 M formic acid pH 2.4. The resin can also resolve aminoacyl tRNA on either the analytical or preparative scale.

INTRODUCTION

In recent years several chromatographic procedures have been developed for the separation and detection of nucleic acid components. Two-dimensional thin-layer chromatography (TLC) on cellulose or silica gel, coupled with tritium post labeling[1] is an extremely sensitive method, which has recently been applied to the complete nucleotide sequence analysis of tRNA[2,3]. Since this TLC procedure requires several days before the results can be analyzed, high-performance liquid chromatography (HPLC) has often been employed as an alternative when rapid analysis is desired. By using small particle diameter ion-exchange resins such as Aminex[4–8] and reversed-phase resins (RPC-5)[9,10] the complete analysis of nucleic acid components can be obtained within a few hours. We now wish to report the isocratic separation of nucleosides and nucleotides on a new polystyrene–divinylbenzene 8% cross-linked quaternary amine anion exchanger, Chromex DA-X8-11, and its application to RNA sequence analysis. In addition, we have also investigated the elution profiles of Walker 256 mammary tumor and rat liver tRNA^{Tyr} on Chromex DA-X8-11 to demonstrate the feasibility of this resin for determining alterations in the chromatographic behavior between normal and tumor aminoacyl tRNAs[5,11–14].

EXPERIMENTAL

The anion-exchange resin, DA-X8-11 (Lot No.: 71-81), was obtained from Durrum (Sunnyvale, Calif., U.S.A.). Nucleosides and nucleotide monophosphates, obtained from Calbiochem (La Jolla, Calif., U.S.A.), were dissolved in deionized distilled water. In some cases dilute HCl was added to enhance the solubility of purine nucleosides.

Chromatographic methods

Column chromatography was carried out in a stainless-steel jacketed column (30 × 0.5 cm I.D.) which was prepared by joining Swagelock end fittings (Akron Valve and Fitting, Akron, Ohio, U.S.A.) onto 316 stainless tubing (Tube Sales, Rocky River, Ohio, U.S.A.). A stainless-steel fritted end (Swagelock reducing union with an SR-17 snubber) served as the bed support. The column temperature was maintained at 50° by a Haake circulating water bath.

A Milton Roy pump (Mil-Roy-D) with a maximum pressure rating of 1000 p.s.i. was employed to obtain flow-rates of 0.5–1.0 ml/min. Typical eluent pump pressures measured with a WIKA pressure gauge (O. W. Heyman, Cleveland, Ohio, U.S.A.) ranged from 500 to 1000 p.s.i. Samples (25–50 μi, 0.2–0.4 A_{260} units* for nucleosides and nucleotides) were introduced using an off-column septum sample injector by means of a Hamilton syringe. The effluent was monitored at 254 nm with an Altex UV detector equipped with a 20-μl flow cell. Full-scale detector output ranged from 0.01 to 2.54 absorbance units and was displayed on a Laboratory Data Control-Servographic (Riviera Beach, Fla., U.S.A.) strip-chart recorder (10 mV) at a chart speed of 12 in./h.

The resin was slurried in 1.0 M ammonium carbonate, poured into the column, and packed by pumping with the same buffer for 60 min at a flow-rate of 1.0 ml/min. Equilibration was with 0.05 M formic acid (pH 2.4) for nucleic acid analysis under acidic conditions, 1.0 M ammonium carbonate (pH 8.8) for analysis under basic conditions or 0.1 M NaCl, 0.01 M sodium acetate (pH 4.5), 0.01 M MgCl$_2$, and 0.001 M sodium thiosulfate for aminoacyl tRNA analysis. All buffers were throughly degassed before use.

Analysis conditions

tRNA hydrolysis. E. coli tRNA^{Met} (0.3 mg) was hydrolyzed to nucleosides using a mixture of pancreatic ribonuclease, snake venom phosphodiesterase, and alkaline phosphatase[7]. Nucleotide 3′ isomers were obtained from a ribonuclease T$_2$ digest of tRNA^{Met} (0.3 mg)[15].

Eluted solutes were identified by comparing observed retention times with those of standard nucleosides or nucleotides. The identification of 3′ nucleotide isomers was verified by examination of both the elution positions and retention times of a 3′ nucleotide mixture obtained from a ribonuclease T$_2$ digestion of tRNA^{Met} as well as by observing the UV absorption curve of each eluted peak on a Cary Model 116C recording spectrophotometer.

Aminoacylation and chromatography of tRNA. Preparation, aminoacylation, and chromatography of tRNA isolated from rat liver and Walker 256 mammary carcinosarcoma tissue was accomplished as previously described[5,16,17]. Preparative aminoacylation[16] employed [^{14}C]tyrosine (50 μCi/μmole from Schwarz/Mann, Orangeburg, N.Y., U.S.A.) for tumor tRNA and [^{3}H]tyrosine (250 μCi/μmole from New England Nuclear, Boston, Mass., U.S.A) for liver tRNA. After incubation at 37° for 30 min, the aminoacyl tRNA was isolated by phenol extraction and ethanol precipitation[14].

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* A_{260} unit is defined as that amount of material per ml of solution which produces an absorbance of 1 in a 1-cm light path at 260 nm.

Approximately 20,000 cpm ³H-labeled rat liver tRNA^Tyr and 10,000 cpm ¹⁴C-labeled Walker tRNA^Tyr were combined by dissolving in 250 µl of buffer solution B (0.01 M sodium acetate pH 4.5, 0.01 M MgCl₂, and 0.001 M sodium thiosulfate) containing 0.1 M NaCl. After application of the sample, elution was performed at room temperature with a 73 ml concave gradient (42 ml buffer solution B containing 0.1 M NaCl and 31 ml of buffer solution B containing 1.2 M NaCl) and the flow-rate was maintained at 0.5 ml/min with a Milton-Roy pump. Fractions (0.5 ml) were collected in 2-ml (half-dram) vials (Rochester Scientific, Rochester, N.Y., U.S.A.). After the addition of 1.5 ml of Aquasol (New England Nuclear), radioactivity (¹⁴C and ³H) was determined by dual-label liquid scintillation counting in a refrigerated Nuclear-Chicago Isocap 300.

RESULTS AND DISCUSSION

Separation of nucleosides and nucleotides under basic conditions by anion-exchange chromatography

Chromatographic separations of nucleosides and nucleotides at pH 8.8 are shown in Figs. 1a and b. With the exception of the overlapping 2′,3′ CMP-UMP pair, all components are sufficiently resolved to permit identification and quantitation. Theoretically, an improved resolution of the overlapping CMP–UMP pair could be
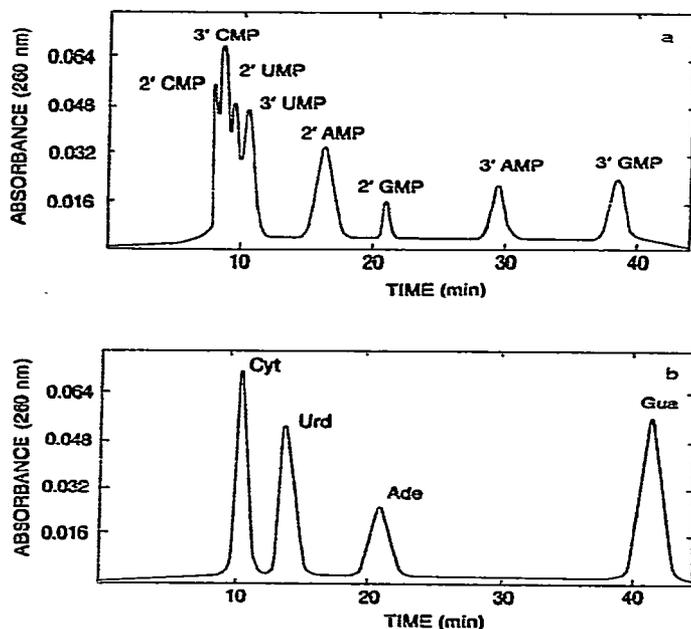

Fig. 1. (a) Separation of a mixture of 2′ and 3′ isomers of CMP, UMP, AMP and GMP on Chromex DA-X8-11. The resin bed was maintained at 50° during elution with 1.2 M ammonium carbonate pH 8.8 at a flow-rate of 1.0 ml/min. (b) Separation of cytidine, adenosine, uridine and guanosine nucleosides on Chromex DA-X8-11. Conditions were identical with those described in (a) except that 1.0 M ammonium carbonate pH 8.8 was the eluent.

achieved by choosing a higher eluent pH, which would increase the extent of the enolized form of UMP (pK_a 9.43)[18] and increase its retention time. Experiments aimed at improving the resolution of the CMP–UMP pair are presently in progress. Alternatively, by varing the ionic strength an improved CMP–UMP separation could be obtained since formation of the negatively charged enolized uracil base in the nucleotide is substantially favored at a higher ionic strength[18].

The elution order of the nucleosides is Cyt, Urd, Ade, Gua; this result is consistent with their ionic charges at pH 8.8. Cyt and Ade exist as non-ionized species (pK_a 4.15 and 3.6)[18] and therefore elute earlier. Urd (pK_a 9.2) and Gua (pK_a 9.25) exhibit a partial negative charge due to ionization of the oxo groups at pH 8.8 and therefore longer retention times. Non-polar interaction between the purine rings of Gua and Ade and the divinylbenzene cross-linked polystyrene matrix may contribute to the increased retention times of those solutes in comparison with their pyrimidine counterparts[7,19]. The same rationale can explain the similar elution order of nucleotide monophosphates.

Separation of nucleosides and nucleotides under acidic conditions by cation-exclusion chromatography

In ion-exclusion chromatography[7] solutes are repelled from the similarly charged exchange groups. In the pH range 2.4–2.8, the nucleoside bases Cyt and Ade are essentially fully protonated, they would be repelled by the resin's quaternary amine exchange groups and should therefore elute earlier. Such a result was obtained in the chromatographic separations for nucleosides and nucleotides at pH 2.8 and 2.4 shown in Figs. 2a and b. By changing the pH of the eluent, the charges on solutes can be altered, thereby optimizing the exclusion of selected species as shown in Fig. 3.

Application of this method to the separation of nucleosides in an enzymatic hydrolyzate of tRNA^{Met} is shown in Fig. 4. Here the modified nucleosides, pseudo-uridine and ribothymidine are resolved from the four unmodified nucleosides and can easily be quantitated. We are currently using this HPLC analytical technique to rapidly locate specific fragments from a ribonuclease T_1 digest of crude *E. coli* and mammalian tRNA for studies on the role of modified nucleotides in tRNA.

Both cation-exclusion and anion-exchange chromatography on Chromex DA-X8-11 can be used to separate nucleic acid components. Isocratic elution does not require the re-equilibration needed when gradient elution is used. In addition, the four nucleotides or nucleosides can be resolved more rapidly and at lower eluent pump pressures (500–1000 p.s.i.) than other ion-exchange procedures[7,20,21]. Our separations (Figs. 1, 2, and 4) are comparable to those obtained when similar solutes are chromatographed on Aminex resins. Shorter retention times are observed for nucleoside separations (25 min) using Chromex DA-X8-11, significantly enhancing the ease of analysis compared to chromatography on Aminex A-25[7], A-14[19], and A-6[22] under similar conditions.

Chromatography on aminoacyl-tRNA^{Tyr}

Fig. 5 shows the elution profiles obtained by co-chromatography of Walker 256 rat liver tRNA aminoacylated with [^{14}C]- and [^{3}H]tyrosine, respectively. As reported earlier[5], chromatography on Aminex A-28 resolves at least two isoaccepting species of tRNA^{Tyr}. As shown in Fig. 5 the two isoaccepting species of tRNA^{Tyr}
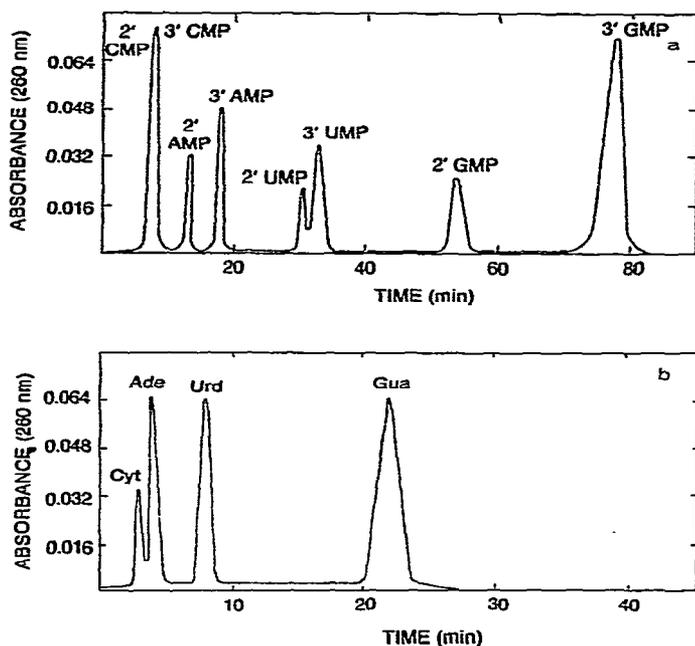
Fig. 2. (a) Separation of a mixture of 2′ and 3′ isomers of CMP, UMP, AMP and GMP on Chromex DA-X8-11. The resin bed was maintained at 50° during elution with 0.3 M ammonium formate pH 2.8 at a flow-rate of 1.0 ml/min. (b) Separation of a mixture of cytidine, adenosine, uridine and guanosine nucleosides on Chromex DA-X8-11. The resin bed was maintained at 50° during elution with 0.05 M formic acid pH 2.4, at a flow-rate of 0.5 ml/min.
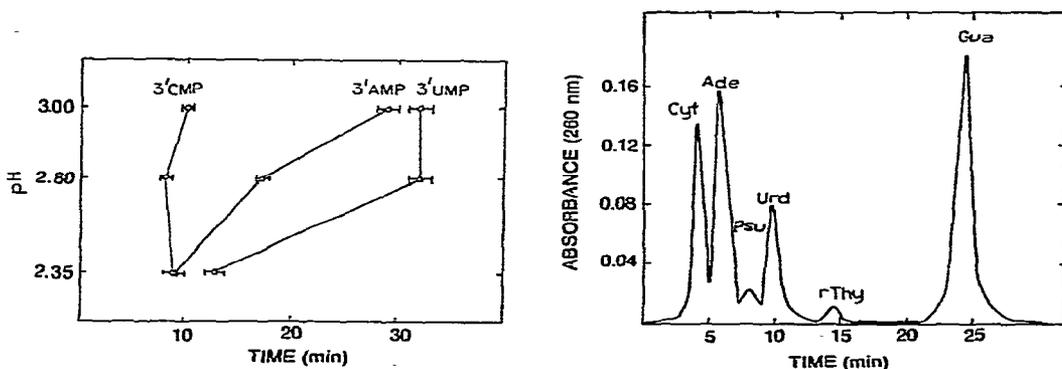

Fig. 3. Effect of pH on retention times for 3′ CMP, AMP and UMP. Bar indicates peak width at half height. Conditions are the same as those reported in Fig. 2.

Fig. 4. Separation of a nucleoside mixture obtained from the enzymatic hydrolysis of 0.6 A_{260} units of tRNA^{Met} on Chromex DA-X8-11. The resin bed was maintained at 50° during elution with 0.025 M formic acid pH 2.6 at a flow-rate of 0.5 ml/min.
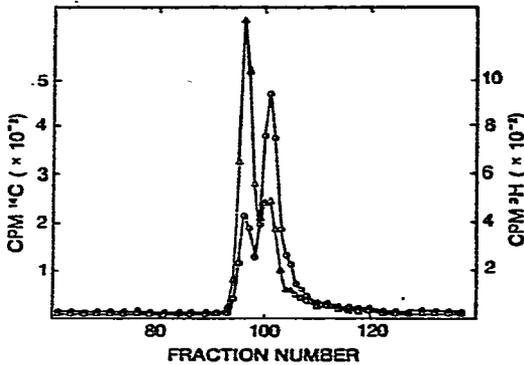
Fig. 5. Chromatography of [^{14}C]Tyr-tRNA^{Tyr} from Walker 256 tumor (O–O) and [^{3}H]Tyr-tRNA^{Tyr} (△–△). 35 A_{260} units of each aminoacyl tRNA were applied to a Chromex column, eluted, and the radioactivity determined as described in Experimental.

are also resolved on Chromex DA-X8-11 but the Chromex resin has the advantage that the required operating pressures (500 p.s.i.) are much less than the 4000 p.s.i. required for Aminex A-28.

·In conclusion, the resolution obtained by Chromex DA-X8-11 chromatography under both ion-exchange and ion-exclusion conditions is comparable to previously reported methods[7,19,22]. However, the major advantage of this particular resin is that the running pressure is greatly reduced at faster flow-rates and thereby, the total analysis time is considerably shortened. In addition this resin can also be employed as a sensitive analytical tool for the determination of alterations in tRNA from normal and tumor tissues with all the advantages mentioned above.

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