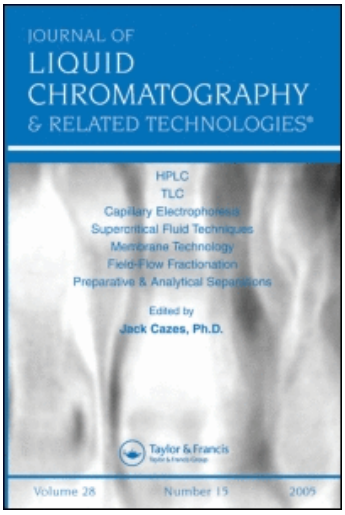

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High-Performance Liquid Chromatography of T-RNA

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF T-RNA

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ABSTRACT

Fractionation of amino acid specific tRNAs by descending salt gradient on a reversed-phase high-performance liquid chromatography column is demonstrated. Stationary phase variables such as n-alkyl chain length and silica type are discussed in terms of stability and selectivity. Mobile phase parameters including salt concentration, organic modifier, pH, and temperature are investigated. The concentration of organic modifier to adjust elution profiles can be critical in terms of tRNA retention.

INTRODUCTION

Fractionation of tRNAs has been investigated by many laboratories. The earliest method devised was liquid-liquid countercurrent distribution (1). Later, chromatography materials such as hydroxyapatite (2), DEAE-Sephadex (3,4), methylated albumin on kieselguhr (MAK) (5), and benzoylated DEAE-cellulose (BD-cellulose) (6) were used with some success. However, much higher resolution of oligo- and polynucleotides has been obtained with 1)

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reversed-phase chromatography (RPC-5) adsorbants (7), based on polychlorotrifluoro-ethylene beads (Plascon 2300) coated with a trialkylquaternary amine (Adogen 464), and 2) Sepharose 4B beads (2,8,9), which are carbohydrate polymers. Elution from both columns utilizes salt gradients, ascending for RPC-5 and descending for Sepharose 4B. Most interestingly, the common denominator of selectivity in both systems involves an ionically facilitated hydrophobic interaction. Narihara et al (10) have adapted a trialkylamine coating onto silica instead of Plascon beads via a silicone oil interface. The system allowed increased flow rates while retaining the selectivity of RPC-5 type supports.

This paper reports an increased flow rate system utilizing high-performance liquid chromatography (HPLC)[1] columns. The concept used for fractionation is a hybrid based on siliceous support materials with reversed-phase character (7) and a mobile phase similar to that used for Sepharose 4B chromatography (2).

Reversed phase liquid chromatography (RPLC) has been extensivley used in the life sciences because many biological molecules are at least slightly hydrophobic. To accomplish elution of hydrophobic species from RPLC columns it is common practice to use increasing concentrations of organic solvent. This technique works well with small molecules but has a disadvantage with proteins and polynucleotides: organic solvents in greater than 3-10% concentration may alter or destroy secondary, tertiary and quaternary structure of biopolymers (11). The general structure of tRNA species is maintained through base pairing and a variety of stacking interactions that stabilize its tertiary structure (12).

The literature cited above would suggest that a very weakly hydrophobic high-performance column should allow the control of tRNA retention with descending salt gradients and little or no organic solvent. It was the objective of this research to: 1) identify HPLC bonded phases that allow retention of tRNA when high salt concentration is used in the aqueous mobile

phase, then release tRNA when the salt concentration is reduced; 2) optimize the mobile phase in terms of resolution and selectivity; and 3) select the best silica matrix for the optimal mobile phase/bonded phase system. The method developed consists of a descending salt gradient on a reversed-phase column (DSG-RP). Fractionations of various tRNA isoacceptor species have been achieved in an hour or less.

DSG-RP was used to fractionate both yeast and E. coli tRNA mixtures. Alkylsilane bonded phases of the C_1, C_2, and C_4 type all worked well on macroporous, microparticulate silica to effect tRNA separations. A very low concentration of organic modifier such as propanol, ethanol, or methanol was found to enhance the fractionation process. Other variables such as initial salt concentration, temperature, and pH also affected DSG-RP selectivity.

MATERIALS AND METHODS

Hypersil (5μm) was purchased from Shandon Southern Instruments (Sewickley, PA). Vydac TP (5μm) was obtained from The Separations Group (Hesperia, CA). LiChrospher Si 500 (10μm) was purchased from E. Merck (Darmstadt, West Germany). n-Alkylchlorosilanes were purchased from Petrarch Systems (Levittown, PA). 1-Propanol was obtained from Burdick and Jackson Laboratories (Muskegon, MI). 2-Propanol was purchased from Fisher Scientific Company (Fair Lawn, NJ). Methanol was obtained from J. T. Baker Chemical Company (Phillipsburg, NJ). All solvents were HPLC grade. Absolute ethyl alcohol was purchased from U. S. Industrial Chemicals Company (New York, NY). Transfer ribonucleic acid (tRNA) No. R-9001 Type X from baker's yeast with amino acid acceptor activity for glutamic acid, phenylalanine, valine, and alanine was purchased from Sigma Chemical Company (St. Louis, MO). tRNA from Escherichia coli MRE 600 (RNase negative) specific for phenylalanine (CAT. No. 109 673), valine (CAT. NO. 109 720) and a mixture (CAT. No. 109 517) of lysine, phenylalanine, serine, and valine activity was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Preparation of Stationary Phases

n-Alkylation of silicas via silylation was achieved by methods analogous to those previously reported (13,14).

Column Packing

Supports were slurry packed (2% w/v) into columns with 2-propanol at 8000 psi by means of a pneumatic pump, (Haskel, Burbank, CA). LiChrospher Si 500 was packed at 4000 psi (10). All columns were 0.41 x 5.0 cm LiChroma stainless-steel precision tubing (Anspec Co., Inc., Warrenville, Ill) with 2.0 μm frits. A precolumn (0.41 x 4.5 cm) was fitted to the slurry vessel and connected to the analytical column to channel microparticulate silica into the column during the packing process. Allowed packing time was ca. 10 min.

High-Performance Liquid Chromatography

Analyses by HPLC were done using a Varian Vista System (Varian Associates, Walnut Creek, CA). The system was fitted with a Valco model 9080 sample injector (Anspec Company, Inc., Warrenville, IL) with a 100μl injection loop.

Mobile Phase Conditions

A binary gradient system was used for all tRNA separations. Buffer A contained ammonium sulfate and 0.2M potassium phosphate. Buffer B was 0.2M potassium phosphate. Initial ammonium sulfate concentrations, amount and type of organic modifier, temperature, and pH varied and are described in the text. Gradients were from 0 to 100% buffer B in 40 or 80 min. after which 100%B was left running isocratically until solute elution was complete.

Sample Preparation

Fresh tRNA samples of about 2μg/μl concentration were prepared every 2-3 weeks using buffer A as diluent. It was found that slightly better resolution

was achieved when tRNAs were prepared, stored, and injected in solutions not containing organic modifiers.

Recovery Determination

The tRNA profile in Fig. 6a was collected. A blank gradient was then collected for a background absorbance zero. The amount of tRNA injected onto the column in Fig. 5a was directly added to the collected blank gradient and a comparison was made. Recovery is reported as relative absorbance.

RESULTS AND DISCUSSION

Spencer (2,8) has shown that Sepharose 4B can be used for descending ammonium sulphate gradient elutions of tRNAs. The mechanism of retention is thought to be two-fold. First, the high ionic strength of the initial buffer induced "interfacial precipitation" of tRNAs, which could then be released as the salt concentration was reduced. Second, solubilized tRNAs experienced an "adsorptive retardation" through hydrophobic interactions with the stationary phase as they were eluted from columns. Manipulation of the latter mechanism has been shown to be more important for tRNA fractionation (8). The present study investigates this retention and elution protocal with regard to the design of a silica-based HPLC column.

Stationary Phase

Since Spencer found that relatively hydrophilic Sepharose 4B (a polymer of alternating D-galactose and anhydrogalactose residues) was sufficiently lipophilic for descending salt gradient chromatography of tRNAs, it was concluded that a short alkyl ligand attachment to silica would provide an optimal HPLC material. Short chain length supports have already seen some utility in the separation of biopolymers (15,16). The C_8 and C_{18} (ODS) ligands are used most often, but recent reports (16) have indicated low recoveries for some large solutes (>40,000 daltons) such as proteins. It has

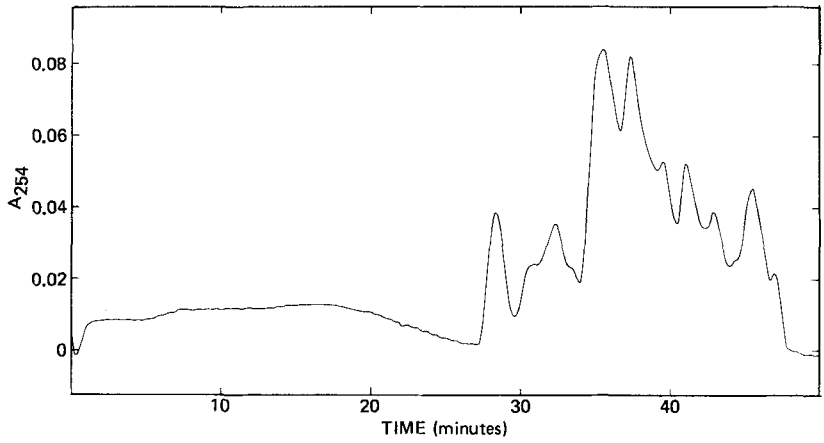

FIGURE 1 Fractionation of yeast tRNA on a reversed-phase column (0.41 x 5cm) packed with TMCS coated Vydac (5μ) silica. Buffer A, 2.0M ammonium sulfate and 0.2M potassium phosphate pH 7, Buffer B, 0.2M potassium phosphate pH 7. Gradient was 0-100% buffer B in 40 min. then 100% B for an additional 15 mins. Flow, 0.7 ml/min; sample, 50μg in 100μl buffer A; temperature, ambient.

been demonstrated that protein loadability (17), recovery (16), and resolution (17) could be increased by employing shorter n-alkyl ligands. Another advantage, relative to longer chains, is higher efficiency due to better mass transfer (18). Therefore, a trimethylsilylated silica support was prepared for these studies. Fig. 1 shows an elution profile of a yeast tRNA mixture high in glutamic acid, phenylalanine, valine, and alanine acceptor activity. tRNAs did not elute until near the end of the 40 min. gradient, which indicated strong retardation forces were still apparent. In an effort to negate the interfacial precipitation contribution to the retention mechanism (2) the column temperature was increased to 55°C- just below the 50-90°C transition range of tRNA melting (19). tRNAs eluted during the 40 min. descending gradient but the useful lifetime of trimethylchlorosilane (TMCS) bonded phase was approximately 2 hrs. at this temperature. A polymeric C_1

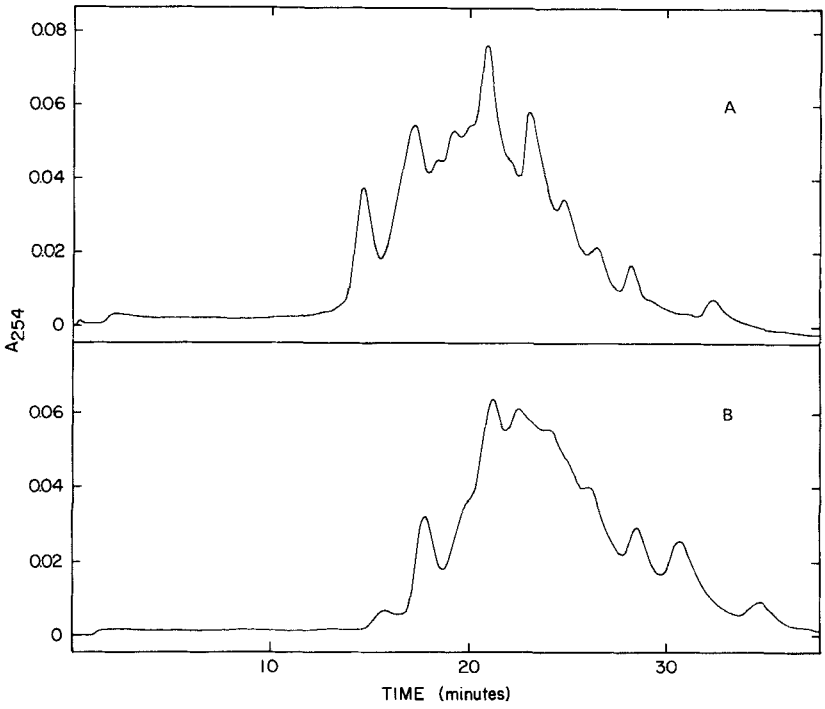

FIGURE 2 Effect of temperature on retention. Same conditions in Fig. 1 except packing was polymeric C_1 coated and loading was 200µg tRNA. A, gradient ran at 55°C; B, 24°C.

coating proved to be more temperature stable. As shown in Fig. 2 the yeast tRNA profile eluted about 3 min. earlier at 55°C as opposed to room temperature. This polymeric C_1 column reproduced four 55°C gradient elution profiles prior to the 24°C run in Fig. 2b. Polymeric C_1 columns could be run for about ten gradients under conditions of Fig. 2a before thermal exposure erroded the bonded phase and diminished resolution. Polymeric C_2 and C_4 coatings were much more stable at 55°C and were employed for the remainder of the study. The C_4 column was used for approximately 45 gradients, 20 of which were at 55°C. At room temperature, short n-alkyl chain columns have reportedly lasted over 200 hrs. (16).

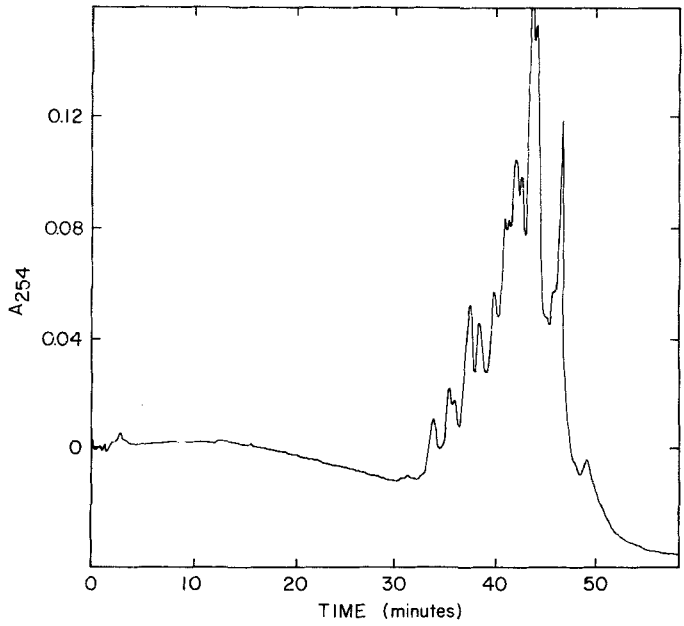

FIGURE 3 Fraction of yeast tRNA on a polymeric C_2 coated packing. Elution conditions as in Fig. 1 except temperature was 55°C.

Mobile Phase

Identification and manipulation of adsorptive retardation contributions on agarose media have been done by pH, temperature, column dimension and gradient slope variations (2,8). We have found that another parameter, addition of small amounts of alcohol to the mobile phase, can alter selectivity in tRNA fractionations (compare Fig. 3 with Figs. 4a and 4b). Interestingly, the less hydrophobic tRNAs have a marked difference in fractionation pattern depending upon whether 1% 1- or 2-propanol is used, although there seems to be no selective difference for the more hydrophobic tRNAs eluted in the last third of the gradient profiles (Figs. 4a and 4b). In general, peaks eluted faster using 1-propanol as opposed to 2-propanol. This same effect has been noted in reversed-phase peptide separations (20). In that type of system protein adsorption-desorption is thought to be a result of

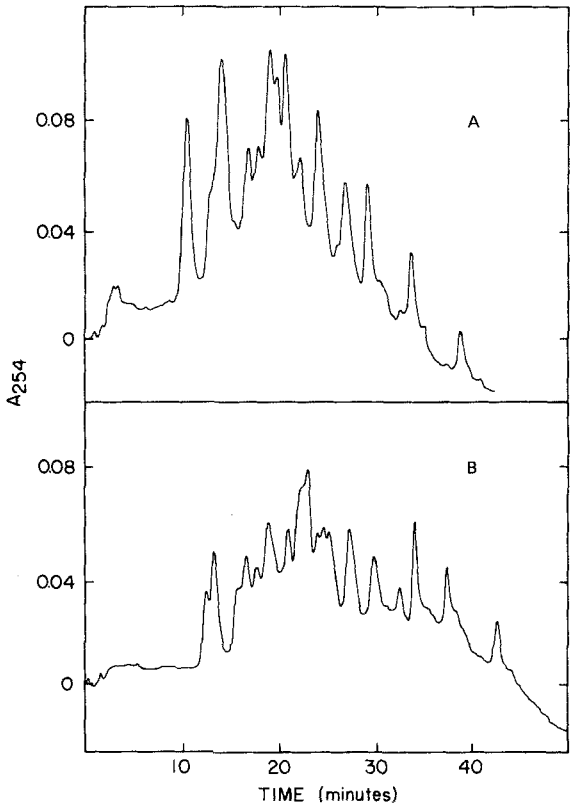

FIGURE 4 Beneficial effect of organic modifier on tRNA retention and resolution. Conditions as in Fig. 3 except 1% propanol was added to both buffers. A, 1-propanol; B, 2-propanol.

multisite binding and release mediated by a critical concentration of organic modifier (14,21). The effect an organic modifier has on DSG-RP chromatography may be somewhat analogous in that only a small amount of propanol in the mobile phase can cause drastic reductions in tRNA retention times. Under conditions of higher initial salt concentration, addition of 1% (v/v) 2-propanol to the mobile phase for fractionation of tRNAs gave excellent results, whereas addition of 2.5% resulted in non-retention of all species (Table 1). The same type of phenomenon was observed with ethanol and

TABLE 1

Effect of Organic Modifier on Retention

Organic Modifier	Percent Modifier in Mobile Phase[a]	
	Optimal tRNA Profile	No tRNA Retention
2-Propanol	1%	2.5%
Ethanol	2	4
Methanol	5	8

[a]All elution profiles are E. coli tRNA on a C_2 column at 55°C and salt gradient conditions as in Fig. 4a.

methanol. The data in Table 1 imply there is a narrow concentration range where organic modifiers may enhance resolution, with an upper threshold which, when surpassed, results in a sharp reduction of tRNA adsorptive retardation. It is apparent from the above examples that increased initial salt concentration in DSG-RP raises the critical concentration of organic modifier.

An organic modifier gradient was investigated next. An ascending 2-propanol gradient (0-2%) run concommitantly with the 2.0M $(NH_4)_2SO_4$ descending salt gradient in Fig. 5a could fractionate E. coli tRNA into 12 peaks (chromatogram not shown), but with a constant 0.5% 2-propanol concentration during the descending salt gradient, about 20 peaks were present (Fig. 5a). It was apparent that the use of organic modifiers worked better in an equilibrium rather than dynamic process. Therefore the investigation continued utilizing constant organic modifier concentrations during gradients.

Descending salt gradient chromatography of tRNAs on Sepharose 4B requires a high ammonium sulfate concentration (> 2.0M) to initiate interfacial

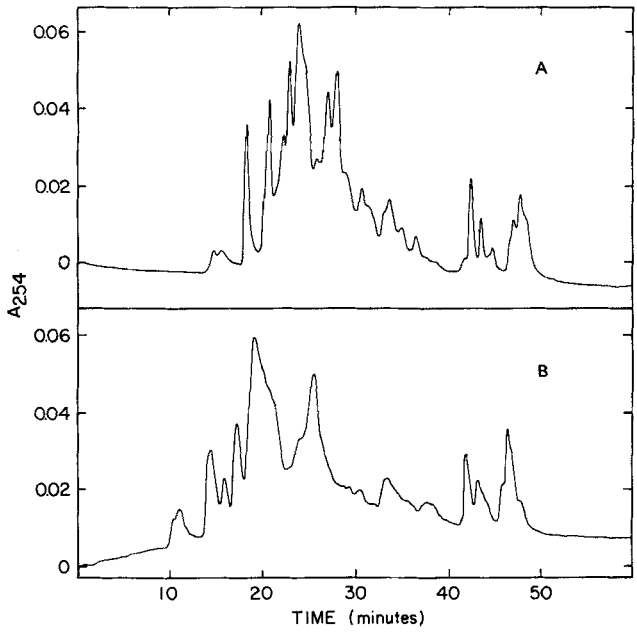

FIGURE 5 Fractionation of E. coli tRNA on a polymeric C_4 coated column. Same elution conditions as in Fig. 1, loads were 186µg, and pH varied. A: pH 7.05. B: pH 5.1.

precipitation and a long column bed (> 15cm) to facilitate absorptive retardation (2,8). 150mg tRNA loads typically require 50-100 hrs for elution. Comparable resolution of tRNAs by DSG-RP can be achieved in about 1 hr. at lower salt concentration (<2.0M) on a 5cm analytical column.

The elution profiles of E. coli tRNA in Figs. 6a-e illustrate that after a descending salt gradient has been run, organic modifier additions can then be used to adjust elution times. It was found for both yeast and E. coli tRNA that resolution at neutral pH was superior to slightly acidic conditions (Fig. 5). Retention times for relatively hydrophobic tRNAs did not vary with pH whereas less hydrophobic tRNAs eluted faster at pH 5.1.

The E. coli tRNA sample used in this study was a heterogeneous mixture of isoacceptors specific for lysine, phenylalanine, serine and valine (Fig. 7a).

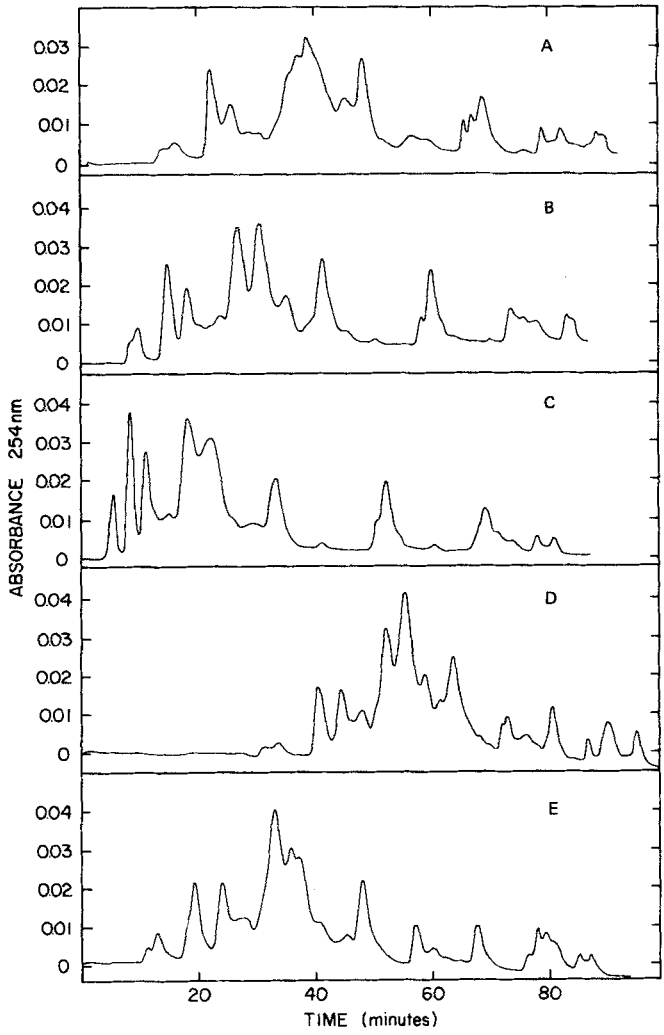

FIGURE 6 Effects of initial salt concentration and % organic modifier on tRNA retentions. 0.41 x 5.0cm polymeric C_2 column. Buffer A, ammonium sulfate and 0.2M potassium phosphate pH 7: Buffer B, 0.2M potassium phosphate pH 7. Gradient was 0-100% buffer B in 80 min. then 100% for an additional 20 min. Flow, 0.7ml/min; temperature, 55°C; injections, 100µl of 180-230µg E. coli tRNA in buffer A. Initial concentration of ammonium sulfate in buffer A and % 2-propanol maintained during the salt gradient: A, 1.5M, no propanol; B, 1.5M, 0.25%; C, 1.5M, 0.5%; D, 2.0M, no propanol; E, 2.0M, 1%.

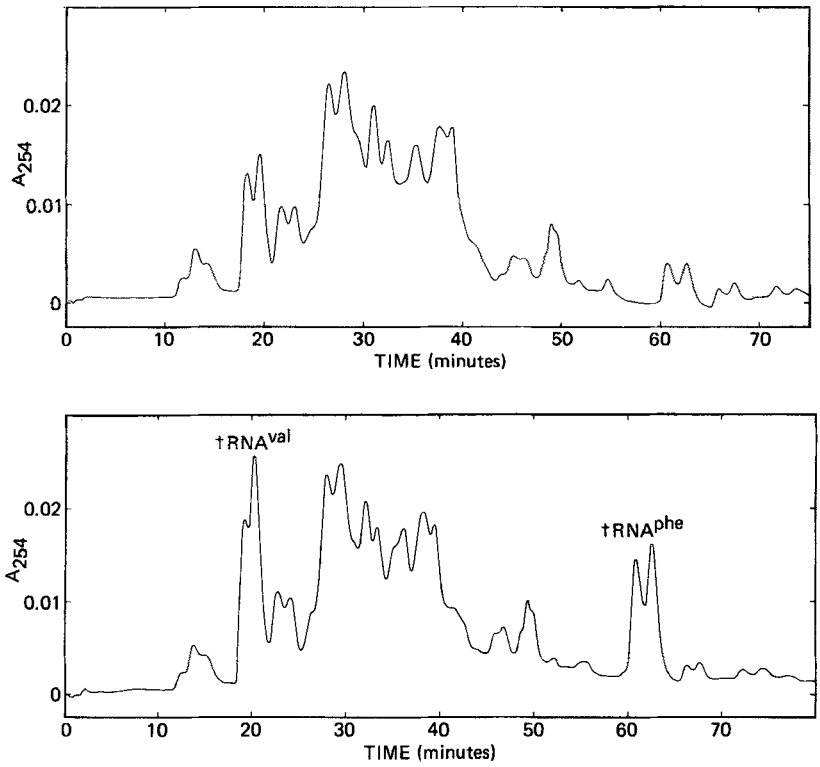

FIGURE 7 Separation of $tRNA^{val}$ from $tRNA^{phe}$ isoacceptors. Conditions as in Fig. 6D but with 0.75% 2-propanol added to buffers. A: 180µg tRNA. B: 180µg tRNA plus addition of commercially purified $tRNA_{val}$ and $tRNA_{phe}$.

Phenylalanine specific isoacceptors would be predicted to elute late in the profile because of their known hydrophobic character on classical reversed-phase supports (22,23), while the relatively less hydrophobic $tRNA^{val}$ should elute early (22,23). Chromatography of commercially purified $tRNA^{phe}$ isoacceptors was shown to elute two peaks (60 and 62 min.) late in the gradient, characteristic of relatively hydrophobic species, while commercially purified $tRNA^{val}$ isoacceptors were identified as

eluting much earlier (19 and 20 min.). A double addition of both species to the E. coli sample is illustrated in Fig. 7b. The recovery determined by absorbance at 254nm for a 180µg analytical load of E. coli tRNA using elution conditions specified in Fig. 5a was 80%.

Silica

An important consideration was the optimal type of microparticulate silica that could serve as the support for the stationary phase. Recent studies have shown that pore-diameter and other inherent silica matrix properties are important for optimizing HPLC fractionation of macromolecules such as peptides (13,14), proteins (14,24-26), and oligonucleotides (27). For example, pore sizes from 100Å for oligonucleotides (27) to 1000Å for large proteins (26) have been espoused as optimal. A qualitative study of resolution using C_2 and C_4 n-alkylsilyated silicas with pore-diameters of 120, 330, and 500Å indicated that tRNAs fractionated best on the 330Å silica (data not shown). From a purely geometrical standpoint, the dimensions of a monoclinic, crystalline form of yeast $tRNA^{phe}$ are 56 x 33.4 x 63.0Å (12), which suggests that utilization of a high-surface area, mesopore (100Å) silica would suffice. Actually, three lines of physical evidence dictate the desirability of macroporosity:

1) In solution tRNA (27,000 daltons) has considerable linear structure (28) which causes the hydrodynamic volume to be comparable to a 60,000 dalton protein (29);
2) A 60,000 dalton sphere or solute that kinetically behaves like one has diffusivity problems in mesoporous silica, resulting in poor efficiency (30);
3) The DSG-RP separation mechanism is partly based on interfacial precipitation where possible multilayer stacking (2) must be considered.

In addition to better selectivity, the 330Å pore-diameter silica was preferred over the 500Å silica on the basis of pressure stability (14) and greater surface area (100 vs. 50 m^2/g).

CONCLUSION

Fractionation of tRNA isoacceptors was achieved by a descending salt gradient on a reversed-phase high-performance liquid chromatography column. This system (DSG-RP) was demonstrated on C_1, C_2, and C_4 type coatings. Taking into consideration the physical nature of tRNA molecules, it was found that macroporous, as opposed to mesoporous, microparticulate silica was desirable. Retention time could be manipulated by 1) initial salt concentration or 2) amount organic modifier added to the mobile phase. The type and amount of organic modifier used were important variables. Resolution was increased at elevated temperature, but at the expense of a concomitant decrease in column lifetime. At room temperature the columns were very stable. Hydrophobic interactions are normally enhanced at a high temperature (2). In this study, elution at 55° as opposed to 24° decreased retention times. Apparently the thermal retardation enhancement was more than offset by the effect increasing temperature had on solubilizing tRNA to negate interfacial precipitation. The same affect has been noted by Spencer for Sepharose 4B (2). The effects of initial salt concentration, type and amount of organic modifier, temperature and pH had, in general, a greater effect on the less hydrophobic tRNAs. The efficacy of this new HPLC method has yet to be explored for separation of larger polynucleotides.

[1]Abbreviations used: HPLC, high-performance liquid chromatography; RPLC, reversed-phase high-performance liquid chromatography; DSG-RP, descending salt gradient reversed-phase.

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REFERENCES

1. Holley, R. W., and Merrill, S. H. (1959) J. Am. Chem. Soc. 81, 753.
2. Spencer, M. (1982) J. Chromatogr. 238, 307-316.
3. Kawade, Y., Okamoto, T., and Yamamoto, Y. (1963) Biochem. Biophys. Res. Commun. 10, 200-203.
4. Cherayil, J. D., and Bock, R. M. (1965) Biochemistry 4, 1174-1183.
5. Sueoka, N., and Cheng, T. Y. (1962) J. Mol. Biol. 4, 161-172.
6. Hecker, L. I., Uziel, M. and Bartnett, W. E. (1976) Nucl. Acids Res. 3, 371-380.
7. Wells, R. D., Hardies, S. C., Horn, G. T., Klein, B., Larson, J. E., Neuendorf, S. K., Panayotatos, N., Patient, R. K., and Selsing, E. (1980) in Methods in Enzymology 65, 327-347.
8. Spencer, M., and Binns, M. M. (1982) J. Chromatogr. 238, 297-306.
9. Hatfield, G. W. (1979) in Methods in Enzymology LIX, 215-218.
10. Narihara, T., Fujita, Y., and Mizutani, T. (1982) J. Chromatography 236, 513-518.
11. Bull, H. B., and Breeze, K. (1980) Biopolymers 17, 2121.
12. Robertus, J. D., Ladner, J. E., Finch, J. T., Rhodes, D., Brown, R. S., Clark, B. R. C., and Klug, A. (1974) Nature 250, 546-551.
13. Pearson, J. D., Mahoney, W. C., Hermodson, M. A., and Regnier, F. E. (1981) J. Chromatography 207, 325-332.
14. Pearson, J. D., Lin, N. T., and Regnier, F. E. (1982) Anal. Biochem. 124, 217.
15. O'Hare, M. J., Capp, M. W., Nice, E. C., N. H. C. Cooke, and B. G. Archer, (1981) International Symposium on HPLC of Peptides and Proteins. Washington, D.C., Paper No. 305.
16. Nice, E. C., Capp, M. W., Cooke, N., and O'Hare, M. J. (1981) J. Chromatogr. 218, 569-580.
17. Pearson, J. D., and Regnier, F. E., J. Liq. Chromatogr., in press.
18. Brown, P. R., and Krstulovic, A. M. (1979) Anal. Biochem. 99, 1-21.

19. Crothers, D. M., Cole, P. E., Hilbers, C. W., and Shulman, R. G. (1974) J. Mol. Biol. 87, 63-88.

20. Mahoney, W. C., and Hermodson, M. A. (1980) J. Biol. Chem. 255, 11199-11203.

21. Barford, R. A., Sliwinski, B. J., Breyer, A. C., Rothbart, H. L. (1982) J. Chromatogr. 235, 281-288.

22. Weiss, J. F., Pearson, R. L., and Kelmers, A. D. (1968) Biochemistry 7, 3479-3487.

23. Dion, R., and Cedergren, R. J. (1978) J. Chromatogr. 152, 131-136.

24. Lewis, R. V., Fallon, A., Stein, S., Gibson, K. D., and Udenfriend, S. (1980) Anal. Biochem. 104, 153-159.

25. Vanecek, G., and Regnier, F. E. (1980) Anal. Biochem. 109, 345-353.

26. Vanecek, G., and Regnier, F. E. (1982) Anal. Biochem. 121, 156-169.

27. Pearson, J. D., and Regnier, F. E. (1983) J. Chromatogr. 255, 137-149.

28. Kim, S.-H., and Quigley, G. J. (1979) in Methods in Enzymology LIX, p. 16.

29. Wehr, C. T., and Abbott, S. R. (1979) J. Chromatogr. 185, 453-462.

30. Pfannkoch, E., Lu, K. C., Regnier, F. E., and Barth, H. G. (1980) J. Chromatogr. Sci. 18, 430-441.