Journal of Chromatography, 463 (1989) 409–417
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 063

MICROPREPARATIVE SEPARATION OF TRANSFER RIBONUCLEIC ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received May 18th, 1988; revised manuscript received October 20th, 1988)

SUMMARY

A method was developed for the micropreparative separation of individual species of tRNA using reversed-phase high-performance liquid chromatography on large pore spherical silica bonded with C_3 alkyl chains. Columns were eluted with linear gradients of decreasing sodium chloride and increasing methanol concentrations. The decreasing salt gradient gradually abolished hydrophobic interactions and a significantly higher selectivity was thus obtained when compared with increasing gradients of salts usually employed in reversed-phase separations of tRNA. The acceptance of tRNA fractions was tested by charging them with fifteen different amino acids. Significantly different separations were obtained with tRNA from *Escherichia coli* and from rat liver. $tRNA^{Glu}$ and $tRNA^{Tyr}$ from *E. coli* were obtained in a pure form, all other tRNAs were more or less contaminated by adjoining tRNAs for other amino acids. Rechromatography under suitable isocratic conditions was required to obtain pure tRNA species from rat liver. Isoaccepting tRNAs for several amino acids were separated from rat liver. The method described seems suitable for preliminary fractionations of complex mixtures of tRNA and for a simple purification of isoaccepting species if the presence of tRNAs for other amino acids is not an hindrance.

INTRODUCTION

Separation of individual species of tRNA has always been a difficult task because of the close similarity in size and composition of tRNA molecules specific for different amino acids. Chromatography on benzoylated DEAE-cellulose[1] was found useful for tRNAs accepting aromatic amino acids (in particular $tRNA^{Phe}$) which become strongly bound to this carrier. On the other hand, the initiator tRNA ($tRNA_F^{Met}$) is retained only slightly on benzoylated DEAE-cellulose and can thus be relatively simply separated[2]. However, other tRNA species were eluted together without any reasonable resolution. Low-pressure chromatography on reversed phases (RPC)[3] provided better separations of individual tRNAs, however rechromatography was required to obtain tRNA species in a satisfactory purity.

High-performance liquid chromatography (HPLC) was shown to give a promising resolution of RNA molecules of different sizes (as reviewed by McLaughlin[4]).

0021-9673/89/$03.50

Several different stationary phases were used for the separation of individual tRNA species. In earlier attempts, a reversed-phase support consisting of inert poly(chlorotrifluoroethylene) beads to which a pellicular layer of trioctylammonium chloride had been adsorbed was used and a very rapid resolution of isoaccepting tRNAs, *i.e.*, different tRNAs accepting the same amino acid for serine and leucine from bacterial materials was obtained[5]. Alkylated silicas with short-chain chlorosilanes appeared to be a promising support material for the separation of tRNA, employing a gradient elution with decreasing ammonium sulphate concentration at elevated temperatures[6]. Using a Vydac C_4 derivatized silica column, tRNA from *Escherichia coli* B was separated into more than 20 peaks. However, only species accepting phenylalanine, tryptophan and valine have been identified[7]. Microparticulate bonded stationary phases were also employed for the separation of model mixtures of $tRNA^{Glu}$, $tRNA^{Lys}$, $tRNA^{Phe}$ and $tRNA^{Val}$ from *E. coli* and a good and rapid resolution has been demonstrated[8].

Further supports employed for the separation of tRNA by HPLC included polystyrene and reversed-phase anion exchangers as well as affinity columns[9]. A support consisting of an octadecasilyl-bonded phase silica aggregated with the tetraalkylammonium chloride was found to be useful for the separation of isoaccepting tRNAs from baker's yeast and a good resolution of $tRNA^{Val}$, $tRNA^{Ile}$, $tRNA^{Ser}$ and $tRNA^{Phe}$ has been reported[10]. Gel permeation high-performance chromatography appeared to be useful for the separation of small tRNA species but is apparently of minor importance for the resolution of individual tRNA species[11].

With the exception of a single paper[12], only four to eight tRNAs for different amino acids were identified in experiments on the separation of individual tRNA species by HPLC.

For our studies on the interaction of chemical carcinogens with tRNA[13], several species of tRNA specific for different amino acids are required. The method developed for this purpose and described in this paper allows a satisfactory separation of at least thirteen tRNAs for different amino acids not only on an analytical but also on a micropreparative scale.

MATERIALS AND METHODS

Chemicals and radiochemicals

Transfer RNA from *E. coli* B was a product of Calbiochem (San Diego, CA, U.S.A.). Transfer RNA from rat liver was isolated as described by Rogg *et al.*[14] and deacylated[2]. Aminoacyl-tRNA synthetases were partially purified from *E. coli* B or rat liver as described by Stanley[2]. L-[U-^{14}C]alanine (120 mCi/mmol), L-[U-^{14}C]arginine (240 mCi/mmol), L-[U-^{14}C]aspartic acid (160 mCi/mmol), L-[U-^{14}C]glutamic acid (200 mCi/mmol), L-[U-^{14}C]glycine (80 mCi/mmol), L-[U-^{14}C]histidine (240 mCi/mmol), L-[U-^{14}C]isoleucine, L-[U-^{14}C]leucine (240 mCi/mmol), L-[U-^{14}C]lysine (240 mCi/mmol), L-[U-^{14}C]phenylalanine (360 mCi/mmol), L-[U-^{14}C]serine (120 mCi/mmol), L-[U-^{14}C]threonine (160 mCi/mmol), L-[U-^{14}C]tyrosine and L-[U-^{14}C]valine (240 mCi/mmol) were from the Institute for Research, Production and Utilisation of Radioisotopes, Prague, Czechoslovakia. [Methyl-^{3}H]methionine (280 mCi/mmol) was obtained from the Institute of Radioisotopes, Hungarian Academy of Sciences, Budapest, Hungary.

High-performance liquid chromatography

A Beckman 345 HPLC system was used consisting of the 114M solvent delivery module, Type 165 variable wavelength detector and Type 450 data system/controller. Columns containing large pore silica bonded with C_3 alkyl chains (Beckman Ultrapore C_3RPSC) (7.5 cm × 4.6 mm I.D.) were used at a flow-rate of 1 ml/min and the absorbance of the eluate was recorded at 260 nm. A linear gradient of buffer B (10 m*M* Tris–HCl buffer, pH 7.55, 10 m*M* magnesium chloride, 200 m*M* sodium chloride), and 8% (v/v) metanol was added to buffer A (10 m*M* Tris–HCl buffer, pH 7.55, 10 m*M* magnesiumchloride, 1 *M* sodium chloride). Pumps were programmed to deliver 0–50% of buffer B during 100 min after the sample injection and 50–100% of buffer B during the next 50 min. Fractions (each 1.0 ml) were collected. Each fraction was supplemented with 1/10 volume of 2 *M* sodium acetate buffer, pH 5.0, followed by two volumes of chilled (−20°C) ethanol. The solutions were mixed on a vortex mixer and left at −20°C overnight. RNA precipitates were then centrifuged at low speed and −10°C, supernatants were discarded and precipitates were dried under a stream of nitrogen.

Agarose gel electrophoresis

This was performed as described by Maniatis *et al.*[15] in the gel electrophoresis instrument GNA-100 (Pharmacia, Uppsala, Sweden).

Charging of tRNA with amino acids

Transfer RNA in individual fractions was dissolved in 0.1 ml of water and charged with amino acids as described by Hradec[13]. Briefly, mixtures containing tRNA, aminoacyl-tRNA synthetases from *E. coli* or from rat liver, ATP and CTP were incubated with individual labelled amino acids for 20 min at 37°C. Thereafter, aminoacyl-tRNA was precipitated, washed and its radioactivity was assayed by liquid scintillation counting.

RESULTS

The elution profile of fifteen individual tRNA species tested was completely different when comparing tRNA of bacterial origin with that from rat liver. These results are summarized in Table I.

With tRNA from *E. coli* at least two tRNAs ($tRNA^{Glu}$ and $tRNA^{Tyr}$) were obtained in a pure form from a single experiment. With the usual loads, 2.5 mg in 50 µl of buffer A, the yields of these individual tRNAs were in the range of 20–50 µg. Moreover, one isoaccepting species of $tRNA^{Leu}$ was only sparingly contaminated with $tRNA^{Ser}$.

On the other hand, no resolution of $tRNA^{Met}$ from $tRNA^{Thr}$ was obtained. There was also no separation of $tRNA_F^{Met}$ (initiator tRNA) from $tRNA_M^{Met}$ (tRNA inserting L-methionine into the inside of the peptide chain).

The other tRNAs were not isolated in a pure form. Thus $tRNA^{Asp}$ showed overlapping with $tRNA^{Thr}$ and $tRNA^{Lys}$, tRNA accepting arginine was contaminated with $tRNA^{His}$, $tRNA^{Val}$ was partially overlapped with $tRNA^{Gly}$ and $tRNA^{Ser}$ with one isoaccepting species of $tRNA^{Leu}$. The complex of $tRNA^{Met}$ and $tRNA^{Thr}$ was contaminated with the other isoaccepting species of $tRNA^{Leu}$. These overlapping

TABLE I

RETENTION TIMES FOR INDIVIDUAL tRNAs FROM *E. COLI* AND RAT LIVER

The width of a particular peak is indicated in brackets (start to end of the peak).

Amino acid	*Retention time (min)*	
	tRNA from E. coli B	*tRNA from rat liver*
(1) Alanine	95 (94.23–96.50) 110 (108.97–113.53)	5 (4.50–7.07) 11 (10.20–11.93)
(2) Arginine	66 (65.00–66.23)	53 (52.00–53.20) 63 (62.33–62.53) 69 (66.93 70.90) 77 (75.50–78.50)
(3) Aspartic acid	57 (56.01–57.90)	27 (26.97–27.17)
(4) Glutamic acid	18 (17.00–19.27)	10 (9.20–10.80)
(5) Glycine	32 (31.03–33.20)	8 (7.07–8.97)
(6) Histidine	69 (68.87–69.90)	– –
(7) Isoleucine	50 (46.00–50.53)	41 (39.60–42.80) 49 (45.63–50.27)
(8) Leucine	43 (42.23–43.30) 116 (115.03–117.20)	24 (23.80–24.37) 33 (32.63–33.83) 51 (49.43–53.20) 75 (74.00–75.90)
(9) Lysine	59 (58.30–62.07)	38 (36.50–39.00) 56 (55.20–56.93) 62 (61.23–62.33)
(10) Methionine	48 (46.00–50.53)	36 (34.97–36.50)
(11) Phenylalanine	97 (96.63–97.80)	100 (99.90–100.23) 112 (108.83–114.07)

TABLE I (*continued*)

Amino acid	*Retention time (min)*	
	tRNA from E. coli B	*tRNA from rat liver*
(12) Serine	108 (105.80–108.93)	37 (36.50–39.00) 94 (93.33–94.87) 109 (108.83–109.53)
(13) Threonine	47 (46.00–50.53)	73 (72.20–73.53) 76 (75.30–76.77) 83 (82.90–83.50)
(14) Tyrosine	144 (140.60–145.87)	16 (15.77–17.53) 33 (32.63–33.83) 40 (39.60–42.80) 88 (87.77–89.43)
(15) Valine	27 (24.63–27.90)	5 (4.50–7.07) 14 (13.57–14.23) 20 (18.53–20.83) 28 (27.17–28.53)

tRNA species were relatively easily purified by rechromatography under isocratic conditions deduced from the appropriate portions of the gradient used for the original separation (results not shown).

In comparison with eukaryotic tRNA, only two isoaccepting species of $tRNA^{Leu}$ and $tRNA^{Ala}$ were separated from bacterial tRNA. The separation of both these species was very good. Results of the resolution of individual bacterial tRNAs are summarized in Fig. 1.

In contrast to bacterial tRNA, not one tRNA species from rat liver was isolated in a pure form during a single experiment.

In spite of this, a good resolution of several isoaccepting tRNAs was obtained. This holds true for $tRNA^{Gly}$, $tRNA^{Ile}$ and $tRNA^{Phe}$, tRNAs for aspartic acid and arginine, for alanine and serine and for glutamic acid and lysine. However, all these tRNA species were contaminated with tRNAs for various other amino acids. In comparison with bacterial tRNA, $tRNA^{Met}$ and $tRNA^{Thr}$ were fully resolved. Moreover, a partial separation of both tRNA species accepting L-methionine was demonstrated.

With the exception of tRNAs for glycine and for aspartic acid, all the other

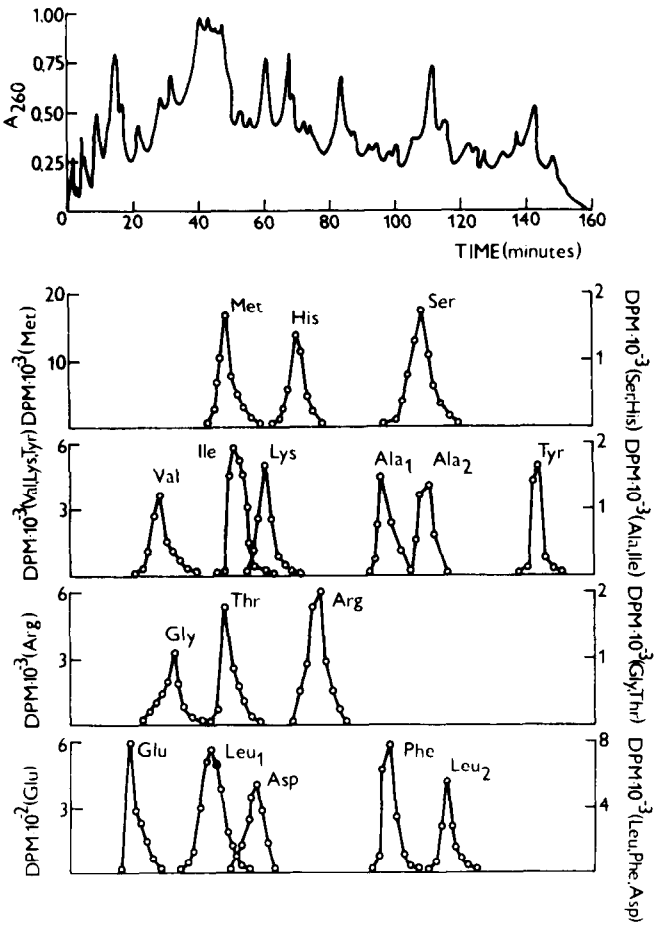

Fig. 1. Separation of total tRNA from *E. coli* by HPLC. A 2.5-mg amount of unfractionated tRNA in 50 μl of the initial buffer (see below) was injected on to a column of large pore silica bonded with C_3 alkyl chains (Beckman Ultrapore C_3RPSC, 75 mm × 4.6 mm I.D.) eluted with a linear gradient of 10 m*M* Tris–HCl buffer, pH 7.55, 10 m*M* magnesium chloride, 200 m*M* sodium chloride and 8% (v/v) methanol added to 10 m*M* Tris–HCl buffer, pH 7.55 containing 10 m*M* magnesium chloride and 1 *M* sodium chloride. 50% of the gradient was delivered during 100 min after the sample injection and the rest during the next 50 min. Details are described in the Materials and Methods section. Top, the A_{260} profile; below, the acceptor activity of fractions for individual amino acids. As described in detail in the Materials and Methods section, tRNA in each third fraction was precipitated with ethanol and after drying it was charged with all fifteen labelled amino acids used in the presence of aminoacyl-tRNA synthetases from *E. coli* and the radioactivity present in aminoacyl-tRNA was assayed. Only amino acids giving a positive charging response are shown.

tRNA species from rat liver were resolved into at least two isoaccepting species. An excellent resolution of at least four isoaccepting tRNAs for tyrosine and serine as well as a partial separation of two species of $tRNA^{Glu}$, $tRNA^{Lys}$, $tRNA^{Phe}$, $tRNA^{Thr}$ and several isoaccepting species of tRNAs for valine, leucine and arginine were obtained. Separations of individual tRNA species from rat liver are demonstrated in Figs. 2 and 3.

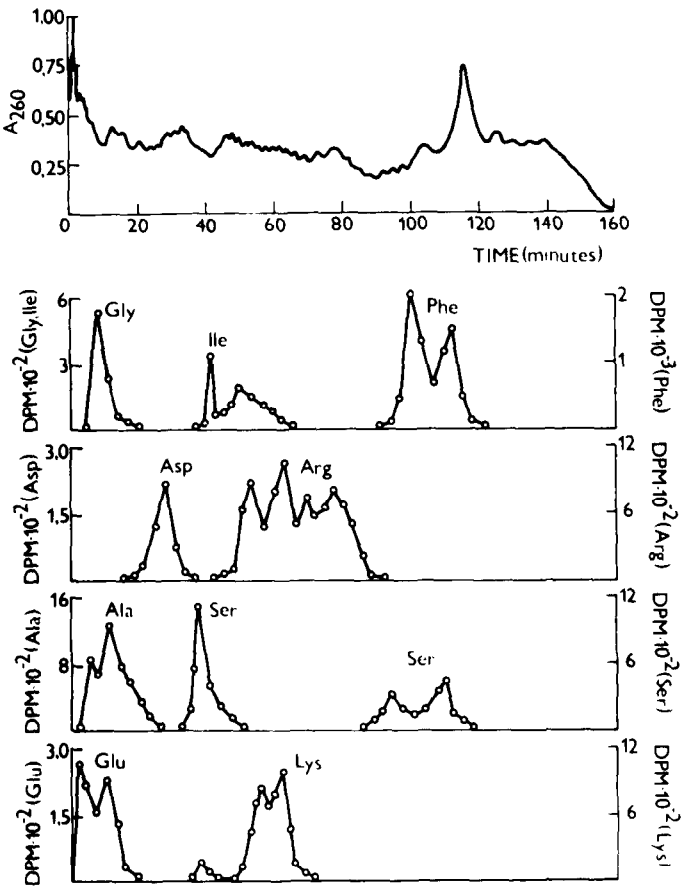

Fig. 2. Separation of unfractionated tRNA from rat liver by HPLC. A 2.5-mg amount of tRNA in 50 μl of the initial buffer was separated on a column of large pore silica bonded with C_3 alkyl chains. See Fig. 1 and the Material and Methods section for experimental details. Fractions were charged with aminoacyl-tRNA synthetases from rat liver.

No charging with any of the amino acids tested was demonstrated in a rather broad range of fractions eluted between 120 and 160 min. Agarose gel electrophoresis revealed that these fractions do not contain tRNA but 5S RNA and further nucleotide impurities of unknown nature (results not shown).

DISCUSSION

Because of an high and rapid resolution of individual RNAs, HPLC is apparently superior to earlier techniques used for the separation of tRNA species. Nevertheless, liquid chromatography at atmospheric pressure may be useful for some special purposes. Thus an excellent separation of isoaccepting tRNA species from *E. coli* was reported using polystyrene anion exchangers[16].

In our present experiments we were able to identify tRNAs for fifteen different

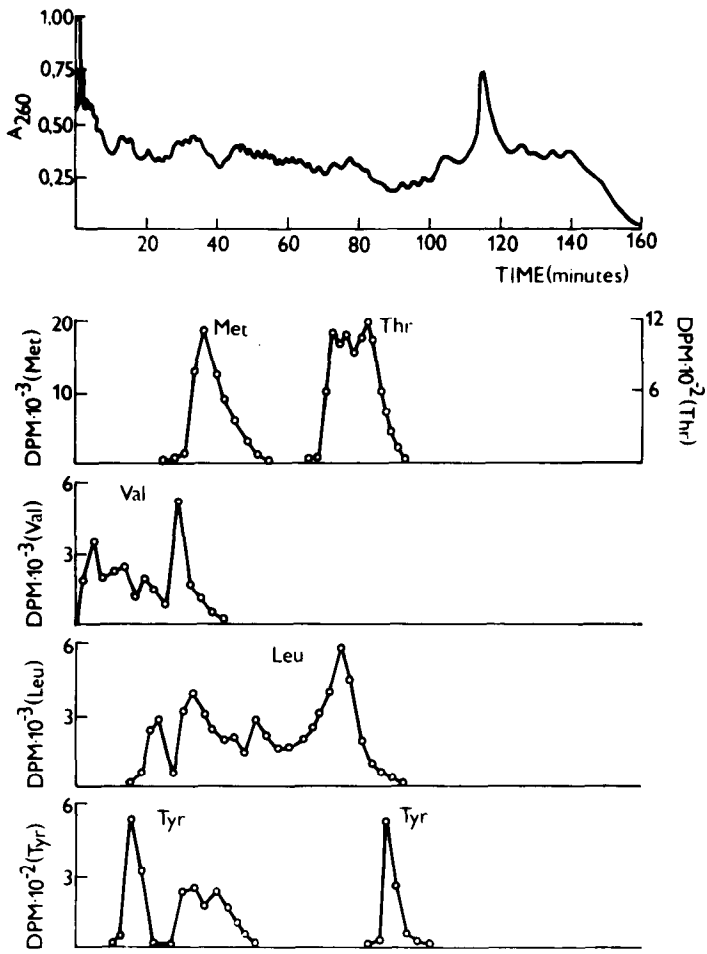

Fig. 3. Separation of unfractionated tRNA from rat liver by HPLC (continued from Fig. 2). Details are given in Fig. 2 and in the Materials and Methods section.

amino acids. This is comparable with the results of Bischoff and McLaughlin[12] who reported the identification of tRNA species for thirteen different amino acids after their separation using an ionic–hydrophobic mixed-mode matrix for HPLC.

The novelty of the method described in this paper lies in the use of short alkyl chains bonded to silica and of a decreasing salt gradient in order gradually to reduce hydrophobic interactions. In our hands, supports containing short alkyl chains were found superior to those with long chains (ODS)[17]. Also, reversed eluting gradients with decreasing concentrations of sodium chloride but increasing contents of methanol were more useful than simpler gradients where only one solvent component was changed.

The procedure described seems to be useful for a micropreparative isolation of tRNA species from *E. coli*. Some species may be isolated pure in a single experiment and several others are purified by rechromatography. On the other hand, it is not

suitable for the purification of tRNA species from eukaryotic cells. Very complex mixtures are obtained in a single experiment and it would be very difficult to use them as a starting material for the purification of individual tRNAs. For the purification of eukaryotic initiator tRNA, a combination of other techniques seems to be more advantageous[18]. However, because of the excellent resolutions of isoaccepting species of tRNA obtained by the present method with tRNA from rat liver, this technique may be useful for the separation of isoaccepting tRNAs for particular amino acids if the presence of tRNAs for other amino acids is not an hindrance. It can also be employed for the removal of 5S RNA and other nucleotide impurities that may be present in preparations of eukaryotic tRNA[14].

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