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Simple and rapid chromatographic method for the separation of major classes of ribosomal ribomononucleotides

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Abstract

We have described a simple and rapid chromatographic method for the analytical and preparative separation of major types of ribosomal ribomononucleotides with Dowex 1-X10 ($HCOO^-$, 37–74 μm) and Dowex 2-X10 ($HCOO^-$, 37–74 μm) columns, by desorption with formiate solutions in 1–2 h. The separation has been achieved for Cp, Ap, Up and Gp, while a mixture of 2′-, and 3′-nucleoside phosphates desorbs as a single peak; with both resins, a successful separation was achieved with a load from 25 μg to 1 mg of ribomononucleotide mixture per ml of packed resin. A complete separation was achieved with Dowex 1, while the separation with Dowex 2 resin was even better. The resins cannot separate unusual nucleosides; therefore, our method is suitable for studies of ribonucleic acids with a low content of unusual nucleosides. Our method has been applied for the quantitative determination of the ribomononucleotide composition of 18S and 28S rRNAs, isolated from mammalian tissues: rat liver, mouse kidney and Ehrlich ascites cells. Dowex 1 and Dowex 2 resins afforded similar or identical ribomononucleotide compositions in all cases; analytical data were in agreement with the literature data. Our method is competitive, in several respects, with modern HPLC techniques for the separation of ribomononucleotides.

Keywords: Ribonucleotides; RNA; Nucleotides

1. Introduction

In our previous work, we developed simple and rapid chromatographic procedures for the separation and purification of mammalian RNAs, employing their selective hydrophobic binding to agar gel [1] or nitrocellulose column [2,3]. Also, we were able, applying CL-Sepharose 4B, to isolate all major types of mammalian nucleic acids (DNA, tRNA, 18S and 28S rRNA) from crude cell extracts, in a single chromatographic step [4]. Despite the fact that the latter method did not afford clean 18S and 28S rRNA, it presented the initial step towards the isolation of ultrapure rRNAs.

In the course of the above work, we were confronted with a need for a simple and rapid chromatographic method for the separation of rRNA ribomononucleotides. Recently, fast HPLC methods were developed, which are able to separate all acid soluble nucleotides and their derivatives in nmol quantities in 30 min [5].

In this work, we have developed a modification of a much-used Cohn's ion-exchange chromatographic method [6]. More precisely, out of numerous modifications of Cohn's method (for review see [7]), we have improved the method of Petrović and Brkić [8],

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which applies a Dowex 1 ($HCOO^-$) column and separates pure Cp and Ap, while the separation of Up from Gp was incomplete; a successful separation of pseudouridine was achieved. In a further development of this method, the height of the column was diminished and the time of separation was reduced from 17 h to 1–2 h; this modification did not separate unusual nucleosides, and the separation of Up from Gp was still incomplete [9].

In the present work, we have used anion-exchange columns Dowex 1-X10 ($HCOO^-$, 37–74 μm) and Dowex 2-X10 ($HCOO^-$, 37–74 μm), and were obtained, using discontinuous desorption with volatile formiate solutions, a complete separation of major ribomononucleotides Cp, Ap, Up and Gp for only 1–2 h; the separation of Up and Gp was complete. Our method is, in several respects, competitive with HPLC techniques for the separation of ribomononucleotides.

2. Materials and methods

Dowex 1-X10 (37–74 μm) and Dowex 2-X10 (37–74 μm) resins were obtained from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate (SDS, for tensio-active work) was obtained from Merck (Darmstadt, Germany), and used without further purification; CL-Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden).

Native 18S and 28S ribosomal RNAs were isolated from extracts of total nucleic acids obtained from rat liver, mouse kidney or Ehrlich ascites cells, as previously described [4]. Further purification of 18S and 28S rRNAs fractions was performed separately.

Small ribosomal 5.0S and 5.8S RNAs were quantitatively removed on CL-Sepharose 4B column (10×0.7 cm) in 0.02 *M* Tris·HCl buffer, (pH 7.0), supplemented with 0.14 *M* NaCl, 0.1 m*M* EDTA and 0.5% SDS; SDS inhibits most RNAses. 18S rRNA appears as a single peak at K_d 0.6 (K_d=distribution coefficient in gel filtration), while contaminating quantities of small rRNAs appear with a 'total volume' in this purification (after 35 ml of buffer).

Purification of 28S rRNA from hnRNA (mixture of nuclear preribosomal and non-processed mRNA) was achieved by gel filtration on CL-Sepharose 4B column (15×1 cm) with the same buffer as above; hnRNA appears with the void volume, while 28S RNA appears at K_d 0.6, as a single peak [4,10,11].

The only remaining contaminations in 18S and 28S RNA are messenger RNAs. Removal of contaminating mRNAs by affinity chromatography on poly-U Sepharose was not attempted, because such a step may cause a serious loss of rRNA [12]. However, contamination with mRNAs may be neglected, since they represent only 1% of total RNA in mammals [13].

Alkaline hydrolysis of rRNAs was performed in 0.5 *M* KOH, at 37°C for 17 h. Thereafter, hydrolysate was acidified with concentrated $HClO_4$ up to 0.5 *M*, and insoluble $KClO_4$ was removed by centrifugation. We have omitted the enzymatic degradation of RNAs which is suitable for rRNA depolymetization to nucleotides [14] or nucleosides [15,16], in order to compare our data with those from the literature [17–19].

The hydrolysate was purified on a column of crystalline charcoal Barnabey-Chenly 188 (4.0×0.5 cm). Acid hydrolysate was passed through the column three times, and the column was washed with 10 ml of bidistilled water. Desorption was achieved with 10 ml of ethanol–ammonia–water (45:3:52, v/v); recovery of nucleotides was 90%, measured from *A* at 260 nm. The eluate was reduced to 2 ml by warming and applied directly to ion-exchange columns; this additional step removes carbonates which may interfere with desorbtion of nucleotides from resins.

Dowex 1 and Dowex 2 were packed in columns of 3×0.7 cm size, and the columns were developed by discontinuous desorption with the following solutions:

1. 0.005 *M* HCOOH, desorption of nucleosides,
2. 0.05 *M* HCOOH (9 ml) Cp,
3. 0.5 *M* HCOOH (15 ml) Ap,
4. 0.1 *M* $HCOONH_4$ (pH 7, 12 ml),
5. 0.1 *M* $HCOONH_4$+0.05 *M* HCOOH (9 ml) Up, and
6. 0.2 *M* $HCOONH_4$+0.5 *M*. HCOOH (18 ml) Gp.

The flow-rate was maximal under gravity at room temperature.

The labeling of rRNAs with ^{32}P was achieved as previously described [4]. Uridine (500 $\mu Ci\ mmol^{-1}$) and [U-^{14}C]-adenosine (570 $\mu Ci\ mmol^{-1}$) were ob-

tained from the Radiochemical Center, (Amsterdam, Netherlands). Rat livers were labeled with 100 μCi i.p. for 24 h. Desorption zones from Dowex columns were collected directly into glass vessels for radioactivity measurements and evaporated to dryness at 100°C. The Bray solution (5 ml) was added and the radioactivity measured in scintillation counter with an automatic determination of quenching; the quenching was not detected in any sample.

3. Results and discussion

Figs. 1 and 2 show the elution diagrams for 28S rRNA ribomononucleotides, isolated from rat liver on Dowex 1 or Dowex 2 columns, respectively. Both elution diagrams indicate a complete separation of major ribomononucleotides Cp, Ap, Up and Gp; Dowex 2 afforded a better separation of Up from Gp.

Hydrophobic interactions and partition chromatography play an important and independent role in determining the distribution coefficient of organic compounds on polystyrene matrices. For example, purine compounds are retarded on polystyrene exchangers more than are equally charged pyrimidine compounds. This has been rationalized by noting the more 'organic' nature of the purines (compared to pyrimidines of similar structure) and the benzenoid nature of the polystyrene matrix [19]. Thus, guanine is more hydrophobic than uracil at pH 7.0 [20], a property which is probably retained in their nucleotide derivatives Up and Gp. Therefore, a complete separation of Up from Gp with our method is, most probably, due to stronger hydrophobic binding of Gp to polystyrene Dowex 1 or Dowex 2 columns at pH 7, compared to Up; the separation is, probably, a combination of hydrophobic and ion-exchange chromatography, although a precise mechanism remains to be established.

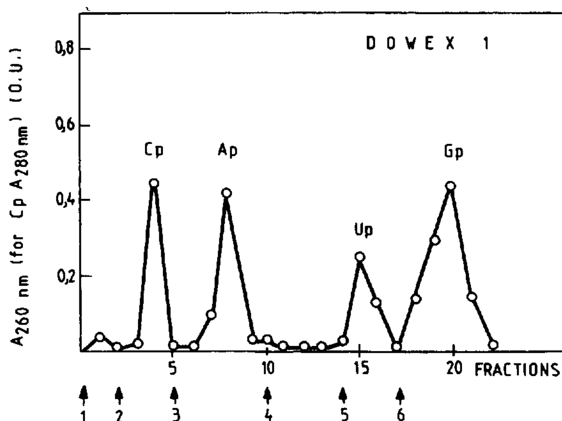

Fig. 1. Dowex 1-X10 ($HCOO^-$, 37–74 μm) column (3×0.7 cm) was loaded with 220 μg of ribomononucleotide mixture obtained by alkaline hydrolysis of 28S rRNA from rat liver, as described in Section 2. The column was developed by discontinuous desorption with following solutions (shown by arrows on abscissa): (1) 0.005 *M* HCOOH, (2) 0.05 *M* HCOOH, (3) 0.5 *M* HCOOH, (4) 0.1 *M* $HCOONH_4$ (pH 7), (5) 0.1 *M* $HCOONH_4$+0.05 *M* HCOOH and (6) 0.2 *M* $HCOONH_4$+0.5 *M* HCOOH. Abscissa also shows the numbering of 3 ml fractions in the eluate.

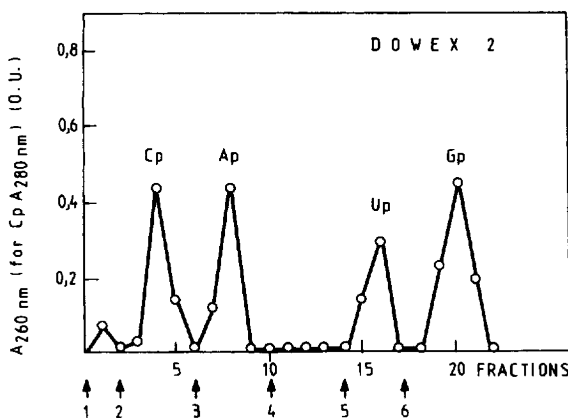

Fig. 2. Dowex 2-X10 ($HCOO^-$, 37–74 μm) column (3×0.7 cm) was loaded with 200 μg of ribomononucleotide mixture obtained by alkaline hydrolysis of 28S rRNA from rat liver; other experimental details as in Fig. 1.

Spectral characteristics of isolated ribomononucleotides were identical. The ratio of absorbancies at 280 nm versus 260 nm were for Cp 1.7–1.9; for Ap 0.23–0.26; for Up 0.29–0.32; and for Gp 0.64–0.7.

Fig. 3 shows the labeling of 28S RNA with [^{14}C]-uridine (as pyrimidine precursor). Radioactivity was detected in Cp and in Up peaks, and there was no Gp contamination with Up. Fig. 4 shows the labeling of 28S RNA with [^{14}C]-adenosine (a purine precursor); radioactivity was detected in Ap and Gp peaks. We have selected 28S RNA for labeling experiments because of its relatively high content of unusual nucleotides [21].

Our method afforded a satisfactory separation with both resins, in a broad concentration range from 26

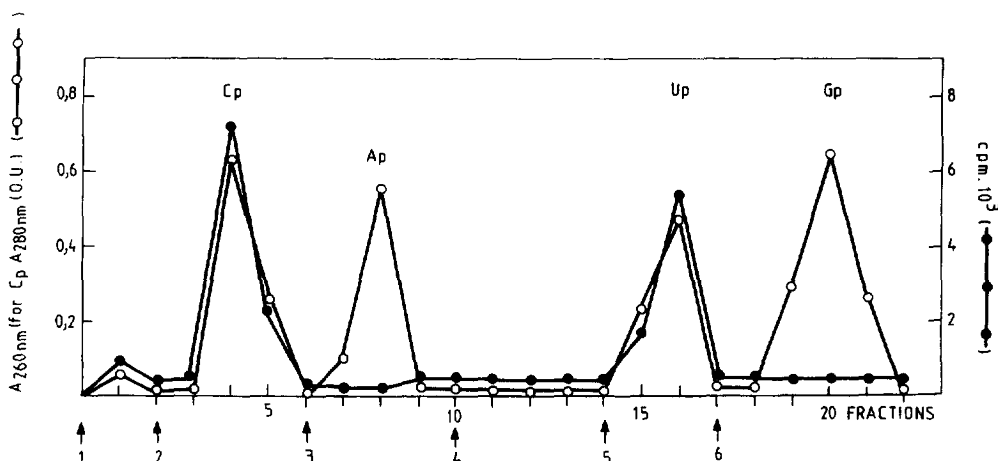

Fig. 3. Fractionation of 450 μg of ribomononucleotides on Dowex 2 column. Pyrimidines were labeled as described in Section 2; other experimental details as in Fig. 1

μg to 1 mg of ribomononucleotide mixture per ml of packed resin; therefore, our method can be applied in analytical, as well as in preparative work. This is a clear advantage over HPLC methods, which require separate columns for preparative and for analytical work. In HPLC analytical work, a nucleotide load is much lower compared to our method; for example, a load of soluble nucleotides is only 5 nmol with an anion-exchange Partisil-10 SAX column or with an anion-exchange Partisil-10 stainless-steel column [5].

In studies with radioactively labeled RNA and radioactively labeled nucleotides, ^{3}H-labeled compounds are most commonly used due to their high specific radioactivity; less common are ^{14}C-labeled, although $H_3{}^{32}PO_4$ with high specific radioactivity was often used. Our method may be applied with

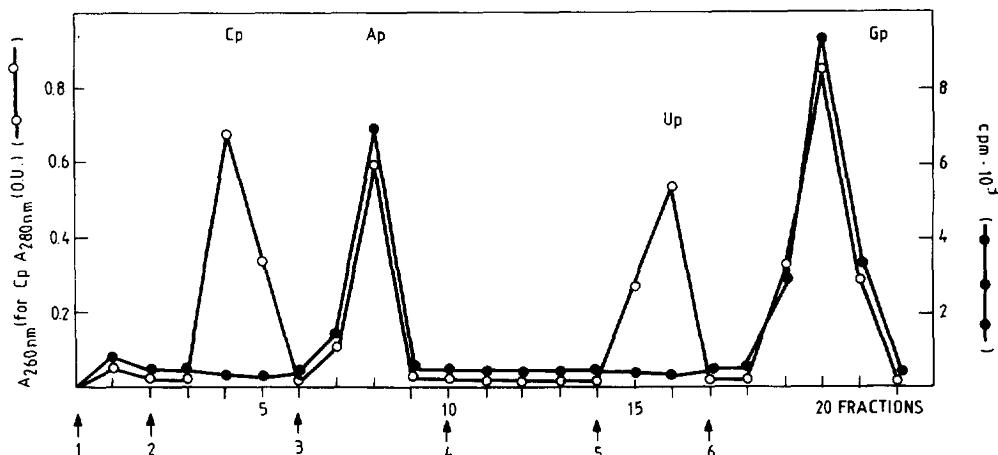

Fig. 4. Fractionation of 480 μg of ribomononucleotides on Dowex 2 column. Purines were labeled as described in Section 2; other experimental details as in Fig. 1.

^{14}C- and ^{3}H-labeled precursors with lower specific radioactivity; thus, for example, we have successfully used $H_3\,^{32}PO_4$ for the estimation of nucleotide composition, with only 1000 Ci/mg P. Compounds labeled with ^{3}H are subject to faster radiolysis, and after a prolonged storage must be purified.

Our method has been applied for the quantitative determination of the nucleotide composition of ribosomal RNAs, obtained from different mammalian cells. Tables 1 and 2 show the nucleotide structure of rRNAs from various mammalian cells, determined by ion-exchange chromatography on Dowex 1 or Dowex 2 columns, respectively. Both tables show similar or identical nucleotide composition for different rRNAs, obtained from different sources. Our analytical results with Dowex 1 and Dowex 2, shown in Tables 1 and 2, are similar or identical with comparable data in the literature, for rat liver [17] or Ehrlich ascites cells [18]. Our method is unable to separate unusual nucleosides and, therefore, their presence was not detected on Dowex 1 or Dowex 2 columns. Our method has been applied to mammalian RNAs, but it is also applicable to other types of ribosomal RNAs, especially those with low content or without unusual nucleosides, such as 5.0S and 5.8S eukaryotic ribosomal RNAs [21].

Our method is applicable to large series of samples. Another advantage of this method is an easy purification and regeneration of resins, which can be used for many years, until they are degraded by autocatalysis; this is not the case with HPLC methods, which require replacement of columns, thus presenting a problem to less affluent laboratories.

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Table 1
Nucleotide composition of ribosomal RNAs, determined by column chromatography on Dowex 1-X10 ($HCOO^-$)

	Cytidilyc acid	Adenylic acid	Uridylic acid	Guanylic acid	G–C (mol%)
18S RNA					
Rat liver					
From absorbancy[a]	25.6	22.6	19.5	31.8	57.4
From radioactivity[b]	25.0	21.2	25.5	29.0	54.0
Ehrlich ascites					
From absorbancy	29.0	24.2	19.1	28.3	57.3
From radioactivity	25.2	25.8	17.1	30.1	55.3
Mouse kidney					
From absorbancy	28.0	23.3	21.2	28.0	56.0
From radioactivity	27.4	25.7	17.2	30.1	57.6
28S RNA					
Rat liver					
From absorbancy	32.4	16.5	15.7	35.5	67.9
From radioactivity	32.9	19.1	18.3	30.7	63.6
Ehrlich ascites					
From absorbancy	32.8	20.2	15.7	31.8	64.6
From radioactivity	33.4	20.2	17.2	30.0	63.4
Mouse kidney					
From absorbancy	31.4	21.2	15.5	32.6	64.0
From radioactivity	32.2	18.9	17.1	31.2	63.3

[a] Calculated from absorbancy with the aid of molar extinction coefficients from Cohn [6].
[b] Calculated from radioactivity, after labeling with $H_3\,^{32}PO_4$ for 24 h with 500 μCi per animal intraperitoneally.

Table 2
Nucleotide composition of ribosomal RNAs, determined by column chromatography on Dowex 2-X10 ($HCOO^-$)

	Cytidylic acid	Adenylic acid	Uridylic acid	Guanylic acid	G–C (mol%)
18S RNA					
Rat liver					
From absorbancy[a]	25.5	22.7	19.4	31.6	57.1
From radioactivity	25.0	21.2	25.5	29.0	54.0
Ehrlich ascites					
From absorbancy	29.0	24.2	20.1	28.2	57.3
From radioactivity	25.1	26.0	18.0	30.4	55.4
Mouse kidney					
From absorbancy	28.0	23.2	21.2	30.0	58.0
From radioactivity	27.4	25.8	17.1	30.7	57.5
28S RNA					
Rat liver					
From absorbancy	32.5	16.5	15.5	35.2	67.7
From radioactivity	34.0	20.1	17.4	30.7	64.7
Ehrlich ascites					
From absorbancy	32.8	20.1	15.4	31.9	64.7
From radioactivity	33.5	20.0	17.0	29.9	63.4
Mouse kidney					
From absorbancy	31.3	21.1	15.4	32.5	63.8
From radioactivity	32.1	18.1	17.2	32.0	64.1

[a] Calculated from absorbancy with the aid of molar extinction coefficients from Cohn [6].
[b] Calculated from radioactivity, after labeling with $H_3{}^{32}PO_4$ for 24 h with 500 μCi per animal intraperitoneally.

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