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Separation of high-molecular mass RNAs by high-performance liquid chromatography on hydroxyapatite

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ABSTRACT

High-molecular-mass RNAs [transfer-(t-), 5S-, 18S- and 28S-RNA] in 25 m*M* sodium acetate buffer (pH 6.0) were separated by high-performance liquid chromatography (HPLC) on hydroxyapatite using a linear gradient (120 min–duration) from 0.03 to 0.147 *M* of phosphate buffer (pH 7.0) containing 0.3 *M* potassium chloride and 1 m*M* sodium azide with a slope of 2 m*M*/ml at a flow-rate of 0.5 ml/min. When the RNAs were dissolved in 4 *M* guanidine isothiocyanate–25 m*M* sodium acetate buffer (pH 6.0)–0.1 *M* β-mercaptoethanol (4 *M* GIT), t-, 5S- and 18S- or 28S-RNAs but not 18S- and 28S-RNAs were separated. RNAs extracted from rat superior cervical ganglia with 4 *M* GIT could be separated. Thus, HPLC on hydroxyapatite is a rapid and accurate means of quantifying and/or preparing high-molecular-mass RNAs such as t- and ribosomal RNAs.

INTRODUCTION

To separate and analyse RNAs of higher molecular mass than transfer RNA (tRNA), agarose or polyacrylamide gel electrophoresis is often used[1]. These methods are simple, but time consuming, and quantification can be difficult. The ceramic-type hydroxyapatite resin, which has a spherical and rigid structure with uniform quality, has been developed[2]. This resin for high-performance liquid chromatography (HPLC) has been used to purify mouse monoclonal antibodies[3] and to separate tRNAs from *Escherichia coli* and *Bacillus subtilis*[4]. HPLC on hydroxyapatite has been used to separate single- and double-stranded DNAs[5] and also RNA and DNA[6]. We now report the separation of t- and ribosomal RNAs and a procedure applicable

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to the quantitative analysis of t- and ribosomal RNAs in the superior cervical ganglion (SCG), using HPLC on hydroxyapatite.

EXPERIMENTAL

Materials

Ribosomal RNAs (18S and 28S) of calf liver (100 absorbance units at 260 nm) were purchased from Pharmacia (Tokyo, Japan), 16S- and 23S-ribosomal RNAs (100 absorbance units at 260 nm), 5S-ribosomal RNA (20 absorbance units at 260 nm) and tRNA of *E. coli* MRE 600 from Boehringer Mannheim Yamanouchi (Tokyo, Japan), [5,6-^{3}H]uridine (38.3–42.0 Ci/mmol) from New England Nuclear (Boston, MA, U.S.A.), Dulbecco's modified Eagle medium, high glucose (DME medium) in powder form from GIBCO (OH, U.S.A.) and a Millex-GV (0.22 μm) filter from Japan Millipore (Yamagata, Japan). Other reagents were obtained from commercial sources.

High-performance liquid chromatography

A packed column (10 cm × 7.5 mm I.D.) of hydroxyapatite (2 or 5 μm) was obtained from Tonen (Tokyo, Japan). A guard column (1 cm × 7.5 mm I.D.) was installed. A Hitachi (Tokyo, Japan) L-6200 intelligent pump with a low pressure gradient system was used. The elution pattern was monitored at 260 nm using a Hitachi L-4200 UV–VIS detector and recordings were made with a Hitachi D-2500 chromato-integrator. To dissolve the RNAs we used 25 m*M* sodium acetate buffer (pH 6.0) or 4 *M* guanidine isothiocyanate–25 m*M* sodium acetate buffer (pH 6.0)–0.1 *M* β-mercaptoethanol (4 *M* GIT). Injection of the sample (50 μl) onto the column was followed by washing for 2 min with 0.01 *M* KH_2PO_4–KOH (pH 7.0) containing 0.3 *M* potassium chloride and 1 m*M* sodium azide. Elution of RNAs was done by a linear gradient (120 min–duration) of phosphate buffer (pH 7.0) from 30 to 147 m*M* with a flow-rate of 0.5 ml/min at ambient temperature (20–25°C), unless stated otherwise.

Electrophoresis in agarose gel

Fractions of each peak were dialysed against 1 *M* guanidine isothiocyanate–25 m*M* sodium acetate buffer (pH 6.0) for 4 h to remove the phosphate, a necessary procedure because a high concentration of phosphate interferes with the ethanol precipitation of RNA. RNAs in each sample were precipitated in 75% ethanol and then dissolved in autoclaved (120°C, 20 min) Milli-Q water. Dissolved RNA samples were denatured with glyoxal and dimethyl sulphoxide, followed by electrophoresis[7] in a 1.5% agarose gel submerged in buffer (0.01 *M* NaH_2PO_4–NaOH, pH 7.0) at 3–4 V/cm for 5 h. At the end of the run, the gel was stained with aqueous acridine orange (20 μg/ml)[8] for 30 min and photographed under UV light (λ_{max} = 254 nm) after destaining overnight with Milli-Q water.

Labelling of synthesized RNA and extraction of RNA from rat superior cervical ganglion

Superior cervical ganglia (SCG) were removed from Wistar albino rats (20 days old). Synthesized RNA was labelled with [^{3}H]uridine, in organ culture in which DME medium was used together with 100 units/ml of penicillin G, 100 μg/ml of streptomycin sulphate and 0.25 μg/ml of amphotericin B (fungizone). Four SCGs were in-

cubated at 37°C under oxygen–carbon dioxide (95:5) in 300 μl of the culture medium with [^{3}H]uridine (20 μCi) for 20 h. At the end of incubation, the SCGs were immediately homogenized in 4 *M* GIT.

Chromatogram of rat SCG extract

The homogenate was centrifuged at 18 500 *g* for 25 min and the supernatant was injected onto the hydroxyapatite (5 μm) column (10 cm × 7.5 mm I.D.) after passage through a 0.22-μm filter. The column was washed with 0.01 *M* KH_2PO_4–KOH (pH 7.0) containing 0.3 *M* potassium chloride and 1 m*M* sodium azide for 5 min with a flow-rate of 2 ml/min at ambient temperature (20–25°C) and 0.2-min fractions were collected. A 100-μl volume of each of fraction in a microvial was mixed with 4 ml of Atomlight. Radioactivity was measured using a Packard Tri-Carb 460C liquid scintillation spectrophotometer.

Determination of RNA content

Absorbance measured in a cuvette with a 1-cm path length was considered to indicate an RNA with a concentration of approximately 40 μg/ml[9].

RESULTS AND DISCUSSION

Chromatogram of tRNA

An appropriate concentration gradient (80 min–duration) of phosphate for chromatography of tRNA was from 0.03 to 0.11 *M* with a slope of 2 m*M*/ml at a flow-rate of 0.5 ml/min (Figs. 1A and 2A). tRNA dissolved in 25 m*M* sodium acetate buffer (pH 6.0) (Fig. 1A) or 4 *M* GIT (Fig. 2A) was separated into several peaks, as noted by Yamakawa *et al.*[4]. The molecular mass of each peak corresponded to that of tRNA on agarose gel electrophoresis (lanes 1,2 and 3 in Figs. 1D and 2D). Hence, these multiple peaks presumably indicate the heterogeneity of tRNA corresponding to each amino acid.

Chromatogram of 5S-RNA

An appropriate concentration gradient (80 min–duration) of phosphate for chromatography of 5S-RNA was the same as that for tRNA (Figs. 1B and 2B). 5S-RNA dissolved in 25 m*M* sodium acetate buffer (pH 6.0) (Fig. 1B) was separated into one major peak and one minor peak, but 5S-RNA dissolved in 4 *M* GIT (Fig. 2B) separated into one major and two minor peaks. The molecular mass of each peak corresponded to that of 5S-RNA on agarose gel electrophoresis (lanes 1 and 2 in Figs. 1E and 2E). Hence these multiple peaks are indicative of contamination, multiplicity of 5S-RNA or minor degradation of native 5S-RNA. Four variants of 5S-RNA have been found in *E. coli* A19[10]. The multiple peaks may correspond to these variants of 5S-RNA.

Chromatogram of 18S- and 28S-RNA

An appropriate concentration gradient (80 min–duration) of phosphate for the separation of 18S- and 28S-RNAs dissolved in 25 m*M* sodium acetate buffer (pH 6.0) was from 0.07 to 0.15 *M* with a slope of 2 m*M*/ml at a flow-rate of 0.5 ml/min (Fig. 1C). 18S- and 28S-RNAs each gave single symmetrical peaks. Elution of 28S-RNA

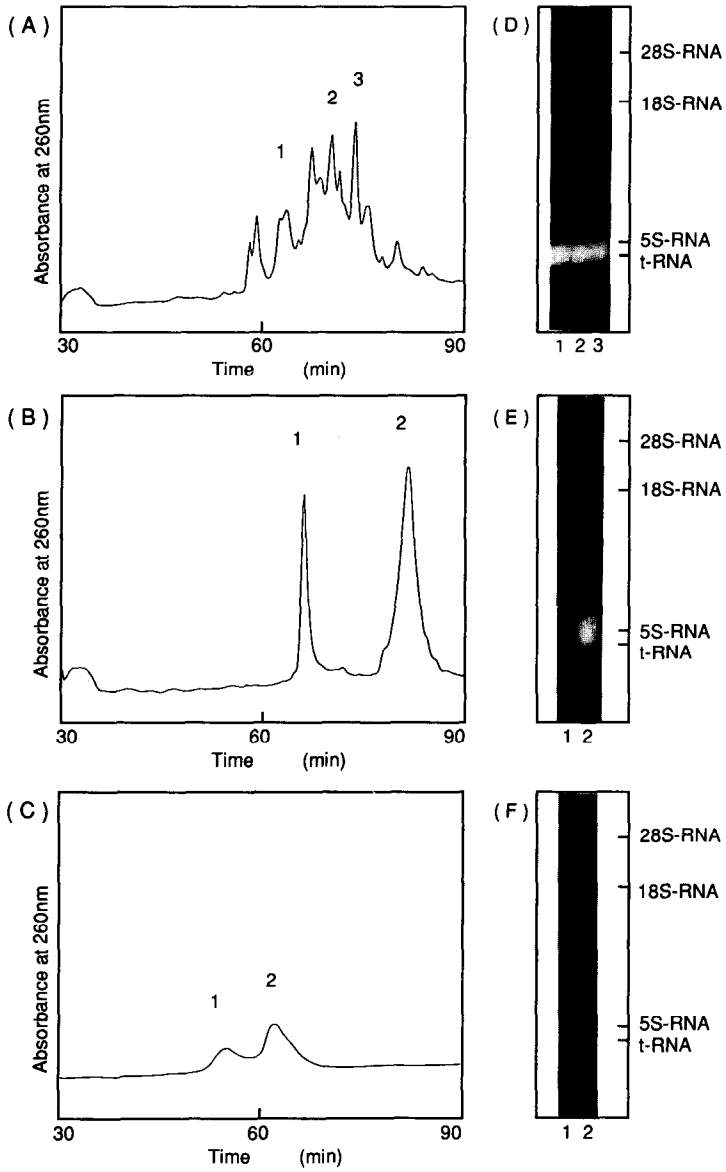

Fig. 1. Chromatograms of t-, 5S-, 18S- and 28S-RNAs on hydroxyapatite. A packed column (10 cm × 7.5 mm I.D.) of hydroxyapatite (2 μm) was used. Washing buffer at sample application was 0.01 *M* KH_2PO_4–KOH (pH 7.0) containing 0.3 *M* KCl and 1 m*M* NaN_3. Elution of RNA was done by a linear gradient (80 min–duration) of phosphate buffer (pH 7.0) containing 0.3 *M* KCl and 1 m*M* NaN_3 at a flow-rate of 0.5 ml/min at ambient temperature (20–25°C). The concentration gradient was varied as described below. RNAs were dissolved in 25 m*M* sodium acetate buffer (pH 6.0). (A) Chromatogram of tRNA from *E. coli* MRE 600. tRNA (1.2 μg) was injected onto the column and eluted by a linear gradient from 0.03 to 0.11 *M* of phosphate buffer (pH 7.0). (B) Chromatogram of 5S-RNA from *E. coli* MRE 600. 5S-RNA (0.1 μg) was injected onto the column and eluted by a linear gradient from 0.03 to 0.11 *M* of phosphate buffer (pH 7.0). (C) Chromatogram of 18S- and 28S-RNAs. 18S- and 28S-RNA mixture (0.5 μg) was injected onto the column and eluted by a linear gradient from 0.07 to 0.15 *M* of phosphate buffer (pH 7.0). (D) Electrophoresis in agarose gel of tRNA. Peak fractions 1, 2 and 3 from tRNA chromatogram A were electrophoresed in 1.5% agarose gel. The gel was stained with acridine orange and photographed under UV light (λ_{max} = 254 nm). (E) Electrophoresis in agarose gel of 5S-RNA. Peak fractions 1 and 2 from 5S-RNA chromatogram B were electrophoresed in 1.5% agarose gel. The gel was stained with acridine orange and photographed under UV light (λ_{max} = 254 nm). (F) Electrophoresis in agarose gel of 18S- and 28S-RNAs. Peak fractions 1 and 2 from 18S- and 28S-RNA chromatogram C were electrophoresed in 1.5% agarose gel. The gel was stained with acridine orange and photographed under UV light (λ_{max} = 254 nm).

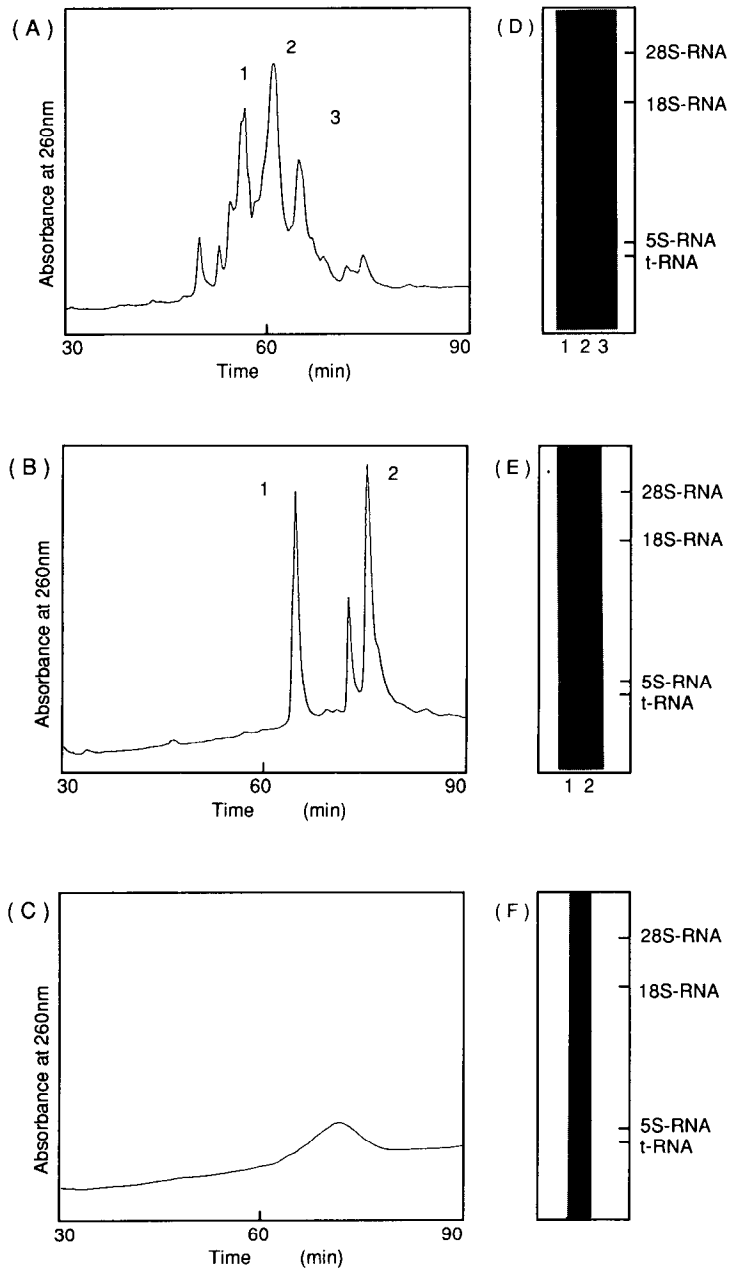

Fig. 2. Chromatograms of t-, 5S-, 18S- and 28S-RNAs on hydroxyapatite. RNAs were dissolved in 4 *M* GIT. (A) Chromatogram of tRNA from *E. coli* MRE 600. (B) Chromatogram of 5S-RNA from *E. coli* MRE 600. (C) Chromatogram of 18S- and 28S-RNAs. (D) Electrophoresis in agarose gel of tRNA. Peak fractions 1, 2 and 3 from tRNA chromatogram A were electrophoresed in 1.5% agarose gel. (E) Electrophoresis in agarose gel of 5S-RNA. Peak fractions 1 and 2 from 5S-RNA chromatogram B were electrophoresed in 1.5% agarose gel. (F) Electrophoresis in agarose gel of 18S- and 28S-RNAs. Peak fraction of 18S- and 28S-RNA chromatogram C was electrophoresed in 1.5% agarose gel. Other conditions in Fig. 1.

followed that of 18S-RNA (Fig. 1F). However, when the 18S- and 28S-RNA mixture was dissolved in 4 *M* GIT, separation was not feasible (Fig. 2C and F). 16S- and 23S-RNAs dissolved in 25 m*M* sodium acetate buffer (pH 6.0) were also separated using a linear gradient (80 min–duration) from 0.07 to 0.15 *M* of phosphate buffer (pH 7.0) with a slope of 2 m*M*/ml at a flow-rate of 0.5 ml/min (data not shown). However, when the mixture was dissolved in 4 *M* GIT, they could not be separated. A mixture of 16S-, 18S-, 23S- and 28S-RNAs in 25 m*M* sodium acetate buffer (pH 6.0) could not be separated using our experimental procedures; only a single broad peak appeared (data not shown).

Separation of t-, 5S-, 18S- and 28S-RNAs

5S-RNA was separated from tRNA using a linear gradient of phosphate from 0.03 to 0.11 *M* (Fig. 3B), that is, 5S-RNA was eluted following tRNA. For the separation of t- and 5S-RNAs, the addition of a salt such as potassium chloride or sodium chloride was essential (Fig. 3A and B). The first minor peak of 5S-RNA

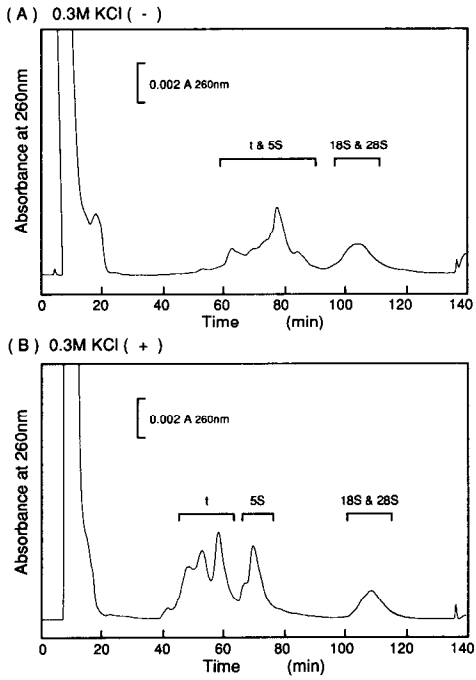

Fig. 3. Effect of salt on separation of t-, 5S-, 18S- and 28S-RNAs on hydroxyapatite. A packed column (10 cm × 7.5 mm I.D.) of hydroxyapatite (2 μm) was used. RNAs were dissolved in 4 *M* GIT. (A) Washing buffer at sample application was 0.01 *M* KH_2PO_4–KOH (pH 7.0) containing 1 m*M* NaN_3. Elution of RNA was done by a linear gradient (120 min–duration) from 0.03 to 0.147 *M* of phosphate buffer (pH 7.0) containing 1 m*M* NaN_3 at a flow-rate of 0.5 ml/min at ambient temperature (20–25°C). (B) Washing buffer at sample application was 0.01 *M* KH_2PO_4–KOH (pH 7.0) containing 0.3 *M* KCl and 1 m*M* NaN_3. Elution of RNA was done by a linear gradient (120 min–duration) from 0.03 to 0.147 *M* of phosphate buffer (pH 7.0) containing 0.3 *M* KCl and 1 m*M* NaN_3 at a flow-rate of 0.5 ml/min at ambient temperature (20–25°C).

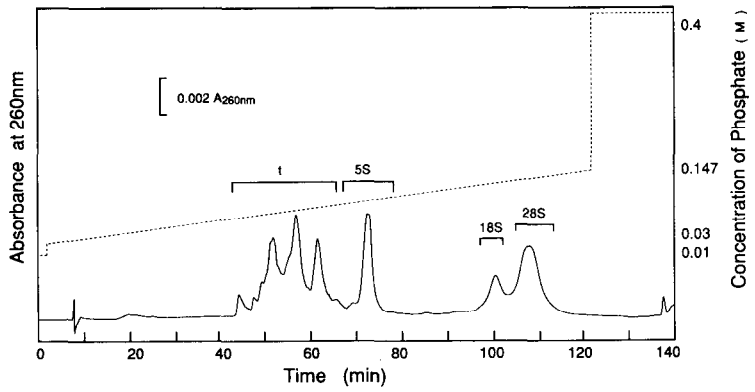

Fig. 4. Separation of t-, 5S-, 18S- and 28S-RNAs on hydroxyapatite. A packed column (10 cm × 7.5 mm I.D.) of hydroxyapatite (2 μm) was used. Washing buffer at sample application was 0.01 *M* KH_2PO_4–KOH (pH 7.0) containing 0.3 *M* KCl and 1 m*M* NaN_3. Elution of RNA was done by a linear gradient (120 min–duration) from 0.03 to 0.147 *M* of phosphate buffer (pH 7.0) containing 0.3 *M* KCl and 1 m*M* NaN_3 at a flow-rate of 0.5 ml/min at ambient temperature (20–25°C). RNAs were dissolved in 25 m*M* sodium acetate buffer (pH 6.0).

overlapped with the later peak of tRNA, even in the presence of 0.3 *M* potassium chloride (Fig. 3B). The mixture of t-, 5S-, 18S- and 28S-RNAs in 25 m*M* sodium acetate buffer (pH 6.0) was separated using a linear gradient (120 min–duration) from 0.03 to 0.147 *M* of phosphate buffer (pH 7.0) with a slope of 2 m*M*/ml at a flow-rate of 0.5 ml/min (Fig. 4), except that a minor peak of 5S-RNA overlapped with that of tRNA, as described above. When the mixture of t-, 5S-, 18S- and 28S-RNAs dissolved in 4 *M* GIT was chromatographed on hydroxyapatite using a linear gradient (120 min–duration) from 0.03 to 0.147 *M* of phosphate buffer (pH 7.0) with a slope of 2 m*M*/ml at a flow-rate of 0.5 ml/min, 18S- and 28S-RNAs could not be separated, but t- and 5S-RNAs could be separated (Fig. 5), except for a partial overlap, as described above. The elution sequence of t-, 5S-, 18S- and 28S-RNAs was from a

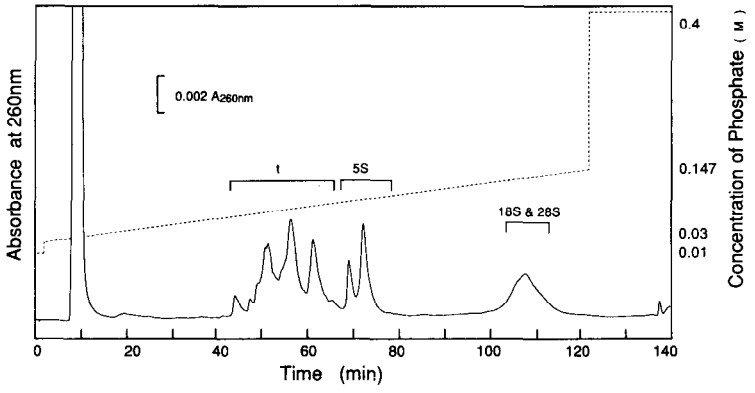

Fig. 5. Separation of t-, 5S-, 18S- and 28S-RNAs on hydroxyapatite. RNAs were dissolved in 4 *M* GIT. Other experimental conditions in Fig. 4.

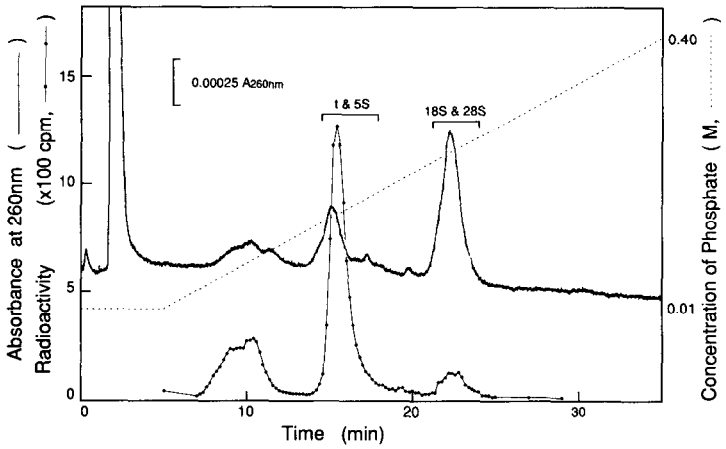

Fig. 6. Chromatogram of rat SCG extract. SCGs from 20-day-old rats were incubated with [^{3}H]uridine for 20 h. The extract of SCG with 4 *M* GIT was injected onto a hydroxyapatite (5 μm) column (10 cm × 7.5 mm I.D.). The column was washed with 0.01 *M* KH_2PO_4–KOH (pH 7.0) containing 0.3 *M* KCl and 1 m*M* NaN_3 for 5 min at a flow-rate of 2 ml/min. Elution of RNA was done by a linear gradient (30 min–duration) from 0.01 to 0.4 *M* of phosphate buffer (pH 7.0) containing 0.3 *M* KCl and 1 m*M* NaN_3 at a flow-rate of 2 ml/min at ambient temperature (20–25°C); 0.2 min fractions were collected. The radioactivity in each of these fractions was determined using a liquid scintillation spectrophotometer.

lower to a higher molecular mass, that is, a higher concentration of phosphate was necessary to elute the higher molecular mass of RNA, presumably because the phosphate moiety in nucleic acid interacts with the Ca^{2+} moiety in hydroxyapatite, and the interaction is strengthened, depending on the amount of nucleic acid in RNA.

Separation of t- and ribosomal RNAs in rat SCG

GIT 4 *M* is useful for the complete inhibition of endogenous nucleases and deproteinization from RNA[11]. Thus, extraction of RNAs in rat SCG was effected using 4 *M* GIT and the extracted RNAs were used for hydroxyapatite column chromatography with a linear gradient (30 min–duration) from 0.01 to 0.4 *M* of phosphate buffer (pH 7.0) with a slope of 6.5 m*M*/ml at a flow-rate of 2 ml/min (Fig. 6). The t- and 5S-RNAs were completely separated from 18S- and 28S-RNAs. The radioactivity of [^{3}H]uridine corresponded to the absorbance at 260 nm. Hence HPLC on hydroxyapatite can be used for the analysis and/or preparation of high-molecular-mass RNAs such as t- and ribosomal RNAs. Separation between t- and 5S-RNAs and between 18S- and 28S-RNAs is in progress.

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