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SINGLE-STEP SEPARATION OF MAJOR AND RARE RIBONUCLEOSIDES AND DEOXYRIBONUCLEOSIDES BY HIGH-PERFORMANCE LIQUID CATION-EXCHANGE CHROMATOGRAPHY FOR THE DETERMINATION OF THE PURITY OF NUCLEIC ACID PREPARATIONS

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SUMMARY

A method is described for the separation of 13 major and minor ribo- and deoxyribonucleosides in a single chromatographic run using high-performance liquid chromatography on strongly acidic cation-exchange columns. The method proved useful for the routine determination of small amounts of ribonucleic acid impurities in deoxyribonucleic acid preparations and *vice versa*. About 3% or even less of nucleic acid contamination in a given sample can be easily detected and quantitatively determined under the conditions used.

INTRODUCTION

The quantitative determination of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) impurities in DNA or RNA samples during the isolation procedure still represents a difficult task, particularly if small amounts of DNA or RNA (less than 10^{-4} g) have to be processed and if the impurity is 3% or less of the total sample.

Purification of DNA preparations from contaminating RNA is commonly achieved by CsCl centrifugation[1], and the amount of RNA which is found at the bottom of the centrifuge tube may then be determined quantitatively. The separation of contaminating DNA from RNA or of DNA and rRNA from tRNA can be achieved by selective precipitation or extraction procedures[2,3], by chromatographic methods[4,5] or by polyacrylamide gel electrophoresis[6]. The fractions containing the impurities or minor components are pooled and quantitatively determined. These methods, however, are more or less time consuming and require at least two subsequent steps of processing, one for the separation and another for the quantitative determination of the minor residues.

In the course of our recent work on the isolation and characterization of DNA[7]

and RNA[8] we developed a simple and rapid method for the determination of RNA impurities in DNA preparations and *vice versa*. The procedure depends on the separation and quantitative determination of ribo- and deoxyribonucleosides in a single chromatographic run using high-performance liquid cation-exchange chromatography. Prior to this the nucleic acid preparation is enzymatically hydrolysed by ribo- and deoxyribonucleases, by phosphodiesterase and phosphatase.

In this paper the feasibility of this method in routine analytical work and its sensitivity are demonstrated by means of an RNA preparation from *Escherichia coli* and a DNA preparation of the sponge *Geodia cydonium*.

MATERIALS AND METHODS

DNA of the sponge *Geodia cydonium* was isolated according to Hönig *et al.*[9] from sponges harvested in the Northern Adriatic near Rovinj (Yugoslavia). RNA was isolated according to Aviv and Leder[10] from *E. coli* ATCC 11303.

Ribonuclease I (RNase I, EC 3.1.4.22) was purchased from Miles Laboratories, Slough, Great Britain, ribonuclease T_1 (RNase T_1, EC 3.1.4.8), deoxyribonuclease I (DNase I, EC 3.1.4.5, 1000 U/ml), snake venom phosphodiesterase (PDase, EC 3.1.4.1, 2 mg/ml), and alkaline phosphatase (APase, EC 3.1.3.1, 1 mg/ml) were from Boehringer, Mannheim, G.F.R.

Strongly acidic cation-exchange resin (type M-71, particle diameter 10–12 μm) was purchased from Beckman, München, G.F.R. The reagents, obtained from E. Merck, Darmstadt, G.F.R., were of the highest available purity.

Adenosine (rAdo), cytidine (rCyd), guanosine (rGuo), inosine (rIno), uridine (rUrd), deoxyadenosine (dAdo), deoxycytidine (dCyd), deoxyguanosine (dGuo), deoxyinosine (dIno), and deoxythymidine (dThd) were obtained from Papierwerke Waldhof-Aschaffenburg, Mannheim, G.F.R., pseudouridine (Ψrd, natural isomer), ribothymidine (5-methyluridine, me^{5}Urd), and 5-methyldeoxycytidine (me^{5}dCyd) from P. L. Biochemicals, Milwaukee, Wisc., U.S.A.

For the preparation of reference solutions the (deoxyribonucleosides were dissolved in standard buffer solutions at pH 6.0 and 7.0. The solutions, which exhibited absorbances of 2 to 3 at λ_{max}, were diluted before use with 0.4 *M* ammonium formate, pH 4.6, in the ratio 1:10.

The isolated DNA and RNA samples were separately dissolved in 0.01 *M* Tris–HCl + 0.1 *M* NaCl, pH 7.9, at a concentration of 0.5 mg/ml. A 1.0 ml sample of each solution was digested with 10 μg each of RNase I and RNase T_1 for 4 h at room temperature. Then 200 μl of DNase I, 100 μl of PDase, and 200 μl of APase, together with 1.0 ml of Tris–HCl, pH 9.4, and 50 μl of 0.6 *M* $MgCl_2$ solution were added and the mixture reincubated for 4 h at 37 °.

The ribo- and deoxyribonucleosides were separated in a Varian LCS-1000 liquid chromatograph, equipped with a 254 nm UV flow-cell detector and a diaphragm-piston pump (Orlita, Giessen, G.F.R.). A 150 × 0.18 cm I.D. stainless steel tube was filled with cation-exchange resin according to Scott and Lee[11], and the column eluted with 0.4 *M* (with respect to the NH_4^+ concentration) ammonium formate, pH 4.6, at a linear flow velocity of 4.7 cm/min (flow-rate 7.2 ml/h) at 40° column oven temperature.

RESULTS AND DISCUSSION

The total separation of the major ribo- and deoxyribonucleosides from each other, from inosine and deoxyinosine and some rare nucleosides within a single column-chromatographic run has not previously been reported.

As compared to similar devices which have been reported earlier for the separation of the major deoxyribonucleosides and of deoxyinosine[12], for the major and some rare ribonucleosides[13], for a minor deoxyribonucleoside from DNA hydrolysates[14], and for 6-thiopurine ribonucleosides and bases[15], only the column length and the column temperature had to be changed to 150 cm and 40°, respectively. By these means the nucleoside pairs dIno–rGuo, rCyd–dAdo, and particularly dGuo–rAdo, were separated. The separation of Ψrd–rUrd–dThd–rIno and dCyd–me^{5}dCyd was easily achieved on even shorter columns. The me^{5}Urd fraction, however, if added to the reference sample, eluted in between rUrd and dThd without resolution from either nucleoside. An elution pattern as obtained from the separation of 13 ribo- and deoxyribonucleosides on the 150 cm column is shown in Fig. 1.

The elution pattern of a separation of the deoxyribonucleosides obtained from an enzymatic hydrolysis of DNA from *Geodia cydonium* which was slightly con-

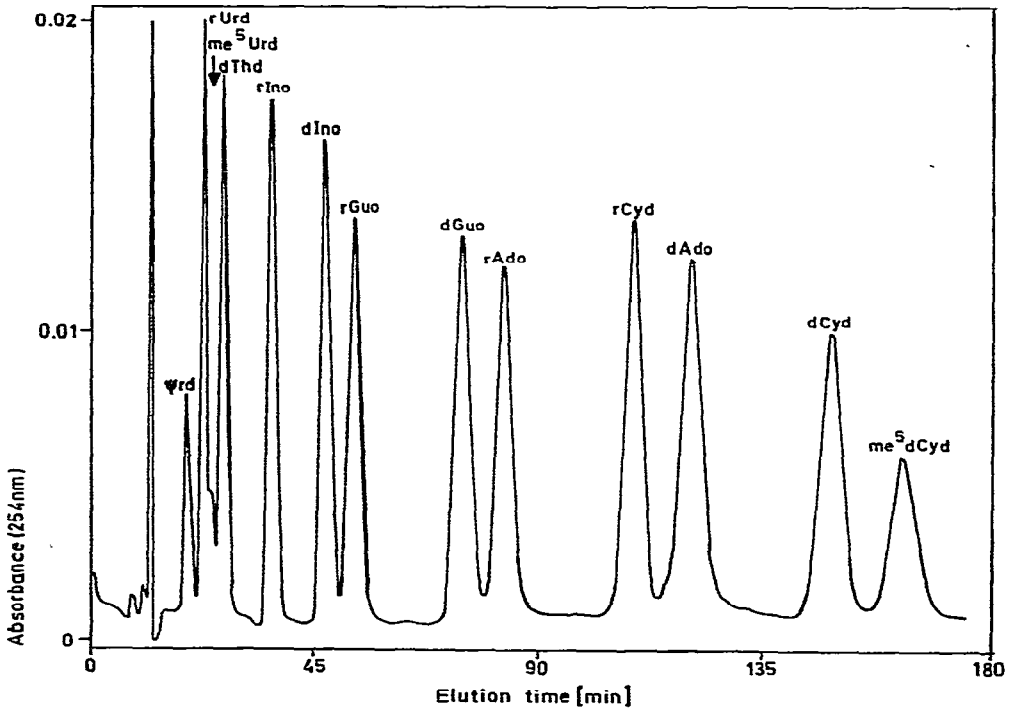

Fig. 1. Elution pattern obtained from the separation of a synthetic mixture of 13 major and rare ribo- and deoxyribonucleosides (for chromatographic conditions see Materials and Methods). Injected volume: 28 μl. Amounts of nucleoside (pmole): ψrd, 90; rUrd, 200; me^{5}Urd, 55; dThd, 240; rIno, 200; dIno, 200; rGuo, 180; dGuo, 220; rAdo, 220; rCyd, 660; dAdo, 220; dCyd, 580; me^{5}dCyd, 450.

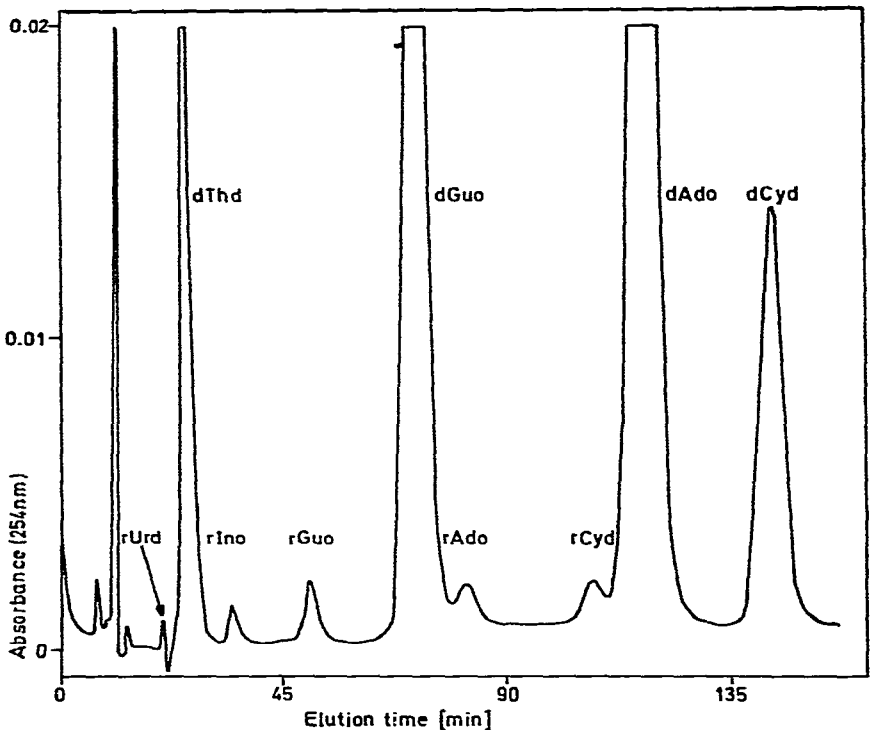

Fig. 2. Elution pattern obtained from the separation of a *Geodia cydonium* DNA hydrolysate containing *ca.* 3% of RNA impurities (for chromatographic conditions see Materials and Methods). Injected volume: 15 μl.

taminated with RNA is shown in Fig. 2. A concentration of *ca.* 3% of RNA in the DNA sample is easily detectable.

The elution pattern of a separation of the ribonucleosides obtained from an enzymatic hydrolysis of RNA from *E. coli* which contained *ca.* 3% of DNA is shown in Fig. 3. As the RNA preparation contains a relatively high amount of rare bases, additional peaks (Ψrd and the unidentified compounds A and B) are obtained in the elution diagram together with the major ribo- and deoxyribonucleoside fractions. To demonstrate the sensitivity of detection 150 pmole of dAdo was added to the sample which was injected onto the column.

As compared to a method of Schrecker *et al.*[16] who used thin-layer chromatography for the separation of deoxyribo-, ribo- and arabinonucleosides, the method described here is much more sensitive. Even unlabelled ribo- and deoxyribonucleosides can be detected in amounts less than 30 pmole.

Aoyagi *et al.*[17] recently described a method for the separation of ribo- or deoxyribonucleosides which, however, required previous group separation[18] if the ribo- and deoxyribonucleoside of a given base were to be distinguished.

A separation method for ribo- and deoxyribonucleosides described by Duch and Laskowski[19] working with an Aminex A-7 column (25 × 0.24 cm I.D.) which is eluted with ammonium formate of various molarities at pH 4.55 gave poor separation of rUrd and dThd. Furthermore, the elution diagrams showed broad peak

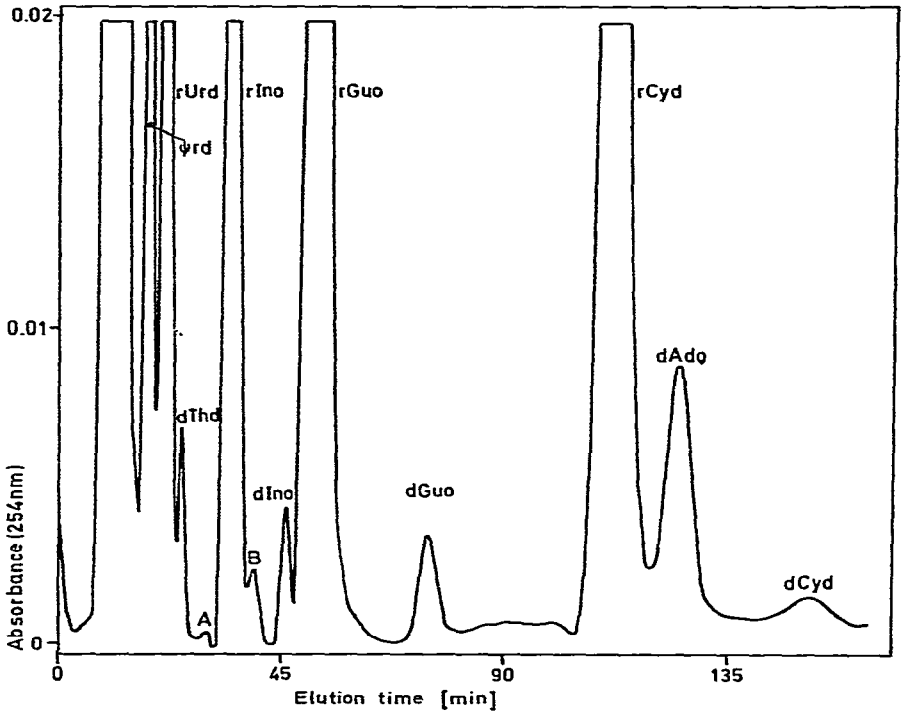

Fig. 3. Elution pattern obtained from the separation of an *E. coli* RNA hydrolysate containing *ca.* 3% of DNA impurities (for chromatographic conditions see Materials and Methods). Injected volume: 30 μl; dAdo (150 pmole) was added to the injected sample.

shapes although the separation was finished within 60 min. Owing to this disadvantage quantitative measurements had to be carried out by cutting the peaks and weighing the paper. Obviously the system was not optimized with respect to the molarity of the eluent, resulting unfavourable values for the capacity factors of strongly retained compounds[20].

The system described here clearly separates rUrd from dThd, and the major ribo- and deoxyribonucleosides from each other, as well as from Ψrd, rIno, dIno, me^{5}Urd, and me^{5}dCyd. Even at the end of the elution diagram, peak widths are sufficiently narrow although a total separation lasts for *ca.* 3 h. Hence, quantitative determinations of ribo- and deoxyribonucleosides are easily obtained with small standard errors using simple peak-height measurements if an integrator is not available. The sensitivity of this method for the determination of impurities of RNA in DNA samples and *vice versa* was found to be better than 0.07% of a nucleoside.

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