An Isocratic High-Pressure Liquid Chromatographic Purification Method for Radioactively Labeled Deoxyribonucleoside Triphosphates

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A method is described for the rapid purification of radioactively labeled deoxyribonucleoside triphosphates from their spontaneously emerging hydrolysis products deoxyribonucleoside diphosphate, deoxyribonucleoside monophosphate, and deoxyribonucleoside. The separations which are finished within 3 min or less are carried out on a 0.1×5 cm column filled with LiChrosorb-NH_2, using isocratic elution with 0.025 M potassium phosphate, pH 6.8, in a high-pressure liquid chromatograph at room temperature and a flow rate of 30 ml·h^{-1} (flow velocity 63.7 cm·min^{-1}).

Introduction

The determination of deoxyribonucleoside triphosphates (dNTP) in organic materials has become an easy and rapid task since the development of the enzymatic DNA-polymerase assay for these compounds by Solter and Handschumacher [1], Skoog [2] and Lindberg and Skoog [3] using either DNA [1] or synthetic polynucleotides (poly dIC and poly dAT) [2, 3]. These methods are based on the observation that a continued DNA synthesis depends on the presence of the four dNTPs at a time.

One dNTP in the enzyme assay is offered in a limiting amount whereas the other components are present in excess. The DNA synthesis proceeds until the limiting dNTP has become totally incorporated into the newly synthesized DNA or polynucleotide strand.

One of the dNTPs which are present in excess amount bears a radioactive label in the purine or pyrimidine moiety. Using synthetic polynucleotides for the assay the amount of radioactivity which is incorporated into the newly synthesized DNA is proportional to the amount of the limiting component. Using DNA as a primer of the assay reaction this amount is correlated to the (A + T)/(G + C)-ratio of the used DNA.

The sensitivity of the determination of a dNTP by the DNA-polymerase assay strongly depends on a high specific activity of the radioactively labeled dNTP added in excess. The radioactivity which is incorporated into the acid insoluble polynucleotide represents a measure proportionate to the amount of the limiting dNTP.

As only dNTPs serve as substrates for the polymerase action the bulk amount of radioactivity which is added to the enzyme assay must be adjoined to these compounds.

dNTPs, however, permanently undergo spontaneous hydrolysis and the corresponding dNTPs and dNMPs are formed. If too much of the radioactivity of a dNTP sample is adjoined to dNDPs and dNMPs poor incorporation rates into the polynucleotides are obtained leading to incorrect results. This fact must be taken into account despite the label belongs to a purine or pyrimidine base or to the α-phosphate group of the dNTP.

The dNTPs commonly are separated from contaminating dNDPs, dNMPs, dNuc, and the pyrophosphate by chromatography on either DEAE-cellulose or DEAE-Sephadex columns according to Weimann and Khorana [4] and Wehrli *et al.* [5].

In the course of estimating the dNTP pools in various tissues we needed only small amounts of radioactively labeled dNTPs with high specific activity. As the spontaneous hydrolysis of purified dNTPs, however, occurs even in samples stored at low temperature (−4 to 10 °C) [6] we developed a rapid purification method for each dNTP in order to obtain highly pure dNTP preparations daily. The

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Abbreviations: dNuc, deoxyribonucleoside; dNMP, deoxyribonucleoside monophosphate; dNDP, deoxyribonucleoside diphosphate; dNTP, deoxyribonucleoside triphosphate; DNA, deoxyribonucleic acid; poly d(IC) or poly d(AT), copolymer chain of alternating hypoxanthine and cytosine or adenine and thymine residues bound to 2′-deoxyribose associated with another such chain by base-pairing; HPLC, high-pressure liquid chromatography.

method of Wehrli *et al.*[5] for this purpose proved to be rather difficult and time consuming.

In this paper we report a simple and very rapid purification method for small amounts of labeled dNTPs using isocratic elution in a high-pressure liquid chromatographic device.

Materials and Methods

A Varian LCS 1000 liquid chromatograph was used for the separations. Radioactivity measurements were carried out in a Hewlett-Packard Tri-Carb liquid szintillation counter.

LiChrosorb-NH_2 (particle diameter 10 μm) which served as the stationary phase of the chromatographic system was obtained from Merck, Darmstadt (West Germany).

A 0.1×5 cm stainless steel tubing was filled with a thick slurry of LiChrosorb-NH_2, dissolved in 0.025 M potassium phosphate, pH 6.8, according to the method of Scott and Lee[7].

Then a flow rate of 30 ml·h^{-1} (flow velocity 63.7 cm·min^{-1}) was adjusted at a column pressure

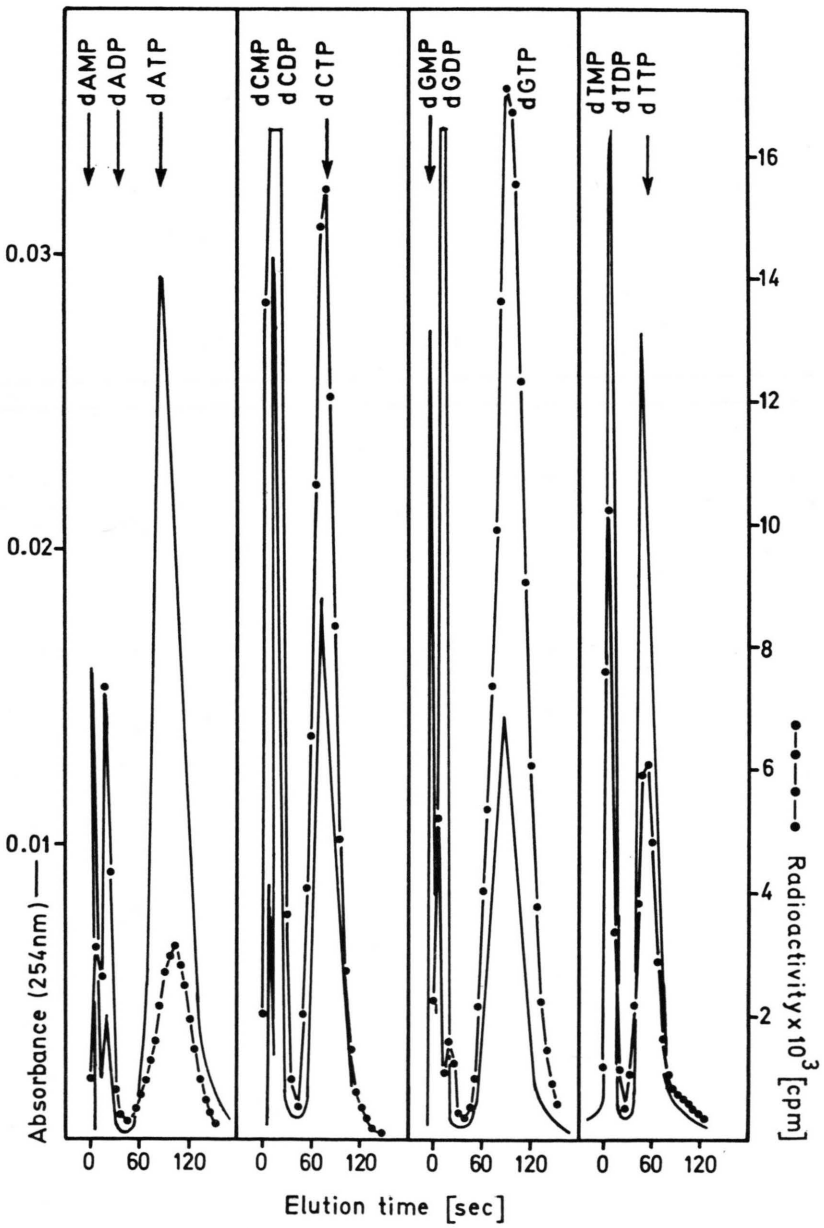

Fig. 1. Elution patterns and radiochromatograms of the separation of each dNTP from its dNMP and dNDP as obtained on the 5×0.1 cm column with LiChrosorb-NH_2 as the stationary phase, eluent 0.025 M KH_2PO_4, pH 6.8 (further details see Materials and Methods). Sample sizes:

dATP (2.23 nM+34.1 nCi);
dCTP (2.38 nM+139 nCi);
dGTP (2.52 nM+119 nCi);
dTTP (4.22 nM+101 nCi).

of about 800 psi. All separations were carried out at room temperature.

The samples were directly injected onto the column using a Reeve-Angel injection port and a 10 µl Hamilton syringe.

The column effluent was fractionated into liquid szintillation vials (0.14 ml per vial). The radioactivity measurements were carried out as described elsewhere [8].

The four unlabeled dNTPs (dATP, dCTP, dGTP, and dTTP) were purchased from Serva-Feinbiochemica, Heidelberg (West Germany). Reference samples contained 235, 213, 179, and 223 pmoles per µl of the dATP, dCTP, dGTP, and dTTP solution, respectively.

The ^{3}H-labeled dNTPs, [8-^{3}H]dATP (24 Ci/mmol), [5-^{3}H]dCTP (27 Ci/mmol), [8-^{3}H]dGTP (6.2 Ci/mmol), and [methyl-^{3}H]dTTP (47 Ci/mmol), were purchased from The Radiochemical Centre, Amersham (England), dissolved in aqueous ethanol.

An aliquot of each solution was diluted with ethanol/water (1:1, V:V) until the radioactivity reached 34.1, 677, 575, and 503 nanoCi per 10 µl in the labeled dATP, dCTP, dGTP, and dTTP solution, respectively.

Results and Discussion

Fig. 1 shows the elution patterns and the radiochromatograms obtained from the 0.1 × 5 cm column of the separations of each of the dNTPs from its hydrolysis products dNuc, dNMP, and dNDP. The contaminants are hardly separated from each other. The dNTPs, however, are well separated from the contaminants.

Gere [9] and Shmukler [10] separated the ribonucleoside mono-, di- and triphosphates from each other by HPLC in 4 and 25 min, respectively, using a gradient elution method.

As compared to these techniques our method proved superior with respect to either the speed and the isocratic elution device. Each separation is finished within 2.5 min and no regeneration of the column is necessary. So the four dNTPs may successively be purified within 10 min.

The retention times of the dNTPs strongly depend on the molarity and pH value of the eluent. Lowering the pH value to 6.2 or the molarity to 0.010 M the dNDPs and dNTPs are totally retained on the column. At pH values and molarities higher than 7.0 and 0.040 M, respectively, no separation of the three phosphorylated compounds can be achieved.

10 to 30 nCi of radioactivity which are commonly required for the dNTP polymerase assay with either activated DNA [1, 11] or poly dIC [2] and poly dAT [3] represent extremely small amounts of substance as the ^{3}H-labeled dNTPs used for this purpose show very high specific activities of about 20 to 50 Ci/mmole. Due to this fact about 2 to 5 µCi of radioactivity bound to 200 to 500 pmol of dNTP can be purified from contaminant radioactivity on the 5 cm column without loss of separation efficiency as the column hardly becomes overloaden.

If bulk separations of high amounts of dNTPs are required a 20 cm column with 0.2 cm I.D. may be used at the same elution conditions [12].

A sample of 10 to 50 µl of volume which is injected onto the column after the separation procedure yields a volume of 500 to 700 µl. This is easily reduced to 50 µl by evaporation to dryness under reduced pressure. The sediment then is redissolved in 50 µl of destilled water. The molarity of the solution, however, by this procedure raises to 0.25 to 0.35 M. As the DNA polymerase assay needs a 0.040 M phosphate buffer, pH 7.4, the high molarity should be taken in account when the reaction mixture, commonly 80 to 100 µl, is prepared.

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