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Improved sample preparation method for high-performance liquid chromatography of deoxyribonucleoside triphosphates from cell culture extracts

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SUMMARY

The accurate determination of deoxyribonucleoside triphosphates in cells is difficult owing to the high concentrations of interfering ribonucleoside triphosphates. The latter can be degraded to their respective bases by periodate oxidation of cell extracts. However, the large amount of bases so produced can interfere with subsequent high-performance liquid chromatographic (HPLC) analysis. The use of a weak ion-exchange cartridge to partially purify and concentrate deoxyribonucleoside triphosphates in periodate-treated cell extracts, prior to HPLC, thus allowing accurate determination is described. The recovery of the deoxyribonucleoside triphosphates is >95%, and >90% of the interfering bases are removed.

INTRODUCTION

The measurement of nucleoside triphosphate pools in mammalian cells is of importance in studies on aspects of DNA and RNA synthesis and regulation. Many anticancer and antiviral drugs interfere with DNA and RNA precursors and with DNA and RNA synthesis. High-performance liquid chromatography (HPLC) has been used for the direct separation and quantification of nucleotides in cell extracts. However, the determination of deoxyribonucleoside triphosphates (dNTP) in cell and tissue extracts is difficult because ribonucleoside triphosphates (NTP), which normally co-elute with dNTP, are present in concentrations several orders of magnitude higher than those of dNTP[1].

Attempts have therefore been made to remove NTP from cell extracts prior to HPLC of dNTP. Boronate affinity chromatography has been used to retain NTP selectively in cell extracts, but the procedure is lengthy and a lyophilization step is required prior to HPLC of the dNTP[2].

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The selective degradation of NTP to their corresponding bases by periodate and methylamine has been used to remove interfering NTP prior to HPLC of the remaining unaffected dNTP[1]. The degradation of NTP by the periodate oxidation procedure has been used extensively. Although the method is rapid and efficient, we experienced problems with the separation of the large amount of bases produced from the NTP in the periodate oxidation (which eluted early in the chromatogram) from the dNTP of interest.

The large amount of bases absorbed UV radiation, making accurate measurement of the small dNTP peaks difficult, and also shortened the life of the column. Hence a method for removing these interfering bases and for purifying dNTP in periodate-treated cell extracts prior to HPLC is required.

A number of different procedures for partially purifying dNTP in periodate-treated extracts prior to HPLC have previously been published. These include calcium fluoride coprecipitation[3], acetonitrile precipitation[3], silica cartridge separation[4] and strong anion-exchange (SAX) cartridge separation[3]. All these procedures give good recoveries using standard aqueous solutions of nucleotides but, except for the SAX cartridge procedure, give much poorer recoveries with periodate-treated cell extracts. We have previously shown that the SAX cartridge procedure shows consistently high recoveries of dNTP both using standard aqueous solutions and periodate-treated cell extracts[3]. Unfortunately, the procedure involved elution with 1 *M* hydrochloric acid, which must therefore be quickly neutralized to avoid any acid hydrolysis of the dNTP. A further disadvantage was that the elution volume was between 2 and 5 ml to achieve 100% recovery of the dNTP, which meant that a lyophilization step was necessary prior to HPLC, which might affect the recovery. We have now investigated the use of weak anion-exchange (aminopropyl) cartridges for the partial purification and concentration of dNTP in periodate-treated cell extracts prior to HPLC. The results show that dNTP in periodate-treated cell extracts can be quickly and simply partially purified prior to HPLC using weak anion-exchange cartridges.

EXPERIMENTAL

Chemicals

All nucleotides were of the highest purity available and were purchased from Sigma (St. Louis, MO, U.S.A.). The purity of the standard nucleotides was determined by HPLC as described below. All standard nucleotides used were at least 98% pure.

Chromatographic equipment

All HPLC analyses were performed on a chromatograph from Waters Assoc. (Milford, MA, U.S.A.). The absorbance at 254 and 280 nm was measured with a Model 440 dual-wavelength absorbance detector. Integration of the peaks was performed with a Shimadzu C-R3A integrator with an FDD-IA floppy disc drive.

Chromatographic procedure

Nucleotides were separated isocratically by ion-exchange HPLC using a strong anion-exchange column (Partisil 10 SAX, 250 × 4.6 mm I.D.) (Whatman, Clifton, NJ, U.S.A.). The mobile phase was 0.6 *M* KH_2PO_4 adjusted to pH 3.25 with phosphoric acid. A flow-rate of 2 ml/min was used.

Cell extraction procedure

CEM cells (a human CD4 + lymphocyte cell line) were grown at 37°C in RPMI medium supplemented with 10% heat-inactivated foetal calf serum in a humidified atmosphere of 5% carbon dioxide in air. Cells (1×10^8) were harvested by centrifugation at 1000 *g* for 10 min. The medium was decanted and the pellet completely drained by inversion on tissue paper for 30 s. A volume of cold 12% trichloroacetic acid approximately equal to the volume of the cell pellet (600 μl) was added to the pellet and the solution (total volume *ca.* 1.2 ml) was vortex mixed for 20 s. The extract was kept on ice for 10 min, then mixed again. The precipitate was removed by centrifugation at 1000 *g* for 10 min. The supernatant (volume *ca.* 1 ml) was removed and 1.1 volume of a freshly made 0.5 *M* solution of tri-*n*-octylamine in Freon was added to neutralize the pH of the supernatant[5]. The solution was vortex mixed for 20 s and then centrifuged at 1000 *g* for 2 min to separate the phases. The neutralized aqueous phase (volume *ca.* 1 ml) containing the nucleotides extracted from $1 \cdot 10^8$ cells was carefully decanted and frozen at −20°C.

Periodate oxidation procedure

The ribonucleotides in the neutralized cell extracts were degraded to bases by the periodate oxidation procedure developed by Garrett and Santi[1]. A 40-μl volume of a 0.5 *M* aqueous solution of sodium periodate was added to 1 ml of neutralized extract (containing nucleotides extracted from 1×10^8 cells) and the solution was gently mixed. After incubation at 37°C for 3 min a 50-μl volume of a 4 *M* aqueous solution of methylamine (pH adjusted to 7 by addition of phosphoric acid) was added and the solution gently mixed. The solution was incubated at 37°C for a further 30 min and the reaction was then terminated by the addition of 10 μl of a 1 *M* aqueous solution of rhamnose. The sample (volume *ca.* 1.1 ml) was then put on ice. The entire 1.1-ml sample was applied to the cartridge in the next step.

Weak anion-exchange cartridge procedure

Weak anion-exchange cartridges (Sep-Pak and Sep-Pak Light aminopropyl cartridges) were obtained from Millipore–Waters (Milford, MA, U.S.A.). The cartridge was washed with 3 ml of water before the sample was applied. A 1-ml volume of a standard solution of deoxynucleotides in water (25 μ*M* each of dATP, dGTP, dCTP and dTTP) was then applied to the cartridge. For experiments with cell extracts, 1.1 ml of a periodate-treated extract from $1 \cdot 10^8$ cells was applied to the cartridge instead. When the recovery of the deoxynucleotides was being measured, the cell extract was "spiked" with a predetermined concentration of standard deoxynucleotides in excess of the intracellular concentration (50 μ*M* each of dATP, dGTP, dCTP and dTTP); otherwise, when the intracellular dNTP concentration of CEM cells was being measured the cell extracts were not "spiked". The cartridge was then rinsed with 3 ml of water to remove unretained components. Finally, the cartridge was eluted with 1 ml of 5 *M* sodium chloride solution. A 100-μl volume of the 5 *M* sodium chloride fraction was injected directly onto the HPLC column. The amounts of each dNTP present were determined by integration of the peak at 254 nm and comparison with that given by a known amount of a standard solution of each dNTP injected directly onto the column without use of the cartridge. Peaks were identified by (a) retention time, (b) ratio of absorbance at 280 nm to that at 254 nm and (c) spiking with known

standards. A 300-μl volume of the 3-ml water wash from the cartridge was also injected to check that all the dNTP had been retained on the cartridge prior to the salt elution. The recoveries of each dNTP were determined by calculating the total amount of dNTP eluted in the 1-ml 5 *M* salt fraction and comparing this with the known amount applied to the cartridge in the standard aqueous solution or spiked cell extract. All experiments were performed in triplicate and the mean results with the standard deviations are quoted. The amount of bases present in the sample was determined likewise by integration of the appropriate peaks.

RESULTS AND DISCUSSION

Weak anion-exchange cartridges were used to retain dNTP while the other components of the periodate-treated cell extracts, including the bases that arise from the periodate degradation of NTP, were eluted in the water wash. Only the triply charged dNTP could be expected to have bound to this weak anion-exchange matrix. The NTP cannot bind as they are known to be degraded to bases in the periodate reaction[1]. The dNTP were then eluted with 1 ml of 5 *M* sodium chloride and subjected to HPLC.

The recoveries of each of the four dNTP in the 1-ml salt fraction when applied to the cartridge as an aqueous solution of known concentration and purity were as follows (means of three determinations $\pm$ standard deviation): 97 $\pm$ 0.61% for dATP; 96 $\pm$ 1.4% for dGTP; 98 $\pm$ 2.1% for dCTP; and 98 $\pm$ 0.72% for dTTP. No dNTP was detectable in the 3-ml water wash from the cartridge, indicating that all the dNTP was retained by the cartridge prior to the salt elution. The recoveries of dNTP in the salt fraction when periodate-treated cell extracts were spiked with known amounts of standard dNTP were similar, indicating that there was no decrease in recovery of dNTP when periodate-treated cell extracts were used instead of aqueous solutions.

Fig. 1 shows a typical chromatogram obtained with and without partial purification of the periodate-treated cell extract with the ion-exchange cartridge. Without use of the cartridge (Fig. 1a) the large amount of bases (produced from NTP in the periodate degradation) eluted early in the chromatogram and obscured the dNTP peaks. Only the dGTP peak at 14.4 min was accurately measured. After partial purification of the periodate-treated cell extract with the ion-exchange cartridge (Fig. 1b) most of the bases were removed and the peaks of dCTP, dTTP, dATP and dGTP could be clearly seen and accurately measured. The total absorbance at 254 nm of the interfering bases eluting with the solvent front at 1–10 min (without use of the cartridge, Fig. 1a) or at 1–5 min (after partial purification with the cartridge, Fig. 1b) was determined by integration of the appropriate peaks and the values were compared to determine how much of the interfering bases had been removed by use of the ion-exchange cartridge. The results showed that 93 $\pm$ 6% (mean of three separate determinations $\pm$ standard deviation) of the interfering bases eluting at the solvent front were removed using the ion-exchange cartridge.

Attempts were made to reduce the salt concentration and volume required for elution of the dNTP by the use of sodium chloride in phosphate buffer (0.6 *M* KH_2PO_4 adjusted to pH 3.5 or 2.2 with phosphoric acid). Lowering the pH to 3.5 or 2.2 did not decrease the salt concentration or volume significantly. However, the use of smaller cartridges (Sep-Pak Light) with one third the amount of sorbent did permit a reduction

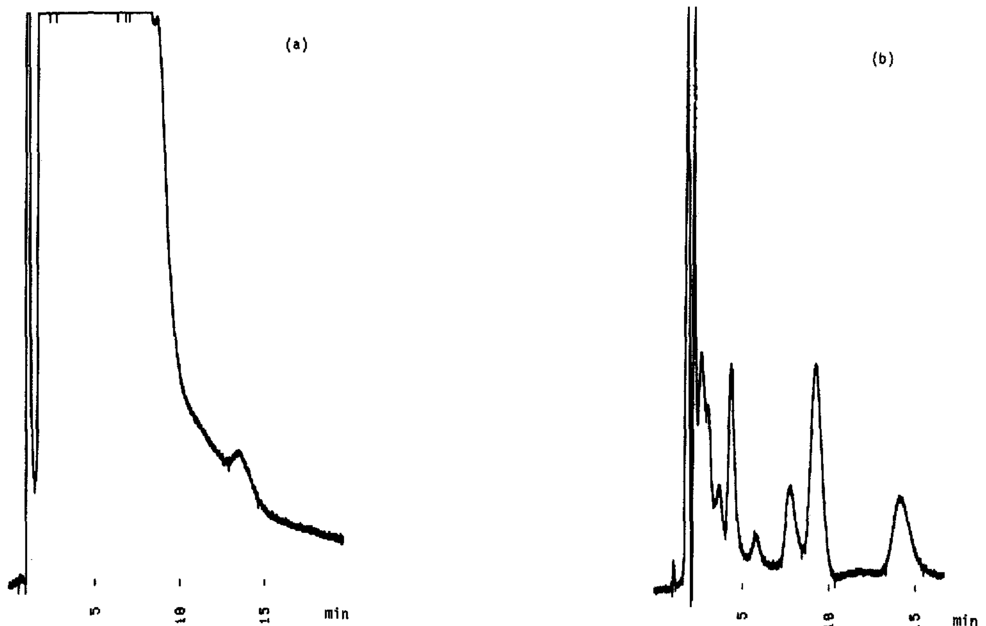

Fig. 1. Chromatogram obtained on injection of a periodate-treated cell extract (a) without further purification and (b) after partial purification on a weak anion-exchange cartridge. Extracts from $2 \cdot 10^7$ cells were injected in each instance. The absorbance at 254 nm was measured. The sensitivity was set at 0.01 a.u.f.s. dCTP, dTTP, dATP and dGTP eluted at 5.9, 7.8, 9.4 and 14.4 min, respectively.

in the elution volume. In this instance the elution volume could be reduced to 0.5 ml without a decrease in recovery. This small volume allowed the concentration of the dNTP in periodate-treated cell extracts and removed the need for lyophilization before HPLC analysis.

Using these cartridges to partially purify the periodate-treated extract, the dNTP content of CEM cells in the late logarithmic phase was measured by HPLC. The dNTP contents in pmol per 10^6 cells of a periodate-treated extract of CEM cells measured by HPLC after partial purification on the ion exchange-cartridge were found to be as follows: dATP, 22.8 ± 5.2; dGTP, 15.7 ± 1.1; dCTP, 3.1 ± 0.7; and dTTP, 18.8 ± 5.7 (means of four determinations $\pm$ standard deviations).

In conclusion, it has been demonstrated that aminopropyl cartridges provide a rapid and reliable means of partly purifying and concentrating dNTP in periodate-treated cell extracts at neutral pH prior to HPLC. The use of these cartridges removes much of the bases produced in the periodate reaction and thus allows the accurate measurement of the dNTP content of cell extracts by HPLC.

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