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Short Communication

Comparison of extraction procedures for high-performance liquid chromatographic determination of cellular deoxynucleotides

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Abstract

For the determination of cellular deoxynucleoside triphosphates (dNTPs) by high-performance liquid chromatography, the choice of extracting agent and periodate oxidation procedure is important for accurate results. Different extraction methods were compared using either cold methanol or trichloroacetic acid for extraction of dNTPs from lymphoblastoid cells. The recoveries of the dNTPs varied with the different extraction methods. A modification of the periodate oxidation procedure for degrading ribonucleoside triphosphates to their bases was also compared with the original method. Both methods gave accurate results when trichloroacetic acid was used as extracting agent, but when methanol was used interfering peaks were present on the chromatogram when the original method was used. These peaks were absent when the modified periodate procedure was used.

1. Introduction

The determination of cellular deoxynucleoside triphosphates (dNTPs) is important for the study of drugs interfering with DNA synthesis. Several anti-human immunodeficiency virus (anti-HIV) drugs are nucleoside analogues and compete with cellular deoxynucleosides for phosphorylation. The triphosphate form of the drug can be incorporated into the viral DNA strand and terminate viral replication [1]. The levels of cellular dNTPs and nucleoside analogue triphosphates are therefore crucial for the antiviral activity.

The levels of cellular dNTPs are usually measured by high-performance liquid chromatography (HPLC) [2–4]. The choice of extraction procedure for the HPLC of dNTPs from cell extracts is important for accurate quantification. Acid extraction of nucleotides using trichloroacetic acid (TCA) or perchloric acid and subsequent neutralization is often used [5–7]. However, some nucleoside analogues such as 2′,3′-dideoxyinosine (ddI) are acid labile, which makes TCA unsuitable as the extracting agent for determining intracellular levels of ddI nucleotides.

We compared different extraction methods based on cold 60% methanol (instead of TCA) for the extraction of cellular deoxynucleotides for HPLC analysis. The recoveries of standard dNTPs were compared with those obtained when TCA was used for extraction.

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Before cellular deoxynucleotides can be determined by HPLC, the large amounts of ribonucleotides present in the cell extracts must be removed by selective degradation. Two different procedures for the degradation of ribonucleotides by periodate oxidation prior to HPLC analysis have been published [2,8]. We compared these two procedures with the different extraction methods. The procedure of Tanaka *et al.* [8] was superior when methanol was used as the extracting agent, but either procedure was suitable when TCA was used.

2. Experimental

2.1. Chemicals

Pure nucleotides were purchased from Sigma (St. Louis, MO, USA).

2.2. Chromatographic equipment

All HPLC analyses were done on a chromatograph from Gilson Medical Electronics equipped with a dual-wavelength spectrophotometer programmed to measure absorbance at 254 and 280 nm. All integration was performed on a Macintosh Classic II computer using Rainin Dynamax HPLC method manager software.

2.3. Chromatographic procedure

Nucleotides were separated on a strong anion-exchange column (Partisil 10 SAX, 250 × 4.6 mm I.D.) (Whatman, Clifton, NJ, USA) using phosphate buffer gradient elution. The two buffers used were 0.02 *M* potassium phosphate (pH 3.5) (buffer A) and 0.80 *M* potassium phosphate (pH 3.5) (buffer B). The gradient was completed in four steps. Step 1 was 100% buffer A for 5 min; step 2 was a 30-min linear increase in buffer B to 100%; step 3 was a constant isocratic 20-min delivery of 100% buffer B; and in step 4 buffer B was decreased to 0% in 10 min. A constant flow-rate of 1.3 ml/min was used throughout.

2.4. Cell harvesting procedure

CEM cells (a human CD4+ lymphoblastoid cell line) were grown at 37°C in a humidified atmosphere of 5% CO_2 in air in RMPI 1640 medium containing 10% heat-inactivated foetal calf serum, penicillin, streptomycin and pyruvate. Cells ($0.9 \cdot 10^8$–$1.3 \cdot 10^8$) were harvested by centrifugation at 1000 *g* for 10 min. The supernatant was decanted and the cell pellet was further drained by inverting the tube on tissue paper for 30 s. For comparison of extraction procedures, cells from the same batch were divided into aliquots for use with the different procedures.

2.5. Methanol extraction procedures

Four different methanol extraction methods were compared. To measure deoxynucleotide recoveries, the cell pellets ($0.9 \cdot 10^8$–$1.3 \cdot 10^8$ cells) were "spiked" with high concentrations (0.05 μmol) of deoxynucleoside triphosphate (dNTP) standards (dATP, dGTP, dCTP, dTTP) in a large excess of intracellular dNTP concentrations. The high concentrations of standards compared with cellular dNTPs is necessary to ensure that the cellular dNTPs present do not contribute significantly to the recoveries measured, thereby giving a falsely high recovery. Also, measuring the recovery of spiked cell extracts rather than of pure solutions of dNTPs gives an indication of losses due to protein binding.

In method 1, 1 ml of cold 60% methanol was added to the "spiked" cell pellet and this mixture was vortex mixed for 20 s. After a 5-min incubation on ice, the sample was mixed again. Cell matter was removed by centrifugation at 1000 *g* for 10 min. The supernatant was carefully removed with a Pasteur pipette and placed on ice. While maintaining the sample on ice, the methanol was reduced to half the volume by evaporation under nitrogen. The sample was then held at −86°C until frozen, followed by

freeze-drying at −50°C overnight. The dried sample precipitate was dissolved in 0.5 ml of cold distilled water and frozen at −20°C until use. The evaporation and freeze-drying steps were necessary to remove the methanol before ion-exchange HPLC could be performed.

Method 2 was the same as method 1 but instead of evaporation, the methanol was diluted by adding 500 μl of distilled water. The sample was then frozen and freeze-dried. This method was used to investigate whether large amounts of dNTPs were lost in the evaporation process.

We investigated whether using a larger volume of methanol for extraction would give a better recovery of dNTPs. In Method 3, 2.5 ml of cold 60% methanol was added to the "spiked" cell pellet and, after mixing, the pellet was left overnight in a freezer at −20°C. Mixing, centrifugation, evaporation and freeze-drying were then followed as in method 1.

Finally, we tried using a larger volume of methanol for extraction, but without evaporation. Method 4 used 2.5 ml of cold 60% methanol and overnight extraction as in method 3. After mixing and centrifuging, the methanol supernatant was removed and diluted by adding 1.5 ml of distilled water. The sample was then frozen and freeze-dried.

2.6. Trichloroacetic acid extraction procedure

For comparison of recoveries with those of the methanol procedures, the cell pellets ($0.9 \cdot 10^8$–$1.3 \cdot 10^8$) were "spiked" with the same concentration (0.05 μmol) of dNTP standards. To these "spiked" cell pellets an equal volume (800 μl) of 12% trichloroacetic acid was added [9] and this solution was vortex mixed for 20 s. The sample was placed on ice for a further 10 min and then remixed. The sample was then centrifuged at 1000 *g* for 10 min to remove the precipitate. The supernatant was removed to a new tube and its volume measured (*ca.* 1 ml). To neutralize the pH of the sample, 1 ml of a fresh 0.5 *M* solution of tri-*n*-octylamine in Freon was added [3,10]. The sample was vortex mixed for 20 s and then centrifuged for 2 min at 1000 *g* to separate the phases. After centrifugation, the neutralized top aqueous layer containing the nucleotides (*ca.* 1 ml) was carefully removed and frozen at −20°C.

2.7. Periodate oxidation procedures

Two different periodate oxidation procedures for the degradation of interfering rNTPs were compared. Procedure 1 was as developed by Garrett and Santi [2]. To each thawed sample tube, 40 μl of a 0.5 *M* aqueous sodium periodate solution were added, gently mixed and then incubated at 37°C for 3 min. After incubation, 50 μl of a 4 *M* aqueous solution of methylamine (adjusted to pH 7.0 with phosphoric acid) were added. The sample was incubated at 37°C for a further 30 min. Following this second incubation, the reaction was terminated by the addition of 10 μl of a 1 *M* aqueous solution of rhamnose. The samples were stored at −20°C until used.

Procedure 2 was a modification of the procedure developed by Tanaka *et al.* [8]. To each thawed sample, 20 μl of a 5 m*M* aqueous solution of deoxyguanosine and 30 μl of a 0.5 *M* aqueous solution of sodium periodate were added. The sample was gently mixed and incubated at 37°C for 5 min. After this incubation, 40 μl of a 4 *M* aqueous solution of methylamine (adjusted to pH 7.0 with phosphoric acid) and 10 μl of a 1 *M* aqueous solution of rhamnose were added. The sample was gently mixed and incubated at 37°C for a further 30 min. The samples were stored at −20°C until used.

2.8. HPLC analysis

A 100-μl volume of all 'spiked" cell extracts was injected into the HPLC system. For both "spiked" and "unspiked" cell extract samples, the dNTPs were identified by their retention times and the ratio of absorbance at 254 nm to that at 280 nm. Recoveries were calculated by comparisons of the peak areas of injected samples with those of pure dNTP standards with known concentrations.

3. Results and discussion

3.1. Comparison of extraction methods

We compared the four different extraction methods using cold 60% methanol and TCA for the extraction of standard dNTPs from spiked cell extracts. The recoveries of standard dNTPs from cells extracted using the different methods are given in Table 1. Comparison of the recoveries shows that the third methanol extraction method, using a larger volume of methanol for extraction and evaporation on ice, gave the highest recoveries of all dNTPs. This indicates that evaporation of the methanol on ice did not cause a loss of dNTPs, and was preferable to dilution of the sample with water (methods 2 and 4). Also, the larger volume of methanol used for extraction in method 3 gave better recoveries than the smaller volume used in method 1. Of the dNTPs, dCTP and dGTP were the most labile and gave the lowest recoveries.

Although the nucleotide pools of extracts of various cells and tissues have been analysed by HPLC, the recovery of nucleotides after extraction has not always been investigated [4–6]. In other instances, standard solutions of pure dNTPs in water have been used for recovery experiments with TCA and periodate, giving slightly higher recoveries than those shown here for TCA [3,8]. We chose to use spiked cell extracts instead of pure solutions for recovery experiments, to reveal whether non-specific binding to cellular proteins could affect the recovery of nucleotides. The results indicate that non-specific binding of nucleotides in cell extracts may result in a lower recovery compared with pure solutions in water [3,8].

3.2. Comparison of periodate oxidation methods

Two different methods for periodate oxidation of rNTPs after methanol extraction of unspiked cells were compared, and chromatograms from each of the methods are shown in Fig. 1. The original periodate oxidation method developed by Garrett and Santi [2] revealed two problematic peaks (Fig. 1a), as described previously [8], one eluting close to dTTP and the other appearing as a shoulder on the dGTP peak. These extra peaks interfered with accurate quantification of the cellular dNTPs.

Fig. 1b shows the results of the modified procedure for oxidation of rNTPs according to Tanaka *et al.* [8]. The two interfering peaks are absent, allowing accurate measurement of cellular dNTP levels. When TCA was used as the extracting agent, however, these interfering peaks were not seen, and the two periodate

Table 1
Comparison of the recoveries of deoxynucleotides using different extraction procedures

dNTP	Mean recovery ± S.E.M. (%)				
	Methanol				TCA method
	Method 1	Method 2	Method 3	Method 4	
dCTP	67 ± 3.8	58 ± 7.3	78 ± 7.0	66 ± 3.4	65 ± 1.8
dTTP	81 ± 0.9	75 ± 3.6	92 ± 2.2	76 ± 2.4	71 ± 3.8
dATP	94 ± 5.9	86 ± 5.8	102 ± 4.7	81 ± 1.7	75 ± 1.9
dGTP	66 ± 6.2	62 ± 3.2	72 ± 5.1	55 ± 9.1	58 ± 2.6

CEM lymphocytes were harvested by centrifugation, spiked with standard dNTPs and extracted using four different procedures based on methanol and one based on trichloroacetic acid. The recovery of dNTPs was determined after periodate oxidation by ion-exchange HPLC. Means of three different experiments ± standard error of the mean (S.E.M).

Table 2
dNTP levels in CEM cells extracted with methanol or TCA

Extraction method	dNTP content (pmol/10^6 cells ± S.E.M.)			
	dCTP	dTTP	dATP	dGTP
Methanol method 3[a]	9.7 ± 1.4	21.9 ± 1.1	55 ± 1.0	8.5 ± 1.4
Methanol method 3[b]	5.4 ± 0.7	19.8 ± 2.2	41.6 ± 1.6	8.0 ± 0.5
TCA method[a]	6.9 ± 0.7	21.7 ± 3.3	33.7 ± 5.1	8.3 ± 1.1

CEM lymphocytes were harvested by centrifugation, extracted with methanol or TCA and subjected to periodate oxidation as described under Experimental. dNTPs were determined by ion-exchange HPLC. Means of three experiments ± S.E.M.
[a] Periodate oxidation according to the method of Garrett and Santi [2].
[b] Periodate oxidation according to the method of Tanaka *et al.* [8].

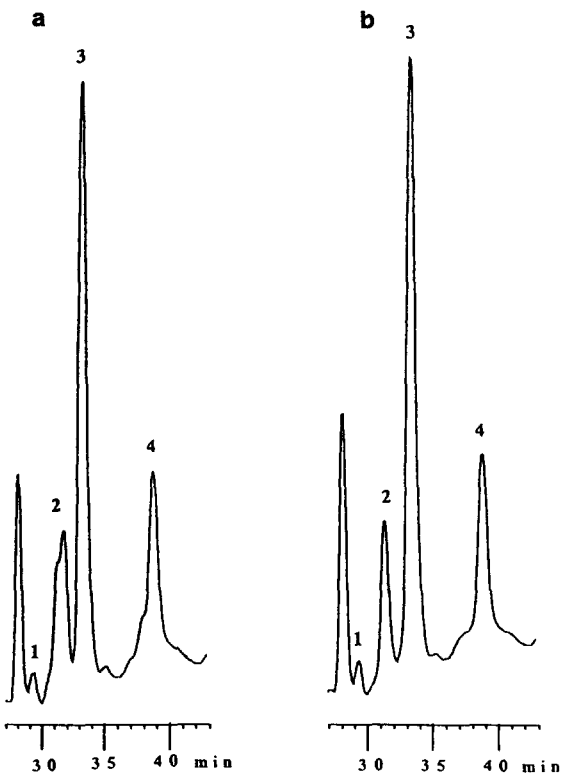

Fig. 1. Portion of the chromatogram showing absorbance at 254 nm obtained on HPLC of CEM cells extracted with methanol (method 3) after periodate oxidation according to (a) Garrett and Santi [2] or (b) Tanaka *et al.* [8]. Peaks: 1 = dCTP; 2 = dTTP and, in (a), an unknown compound; 3 = dATP; 4 = dGTP.

oxidation procedures gave identical chromatograms (data not shown).

3.3. Measurement of dNTPs in CEM cells

Following the results in Table 1, we compared the dNTP pools of unspiked CEM lymphoblastoid cells measured by HPLC after extraction with methanol or TCA, and the results are given in Table 2. Similar pools of dNTPs were measured with both extraction methods, indicating the methanol extraction of cells is a useful alternative to extraction with TCA.

In conclusion, we found that overnight extraction at −20°C with 2.5 ml of cold 60% methanol, followed by evaporation under nitrogen and freeze-drying, gave the best recoveries of dNTPs from CEM cell extracts. The modified periodate procedure of Tanaka *et al.* [8] gave superior results when methanol was used as extracting agent by removing interfering peaks, but did not confer advantages when TCA was used.

4. References

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