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RAPID AND RELIABLE METHOD FOR THE ANALYSIS OF NUCLEOTIDE POOLS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and reliable protocol for the simultaneous separation of ribo-, deoxyribo- and cyclic nucleotides has been developed using high-performance liquid chromatography on a C_{18} μBondapak column and isocratic elution with ammonium phosphate buffer (0.2 M, pH 5.1). Resolution of deoxyribonucleotides has been confirmed by performing resolution before and after periodate oxidation. The general order of elution is ribonucleotides, deoxyribonucleotides and cyclic nucleotides. While periodate oxidation improved the clarity of separation of deoxyribonucleotides by eliminating ribonucleotides, incorporation of methanol in the eluent shortened the retention time of the cyclic nucleotides. The application of this method to a complex biological system is reported.

INTRODUCTION

We have recently reported[1] a rapid and sensitive method for separating ribonucleotides on a C_{18} reversed-phase column using isocratic elution with ammonium phosphate buffer. The elution profiles were dependent on both pH and ionic strength of the buffer. To our knowledge, none of the existing methods affords simultaneous detection of total ribo-, deoxyribo- and cyclic nucleotides using isocratic elution conditions. Some protocols provide for the separation of pyrimidine nucleotides alone[2], purine nucleotides alone[3] and exclusive separation of either ribonucleotides[4] or deoxyribonucleotides[5]. Using an isocratic elution buffer and a reversed-phase column, we now report the resolution of the deoxyribonucleotides and cyclic ribonucleotides from each other and from ribonucleotides. The deoxyribonucleotides and cyclic ribonucleotides elute, in general, later than the corresponding ribonucleotides and, depending on the analysis desired, their elution could be expedited using the same buffer fortified with 10% (v/v) methanol. Combining periodate treatment to

remove ribonucleotides, deoxyribonucleotides can be easily distinguished from the ribonucleotides in biological samples. This protocol has been applied in analyzing the ribonucleotide, deoxyribonucleotide and cyclic nucleotide pool from HeLa cells and for the identification of major purine and pyrimidine species.

EXPERIMENTAL

Apparatus

For high-performance liquid chromatography (HPLC), a Model U6K sample injector, M45 solvent delivery system, either a Model 450 variable-wavelength detector or a Model 440 fixed-wavelength detector (Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe automatic recorder (Houston Instruments, Austin, TX, U.S.A.) or a Hewlett-Packard Model 3390A integrator were used. A pre-packed reversed-phase column (30 × 0.4 cm I.D.; particle size 10 μm) containing an octadecyl (C_{18}) chemically bonded stationary phase (Waters Assoc.) was utilized. A pre-column (5 × 0.4 cm I.D.) also packed with C_{18} reversed-phase material was used to protect the main column.

Chemicals

Ribo-, deoxyribo- and cyclic nucleotides were obtained from Sigma (St. Louis, MO, U.S.A.). A 10 mM stock solution of each nucleotide was prepared in distilled water, stored at −70°C and diluted before use. All other chemicals used were of analytical-reagent grade. Methanol of HPLC grade was obtained from Waters Assoc. Ammonium phosphate buffer (0.2 M, pH 5.1) was freshly prepared in doubly distilled water, pre-filtered through a 0.2-μm Millipore filter and degassed extensively before use.

Periodate oxidation

In principle, the method of Neu and Heppel[6] was followed, with minor modifications. Proportional volumes of ribonucleotides and deoxyribonucleotides were mixed to a final concentration of 1.6 μM. Aliquots of 1 ml of this mixture were treated at ambient temperature for 10 min with 80 μl of 1 M sodium periodate, after which 10 μl of 4 M cyclohexylamine were added and the resultant mixture was incubated at 45°C for 90 min. After incubation, 10 μl of 0.5 M rhamnose were added to remove excess of iodate ions. A 10-μl aliquot was chromatographed from the periodate-oxidized samples.

Silica cartridge chromatography

In order to avoid confusion among ribonucleotides (cytosine and uracil plus thymine series) and free bases which elute with a very close retention time with that of periodate and cyclohexylamine, samples were fractionated according to Lothrop and Uziel[7] through silica cartridge (Waters Assoc.) after periodate oxidation. The resultant free deoxyribonucleotides were then chromatographed on a C_{18} column as for the purine series.

Preparation of HeLa cell extracts

HeLa cell cultures were grown in Hank's MEM containing 10% newborn calf serum at 37°C until confluent. The medium was discarded and the cells were washed three

times with chilled PBS and extracted in 6 ml of 0.66 M formic acid at 4°C for 15 min. The extract was centrifuged (4°C, 3000 r.p.m., 10 min), the supernatant passed through a C_{18} cartridge (Waters Assoc.) at a flow-rate of 1 ml/min, the cartridge washed with 15 ml of distilled water and the pooled sample was lyophilized. The dry material was extracted in 1 ml of distilled water and filtered through a 0.2-μm Millipore filter before chromatography.

Chromatographic conditions

A μBondapak C_{18} column was used in these studies. The column was washed daily with doubly distilled water, followed by methanol–water (30:70), and preserved in the latter between use. Before use, it was equilibrated with water and ammonium phosphate buffer (0.2 M, pH 5.1). The samples were chromatographed at room temperature at a flow-rate of 1 ml/min, 1000 p.s.i., a chart speed of 1 cm/min and monitored at 254 nm at 1.0 a.u.f.s.

RESULTS AND DISCUSSION

From Fig. 1 it is clear that good separation of each ribonucleotide and deoxyribonucleotide in each purine and pyrimidine series is achieved on a C_{18} column using isocratic elution with ammonium phosphate buffer (0.2 M, pH 5.1) at 1 ml/min flow-rate as previously described for the separation of ribonucleotides[1]. Following periodate treatment of the ribonucleotide–deoxyribonucleotide mixture, only deoxyribonucleotides and resulting free bases from ribonucleotides were detected.

In the chromatography of ribonucleotides, the order of elution is triphosphate followed by the diphosphate, with the monophosphate being retained for a relatively longer period. A similar pattern emerges with the deoxynucleotides. The order of
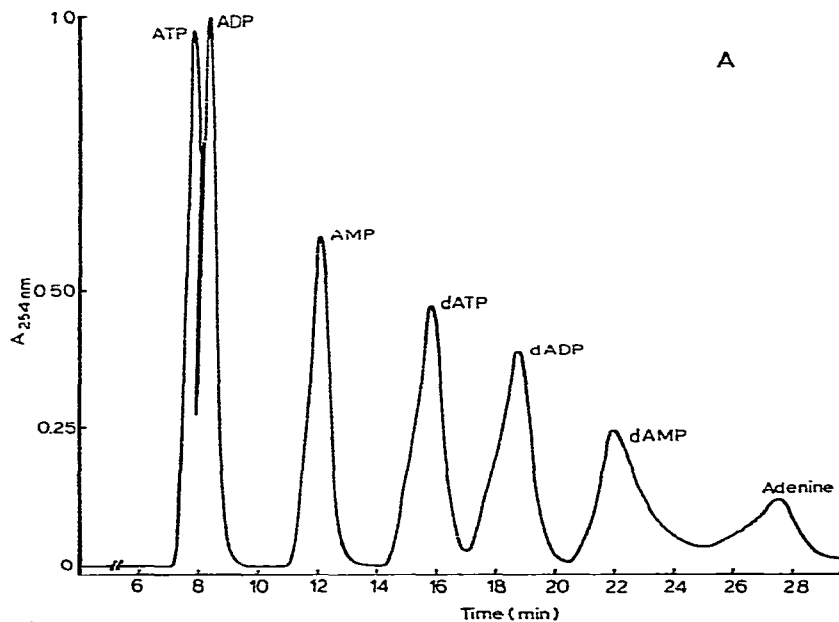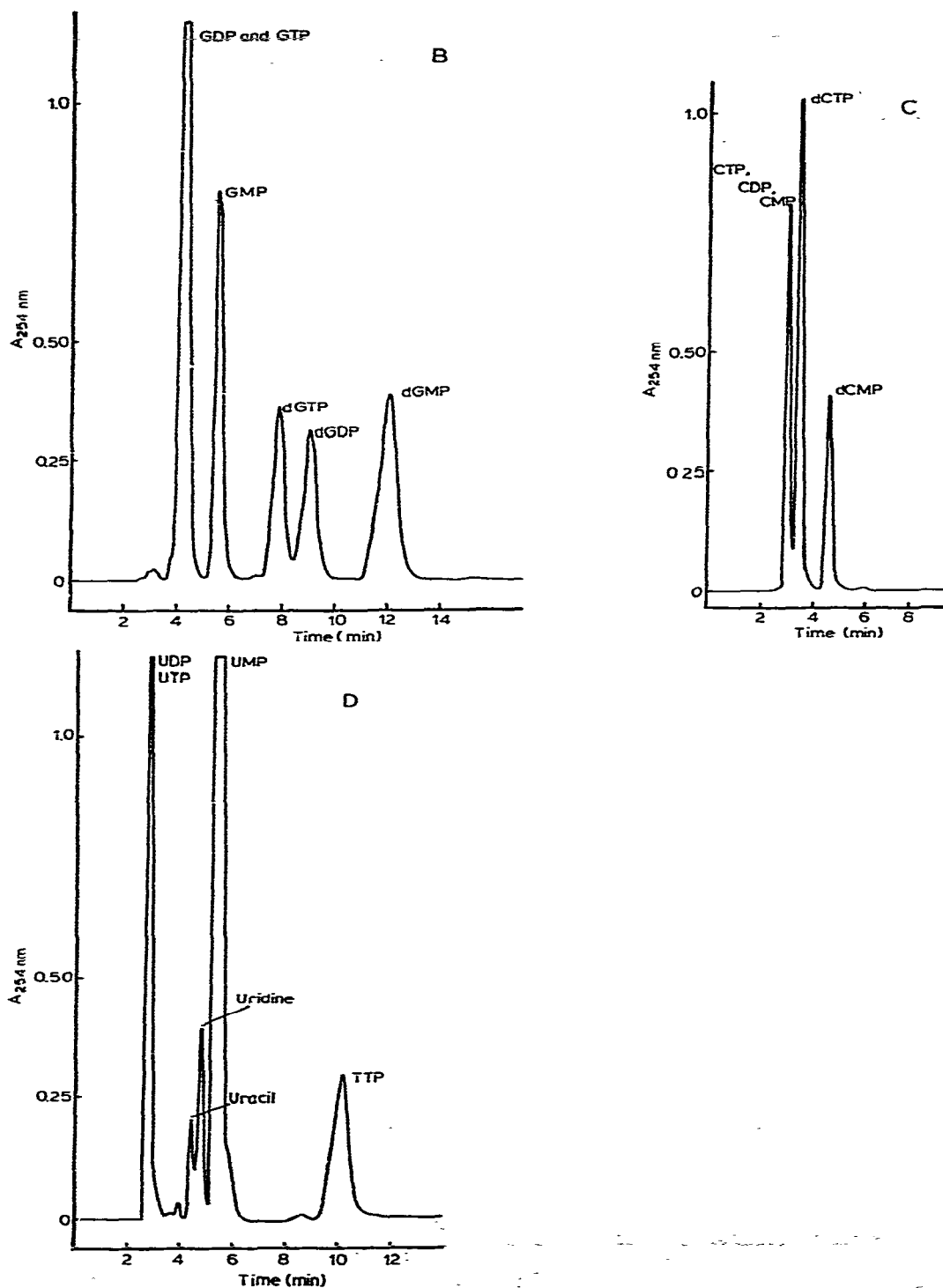

Fig. 1.

Fig. 1. Chromatogram of nucleotide standards on a μBondapak C_{18} column before periodate oxidation. A, Adenine series; B, guanine series; C, cytosine series; and D, uracil plus thymine series. Flow-rate, 1 ml/min.

elution of the ribonucleotides (uracil nucleotides before cytosine nucleotides before guanine nucleotides before adenine nucleotides) is also maintained for the deoxyribonucleotides. After periodate treatment, we have noted a minor shift in the elution pattern of the deoxyribonucleotides, the retention time of the adenine and guanine deoxyribonucleotides being slightly shorter than before the treatment.

Cyclic ribonucleotides under our conditions have a long retention time and are reasonably separated from the ribonucleotides and deoxyribonucleotides (cCMP at 5.9 min, cGMP at 36.4 min and cAMP at 112 min). However, the time of elution of cAMP is longer than normally desired with an HPLC system; modification of the eluent by the addition of differing amounts of methanol expedites the elution without affecting resolution (Fig. 2). The order of elution is consistent with that of the ribotides and deoxyribotides, *i.e.*, the cyclic ribotide of cytosine is retained least, that of guanine intermediate and adenine longest. From Fig. 2, it is also clear that the retention times of CTP, GTP and ATP are reduced by the incorporation of methanol in the eluent.
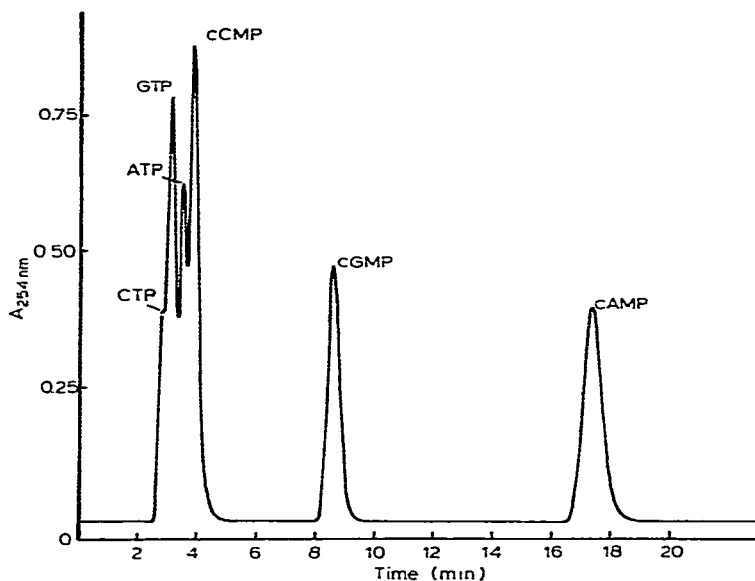

Fig. 2. Chromatogram of cyclic nucleotide standards on a μBondapak C₁₈ column. The eluent was ammonium phosphate buffer (0.2 M, pH 5.1) containing 10% (v/v) methanol. Flow-rate, 1 ml/min.

The elution pattern of cytosine, uracil and thymine ribonucleotides and deoxyribonucleotides is similar, if not identical, making the resolution between tri- and diphosphates markedly difficult unless modifications are introduced in the eluting agent. This is in agreement with our previous results[1]. Yet from Fig. 1 it is obvious that each pyrimidine ribotide separates well from its corresponding deoxyribotide.

Five points emerge from this elution pattern: (i) the D-2-deoxyribose entity is retained on the column more tightly than the D-ribose entity, probably owing to the higher electronegativity of the OH group; (ii) the purine entity is retained on the column more tightly than the pyrimidine entity, probably owing to its higher charge;

(iii) the elution pattern in each series is clearly a function of the degree of protonation of the PO_4 group; the entity with the most PO_4 groups elutes earlier and the entity with the least elutes later; (iv) the degree of protonation is disproportionately masked (possibly for stereospecific reasons) by the number of phosphate groups, as is evident by the distance between the elution of tri- and diphosphates and monophosphates; and (v) cyclization of the monophosphates greatly increases their retention on the column. Thus the affinity of the nucleotides for the C_{18} column is not simply a function of the charge on the PO_4 group alone.

In order to test the applicability of this system to a biological sample, the total acid-soluble extract of HeLa cells was chromatographed as described under Experimental. Fig. 3 shows the nucleotide profiles. Although tentative identification of major peaks could be made by running standard nucleotides individually and by adding a standard to the mixture and confirming the peak by an increase in peak height, it was obvious that certain peaks contained more than one component. Measurement of differential retention times using phosphate buffer with and without 10% (v/v) methanol permitted their further identification. This approach proved exceedingly effective in the identification of cyclic nucleotides in HeLa cells (Fig. 4). Periodate oxidation allowed the clear identification of deoxyribonucleotides from ribonucleotides (Fig. 5). The periodate-treated sample also allowed the detection of bases whose retention times and peak areas remained unchanged as a result of periodate oxidation. The recovery of nucleotides was quantitative and the sensitivity of this method allowed us to measure samples at the level of nanomoles. Minor changes in retention time have been noted between columns, but the order of elution was consistent. A similar strategy of identification in conjunction with silica cartridge fractionation has been used successfully to identify and measure nucleotide concentration in *Stigmatella aurantiaca* and *Xenopus laevis* development[8].
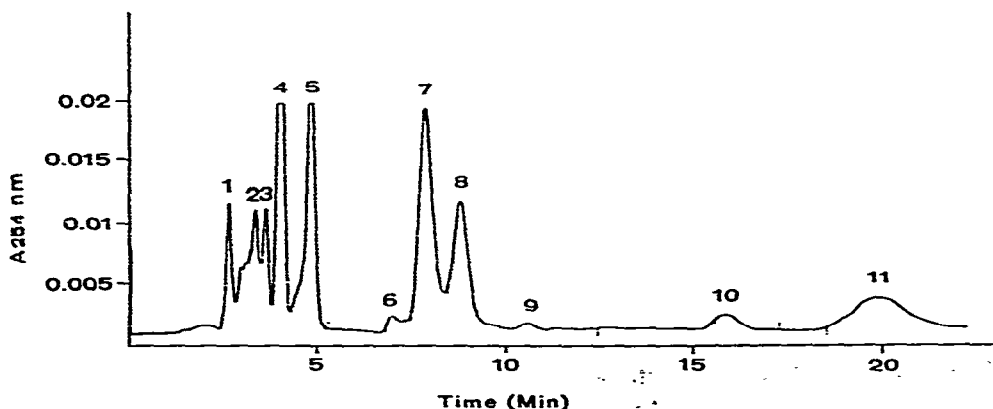

Fig. 3. Elution profile of total acid-soluble nucleotide pool from HeLa cells. All the peaks are identified individually: 1 = unidentified peak; 2 = UTP; 3 = CTP and CDP; 4 = GTP and UDP; 5 = GDP and UMP; 6 = GMP; 7 = ATP; 8 = ADP; 9 = hypoxanthine and guanine; 10 = AMP; 11 = dATP. Flow-rate, 1 ml/min.

Recent efforts to establish the applicability of metal chelate affinity chromatography to achieve the fractionation of AMP or GMP from their respective deoxy homologues were unsuccessful[9]. Besides the need to use a gradient, other procedures
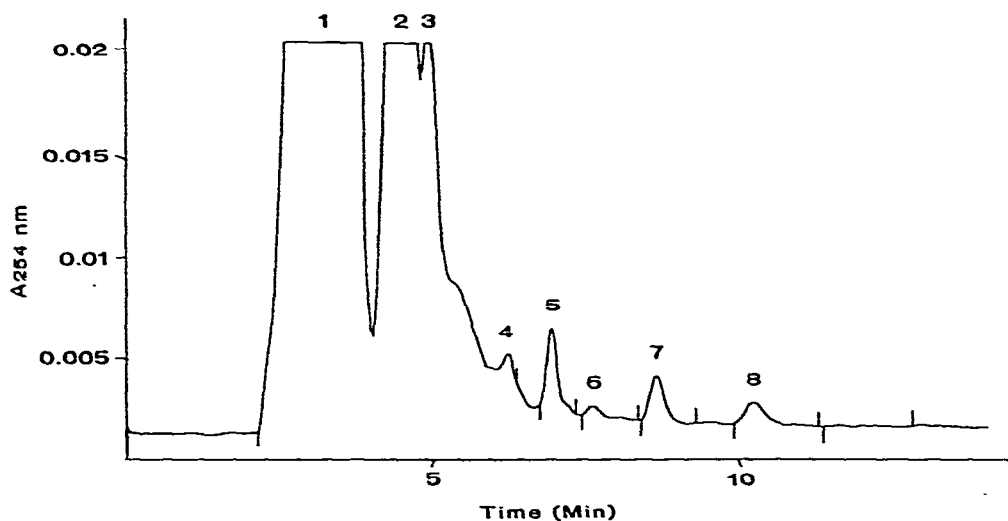
Fig. 4. Analysis of cyclic nucleotides in HeLa cells. Peak 1 contains at least four peaks as indicated by the integrator, composed of cytosine, uracil, cytidine and all nucleotides; peaks 2 and 3 contain hypoxanthine, guanine, and uridine; 4 = thymine; 5 = cGMP; 6 = guanosine; 7 = adenine; 8 = cAMP. Flow-rate, 1 ml/min.

showed coelution or overlapping elution of AMP, TMP and UMP[10]. The use of pH gradient[11], molarity gradient[12] or pH and molarity gradient techniques[13] required an increased time and buffer for equilibration between analyses and gradient programming for optimal resolution. None of these drawbacks are present in our procedure.
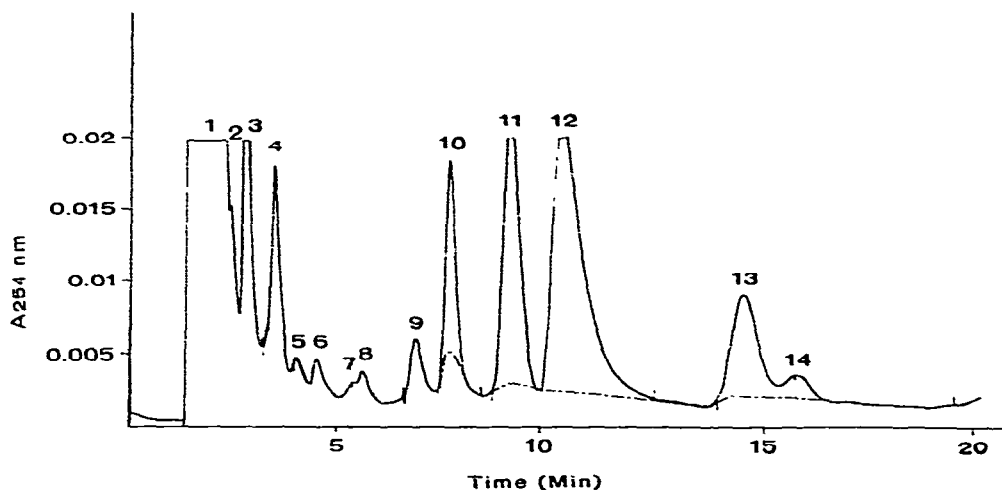

Fig. 5. Elution profile of periodate-treated nucleotide pool from HeLa cells. The cell extract was treated with periodate as described under Experimental. All peaks are identified individually: 1 = periodate; 2 = cyclohexylamine; 3 = uracil; 4 = unidentified; 5 = residual ATP; 6 = residual ADP; 7 = hypoxanthine; 8 = guanine; 9 = dGMP; 10 = dATP; 11 = dADP; 12 = adenine; 13 = dAMP; 14 = unidentified. Broken lines under peaks 10, 11, 13 and 14 represent the actual amounts and solid lines represent the coelution experiment. The increased adenine under peak 12 is due to periodate oxidation. Flow-rate, 2 ml/min.

It is convenient as it uses an isocratic mode of elution, thus avoiding the problems of gradient elution. It is simple, as it does not need the prior elaborate enzymatic treatments required in other systems[14]. Finally, it is comprehensive, as it affords clear and rapid separations of ribo-, deoxyribo- and cyclic nucleotide pools in cells under different pathological states. In the present instance, it has proved reliable and useful in the analysis of HeLa nucleotide pools.

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