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Note

Rapid separation of nucleoside mono-, di- and triphosphates on ion-exclusion/exchange columns

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Despite the popularity and widespread use of ion-exchange chromatography for the separation of a variety of nucleic acid types[1,2], the more recently introduced techniques of ion-exclusion chromatography[3–5] have some advantages over the former, especially with respect to speed and general simplicity. Using ion-exclusion chromatography, it has been shown that bases[4], nucleosides[4,5] and mononucleotides[3,5] may be separated satisfactorily. More recently the use of commercial high-performance liquid chromatography (HPLC) instrumentation has been widely reported[6,7]. This communication details the rapid separation of nucleoside mono-, di- and triphosphates by a combination of ion-exclusion/exchange methods using very simple apparatus. In view of the fact that the speed and efficiency of nucleoside polyphosphate separations achieved by this method, using routine and inexpensive apparatus, approximates that acheived with HPLC, it is likely that this method could become an attractive alternative for many investigators.

MATERIALS AND METHODS

All 5′ nucleoside and deoxynucleoside phosphates were purchased from Sigma (St. Louis, Mo., U.S.A.). The column packing was Bio-Rad AG 50W-X4 (minus 400 mesh), purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.). It was prewashed with 1 *M* HCl and 1 *M* NaOH until the pH 1.15 effluent had an optical density of 0.05. The resin was contained in a glass column (60 × 1.1 cm) fitted with a threaded inlet tube and gasket (Ace Glass Co., Vineland, N.J., U.S.A.). A Buchler monostaltic pump was used to maintain a flow-rate of *ca.* 1 ml/min.

Nucleoside phosphate mixtures (25–100 A_{260} units) in 100–500-μl volumes were applied carefully to the AG 50W-X4 resin (58 × 1 cm prequilibrated with HCl, pH 1.15) and washed in with a few ml of pH 1.15 HCl solution. The column was eluted for *ca.* 5 min with pH 1.15 HCl, then without stopping the pump, the reservoir flask was changed to 1 *M* NaCl in pH 1.15 HCl until the final mononucleotide had emerged. The tri- and diphosphates generally elute from the column within 10–20 min, respectively; the monophosphates require 2–2.5 h. The flow-rate was 1 ml/min. Regeneration of the column to the pH 1.15 HCl form required *ca.* 1 h washing. The column contracts to approximately half-length when equilibrated with 1 *M* NaCl–pH 1.15 HCl compared to its starting length. Regeneration of the column was considered complete when it had regained its original length.

Recovery of individual nucleoside phosphates was close to quantitative; since the commercial tri- and diphosphates are often partially hydrolysed when received, the degree of breakdown of a particular compound had to be determined independently either by paper chromatography (isobutyric acid–1 *M* NH_3–0.1 *M* EDTA, 100:60:1.6) or by thin-layer chromatography (*n*-propanol–ammonia–water, 55:10:35) to achieve proper quantitation. [γ-^{32}P]ATP was synthesized enzymatically[8] and was kindly given to us by Dr. E. Jay, Chemistry Department, University of New Brunswick.

RESULTS AND DISCUSSION

Table I shows the net charges at pH 1.15 calculated for the mono-, di- and triphosphates of the common 5′ ribonucleosides; with the exception of the uridine phosphates the compounds fall into two categories: the negatively charged tri- and diphosphates and the positively charged monophosphates.

TABLE I

NET CHARGES CALCULATED FOR RESPECTIVE NUCLEOSIDE PHOSPHATES AT pH 1.15 FROM pK_a VALUES GIVEN IN REF. 9

Compound	*Charge*	*Compound*	*Charge*
AMP	+0.36	GMP	+0.21
ADP	−0.28	GDP	−0.48
ATP	−0.92	GTP	−1.22
CMP	+0.31	UMP	−0.59
CDP	−0.39	UDP	−1.18
CTP	−1.07	UTP	−1.77

Fig. 1 shows the elution patterns of the four ribonucleoside mono-, di- and triphosphates. The arrow indicates the addition of 1 *M* NaCl–HCl, pH 1.15. The tri- and diphosphates of adenosine, cytosine and guanosine emerge in the sequence predicted by ion exclusion; their respective cationic mononucleotides emerge much later, after having been displaced by Na^+. As may be seen in Fig. 1D, these elution conditions are unable to separate uridine tri- and diphosphates from each other.

From Table I it may be seen for the tri- and diphosphates resolvable by this method, that there is a 2.5 to 3-fold difference in the magnitude of their respective negative charges as contrasted to the smaller (1.5-fold) charge difference in the case of UTP and UDP. The 2-fold difference in charge between UMP and UDP is apparently sufficient for their resolution.

An average separation requires 2 –2.5 h; if multiple runs are to be made regeneration of the column to pH 1.15 HCl requires *ca.* 1 h. In our hands comparable separations using conventional salt gradient–ion-exchange methods are much slower. It is to be emphasized that the equipment required to run these column is minimal and inexpensive, relative to costs incurred with new HPLC instrumentation. This difference extends even to the resins employed in the respective methods, the pellicular resins used in HPLC being 1–2 orders of magnitude more expensive[10] for comparable sized columns.

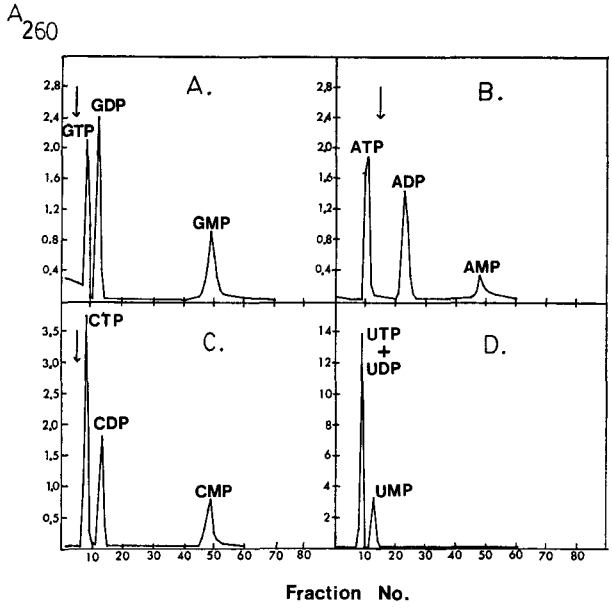

Fig. 1. Elution profiles of ribonucleoside mono-, di- and triphosphates. Conditions of sample application and elution as described under Materials and methods; *ca.* 10 A_{260} units of each derivative applied. Arrow indicates the switch over to 1 *M* NaCl–HCl, pH 1.15. Fractions of 2.5 ml were collected every 2.5 min.

[γ-^{32}P]ATP was prepared enzymatically using standard procedures[8]; when mixed with adenosine mono-, di- and triphosphates it co-eluted with unlabeled ATP, showing a pattern similar to that in Fig. 1B.

Deoxynucleoside mono-, di- and triphosphates behaved generally as the ribo-derivitives in Fig. 1. The deoxypyrimidine derivitives, *i.e.*, the mono-, di- and triphosphates of deoxycytosine and deoxythymidine, were stable under these chromatographic conditions and gave elution patterns similar to those in Fig. 1C and D, respectively. The deoxypurine derivitives, *i.e.*, the mono-, di- and triphosphates of deoxyadenosine and deoxyguanosine, were unstable at pH 1.15. Qualitatively they did show patterns similar to those in Fig. 1A and B; the quantitation, however, was unreliable. Other compounds such as the 5′-5′ pyrophosphate of pT and 3′-5′ cyclic pT have been run on these columns and elute in a predictable way.

In summary, this combined ion-exclusion–ion-exchange approach to the separation of nucleoside mono-, di- and triphosphates constitutes an improvement over conventional ion-exchange methods in terms of speed and simplicity; in addition the speed and quality of separation realized in this method approaches that achieved with HPLC at a fraction of the cost.

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