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SIMPLE ANION-EXCHANGE CHROMATOGRAPHY FOR THE DETERMINATION OF ADENINE NUCLEOTIDES BY USING AG MP-1 RESIN

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SUMMARY

A simple ion-exchange chromatography with AG MP-1 resin (a strong basic anion-exchange resin of macroporous copolymer of styrene and divinylbenzene) for the assay of adenine nucleotides is described. AMP, ADP and ATP can be quantitatively resolved in a linear HCl gradient (0–0.08 *N*).

A milder HCl gradient between 0 and 0.02 *N* is able to separate cyclic AMP from AMP. The described method is not only useful in the purification of the nucleotides but also convenient in the detection of other contaminated nucleotides.

INTRODUCTION

Conventional ion-exchange chromatography using an anion-exchange resin, Dowex 1, for the analysis of adenine and other nucleotides[1–5] has the disadvantage of eluting the individual nucleotide in a large volume and is time consuming. At present, this technique has been replaced by high-performance liquid chromatography for its sensitivity and speed. However, the latter method requires expensive chromatographic equipment, and the column using pellicular ion-exchange systems has limited capacity[6,7]. Recently, Khym[8,9] described a method using a short column of Aminex A-27 (a conventional styrene-type anion-exchange resin of small particle size, 12–15 μm), to operate at a lower pressure and low-cost chromatographic equipment. In this paper, we revive conventional ion-exchange chromatography using AG MP-1 (a strong basic anion-exchange resin of a macroporous styrene-type anion-exchange resin) for the determination of adenine nucleotides. This not only improves but also simplifies the procedures of the conventional chromatographic technique, and can be used conveniently for the quantitation of adenine nucleotides in biological fluids and tissues, and assay of many enzymatic reactions which involve adenine nucleotides as substrates. The technique is also useful in the purification of nucleotides in a large quantity.

EXPERIMENTAL

Chemicals

AG-1 anion-exchange resin (200–400 mesh) and AG MP-1 anion-exchange resin (200–400 mesh) were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Adenine nucleotides were obtained from Sigma (St. Louis, Mo., U.S.A.) or ICN Pharmaceuticals (Plainview, N.Y., U.S.A.).

Procedure

A 2-g amount of AG MP-1 resin was packed in a small column with distilled water (3.5 × 0.9 cm). A thick filter paper disc was then placed on the top of the resin. The elution was done by a linear gradient of HCl by interconnecting two 250-ml flasks filled to the 225-ml mark, one with distilled water and the other with 0.3 *N* HCl. The eluent was delivered to the column by a peristaltic pump from the mixing flask or by hydrostatic pressure at a flow-rate of 2.5 ml/min. The nucleotides in the effluent were continuously monitored through a flow cell of 10 mm light path at 257 nm using a Beckman double beam spectrophotometer.

After a run, the column was regenerated by washing the column with water for at least 20 min. Usually two columns were used alternatively so that while one was in use, the other was washing. The flow-rate was measured at the outlet of the flow cell.

Quantitation of peaks

Two methods were used. One was by comparing the area of each peak with the respective peak of standard nucleotide. A calibration curve was made with each standard nucleotide for each column.

The other method was by peak height and half-height width of the peak according to the equation[6,9]:

$$\text{Amount (nmoles)} = \frac{A_{\text{max.}} \cdot W_{\frac{1}{2}} \cdot F \cdot 1066}{\varepsilon_{257}}$$

where $A_{\text{max.}}$ is the maximal absorbance, $W_{\frac{1}{2}}$ is the width measured at the middle of peak (min), F is the flow-rate in ml/min and ε_{257} is the molar extinction coefficient of each nucleotide at 257 nm, 1066 is an equation constant.

The molar extinction coefficients used are 15 for AMP and ADP and 14.7 for ATP at 257 nm and pH 2 (ref. 10). The total adenine nucleotides in a standard solution was calculated by measurement of the total absorbance at 259 nm using the molar extinction coefficient of $15.4 \cdot 10^3$ in a neutral solution.

RESULTS AND DISCUSSION

Comparison of elution pattern using AG-1 and AG MP-1 resins

AG-1 resin was analytical grade of Dowex 1 resin which was generally used in the conventional ion-exchange chromatography for nucleotides. It gave broadening, tailing and unsymmetrical peaks. On the contrary, AG MP-1 resin gave sharp peaks at milder HCl concentration, as shown in Fig. 1.

AG-1, AG MP-1 and Aminex A-27 are all strongly basic anion-exchange

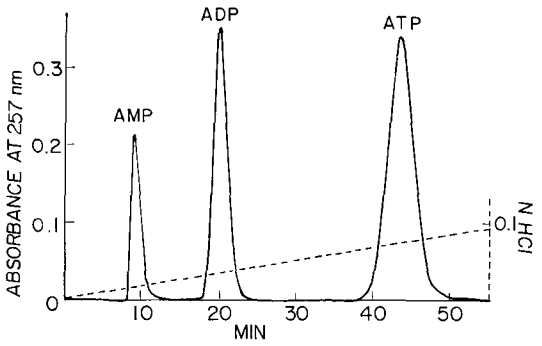

Fig. 1. Chromatogram for adenine nucleotides on AG MP-1 resin column. This curve represented a typical chromatogram for AMP, ADP and ATP. The peaks corresponded to 53.17 nmoles of AMP, 124.78 moles of ADP and 221.52 nmoles of ATP as calculated with the given equation. The broken line was the concentration of HCl gradient.

resins with quaternary ammonium groups attached to a styrene–divinylbenzene copolymer lattice. Aminex A-27 used by Khym[8,9] is a finely sized spherical beads of AG-1 resin while AG MP-1 is a highly crosslinked copolymer with high content of divinylbenzene.

Quantitation

The quantity of a nucleotide is linearly proportional to the area of peak obtained from the chromatogram. The difference obtained from two similar columns used was minimal. Since the same size of column and amount of resin were used, the difference was mainly caused by the flow-rate. If the results were corrected for the flow-rate of each column and calculated according to the equation given under Experimental, an identical curve could be obtained.

Purities of commercial adenine nucleotides

Table I shows the results of adenine nucleotides analyses from Sigma and ICN Pharmaceuticals. All were obtained as crystalline sodium salts. The determined purities for ADP were far from the manufacturers' specifications.

It was reported that solid ADP in storage would convert partially into AMP and ATP[11]. It is evident from Table I that this conversion is extensive during the storage in refrigerator. The first two lots of ADP were obtained from Sigma, 2 years and 1 year ago, respectively. The last ADP was freshly obtained from ICN Pharmaceuticals.

Modification of the elution gradient for the analysis of cyclic adenine monophosphate

Using HCl gradient elution described in the experimental protocol, cyclic AMP was eluted from the column at the position close to AMP. To separate cyclic AMP from AMP, the HCl gradient was modified as follows. The elution was started with 225 ml water in a mixing flask interconnecting to another flask containing 225 ml HCl. As shown in Fig. 2, this HCl gradient of lower concentration eluted AMP and cyclic AMP separately out of the column. After elution for 30 min, the mixing flask was disconnecting from the other flask and connecting to a flask containing 190 ml

TABLE I

CHROMATOGRAPHIC ANALYSIS OF PURITY OF NUCLEOTIDES

C.P. = Chromatographically pure.

Source of nucleotide	*Lot No.*	*Manufacturer's specification (%)*	*ATP (%)*	*ADP (%)*	*AMP (%)*
ATP, Sigma Equine muscle	76C-7360	99–100	98.00	1.56	0.45
ATP, ICN	8073	C.P.	96.04	1.66	2.30
AMP, ICN Muscle	6340	C.P. (99–100)	0	0.46	99.54
ADP, Sigma Grade III Yeast	86C-7530	95–99	3.63	74.00	22.50
ADP, Sigma Grade III Yeast	113C-7070	95–99	2.30	84.20	13.40
ADP, ICN	2923	—	1.84	91.19	6.97

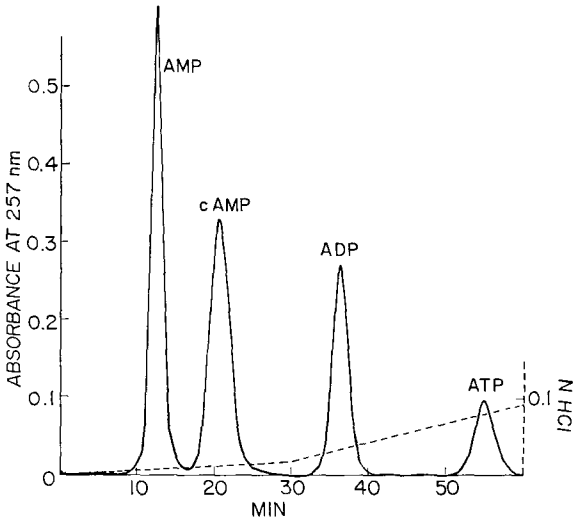

Fig. 2. Chromatogram for adenine nucleotides using HCl gradient of lower concentration. The peaks were identified as AMP, cyclic AMP, ADP and ATP. Amounts of each nucleotide as calculated with the equation were: AMP, 25.58; cyclic AMP, 192.72; ADP, 122.15; and ATP, 56.85 nmoles. Two-step HCl gradient was used. The milder HCl gradient could separate cyclic AMP from AMP.

of 0.4 *N* HCl. This last gradient eluted out ADP and ATP separately in 30 min. The resulting chromatogram is shown in Fig. 2.

Sample volume and salt effect

In a neutral solution containing low salt concentration, nucleotides can be applied to the column in a large volume. In 5 ml 0.02 *M* Tris–HCl buffer, pH 7.4, NaCl at a concentration lower than 0.2 *M* did not interfere with the absorption of AMP to the column. However, 0.25 *M* NaCl resulted in 8% loss of AMP; while 0.4 *M*, 80%.

Nucleotides in 3% trichloroacetic acid were not absorbed on the column; however, they were completely absorbed after the solution was neutralized with Tris-base. For the analysis of solution containing high concentration of protein, it was, therefore, treated as follows before applying to the column. To a sample solution, an equal amount of ice-cold 6% trichloroacetic acid was added. After thoroughly mixed, the mixture was centrifuged to remove the precipitated protein. An aliquot of the supernatant solution was then neutralized with 2 *M* Tris-base (5:1, v/v), and applied to the column. Running the column for 10 to 20 min with water, and then HCl gradient was started as described.

Stability of the column

The column was very stable. Repeated use of a column for several hundred times over a half year did not result in any appreciable change in efficiency. Repacking was not necessary. When it became hard to apply samples because of continuous use for biological fluids, the column was treated in the following manner. The paper disc was removed, the resin bed was stirred and resettled, the column was washed with distilled water and a new filter paper was re-installed.

Purification of ATP using a large column

A large column (14 × 2.6 cm) packed with 50 g of AG MP-1 resin was used for the purification of ADP. A 236-g amount was dissolved in 2 ml of distilled water and absorbed on the column. The HCl gradient was established with 500 ml each of water and 0.3 *N* HCl. The flow-rate was 250 ml per h and 10-ml fractions were collected.

The resulting chromatogram is shown in Fig. 3. The nucleotides in the corresponding peak fractions were 93.4, 308 and 1.4 μmoles for AMP, ADP and

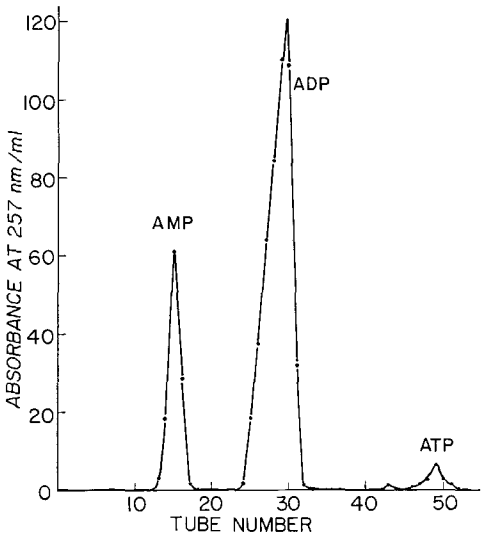

Fig. 3. Purification of ADP in a large chromatographic column. The absorbance was measured at 257 nm by diluting 10–50 μl of the effluent to 1 ml. The peaks were identified as ADP, AMP and ATP, respectively, by an analytical chromatography.

ATP, respectively. Two fractions, tubes 29 and 30, containing high ADP were combined and neutralized to pH 7.4 with Tris-base. Analytical column chromatography showed that it was pure and contained 7.78 m*M* ADP. Other ADP fractions, 25–28 and 31 were combined, neutralized and concentrated by ultrafiltration on a Diaflo UM-05 membrane to 9 ml. This was also chromatographically pure and contained 17 m*M* ADP. AMP fractions, tubes Nos. 14, 15 and 16, were combined, neutralized and concentrated by the ultrafiltration to 10.2 ml. The resulting solution was analyzed by the analytical chromatography to contain 9.25 m*M* AMP and to be pure. 97% was recovered by the ultrafiltration.

As shown in Fig. 3, two small peaks, tubes 43 and 49, corresponding to ATP peak were obtained. They could only be partially separated by analytical chromatography after being neutralized with Tris-base. This indicated a trace contamination of other nucleotides which could not be detected by the analytical chromatography. The first peak corresponded to 0.025% and the second peak to 0.35% of ATP calculated by the total absorbance. Therefore, this procedure is not only convenient and efficient for the purpose of purification but also can be used for the detection of trace amounts of contaminated nucleotides.

Since isolated nucleotides were in dilute HCl solutions, they could be converted to desirable cationic salts by neutralizing with proper bases. The neutralization could also be accomplished by passing the acid solution through Chelex 100 resin preconditioned to a desirable cationic form, and washing the resin with distilled water. Nucleotides were recovered in the void volume.

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