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Note

Operational parameters of anion-exchange chromatography using AG MP-1 resin for rapid assay of adenine nucleotides

SIDNEY S. CHEN* and DAR-SAN HSU

Department of Obstetrics & Gynecology, Long Island Jewish-Hillside Medical Center, 270-05 76th Avenue, New Hyde Park, NY, 11042 and Health Sciences Center, State University of New York, Stony Brook, NY 11794 (U.S.A.)*

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A conventional anion-exchange chromatography for adenine nucleotides using AG MP-1 resin has been described previously[1]. This system is not only simple but also has many potential applications. Among the applications are assays for enzymatic reactions involving adenine nucleotides[2], the determination of adenylate charge[3] and further extension for the analysis of other nucleotides. The operational parameters which may modify and optimize this system are described in the report.

EXPERIMENTAL

Materials

AG MP-1 anion-exchange resin (Cl^-, 200–400 mesh) was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). It contained 55–65% of water and had a total capacity of 4.2 mequiv./g dry weight. Adenine nucleotides were obtained from ICN Pharmaceuticals (Irwine, CA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.).

Determination of apparent affinity

An exact amount of the resin (100 mg) was weighed and packed into a column. A neutral nucleotide solution containing 2 m*M* was then slowly pumped through the column until the effluent solution contained the same concentration of the nucleotide as the original solution. The column was then washed with water to remove the free nucleotide. The total nucleotide bound to the resin was eluted out with 5 ml of 0.3 *N* HCl and was measured by reading the absorbance at 257 nm.

Determination of distribution coefficient

The chromatography was performed essentially according to the procedure described previously[1], except that the scanning was done on Gilson Holochrome fitted with a 40-μl flow cuvette of 10-mm light path. A 2-g amount of the resin was packed with water in a small column without any pretreatment. It gave a packed volume of 1.7 ml/g of the resin. After washing the column with water, 0.1 μmole of each nucleotide was absorbed into the bed. The column was then eluted with water at a flow-rate of 2 ml/min using a peristaltic pump. After 10 min, the upper column water

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was replaced with different concentrations of HCl and eluted with the same. The total elution volume from the start of elution to the peak of each nucleotide was determined from the recorded chromatogram and the flow-rate.

The distribution coefficient was calculated and the operational parameters were evaluated[4–6].

RESULTS

Apparent exchange capacity for adenine nucleotides

The apparent affinities for AMP, ADP and ATP were 2.1, 2.5, and 1.8 mmoles/g dry resin. Approximately 1 mole of the adenosine nucleotide could exchange with 2 moles of Cl^-.

Distribution coefficients and HCl concentrations

The distribution coefficient (K_d) was calculated from the retention volume (V_R) of each peak and void volume (V_0) according to the equation, $V_R = V_0 + mK_d$, where m is the weight of resin. The void volume was determined by the retention volume of adenosine which was not retained by the resin, and the volume did not change with the concentration of HCl. It was 2.1 ml for the 2-g resin column used. The distribution coefficients at different HCl concentrations are shown in Fig. 1. It is clear that adenosine nucleotides can be separately eluted out from the column at different HCl concentrations. Therefore, it is suggested that proper selection of stepwise elution mode is more advantageous than the gradient elution as previously described[1].

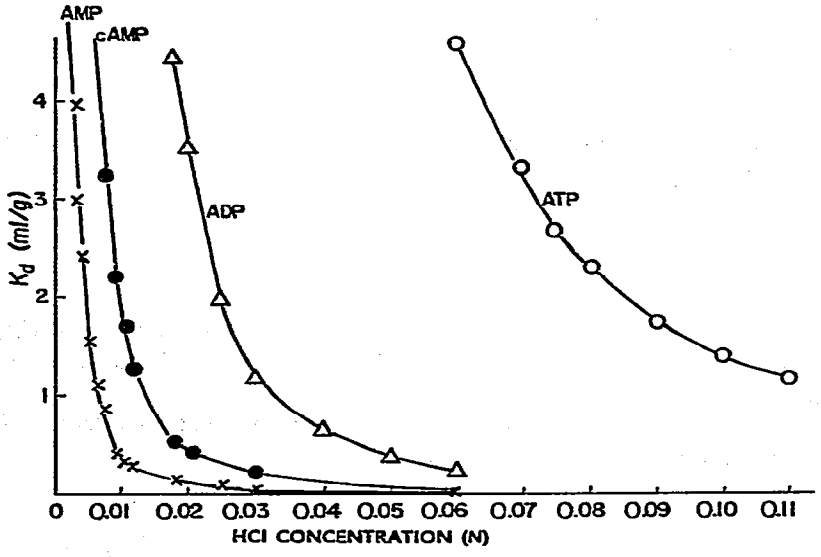

Fig. 1. The distribution coefficient (K_d) *versus* HCl concentrations.

Distribution coefficient and band broadening

The broadening of each nucleotide band measured as the peak width at half height was proportional to the distribution coefficient. The results are shown in Fig. 2. At the operational range of distribution coefficients 0.2–8, the relationship

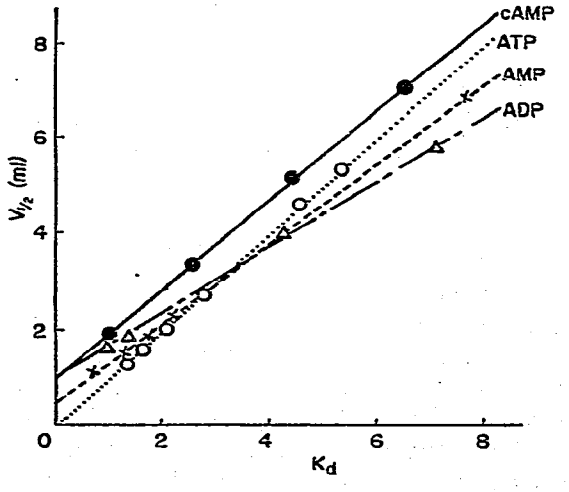

Fig. 2. The width at half height peak *versus* distribution coefficient.

between the volume at half height of peak ($V_{\frac{1}{2}}$) and the distribution coefficient for each nucleotide can be expressed by the following equations:

$$V_{\frac{1}{2}} \text{ for AMP} = 0.4 + 0.8\,K_d$$
$$V_{\frac{1}{2}} \text{ for cAMP} = 0.94 + 0.92\,K_d$$
$$V_{\frac{1}{2}} \text{ for ADP} = 0.9 + 0.68\,K_d$$
$$V_{\frac{1}{2}} \text{ for ATP} = K_d$$

If K_d is known the maximum absorbance at the peak ($A_{\text{max.}}$) can be estimated from the equation as previously described[1].

$$A_{\text{max.}} = \frac{\text{nmoles nucleotide} \times \varepsilon_{257}}{V_{1/2} \times 1066}$$

Since the Gaussian peak width expressed in volume (V_b) is related to the variance of the peak (σ), $V_b = 4\sigma$, and $V_{\frac{1}{2}} = 2\sigma\sqrt{2 \ln 2}$, the total volume of each peak is equivalent to $1.7\,V_{\frac{1}{2}}$.

The performance of the column is therefore calculated from the above relationship according to the equation:

$$N = \frac{16V_R^2}{V_b^2} = \frac{5.545V_R^2}{V_{1/2}^2}$$

At $K_d = 1$, the numbers of theoretical plates (N) for AMP, cAMP, ADP and ATP are 65, 25, 37 and 93 at a flow-rate of 2 ml/min, respectively; corresponding to 1640, 632, 935 and 2340 plates per meter, respectively.

Resolution

In a previous paper[1] the separation of AMP and cAMP and cAMP was accomplished by a very mild HCl gradient. Fig. 3 shows that they were easily separated by an isocratic elution at various HCl concentrations. The experimental results were compiled in Table I. The resolution (R_s) of two peaks was calculated from the equation $R_s = \Delta V_R / \overline{V}_b$, where ΔV_R is the retention volume difference, and $\overline{V}_b$ is the mean peak width. At HCl concentrations higher than 0.012 N, $R_s < 1$, the resolution was incomplete using a column bed of 40 × 9 mm. By increasing the bed height to 60 mm, using 3 g resin, they were completely separated.

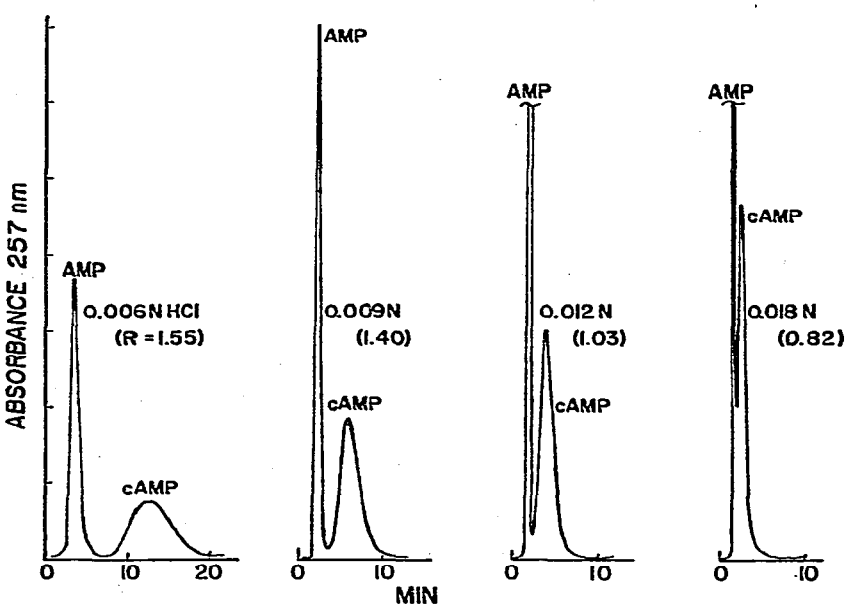

Fig. 3. Chromatographic profile for the separation of AMP and cAMP by isocratic elution at different concentrations of HCl. The column size was 40 × 9 mm.

The numbers of theoretical plates required to give $R_s = 1$ are also given in Table I. They were calculated according to eqn. 1:

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{K'}{1 + K'}\right)\sqrt{N} \qquad (1)$$

where R_s is the separation factor of the two peaks. α is the ratio of the two K_d values, and K' is the average of the two K_d values.

TABLE I

COLUMN PARAMETERS OBTAINED FOR AMP AND cAMP

HCl (N)	Nucleotide	V_R(ml)	K_d(mlg)	$V_\pm$(ml)	N	R_s	$N_{req.}$*
0.006	AMP	6.4	2.15	2.13	50	1.55	35
	cAMP	23.8	10.85	11.10	26		
0.0075	AMP	4.70	1.30	1.45	58	1.48	39
	cAMP	15.30	6.55	7.00	26		
0.009	AMP	3.58	0.74	1.16	53	1.40	44
	cAMP	11.00	4.45	5.11	26		
0.012	AMP	3.30	0.55	0.86	70	1.09	69
	cAMP	7.23	2.57	3.37	26		
0.018	AMP	2.48	0.19	0.55	112	0.82	92
	cAMP	4.19	1.05	1.91	26		

*$N_{req.}$ = the number of theoretical plates calculated according to eqn. 1 by assuming $R_s = 1$.

DISCUSSION

Because the exchange capacity of the resin is high, and K_d values of AMP, ADP and ATP vary greatly with HCl concentration, these three nucleotides can be easily separated on a small column by a stepwise elution using different concentrations of HCl. The HCl concentration determines the retention time and the spreading of the peak. Thus, the speed and the sensitivity of the analysis increase. The mode of elution can be therefore favorably selected according to the relative contents of the three nucleotides in a solution to be analysed. Several examples are illustrated as follows.

If the relative amounts of AMP, ADP and ATP of a solution do not vary greatly, the analysis is better accomplished by a three-step elution. Choosing HCl at higher concentration to give a lower K_d at each step not only facilitates the analysis but also increases the sensitivity. For instance, if each HCl concentration is selected at a K_d smaller than 1, the retention volume for each nucleotide is less than 4 ml, and the peak width at half height of the peak is less than 2 ml for a column size of 40 × 9 mm. The values of these parameters can be estimated from Figs. 1 and 2, or calculated from the equations given in the text. At a flow-rate of 2 ml/min, the total analysis can be accomplished in less than 10 min. At the same time as little as 100 pmoles of each nucleotide can be determined by the system described. Thus, the sensitivity and speed can be comparable with that of high-performance liquid chromatography. However, this system is much more convenient and the sensitivity may be higher because the loading capacity is much higher in this system.

For the analysis of a solution containing the nucleotides in high disproportion, a two-step elution mode can be considered. For instance, if the amount of AMP is very small, AMP and ADP can be eluted out in one step with an HCl concentration

which elutes AMP out at near void volume (low K_d), and ATP at high K_d. By the same token, if the ADP content is much smaller than that of ATP, after AMP is eluted from the column, ADP and ATP are eluted out in one step by the similar principle. This one- step elution principle is, therefore, favorable for the assay of the initial rate of many enzyme reactions involving ATPase and many kinases which yield ADP or AMP as a product. For a reaction which produces ATP, after ADP or AMP is eluted at a relatively low HCl concentration, ATP is eluted at a high HCl concentration.

AMP and cAMP have close K_d values; however, their difference is still large enough to favor an isocratic elution. As shown in Table I, the ratio of the K_d values (α) did not vary with HCl concentration. The resolution is improved by either using a lower HCl concentration to give a large K value or a longer column to increase the number of theoretical plates. It is clear from Fig. 3 that using a column resin bed of 40 × 9 mm and 0.009 M HCl as eluent is suitable for the assay of cAMP phosphodiesterase.

In short, this system can be easily modified and optimized by proper selection of HCl concentration, mode of elution and column size for assays of nucleotides.

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