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Note

Analysis of adenine nucleotides by high-performance liquid chromatography

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The cellular levels of adenine nucleotides, especially of ATP, are a good index of the energy status in living cells, and the level of ATP is well known to decrease with a concomitant increase in AMP and ADP in ischemic tissues[1,2]. It is therefore important to determine accurately the level of adenine nucleotides in a small sample of animal tissues.

Adenine nucleotides have been determined by several different methods. The enzyme assay method[3] is specific for ATP, ADP and AMP. However, it is difficult to treat many samples simultaneously and the sensitivity is not sufficient to determine ATP in the liver of approximately 100 mg wet weight. Although high-performance liquid chromatographic (HPLC) systems have been developed and used, the many different nucleotides contained in the liver are difficult to separate without the use of gradient elution[4–11], a reversed phase[12] or anion-exchange HPLC system[2,13,14]. When gradient elution is used a long time is required to reequilibrate the column. Furthermore, the sensitivity for separation of these nucleotides is not sufficient in reversed-phase[12] or anion-exchange HPLC system[13,14]. Rapid and sensitive separation of nucleotides was performed on an anion-exchange column[2]. However, the column is expensive and its life is fairly short.

In our previous studies[15–17] we succeeded in completely separating thiamine phosphates by use of a LiChrosorb-NH_2 column. Based on these results, we have estasblished a sensitive normal-phase HPLC system for adenine and guanine nucleotides.

MATERIALS AND METHODS

The HPLC system used consisted of a Model PG 350 D pump, Model VL-611 six-way injector and Model UVIDEC-100-V UV spectrophotometer (JASCO, Tokyo, Japan). Peak areas on the chromatograms were measured with a data processor (Chromatopac C-R2A; Shimadzu, Kyoto, Japan). The column was packed with LiChrosorb-NH_2 (Merck, 150 mm × 4.6 mm, 5 μm).

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The mobile phase consisted of 100 m*M* potassium phosphate buffer, pH 6.5, 100 m*M* ammonium chloride and 20% acetonitrile, which were used after filtration through a 0.45-μm membrane filter and degassed by a vacuum aspirator.

The liver was removed from decapitated mice and immediately frozen in liquid nitrogen. Nucleotides in the tissue (approximately 200 mg wet weight) were extracted with 10 volumes of trichloroacetic acid (TCA) by homogenizing in Polytron 20 ST (Kinematica, Switzland) at 0°C, followed by centrifugation at 10 000 *g* for 10 min at 0°C. TCA in the supernatant was removed by shaking with 5 volumes of diethyl ether and this procedure was repeated until the pH of the water layer had risen to 5. A 5-μl volume of the extract thus obtained was injected on to the column of the HPLC system, chromatographed and detected at 254 nm. Adenine nucleotides (ATP, ADP and AMP) in the same liver extract were simultaneously assayed by the enzymatic method described by Williamson and Corkey[3].

RESULTS AND DISCUSSION

In the previous studies[15,16] using a LiChrosorb-NH_2 column, thiamine phosphates were successfully separated by a solvent system of 90 m*M* potassium phosphate (pH 8.4)–acetonitrile (60:40, v/v). This result led to the idea of separating adenine nucleotides by an HPLC system similar to that for thiamine phosphates.

The conditions for separation of adenine nucleotides were investigated by use of the capacity factor, k', as a parameter

$$k' = (t_R - t_{R_0})/t_{R_0}$$

where t_R is retention time of the peak studied and t_{R_0} is that of the initial peak[18].

The effects of the concentrations of potassium phosphate buffer (pH 6.5, Fig. 1A), acetonitrile (Fig. 1C) and salt (ammonium chloride, Fig. 1D) and of pH (Fig. 1B) were tested. The capacity factors for ATP, ADP and AMP were all between 1 and 10 when one component of the solvent system comprising 100 m*M* potassium phosphate buffer (pH 6.5), 20% acetonitrile and 100 m*M* ammonium chloride or the pH of the buffer was varied. This result shows clearly that this solvent system is suitable as the mobile phase.

Authentic samples of various nucleotides were chromatographed under the standard assay conditions using the mobile phase described above (Fig. 2A). Eight nucleotides (each 300 pmol) including AMP, GMP, NADP, ADP, NADPH, GDP, ATP and GTP were well separated and eluted within 23 min. NAD and NADH were not retained by the column under these conditions and eluted as the first unretained peak.

The good separation of each nucleotide shown in Fig. 2A was evidenced by the resolution factor, R_s, calculated[18] for two adjacent peaks (Table I)

$$R_s = (t_{R_2} - t_{R_1})/(W_1 + W_2)$$

where W_1 and W_2 are the half widths of peaks 1 and 2 and t_{R_1} and t_{R_2} are the corresponding retention times. The R_s values for AMP–GMP, NADP–ADP and ADP–NADPH were greater than 1.0 (Table I), showing the complete separation of

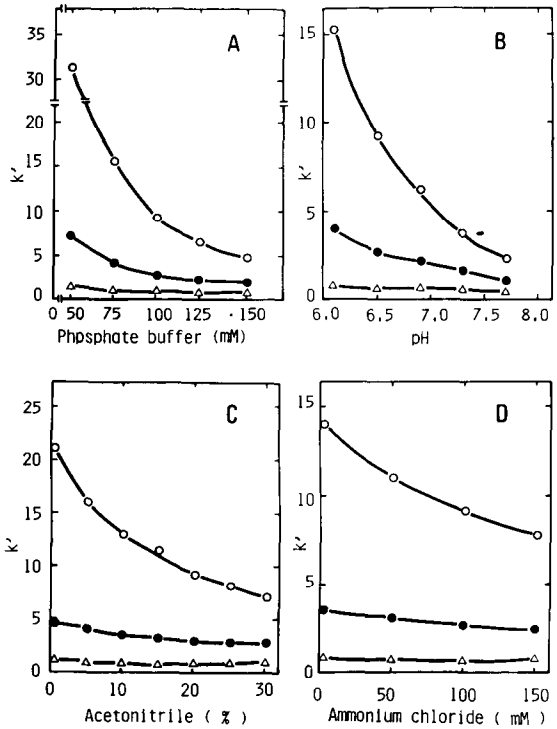

Fig. 1. Effects of the components and the pH of the mobile phase on the capacity factor, k'. The mobile phase consisted of 100 mM potassium phosphate buffer (pH 6.5), 100 mM ammonium chloride and 20% acetonitrile. One component in the mobile phase or the pH of the buffer was varied: (A) phosphate buffer concentration; (B) pH; (C) acetonitrile concentration; (D) ammonium chloride concentration. ○—○, ATP; ●—●, ADP; △—△, AMP.

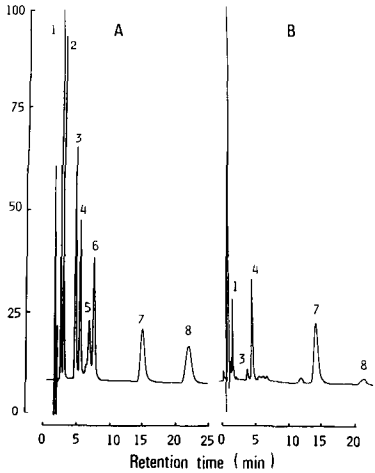

Fig. 2. Chromatogram of nucleotides. (A) Authentic nucleotides (each 300 pmol): 1 = AMP; 2 = GMP; 3 = NADP; 4 = ADP; 5 = NADPH; 6 = GDP; 7 = ATP; 8 = GTP. (B) Mouse liver extract. The numbers correspond to those shown in (A).

TABLE I

PLATE NUMBER AND RESOLUTION FACTOR (R_s)

Calculated as described in Materials and methods.

Compound	*Plate number*	R_s
AMP	2540	AMP–GMP 1.32
ADP	3100	NADP–ADP 1.33
ATP	3270	ADP–NADPH 2.33

these nucleotides from each other, although the R_s value for GDP–NADPH was 0.95. The plate numbers for AMP, ADP and ATP, calculated from the formula $N = 5.54(t_R/W)^2$ (ref. 18), were good enough for complete separation of the nucleotides (Table I).

The recovery of authentic ATP, ADP and AMP analyzed according to the standard conditions was 97.7 ± 4.5, 106.2 ± 3.9 and 102.5 ± 4.5%, respectively. The minimum amount of adenine nucleotides reproducibly detected was 25 pmol for ATP and 10 pmol for ADP and AMP, respectively; the calibration graph was linear to 5 nmol of each adenine nucleotide.

The HPLC system described could be successfully applied to the determination of adenine nucleotides in biological materials. Nucleotides were extracted from mouse liver and subjected to HPLC (Fig. 2B). Five nucleotides including AMP, ADP, ATP, GTP and NADP were identified, although the last two were present in very small quantities. The peaks of the adenine nucleotides detected were completely separated from those of other cellular components. The amount of mouse liver tissue required for the minimum detectable quantity of ATP (25 pmol) is calculated to be 1.5 mg wet weight from the data (Table II) on the content of adenine nucleotides. This HPLC systems seems therefore suitable for the analysis of adenine nucleotides in animal tissues, ischemic tissues, shocked organs[19] or regional samples of the brains from small animals[20].

To demonstrate the accuracy of the HPLC method, adenine nucleotides in the same extract of mouse liver were analyzed simultaneously by the enzymatic method[3]. The contents of AMP, ADP and ATP in normal mouse liver measured by HPLC are given in Table II and these values are closely correlated to those obtained by the enzymatic method[3]. The content of adenine nucleotides in shocked mouse liver or

TABLE II

CONTENT OF ADENINE NUCLEOTIDES IN NORMAL ICR MOUSE LIVER AND ITS CORRELATION TO THAT OBTAINED BY THE ENZYMATIC METHOD

Results are expressed as mean ± S.D. of 9–15 experiments.

Compound	*Content (μmol/g wet weight)*	*HPLC/enzyme assay (%)*
AMP	0.42 ± 0.17	110.0 ± 13.0
ADP	0.93 ± 0.10	100.6 ± 7.6
ATP	2.93 ± 0.17	97.6 ± 4.5

ischemic rat brain was measured in an identical manner by this HPLC system[19,20] as well as by the enzymatic method[3]. The ressults show that the HPLC system is a reliable analytical method for adenine nucleotides and is time- and cost-saving compared to the conventional enzymatic method.

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