HPLC Determination of Adenine Compounds as Fluorescent Derivatives in Canine Red Blood Cells

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Since early reports of the adenosine 5'-triphosphate (ATP) requirement for maintenance of viability [10], morphological structure [9, 10] and cell volume [15] in red blood cells, several functions of ATP in red blood cells have come to light. For example, phosphorylation of membrane protein by protein kinase requires ATP as a substrate [6]. Na, K-pump, and Ca pump in red cell membrane, which regulate the cellular cation composition, require ATP for fuel [5, 13]. In our experiments on ion flux in red blood cells [4], it was necessary to measure the concentration of cellular ATP and its metabolites for the monitoring of cell conditions. Not only high sensitivity but also high specificity was required when the cells were treated with several reagents and incubated in various media.

There are 3 ways to perform ATP measurements in a biological sample. The first is using an ATP-specific enzyme as an indicator for ATP; coupling to oxidation or reduction of pyridine nucleotide [2, 16] or luciferase-luciferin [3]. The second is with the use of NMR [8]. Though cellular ATP could be detected by NMR in intact cells, it was difficult to obtain the absolute concentration. The third is to separate ATP using chromatography and to determine ATP by u.v. absorption [1]. With the advent of high-performance liquid chromatography (HPLC), precise determination and high resolution of the compound became feasible [14]. Furthermore, by using HPLC, separation and measurements for ATP, ADP, AMP, adenosine and adenine could be down simultaneously. Recently, Sonoki *et al.* measured the fluorescence of 1, N⁶-etheno derivatives produced by a reaction of adenine compounds with chloroacetaldehyde (CAA), after separation by HPLC [13]. Fluorescent-labelling improved not only sensitivity but also the specificity to adenine compounds. Here, HPLC analyses of fluorescent derivatives of adenine compounds in red blood cells are performed and used to measure their contents in canine red blood cells.

The equipment used was a Japan Spectroscopic (JASCO) Model 800-MP-15 HPLC with a JASCO FP-210 spectrofluoro monitor; the column used was vinyl alcohol copolymers Asahipak GS-320H (250 mm × 7.6 mm) with dual partition and gel permeation chromatography (13). The column effluent was monitored fluorometrically at an excitation wavelength of 290 nm and at an emission wavelength of 415 nm. Chromatograms were recorded on a JASCO Model 805-GI graphic integrator, while

fluorescence spectra were obtained on a JASCO FP-770 spectrofluorometer.

Blood samples were obtained from 5 clinically healthy mongrel dogs by venipuncture with heparin as an anti-coagulant. After centrifugation at 9,000 g for 2 min, the pellets were added to 2 volumes of hemolysing solution (10 mM phosphate buffer, pH 7.2, containing 0.5 mM EDTA, 3 mM mercaptoethanol). Packed cell volume of the blood and hemoglobin concentration of the both blood and hemolysate were measured for calculation of cellular contents of adenine compounds. Two hundred μ l of the hemolysate was added to a 1.3 ml 6% perchloric acid solution, and left stand for 5 min on ice. Then the mixture was centrifuged at 9,000 g for 2 min and the pH of the supernatant was adjusted to 5.0 by titrating with 1 M K₂CO₃. The protein-free supernatants were stored at -20°C until analysis. To 150 μ l of the protein-free supernatants, 100 μ l each of 0.2 M phosphate buffer pH 5.0 and 0.1 M CAA were added. Then the mixture was heated to 70°C for 60 min in a sealed reaction tube. Thereafter, 700 μ l of 0.1 M Tris/HCl pH 7.4 was added to the reaction mixture, and the diluted mixture was washed with 3 volumes of water-saturated diethyl ether to remove the excess CAA. Usually, a 5 μ l-aliquot of the aqueous phase was injected into the HPLC system, and the injection volume was increased to 100 μ l in some experiments. Elution was carried out at a flow-rate of 1 ml/min at 40°C for 5 min with 0.2 M phosphate buffer pH 5.0, followed by 0.2 M phosphate buffer pH 5.0-methanol (70:30, v/v).

As shown in Fig. 1-A, ATP, ADP, AMP, adenosine and adenine, which were previously added to the hemolysate and treated by the above procedure were clearly separated from each other. The HPLC chromatograms for the samples from fresh cells and the cells after 3-hr incubation with 2 deoxy-D-glucose were also shown in Fig. 1-B and 1-C, respectively. To obtain calibration lines for the adenine compounds, each standard sample was added to hemolysate, and the additions were measured by the above procedure. The values of adenine compounds were calculated by subtracting the concentration in the hemolysate without standard samples from the concentration in each hemolysate with standard samples.

As shown in Fig. 2, the calibration line for ATP was linear from 13 to 400 nmol/ml hemolysate, and the range corresponded to 39 to 1,250 nmol/ml red blood cells (0.10 to 3.26 nmol/mg Hb). The linear range for ADP was from 11 to 350 nmol/ml hemolysate, which corresponded to 33 to 1,100 nmol/ml red blood cells (0.09 to 2.87 nmol/mg

Fig. 1. Chromatogram of 1, N^6 -etheno derivatives of adenine compounds in red blood cells, in which the compounds were previously added (A, 98 nmol/ml hemolysate for ATP, ADP and AMP, and 109 nmol/ml hemolysate for adenosine and adenine), or not added (B), and in the cells after 3 hr-incubation with 10 mM 2 deoxy-D-glucose (C). a, 1, N^6 -etheno-ATP; b, 1, N^6 -etheno-ADP; c, 1, N^6 -etheno-AMP; d, 1, N^6 -etheno-adenosine; e, 1, N^6 -etheno-adenine. In the experiment A, the difference of the contents of endogenous adenine compounds in red blood cells were reduced by the incubation in 10 mM Tris/MOPS, 140 mM NaCl at 37°C for 3 hr, prior to the addition of the adenine compounds. Fresh red blood cells were used in the experiment B. The peak heights among in A, B and C could not compare, because the gains of the integrator were different among in each chromatogram.

Hb); the linear range for AMP was from 18 to 430 nmol/ml hemolysate, which corresponded to 55 to 1,340 nmol/ml red blood cells (0.14 to 3.50 nmol/mg Hb). Cellular ATP contents in several mammalian species varied from 365 to 2,120 nmol/ml red blood cells [14], and ADP contents varied from 62 to 125 nmol/ml red blood cells. Though the above ranges of calibration lines were available for usual measurement to monitor cell condition, the content of AMP was around the lower limit of the range, and adenosine and adenine could not be detected in normal cells. Therefore, the ranges were lowered by increasing the injection volume (100 μ l) to HPLC for lower AMP, adenosine and adenine (Fig. 2). The range for lower AMP was 2.9 to 37 nmol/ml hemolysate, which corresponded to 8.7 to 111 nmol/ml red blood cells (23 to

Fig. 2. Calibration lines of ATP (A), ADP (B), AMP for higher range (C), AMP for lower range (D), adenosine (E) and adenine (F). In the abscissa, the concentration of adenine compounds was indicated as nmol/ml hemolysate. In the ordinate, the fluorescence intensity was indicated as a peak area which was calculated by a graph integrator.

290p mol/mg Hb); the range for adenosine was from 3.2 to 37 nmol/ml hemolysate, which corresponded to 9.6 to 116 nmol/ml red blood cells (25 to 290p mol/mg Hb); and the range for adenine was from 2.9 to 37 nmol/ml hemolysate, which corresponded to 8.7 to 111 nmol/ml red blood cells (23 to 290 pmol/mg Hb). Furthermore, the sensitivities could be set higher if the preparation procedure was changed. Actually, the range of detection for 1, N^6 -etheno derivatives of adenine compounds per se was around 1 to 40 pmol per 10 μ l injection [14], and the injection volume could be increased to 200 μ l for the complete separation of the adenine compounds.

In canine red blood cells, ATP, ADP and AMP contents in the fresh cells were 506 ± 79 , 91 ± 17 and 51 ± 2 nmol/ml red blood cells (1.38 ± 0.22 , 0.25 ± 0.05 and 0.14 ± 0.05 nmol/mg Hb, $n=5$), respectively. However, adenosine and adenine could not be detected in the fresh cells. As shown in Fig. 3, the contents of the adenine compounds in the canine red blood cells during incubation time course were measured in 10 mM Tris/MOPS, 140 mM NaCl, pH 7.4, with ATP generating system (5 mM phosphate, 5 mM glucose, and 5 mM inosine) or 10 mM 2 deoxy-D-glucose as a inhibitor for glycolysis. With the ATP generating system, the cellular contents of the adenine nucleotides were not changed. However, with the inhibitor for glycolysis, cellular ATP contents decreased from 495 to 43 nmol/ml red blood cells; cellular ADP remained at almost the same level, and cellular AMP

Fig. 3. Change of cellular contents of ATP (circles), ADP (squares), and AMP (triangles) in canine red blood cells as a function of incubation time at 37°C in 10 mM Tris/MOPS, 140 mM NaCl with ATP generating system (A) or 10 mM 2 deoxy-D-glucose (B). BW, red blood cells before washing.

content increased from 51 to 284 nmol/ml red blood cells during 3-hr incubation. Again, adenosine and adenine could not be detected in any condition.

As shown in the above, the present method is very useful for examination of the ATP metabolism in red blood cells, because of the simultaneous determination of

all adenine compounds, the high specificity, and extreme sensitivity.

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