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Short communication

High-performance liquid chromatographic determination of diadenosine 5′,5‴-p^1,p^4-tetraphosphate with precolumn fluorescence derivatization and its application to metabolism study in whole blood

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Abstract

Diadenosine 5′,5‴-p^1,p^4-tetraphosphate (Ap4A) was converted with chloroacetaldehyde to the fluorescent di-1,N^6-ethenoadenosine derivative within 60 min at 80°C. It was separated by reversed-phase HPLC and detected fluorimetrically (excitation and emission wavelengths of 275 and 410 nm, respectively). The detection limit of Ap4A was ca. 0.2 μg/ml in plasma when 10 μl of the sample was applied to the column. The rate of degradation of Ap4A added to whole blood (5 μg/ml) was examined using this method. Half-lives (means ± S.E., $n = 3$) were 0.88 ± 0.30 min (in rat blood), 13.7 ± 3.6 min (in dog blood) and 17.2 ± 1.4 min (in human blood). A marked species difference in the degradation rate of Ap4A in blood was observed.

1. Introduction

Diadenosine 5′,5‴-p^1,p^4-tetraphosphate (Ap4A) was found in mammalian cells about eighteen years ago [1,2]. It has been reported that platelets are rich in Ap4A and that the Ap4A is released into the plasma when the blood cells are damaged [3–5]. Ap4A inhibits platelet aggregation induced by ADP to reduce the production of thrombus [6,7]. In addition, Ap4A induces the endothelial purinogenic receptor mediated vasodilation of arteries [8]. An Ap4A specific receptor was found on the membrane of mouse heart [9]. On the other hand, Ap4A degrading enzymes were found in porcine [10] and bovine [11] aortic endothelial cells. An Ap4A degrading enzyme which is regulated by divalent cations, such as Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+}, was also found in plasma [11–13]. Furthermore, the degradation of Ap4A in plasma is inhibited by its degradation products, ATP and AMP [14]. Thus, Ap4A is suggested to play several important roles in biological tissues, especially in the biological circulatory system.

Three methods have been mainly used to analyze Ap4A in plasma. The first is TLC, in which isotopically labeled Ap4A is used [7,9,12–15]. The second is HPLC with ultraviolet ab-

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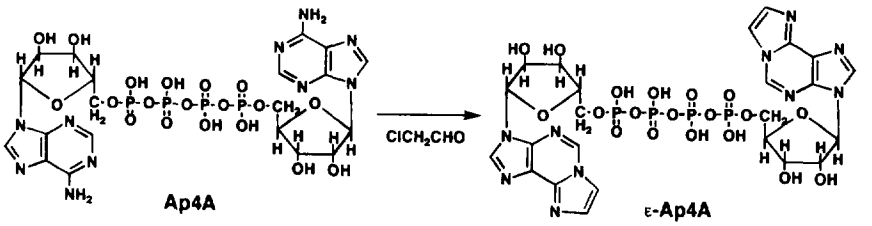

Fig. 1. Derivatization of Ap4A to ε-Ap4A.

sorbance detection [8,16,17]. The third is the luciferine–luciferase method, in which Ap4A is degraded to ATP by alkaline phosphatase and the product ATP is converted to luminescence with luciferine and luciferase [5,11,12,18].

Instead of Ap4A, a fluorescent etheno derivative of Ap4A (di-1,N^6-ethenoadenosine 5′,5‴-p^1,p^4-tetraphosphate, ε-Ap4A) is used as the substrate for the metabolic enzymes to investigate the metabolism of Ap4A [19,20]. The scheme for derivatizing Ap4A to ε-Ap4A is shown in Fig. 1.

The etheno derivatization was thought to be useful for the highly sensitive detection of Ap4A. In this study, an HPLC method for the analysis of Ap4A with precolumn derivatization to ε-Ap4A was developed and its application to the study of Ap4A metabolism in blood was examined.

2. Experimental

2.1. Reagents

Ap4A sodium salt was supplied by Fujirebio (Tokyo, Japan). Perchloric acid (about 70%) and 40% chloroacetaldehyde solution were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were guaranteed or analytical grade commercial products.

2.2. Synthesis of etheno derivatives

A 1-ml volume of saline solution containing Ap4A, ATP, ADP, AMP or adenosine (Ado) (10 μg/ml each), or the EDTA-treated plasma containing Ap4A (2.0 μg/ml) was mixed with 0.5 ml of 10% perchloric acid and the mixture was centrifuged at 7000 *g* for 1 min at room temperature. The supernatant was mixed with 0.5 ml of 2 *M* potassium hydrogen carbonate and the mixture was centrifuged again at 7000 *g* for 1 min at room temperature. After addition of 0.25 ml of 2 *M* sodium acetate buffer (pH 4.5) and 20 μl of 40% chloroacetaldehyde solution to the supernatant, the mixture was kept at 80°C for 60 min. Then, the mixture was cooled in ice water.

2.3. HPLC analysis

HPLC analysis was performed by partial modification of the methods previously reported [19,20]. HPLC was performed on a TSK-gel ODS-80TM (150 × 4.6 mm I.D.; Toso, Tokyo, Japan) and a 10-μl sample was applied to the column. The column was attached to an LC-10AD pump (Shimadzu, Kyoto, Japan) and a Shimadzu RF-535 fluorescence photometer whose excitation and emission wavelengths were 275 and 410 nm, respectively. A Shimadzu CTO-10A column oven was used at 40°C. The mobile phase was a mixture of 900 ml of potassium phosphate buffer (100 m*M*, pH 6.0) and 100 ml of methanol and the flow-rate was 0.7 ml/min. For quantitative calculations a Shimadzu C-R4A data module was employed.

2.4. Ap4A degradation in whole blood

Blood from Sprague–Dawley rats (200–250 g, female), beagle dogs (9.5–11.0 kg, male) or human volunteers (male) was anticoagulated with heparin (final concentration of 5–13 U/ml). In all experiments, fresh blood was used.

Ap4A was added to each blood at a con-

centration of 5 μg/ml. The degradation study was performed at 37°C. At intervals, blood samples were taken and mixed with powders (a mixture of potassium fluoride, heparin sodium and EDTA-2Na) in a vacuum blood tube (Venoject II VP-FH052, Terumo, Tokyo, Japan) to inhibit Ap4A degradation (mainly EDTA inhibits Ap4A degradation [12]). After centrifugation at 1500 *g* for 10 min at 4°C, the plasma obtained was treated for the synthesis of etheno derivatives followed by HPLC analysis.

3. Results and discussion

3.1. Synthesis of etheno derivative of Ap4A and the quantitative analysis by HPLC

The synthesis of ε-Ap4A from Ap4A and aqueous chloroacetaldehyde was reported by Rotllán et al. [19]. However, the reaction time needed in that method was very long: 70 h at 35°C. We found that Ap4A is stable at high temperature; more than 98% of Ap4A remained when incubated at 80°C in water for 6 h and more than 90% of Ap4A remained when incubated at 150°C in pressurized water for 6.5 h (data not shown). At high temperature, the derivatization should be accelerated. Thus, we tried to perform the derivatization of Ap4A to ε-Ap4A at 80°C. The time courses of the reaction are shown in Fig. 2.

Two peaks, with retention times (t_R) of 5.3 and 9.2 min, were detected during the reaction. Since an Ap4A molecule has two adenine parts, the derivatization of Ap4A to ε-Ap4A was thought to occur by a two-step reaction. The di-etheno Ap4A might be synthesized following the mono-etheno Ap4A. As shown in Fig. 2A, the first peak, which has a maximum at ca. 5 min after the start of the reaction, was thought to be the mono-etheno Ap4A (t_R = 5.3 min) and the second peak, which is maximum at 60–70 min after the reaction had started, was thought to be the di-etheno Ap4A (t_R = 9.2 min). In this report, the di-etheno Ap4A was described as ε-Ap4A. In contrast to the case of Ap4A, only one peak was observed when mono-nucleotides, such as ATP, AMP, Ado, guanosine triphosphate (GTP) and guanosine monophosphate (GMP), were converted to the etheno derivatives.

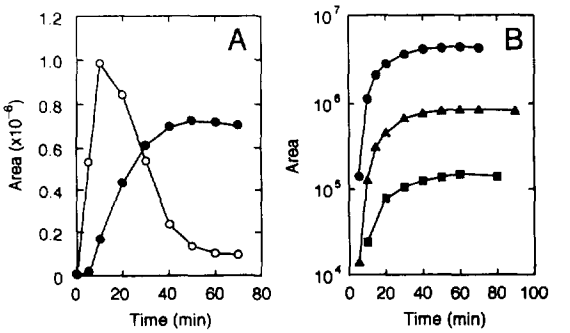

Fig. 2. Time course of reaction of Ap4A to the etheno derivatives. (A) Initial concentration of Ap4A was 100 μg/ml. The areas of peaks (○, t_R = 5.7 min; ●, t_R = 9.2 min) were plotted. (B) Initial concentrations of Ap4A were (■) 1, (▲) 10 and (●) 100 μg/ml. The areas of the latter peak (t_R = 9.2 min) were plotted. The results are shown as the mean of duplicate experiments.

Fig. 2B shows the peak area vs. time relationship of the latter peak at three different Ap4A concentrations. As is evident from the figure, the etheno derivatization of Ap4A reached a maximum level in 50 min at any concentration, suggesting that the derivatization of Ap4A to ε-Ap4A was completed in 50–60 min at 80°C. In addition, the area of the ε-Ap4A peak (t_R = 9.2 min) counted by HPLC integrator after the derivatization was found to be almost constant for 60 h at 37°C.

In plasma, Ap4A was catabolized to ATP and AMP, and ATP was subsequently degraded to ADP, AMP and Ado [10,11,14]. Therefore, Ap4A must be separated from ATP, ADP, AMP and Ado prior to measurement. These compounds also have adenine sites, and can be converted to the etheno derivatives. After the reaction to the etheno derivatives (ε-ATP, ε-ADP, ε-AMP and ε-Ado, respectively), these derivatives were separated from ε-Ap4A by HPLC. A typical chromatogram obtained by HPLC of their mixed saline solution is shown in Fig. 3A. In addition, a typical chromatogram of ε-Ap4A in plasma is shown in Fig. 3B.

ε-Ap4A, ε-ATP, ε-ADP, ε-AMP and ε-Ado

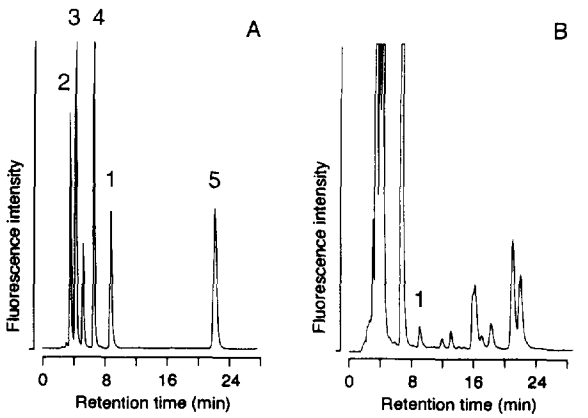

Fig. 3. Typical HPLC chromatograms. (A) Sample of a mixture of Ap4A, ATP, ADP, AMP and Ado (10 μg/ml each in saline). (B) Sample of Ap4A in rat plasma (2.0 μg/ml). Peaks: 1 = ε-Ap4A; 2 = ε-ATP; 3 = ε-ADP; 4 = ε-AMP; 5 = ε-Ado. Sample volume injected: 10 μl.

were separated by HPLC. Furthermore, ε-Ap4A could be clearly detected in spite of the large peak derived from plasma, although the simultaneous detection of the metabolites seems to be difficult under these conditions.

A known amount of Ap4A was added to rat plasma after addition of EDTA to inhibit Ap4A degradation. After the etheno derivatization, the area of the ε-Ap4A peak was plotted against the concentration of Ap4A in the plasma. The C.V. at each concentration was less than 4.5% and the retention time was 9.16 ± 0.01 min ($n = 24$). The linear regression equation determined by the least-squares method was $y = 5346.8x + 1981.2$ ($r = 0.999$). Linearity was obtained up to 16 μg/ml of Ap4A in plasma. Since the peak area at 0.2 μg/ml, but not at 0.1 μg/ml, was significantly different ($p < 0.01$) from the plasma blank value and was on the regression line, the limit of Ap4A detection was estimated to be 0.2 μg/ml in plasma when 10 μl of sample was applied to the HPLC system.

The present method is more sensitive and more convenient than the classical methods. The detection limit of the HPLC method with ultraviolet absorbance detection was 30 pmol [16]. Although the luciferine–luciferase method [18] showed a sensitivity equivalent to that of the present method (about 2 pmol), it is more complex and less convenient.

3.2. Ap4A degradation in whole blood

Ap4A in plasma could be determined by HPLC with fluorescence detection after derivatization to ε-Ap4A. By using this method, Ap4A degradation in whole blood was measured. Whole blood was obtained from rats, dogs and humans. Ap4A was added to a concentration of 5 μg/ml to each blood sample. The degradation experiment was performed at 37°C after addition of Ap4A. The half-life of Ap4A was calculated from a semi-logarithmic plot of Ap4A concentration against incubation time. The half-lives are summarized in Table 1.

The half-life of Ap4A in human blood was similar to that in dog blood, but much longer than that in rat blood. The half-life of Ap4A in rat blood was about one-tenth that of the others.

The half-life of Ap4A in human blood at the initial concentration of 1 μg/ml was 4.87 ± 0.76 min (mean ± S.E., $n = 3$) as determined by the method reported here. Using tritium labeled Ap4A, Lüthje and Ogilvie [14] reported that the half-life of Ap4A in human blood is 5.88 ± 1.88 min (mean ± S.D.) at the initial concentration of 0.7 μ*M* (equal to ca. 0.7 μg/ml). The value obtained in the present study is similar to that obtained by the method with isotopically labeled Ap4A.

The concentration dependence of Ap4A degradation in blood suggests saturation kinetics. The kinetic analysis of Ap4A degradation in blood is currently being studied.

Table 1
Half-lives of Ap4A in rat, dog and human blood

Origin	Half-life (min)
Rat	0.88 ± 0.30
Dog	13.7 ± 3.6
Human	17.2 ± 1.4

The initial concentration of Ap4A was 5 μg/ml. The values are means ± S.E. ($n = 3$).

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