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Short communication

Quantification of carbamylated dehydroascorbate derivative produced from cyanate and dehydroascorbate

Ichiro Koshiishi, Toshio Imanari*

Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi, Inage, Chiba-shi, Chiba 263, Japan

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Abstract

We established a high-performance liquid chromatographic method for separating and quantifying carbamylated dehydroascorbate derivative (CDA), a reaction product of cyanate with dehydroascorbate. The separation of CDA from interfering substances was achieved by anion-exchange HPLC using a TSK gel SAX (250×4.6 mm I.D.) column and 0.12 *M* NaCl eluent. The detection of CDA was achieved through two steps: (1) degradation of CDA to cyanate and amino compounds in alkaline solution, and (2) detection of these products by an indophenol reaction. For the processing of plasma and urine samples, anion-exchange solid-phase extraction was used. The detection limit for quantitative determination was 0.1 μ*M* CDA (*S*/*N*=3). The linear range found applying the optimized conditions was 0.2 to 200 μ*M*. The intra- and inter-day assay precision (R.S.D.) of CDA (10 μ*M*) were 4.8 and 7.2% for rat plasma, and 4.0 and 4.9% for rat urine, respectively. The usefulness of the present method was proved by the application to plasma and urine samples. The study of the biokinetics of CDA in rats revealed that the elimination of CDA is due to urinary excretion. © 1998 Elsevier Science B.V.

Keywords: Carbamylated dehydroascorbate derivative; Cyanate; Dehydroascorbate

1. Introduction

In agriculture, herbicides are generally used for withering plants to control maturity of crops such as potato. Sodium cyanate is well-known to be a herbicide [1,2]. It is relatively unstable in nature and degrades into ammonia and carbon dioxide. Furthermore, it was also reported that cyanate penetrated into leaves and stems of plants and quickly disappeared [3]. Thus, sodium cyanate seemed to be harmless to humans. However, in our latest report, we provided evidence that cyanate penetrated into plants and reacted with dehydroascorbate, producing an end-product termed carbamylated dehydroascorbate derivative (CDA, Scheme 1) [4]. Cyanate may remain in crops not as unaltered form but as CDA.

In order to examine whether CDA is harmless to humans, firstly, a metabolic fate of CDA in mammalians should be elucidated. For this purpose, it is needed to establish a determination method for CDA in biological samples including plasma and urine. CDA is an acidic carbohydrate so that it is detectable by reaction with reagents such as 2-cyanoacetamide [5], guanidine [6], arginine [7], taurocyamine [8], etc.; these are generally used for the fluorimetric detection of carbohydrates. However, the abundant

*Corresponding author.

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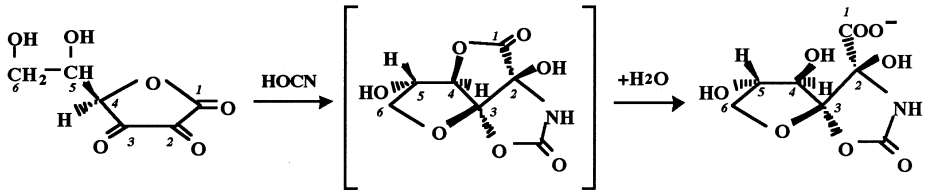


Scheme 1. Production of carbamylated dehydroascorbate derivative (CDA) by the reaction of cyanate with dehydroascorbate under physiological conditions.

carbohydrates present in biological fluids do not permit the sensitive and specific detection of CDA. On the other hand, it is possible to degrade CDA to ammonia quantitatively by heating in alkaline solution and in acidic solution successively, so that CDA is indirectly detectable by the indophenol reaction. The combination of this detection system with anion-exchange chromatography should make it possible to detect CDA selectively, because the amounts of acidic amino compounds in biological samples are relatively low. Along these lines, we established a selective method for determination of CDA in biological fluids using high-performance liquid chromatography (HPLC) with post-column derivatization. Furthermore, the present method was applied to the study of the kinetics of CDA in rats.

2. Experimental

2.1. Materials

Ascorbic acid and sodium cyanate were purchased from Wako Pure Chemicals (Osaka, Japan). Dehydroascorbic acid was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were of reagent grade. TSK gel SAX was obtained from Tosoh (Tokyo, Japan). A standard solution of cyanate (100 m*M*) was prepared from sodium cyanate that had been recrystallized from ethanol.

2.2. Synthesis of carbamylated dehydroascorbate derivative (CDA)

CDA was synthesized as follows [4]: dehydroascorbate was prepared from ascorbic acid by oxidation using cupric ion. Ascorbic acid powder (52 g) was added to 2 l of 0.15 *M* copper(II) acetate solution, and then the reaction solution was stirred vigorously at room temperature. After standing for 10 min, 1 l of 0.75 *M* sodium cyanate solution was added dropwise to the solution, and the reaction mixture was stirred overnight. The reaction mixture was filtered through filter paper (Advantec Toyo, 51A) three times, and the filtrate was passed through Dowex 1-X8 (Cl^- form, 50–100 mesh, 10×5 cm I.D.). The eluate was concentrated to 200 ml by evaporation, and the solution was added dropwise to 800 ml of 99% ethanol. The CDA precipitate was recovered by filtration. The precipitate was dissolved in water, and the volume was adjusted to 200 ml. CDA was crystallized by mixing the solution with 800 ml of 99% ethanol. It was recrystallized four times from ethanol–water. CDA has the molecular formula, $C_7H_8NO_8Na$, and contains two water molecules of crystallization. The results of the elemental analysis were as follows; calculated for $C_7H_8NO_8Na.2H_2O$: C, 28.70%; H, 4.09%; N, 4.78%; O, 54.61%; Na, 7.85%. Found: C, 28.70%; H, 3.89%; N, 4.55%; Na, 7.70%; other elements, 55.16%.

2.3. Determination of cyanate

Cyanate was measured by HPLC with post-column derivatization [9]. The chromatographic conditions were as follows; column, TSK gel SAX (50×4 mm I.D., particle size 5 μm); eluent, 0.3 *M* NaCl. The detection was based on the indophenol reaction for the ammonia which was released by the hydrolysis of cyanate.

2.4. Determination of CDA in biological fluids

CDA in plasma and urine samples was determined by HPLC followed by post-column derivatization

using a UV–Vis detector. Plasma (400 μl) was mixed with 200 μl of 20% trichloroacetic acid, and the mixture was centrifuged at 10 000 *g* for 5 min. The supernatant was neutralized with 1.0 *M* NaOH solution, and passed through a Dowex 1-X8 column (600 μl). The column was washed with 1 ml of water, and CDA was eluted with 0.5 *M* NaCl. The first 300 μl of eluate were discarded and the next 400 μl (CDA fraction) were collected. A portion of the CDA fraction was analyzed by HPLC. Urine sample was passed through a Dowex 1-X8 column (600 μl) without a deproteinization, and was collected and analyzed in the same way as the plasma sample.

The HPLC assembly consisted of a HPLC pump (Hitachi, Tokyo, Japan; L-6000), a sample injector (Rheodyne, CA, USA; 7725), a double-plunger pump (Shimamura Instrument, Tokyo, Japan; PSU-2.5W), a dry reaction bath (Shimamura Instrument; DB-5), a UV–Vis detector (Hitachi; L-4200), Chromato-integrator (Hitachi; D-2500) and PTFE tubing (0.5 mm I.D. and 0.25 mm I.D.). The chromatographic conditions were as follows: column, TSK gel SAX (250×4.6 mm I.D.); eluent, 0.12 *M* NaCl (0.6 ml/min); column temperature, 60°C; reagent I, 0.16 *M* acetic acid containing 2% phenol and 0.01% sodium nitroprusside; reagent II, 0.3 *M* NaOH containing 0.1% sodium hypochlorite; C1, 10 m×0.5 mm I.D.; C2, 15 m×0.5 mm I.D.; C3, 15 m×0.5 mm I.D.; C4, 2 m×0.25 mm I.D.; reaction temperature 80°C; detection, 635 nm.

2.5. Processing of rat blood plasma and urine

Wistar male rats (200–300 g) were used for the study. Polyethylene tubes (0.5 mm I.D.×0.8 mm O.D.) were inserted into the femoral vein and artery of Wistar male rats, and CDA solution was injected into vein through a polyethylene tube. Rat blood (approximately 200 μl) was collected from artery and 0.9 vol. of blood was mixed with 0.1 vol. of 3.8% sodium citrate. After centrifugation at 2000 *g* for 5 min, plasma was subjected to the present method.

Rat urine was collected through a polyethylene tube (non-toxic, medical grade) inserted into the urinary tract. Throughout sample processing, sample solutions were kept below 4°C.

2.6. Preparation of anephric rats

From Wistar male rats (200–300 g), both kidneys were taken out by surgical operation under ether anesthesia. At 1 h after awakening completely, the anephric rats were used for the study.

3. Results and discussion

One of the prominent characteristics of CDA is that it is labile in alkaline solution, producing cyanate (Fig. 1). However, the amount of cyanate released from CDA did not correspond to that of CDA (approximately 60%). CDA degrades along two distinct pathways, (1) the cleavage of the bond between C-2 and N, and (2) the hydrolysis of ester bond of oxazolidone ring, producing cyanate and

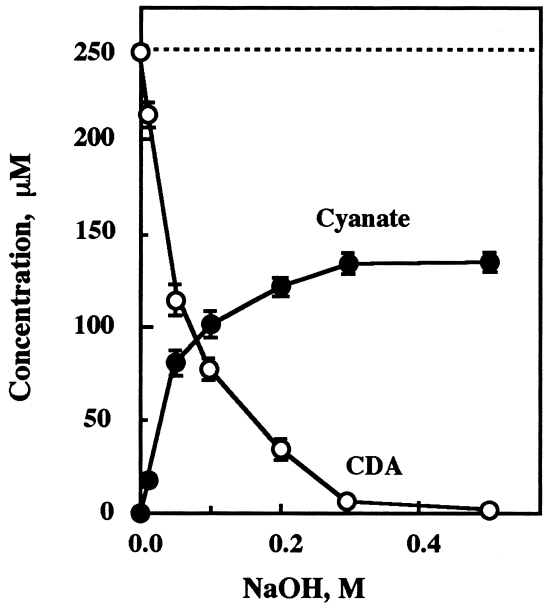
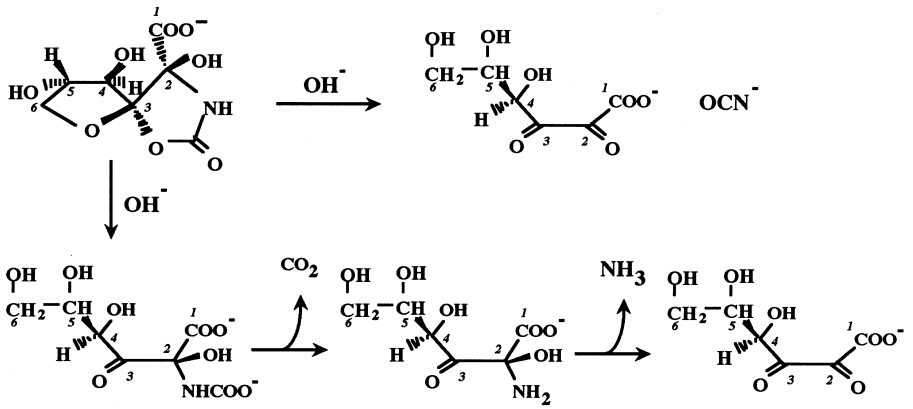


Fig. 1. Release of cyanate from CDA in alkaline solution. CDA solution (500 μ*M*) was mixed with an equal volume of NaOH solution (0.02–1.0 *M*) and the mixture was allowed to stand at 37°C for 30 min. The released cyanate was determined by HPLC with post-column derivatization [9].



Scheme 2. Possible degradation pathways of CDA in alkaline solution, producing cyanate and amino compounds.

amino compounds, respectively (Scheme 2). These degradation products were detected by the modified indophenol reaction [9]. Fig. 2 shows a chromatogram of the CDA breakdown products detected by the modified indophenol reaction. Both cyanate and amino compounds are quantitatively detected as indophenol by this system. This finding suggests that, if the complete alkaline degradation of CDA could be performed in a flow system, it would be possible to determine CDA by HPLC with post-column derivatization. Thus, we examined the degradation of CDA under the alkaline conditions. The result shown in Fig. 3 suggests that the degradation of CDA occurred within 1 min on heating at 80°C in a 15 m*M* NaOH solution. A schematic diagram of the HPLC with post-column derivatization for the analysis of CDA is shown in Fig. 4. The separation of CDA was achieved using an anion-exchange resin packed in a stainless column (TSKgel SAX, 250× 4.6 mm I.D.). The eluted CDA was first degraded to cyanate and amino compounds by passing through the tube 1, and the resultant cyanate and amino compounds were degraded to ammonia by heating in acidic solution in tube 2. Finally, ammonia was detected by the indophenol reaction in tube 3. In order to confirm the reproducibility of the peak area of CDA, standard solutions (1.0 μ*M* and 10.0 μ*M*) were injected repeatedly 10 times. The coefficients of variation (C.V.s) of the peak areas were 4.2% and 2.6%, respectively. The detection limit of this system was 0.1 μ*M* ($S/N=3$), and the correlation between peak responses and injected CDA concentration was found to be linear from 0.2 to 200 μ*M* [$Y=3.41\cdot 10^4X+0.12\cdot 10^4$, $r=0.997$; *Y*, peak area; *X*, CDA concentration (μ*M*)].

In order to apply the present method to biological fluids, we studied the sample processing by anion-exchange solid-phase extraction. The procedure described in Section 2.4 is capable of separating CDA from other amino compounds contained in biological fluids. The present method was applied to plasma and urine samples collected from rats, to which CDA solution was intravenously injected. CDA (0.37 mmol/kg body weight) was injected intravenously to Wistar male rat (230 g), and blood was collected 10 min after injection. A 24-h urine sample was also collected after injection. CDA was stable both in plasma and in urine samples for more than 24 h by keeping them in ice-bath (data not shown). The chromatograms are shown in Fig. 5. Through this pretreatment method, no interfering peak appeared close to the peak corresponding to CDA. Furthermore, the results of the recovery of CDA from plasma (10–100 μ*M* CDA, 96.7±1.2%) and urine (10–100 μ*M* CDA, 97.3±1.5%) indicated that an excellent quantitative correlation existed between the predicted and measured increments of CDA in these samples. The values of the intra-day precision (R.S.D.) of CDA spiked into rat plasma (final concentration, 10 μ*M*) were 4.8% ($N=5$) and 5.3% ($N=5$) at day 1 and at day 2, respectively, and that of the inter-day precision of CDA was 7.2% ($N=10$).

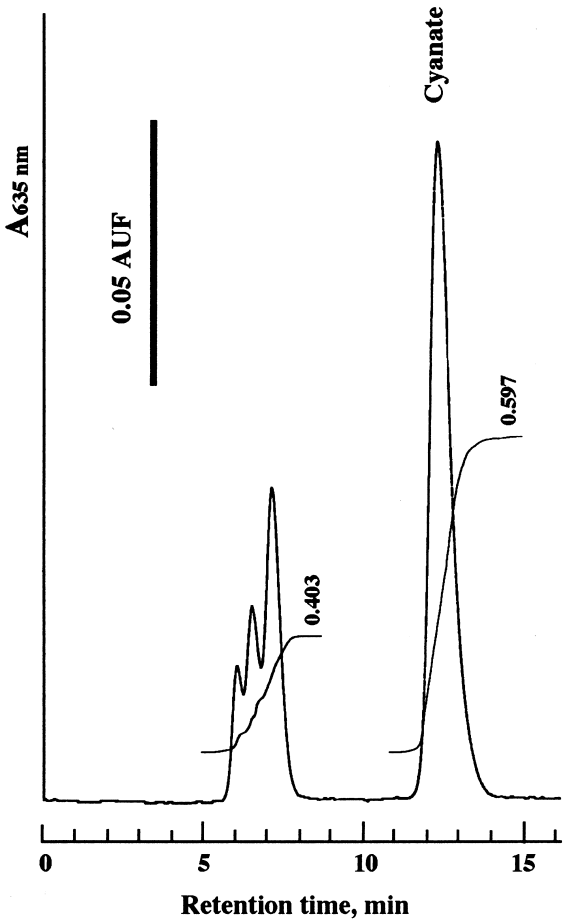


Fig. 2. Chromatographic detection of the products obtained from CDA by alkaline degradation. CDA solution (500 μ*M*) was mixed with an equal volume of 1.0 *M* NaOH solution and the mixture was allowed to stand at 37°C for 30 min. The solution was then submitted to HPLC with post-column derivatization [9]. Cyanate and amino compounds were degraded to ammonia, and the resultant ammonia was detected as an indophenol. The values reported represent the relative peak area.

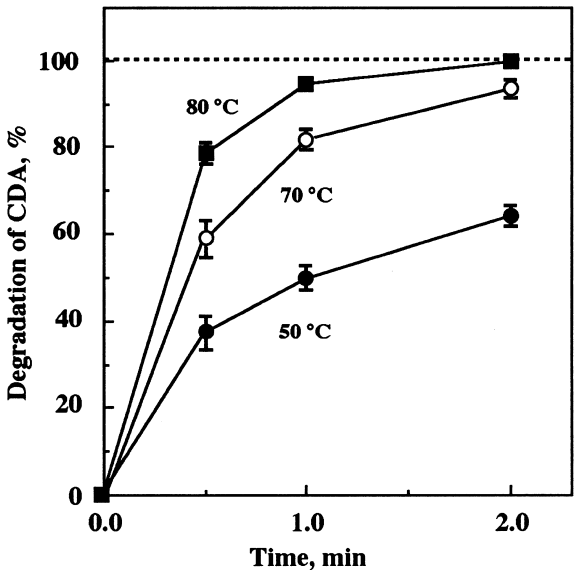


Fig. 3. Effect of temperature on the degradation of CDA to cyanate under alkaline conditions. CDA solution (200 μ*M*) was mixed with an equal volume of 30 m*M* NaOH solution, and the mixture was heated in a water bath at several temperatures. The released cyanate was determined by HPLC with post-column derivatization [9].

The values of the intra-day precision (R.S.D.) of CDA spiked into rat urine (final concentration, 10 μ*M*) were 4.0% ($N=5$) and 3.9% ($N=5$) at day 1 and at day 2, respectively, and that of the inter-day precision of CDA was 4.9% ($N=10$).

To examine the in vivo kinetics of CDA, we injected CDA to rats intravenously, and the time course of CDA levels in plasma and its urinary excretion were studied. As shown in Fig. 6A, the half-life of CDA in circulating blood plasma was approximately 25 min, and more than 99% (99.2% and 99.8%, $N=2$) of administered CDA was excreted to urine for the duration of 24 h after injection. Furthermore, a similar experiment was conducted on the anephric rats (Fig. 6B). The absence of kidneys considerably depressed the disappearance of CDA in blood plasma. These facts suggest that the elimination of CDA is due to urinary excretion.

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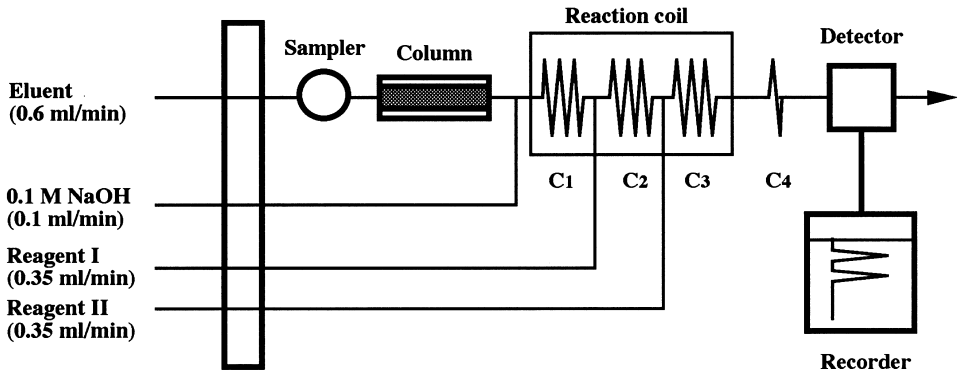

Fig. 4. Schematic diagram of the HPLC system for determination of CDA. The chromatographic conditions were as follows: column, TSK gel SAX (250×4.6 mm I.D.); eluent, 0.12 *M* NaCl (0.6 ml/min); column temperature, 60°C; reagent I, 0.16 *M* acetic acid containing 2% phenol and 0.01% sodium nitroprusside; reagent II, 0.3 *M* NaOH containing 0.1% sodium hypochlorite; C1, 10 m×0.5 mm I.D.; C2, 15 m×0.5 mm I.D.; C3, 15 m×0.5 mm I.D.; C4, 2 m×0.25 mm I.D.; reaction temperature 80°C; detection, 635 nm.

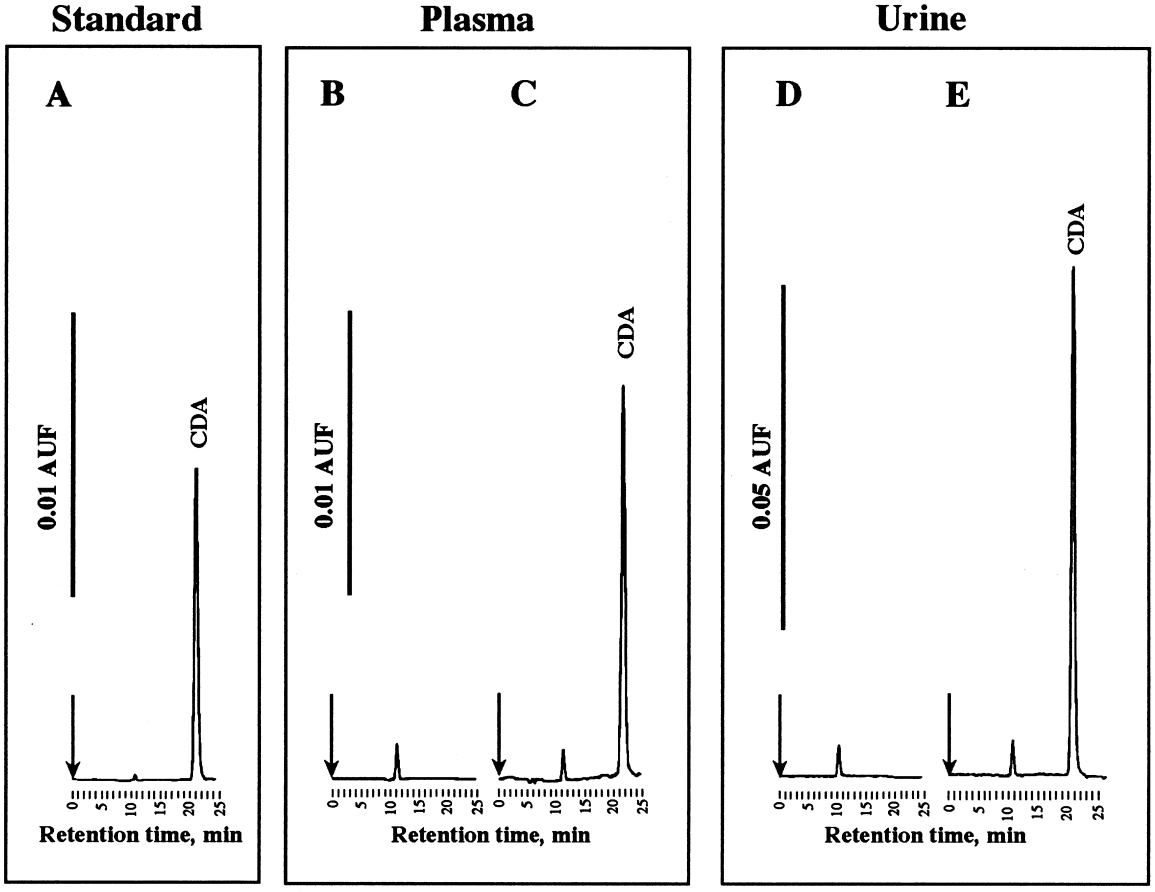

Fig. 5. Chromatograms of rat plasma and urine samples. (A) Standard CDA solution, 20 μ*M*. CDA (0.37 mmol/kg body weight) was intravenously injected into a Wistar male rat (230 g), and blood samples were collected befor injection (B) and at 10 min after injection (C). 24-h urine samples were collected before (D) and after (E) injection.

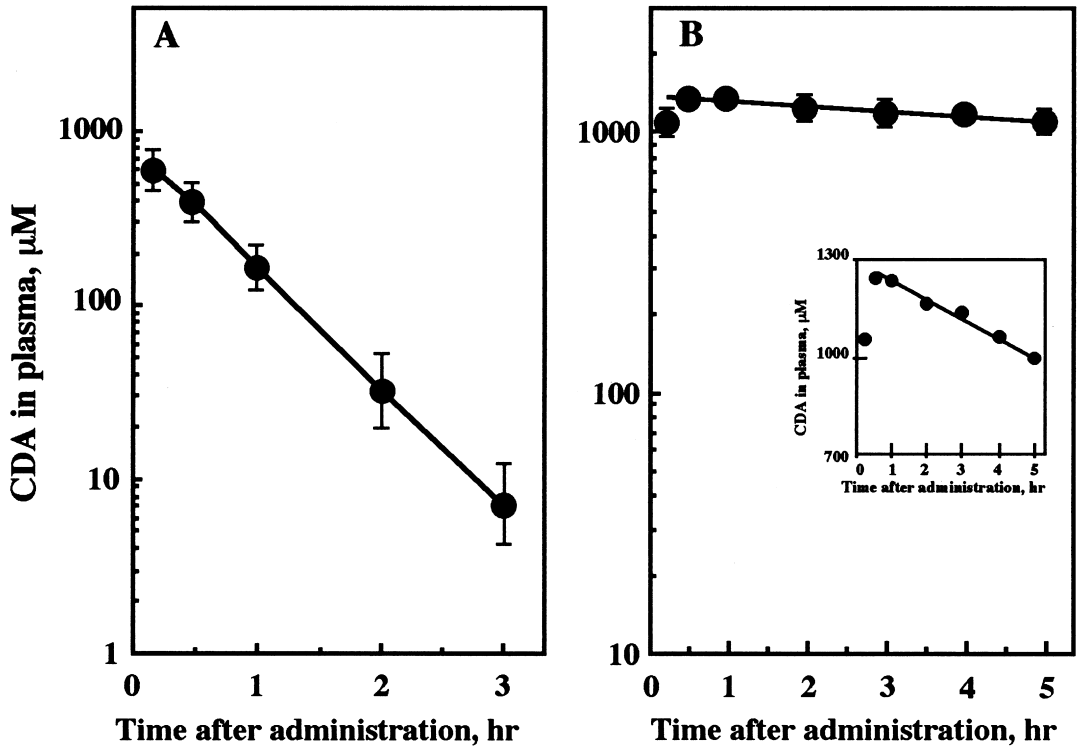

Fig. 6. Time course of CDA level in circulating blood plasma after intravenous administration of CDA to normal rats (A) and anephric rats (B). CDA (0.37 mmol/kg body weight) was administered intravenously to normal rats and anephric rats (Wistar male rat, 200–300 g). At each time after administration, blood was collected from the artery. The error bars represent the range of duplicate determinations.

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