Determination of Nitrate in Biological Fluids Using Nitrate Reductase in a Flow System

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The concentration of nitrate in biological fluids was determined using nitrate reductase (NR) in a flow system. A merging zone method was applied, in which a zone of NR and that of nitrate in separate streams were merged, and allowed to react. The decrease in NADPH caused by the reaction between NR and nitrate was measured at 340 nm. The conditions were as follows: length of the reaction coil used for the enzymatic reaction was 250 cm; 0.1 M PIPES buffer (pH 7.5) was used as the first carrier (C_1); 0.1 M PIPES buffer (pH 7.5) containing 0.1 mM NADPH was used as the second carrier (C_2); the reaction coil was immersed in a water bath maintained at 32°C. Under these conditions, a linear calibration curve (r = 0.994) was plotted for the concentration of nitrate between 1 and 100 μM with a detection limit (S/N = 3) of 0.2 μM. The present method was applied to determine the amount of nitrate in serum, plasma and urine using samples that had not been deproteinized. The concentration of nitrate within each sample was calculated from differences observed in the peak areas obtained in the absence or presence of nitrate reductase. The recovery test of the nitrate added to biological fluids indicated the applicability of the present method to the determination of nitrate in them. The nitrate concentrations determined using this technique correspond well with those of other methods.

Key words —— nitrate, nitrate reductase, flow injection analysis, serum, plasma, urine

INTRODUCTION

Nitric oxide (NO) has recently attracted a great deal of interest due to the discovery that it is a major messenger molecule in a number of physiological processes. The concentration of NO within biological fluids is difficult to determine directly because of its instability. NO undergoes a rapid oxidative degradation to its more stable metabolites, nitrate and nitrite. The latter rapidly reacts with oxyhemoglobin within the vascular system. Therefore, blood nitrate concentrations are used to reflect the amount of NO produced.

It has been established that the concentration of nitrate in biological fluids can be determined from the amount of nitrite produced from the reduction of nitrate with copperized cadmium.[1–3] The nitrite produced as a result of this process can be detected spectrophotometrically as an azodye following diazotization and diazocoupling. In order to do this, deproteinization with NaOH and $ZnSO_4$ solutions is essential. In a previous paper,[4] a direct method using nitrate reductase in a flow system was evaluated with regard to its ability to determine the amount of nitrate in natural water:

$$NO_3^- + H^+ + NAD(P)H \xrightarrow{NR} NO_2^- + NAD(P)^+ + H_2O$$

In the present study, this method was examined to see if it can be used to determine the concentration of nitrate in biological fluids using samples that have not been deproteinized.

MATERIALS AND METHODS

Materials —— Nitrate reductase (NR) from *Aspergillus species* was purchased from Boehringer Mannheim, and NADPH from Oriental Yeast Co. Ltd. Solid state NR (20 units) was dissolved in 2 ml water and the solution was separated into 0.1 ml portions, which were stored at –20°C until they were used. Before use, 0.9 ml of 1/9 M piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.5) was added to each portion in order to make an enzyme solution containing 1 unit of enzyme per 1 ml of 0.1 M PIPES buffer. This solution was injected into the flow system for analysis. Blood was obtained from a 54 year old man and urine from a 23 year old woman (urine1) and from the 54 year old man (urine2), respectively. Plasma was prepared by treatment with heparin. Biological samples were analyzed within a few days after preparation, during which they were stored at 4°C.

Apparatus —— The apparatus used in the present

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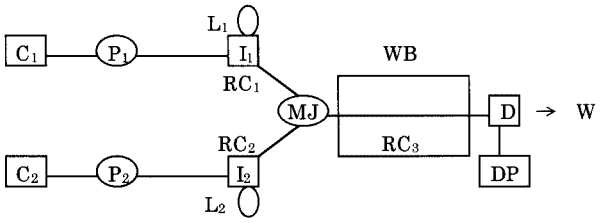

Fig. 1. Apparatus for Determination of Nitrate

C_1, C_2, carrier reservoir ; P_1, P_2, pump; I_1, I_2, sample injector; L_1, L_2, sample loop; RC_1, RC_2, RC_3, reaction coil; MJ, mixing joint; D, spectrophotometer; DP, data processor; WB, water bath; W, waste.

study is the same as that used earlier,[4] the diagram of which is shown in Fig. 1. The two carriers were pumped with Shimadzu LC-10AD pumps (P_1 and P_2) at flow rates of 0.2 ml/min, respectively. NADPH in the merged carrier was monitored at 340 nm by a Hitachi L-4200H. The area of the peak observed was calculated with a Hitachi D-2500 data processor. The flow lines were made of polytetrafluoroethylene (PTFE) tubing (0.5 mm i.d.). The lengths of reaction coils, RC_1, RC_2 and RC_3 were 35, 40 and 250 cm, respectively, and RC_3 was immersed in a water bath (WB) which was thermoregulated at 32°C. The volumes of sample loops, L_1 and L_2 were 50 and 30 μl, respectively. The enzyme solution was injected into the flow system from I_1, and nitrate sample, from I_2. The analytical conditions described above were the same as those applied previously.[4] The first carrier (C_1) was 0.1 M PIPES buffer (pH 7.5) and the second carrier (C_2) was 0.1 M PIPES buffer (pH 7.5) containing 0.1 mM NADPH. The two carriers were merged at a mixing joint (MJ).

To prepare standard nitrate samples for a calibration curve, potassium nitrate was dissolved in C_2 and the solution was diluted with C_2. The biological fluid samples used for analysis contained 0.1 M PIPES buffer (pH 7.5) and 0.1 mM NADPH, which were prepared by diluting original samples with water and 0.2 M PIPES buffer (pH 7.5) containing 0.2 mM NADPH.

RESULTS AND DISCUSSION

When the NR solution and the nitrate solution were injected simultaneously from I_1 and I_2 to the flow system, the two zones of NR and nitrate merged, the latter being completely encompassed by the NR zone. A decrease in NADPH, as a result of the enzymatic reaction, was observed as a negative peak at 2.1 min (Standard in Fig. 2), which was inverted to

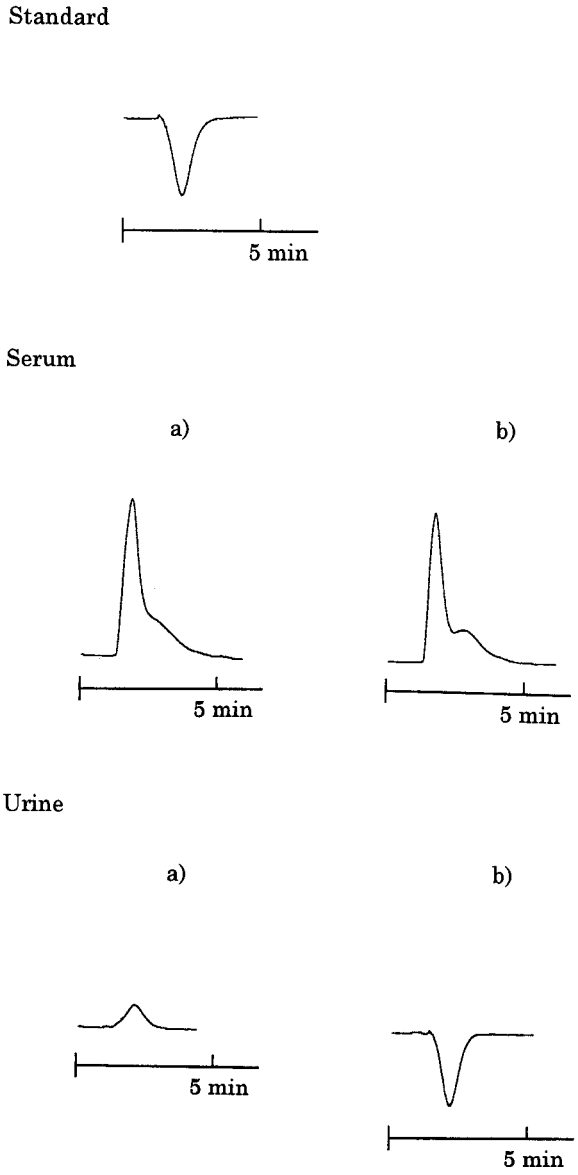

Fig. 2. Peak due to Standard Nitrate Sample and Peaks Observed when Biological Fluid Samples were Merged with C_1(0.1 M PIPES) or C_1 Containing NR

a) C_1, b) C_1 containing NR

a positive peak and its area obtained using a data processor. In the present study, the C_2 carrier contained 0.1 mM NADPH which is higher than the concentration of NADPH used within the C_2 carrier in the previous study (0.02 mM).[4] All other conditions were the same as those in the previous study. Under the conditions used here a linear calibration curve (r = 0.996) was obtained for the rise in nitrate concentration between 1 and 100 μM. The detection limit (S/N = 3) was 0.2 μM. The relative standard deviation of the peak area at 10 μM nitrate was 4.1% (n = 6).

Figure 2 shows the peaks observed when bio-

Table 1. Recovery of Nitrate Added to Biological Fluids

	Added nitrate (μM)	Observed nitrate (μM) mean (±S.D.)	Recovery (%)
Serum	0	42 (±4)	
	104	140 (±11)	94
	208	240 (±6)	95
Plasma	0	35 (±2)	
	103	140 (±6)	102
	206	244 (±7)	101
Urine 2	0	960 (±12)	
	2060	2810 (±22)	90
	4130	5430 (±33)	108

Values are averages of two or three measurements.

Table 2. Nitrate Content in Biological Fluid Compared with Other Methods (μM)

	Present method mean (±S.D.)	Other method
Serum	34 (±1)	41[a)]
Plasma	35 (±2)	29[a)]
Urine 1	370 (±7)	385[b)]
Urine 2	960 (±12)	1020[b)]

Values by the present method are averages of two or three measurements. Samples for analysis by the present method were respectively prepared by 5, 6, 12 and 100 fold dilution of the original serum, plasma, urine1 and urine2 samples. *a*) F-kit (Boehlinger Mannheim). *b*) Method described in reference 5.

logical fluid samples were merged with C_1 or C_1 containing NR, which were injected from I_1. When a serum sample was merged with C_1, a peak due to unknown substances was observed (peak a). On the other hand, when a serum sample containing nitrate was merged with NR, a negative peak as seen in Standard in Fig. 2 overlapped with this peak, resulting in a peak with reduced area (peak b). Thus, nitrate contents could be calculated from the difference in peak areas between the peaks observed when C_1 was injected alone, and those that resulted from the injection of C_1 containing NR.

Table 1 shows the recovery rates for nitrate added to serum, plasma and urine. The results indicate that the present method can be applied to determinations involving the concentration of nitrate in biological fluids. Table 2 indicates the measured contents of nitrate in biological fluids, as determined by the present method, which coincide well with those obtained by other methods.

Deproteinization was not required with this technique to prepare the samples, unlike the nitrate analysis method, in which nitrite levels are detected after nitrate is reduced.[1–3)] Analyzing nitrate levels by the present method requires several minutes, in contrast to the batchwise method using NR,[5)] which requires more than 45 min for analysis.

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