啓明醫大學術誌 第28券 1號
Keimyung Med J
Vol. 28, No. 1, June, 2009

Detection of cyanate, nitrate and carbamylated proteins in the peritoneal dialysate※

Hyun Chul Kim, M.D., Sung Bae Park, M.D., Sung Yeup Han, M.D.,
Eun A Hwang, M.D., Hye Jung Choi, Ph.D., Son Jung Nam, M.S.,
Eunyoung Ha, M.D., You Hee Kim, M.D., Kyo Cheol Mun, M.D.

Dongsan Kidney Institute, Keimyung University,
Daegu, Korea

Abstract : Cyanate is known to contribute to peritoneal injury with morphological changes in the peritoneum. Against carbamylation by cyanate, some proteins including albumin are known to play a protective role by self-carbamylation. Although many studies about cyanate and carbamylated proteins, there are no reports about the detection of the cyanate and carbamylated protein in peritoneal dilaysate. In this study, the cyanate, nitrate, and the carbamylated protein levels in peritoneal dialysate were measure to determine the existence of the uremic toxins in peritoneal dialysate. Cyanate was determined using by the isocratic size exclusion chromatography. Proteins such as albumin, globulin, and hemoglobin were carbamylated with sodium cynate. For the determination of the carbamylated protein in peritoneal dialysate, we performed HPLC system. Nitrite concentration was also assessed in the peritoneal dialysate. In this study, cyanate and nitrate were detected in the peritoneal dialysate. However, carbamylated proteins are not detected in the peritoneal dialysate. According to this study, peritoneal damage during PD may caused by continuous contact with uremic toxins including cyanate and nitrate without proper protection such as proteins.

Key Words : Carbamylation, Cyanate, Nitrate, Peritoneal dialysis

---

※ This work was supported by the research promoting grant from the Fresenius Medical Care, Korea in 2004.
Corresponding Author: Kyo Cheol Mun, M.D., Dongsan kidney Institute, Keimyung University
216, Dalseongno, Jung-gu, Daegu, 700-712 KOREA
Tel: +82-53-250-7786 E-mail: mun@dsmc.or.kr

Introduction

Peritoneal dialysis (PD) is now an established and acceptable mode of treatment for end-stage renal failure (ESRD). During PD, the peritoneal tissues are exposed to numerous harmful agents including urea, cyanate and nitrate. Urea forms cyanate spontaneously at body temperature and pH, and cyanate carbamylates amino acids, peptides, and proteins [1,2]. Generally, cyanate-induced carbamylation leads to changes in protein properties, enzyme activities and lipoprotein modifications [3]. Therefore, cyanate may contribute to peritoneal injury with morphological changes in the peritoneum. Against carbamylation by cyanate, some proteins including albumin are known to play a protective role by self-carbamylation [4].

Although many studies about cyanate and carbamylated proteins have reported [1-3, 5-10], there are no reports about the detection of the cyanate and carbamylated protein in peritoneal dilaysate. In this study, the cyanate, nitrate which are known to contribute the alteration of peritoneum during PD [11], and the carbamylated protein levels in peritoneal dialysate were measured to determine the existence of the uremic toxins in peritoneal dialysate.

Materials and Methods

1. Collection of peritoneal dialysate

Peritoneal dialysate was collected from PD patients (Dong San Hospital, Daegu, Korea). After collection samples were immediately aliquoted and stored at −20℃.

2. Determination of cyanate by high-performance liquid chromatography (HPLC)

Cyanate was determined using the isocratic size exclusion chromatography with the elute buffer, pH 7.4, made with 1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate and 0.4 M sodium chloride. A TSK-GELG3000SW$_{XL}$ (7.8 × 30 cm) column was used and sodium cyanate was used as a standard. The chromatographic separation was isocratic at 1.0 flow rate with a 20 min run time.

3. Carbamylation of protein

Proteins such as albumin, globulin, and hemoglobin were carbamylated with sodium cyanate according to Horkko et al. [10]. Albumin (10 mg/mL) was incubated at of 37℃ with 1 mmol/L sodium cyanate in 0.2 mol/L phosphate buffer, pH 6.5. The reaction will be allowed to proceed for 6 hours. After incubation of the samples, excess sodium cyanate and buffer were removed by dialyzing (membrane cutoff = 12,000 to 14,000 daltones) the carbamylated protein in 10 liters of normal saline solution. The control was incubated under identical conditions except for the omission of sodium cyanate.

4. Determination of carbamylated protein by spectrophotometer

Proteins were used for the reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS). The extent of carbamylation was monitored

by following the loss of free amino groups using TNBS. Fifty microliters of 0.1% TNBS was added to 1 mL of protein solutions and 1 mL of 4% sodium hydrogen carbonate (pH 8.4), and this was incubated for 1 h at 37℃. Absorbance was then measured at 340 nm against a sample blank, and the TNBS reactivity was expressed as a percentage of the absorbance obtained for the noncarbamylated proteins.

5. Determination of carbamylated protein by HPLC

For the determination of the carbamylated protein in peritoneal dialysate, we performed HPLC. Gilson 506B HPLC system equipped with 321 pump, 170 UV-vis detector and 231XL autosampling injector. The system was controlled using Gilson Unipoint 3.0 software. HPLC was carried out using a TSK-GELG3000SWXL (7.8 × 30 cm) column (Tosoh Biosep GmbH, Germany) which is specifically designed to provide size-based separations. The elution was performed at 25 ℃ and at a flow rate of 1.0 mL/min, and with the mobile phase (1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, 0.4 M sodium chloride, pH 7.4). UV detection was performed at 214 nm with 20 min run. All samples and solutions were filtered before use over a 0.45 ㎛ filter.

6. Measurement of nitrite production in peritoneal dialysate

For measuring nitrite accumulation, nitrite concentration was assessed in the peritoneal dialysate described by Green *et al* [12]. Briefly, peritoneal dialysate (100 ㎕) were incubated with an 150 ㎕ of Griess reagent (1% sulfanilamide in 5% phosphoric acid solution, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in water) at room temperature for 10 min in 96-well microplate, followed by spectrophotometric measurement at 550 nm. Standard calibration curves were prepared using sodium nitrite as standard.

Results

1. Determination of cyanate by HPLC

Cyanate was measured by HPLC with size exclusion column. Cyanate was eluted at 11.48 min (Fig. 1).

2. Determination of carbamylated proteins with TNBS

The absorbances of the reaction products of TNBS with proteins was measured. Absorbances of the mixture which suggests

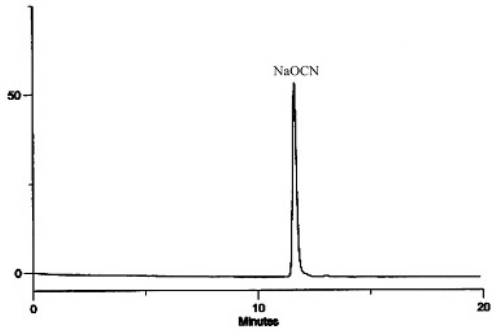

Fig. 1. Chromatogram of cyanate. Column, TSK-GELG3000SW$_{XL}$ (7.8 × 30 cm); eluent, 1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, and 0.4 M sodium chloride, pH 7.4; flow rate, 1 ml/min; UV detection, 214 nm.

the loss of free amino groups in proteins, albumin, globulin, and hemoglobin, were 0.16, 0.14, and 0.20, respectively. These absorbance values are decreased by carbamylation.

3. Determination of carbamylated proteins in the standard solution

Figure 2 shows a chromatograms of proteins before and after carbamylation. Albumin was carbamylated with sodium cyanate, and the resultant carbamylated albumin was detected. Albumin and carbamylated albumin were eluted at 8.29 min and 8.00 min, respectively (A). The retention time of the carbamylated albumin peak appeared to shorter than that of the albumin peak. As shown in Fig. 2, the retention time of protein became decreased after carbamylation by cyanate (Fig. 2B, 2C , and D).

4. Determination of carbamylated protein in peritoneal dialysate

Figure 3 shows a chromatogram of the peritoneal dialysate in patient with ESRD. The retention time of the peak A is 7.66 min and that of the peak B is 8.29 min. Two peaks were detected at the retention time of globulin and albumin, respectively. The peak C was detected at the retention time of sodium cyanate (11.47 min).

5. Measurement of nitrite in peritoneal dialysate

As shown in Table 1, the concentration of nitrite in peritoneal dialysate were ranged from 6.8 μM to 49.4 μM.

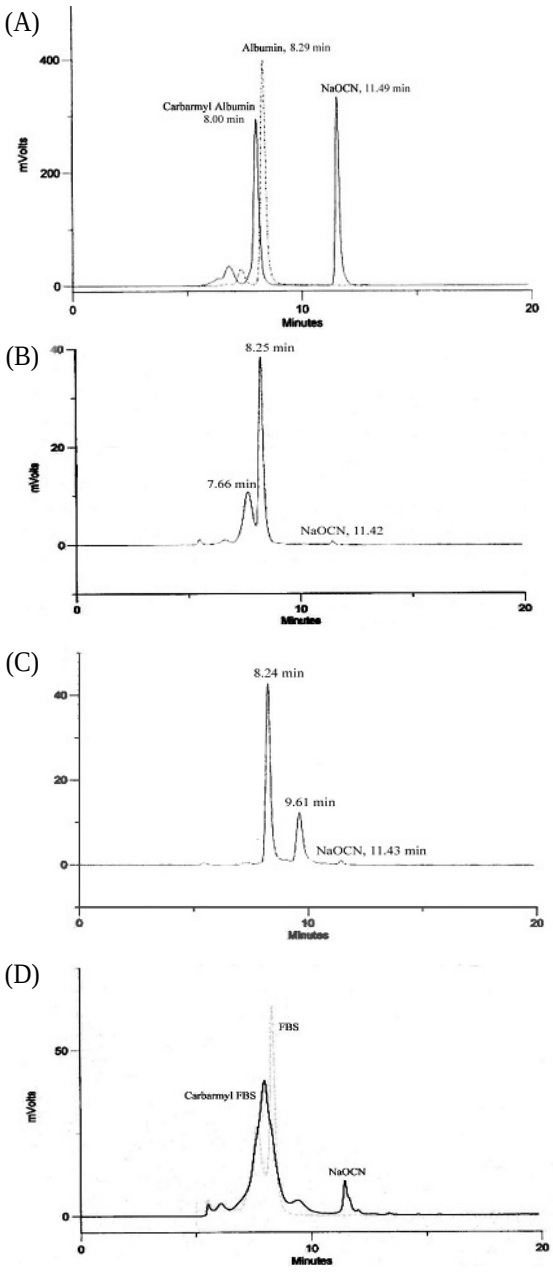

Fig. 2. Chromatogram of carbamylated albumin (A), carbamylated globulin (B), carbamylated hemoglobin (C), and carbamylated FBS (D). HPLC profiles of the albumin before (dotted line) and after (solid line) carbamylation. Column, TSK-GELG3000SW$_{XL}$ (7.8 × 30 cm); eluent, 1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, and 0.4 M sodium chloride, pH 7.4; flow rate, 1 ml/min; UV detection, 214 nm.

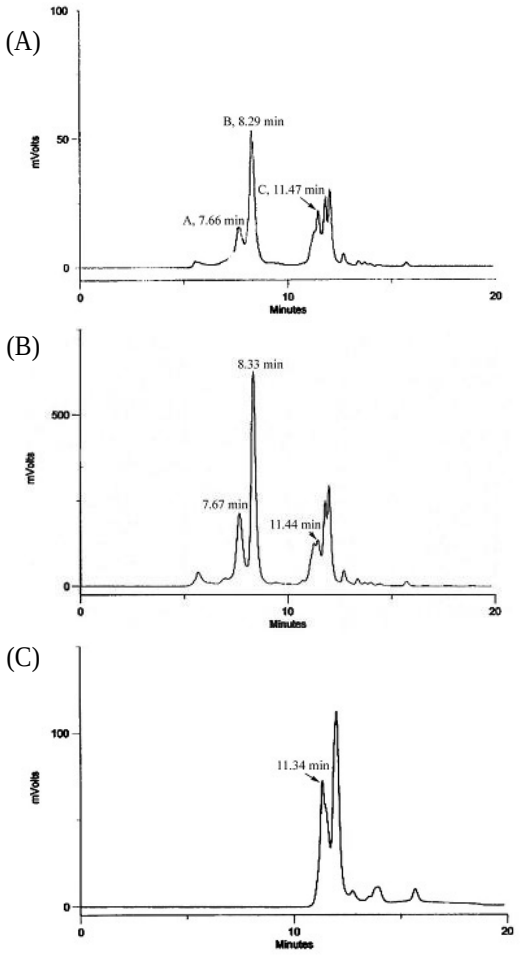

Fig. 3. Chromatogram of the peritoneal dialysate in patient with end-stage renal disease. Column, TSK-GELG3000SW$_{XL}$ (7.8 × 30 cm); eluent, 1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, and 0.4 M sodium chloride, pH 7.4; flow rate, 1 ml/min; UV detection, 214 nm.

Discussion

PD is now an established and acceptable mode of treatment for ESRD. However, long-term PD leads to structural and functional changes in the peritoneum [13,14]. Peritoneal membrane fibrosis is one of the most serious complications in chronic long term PD patients [15]. There are a number of published studies which attempt to address the relationship between morphological changes and clinical events [16,17]. However, the mechanisms are poorly understood and studies of the development of peritoneal fibrosis in PD patients are relatively limited.

In patients with long-term continuous ambulatory peritoneal dialysis (CAPD), peritoneal dysfunction is considered to be due to the loss of peritoneal mesothelial cells and due to subsequent peritoneal fibrosis. During CAPD, various morphological changes take place in the peritoneum including mesothelial denudation, interstitial fibrosis, peritoneal sclerosis, neovascularisation, and vascular alterations. Various components of peritoneal dialysate might play in the occurrence of peritoneal dysfunction in CAPD patient. Advanced glycation end products, growth factors, nitric oxide and oxidant contribute to alterations of the peritoneum during PD [11]. Nevertheless, very little information

Table 1. Concentration of nitrite in peritoneal dialysate

	peritoneal dialysate		
	1	2	3
Nitrite (μM)	6.846 ± 0.001	33.256 ± 0.002	49.410 ± 0.002

concerning the uremic toxins is currently available.

Uremia is the clinical state or syndrome that is reversed by dialysis therapy. Whether or not urine output falls, all patients with uremia accumulate solutes, collectively known as uremic toxins [16]. It is this accumulation of solute, the most abundant of which is urea, that justified the application of dialysis as a treatment for uremia. Most of the solutes known to accumulate in uremia are low in molecular weight and consequently are dialyzable [18]. Although urea is a poor marker of native renal function, it has special significance in ESRD patients because it is the most abundant solute to accumulate and because its accumulation results from both generation, as a result of protein catabolism, and failure of renal excretion [18]. Because urea generation is an index of protein nutrition, monitoring urea levels is potentially doubly important. Other substances proposed as uremic toxins include carbamylated proteins from post-translational modification by high concentrations of urea and cyanate, advanced glycation end products from the Maillard reaction between 3-deoxyglucosone and proteins, ß$_2$-microglubulin, uric acid, *p*-cresol, parathyroid hormone, granulocyte-inhibiting proteins, hydrogen ion and metabolic acidosis, homocystein, and other organic and phenolic acids [18].

During PD, the peritoneum is exposed to waste products, including urea, cyanate, and nitrate. Uremic toxicity is related in part to the accumulation of toxic substances. Against carbamylation by cyanate, some proteins including albumin are known to play a protective role by self-carbamylation [4]. In this study, cyanate and nitrate were detected in the peritoneal dialysate. However, carbamylated proteins are not detected in the peritoneal dialysate.

According to this study, peritoneal damage during PD may caused by continuous contact with uremic toxins including cyanate and nitrate without proper protection such as proteins.

Summary

Cyanate is known to contribute to peritoneal injury with morphological changes in the peritoneum. Against carbamylation by cyanate, some proteins are known to play a protective role. In this study, the cyanate, nitrate, and the carbamylated protein levels in peritoneal dialysate were measure to determine the existence of the uremic toxins in peritoneal dialysate. Cyanate was determined using by the isocratic size exclusion chromatography. Proteins were carbamylated with sodium cynate. For the determination of the carbamylated protein in peritoneal dialysate, we performed HPLC system. Nitrite concentration was also assessed in the peritoneal dialysate. In this study, cyanate and nitrate were detected in the peritoneal dialysate. However, carbamylated proteins are not detected in the peritoneal dialysate. According to this study, peritoneal damage during PD may caused by continuous contact with uremic toxins including cyanate and nitrate without proper protection such as proteins.

References

1. Hagel P, Gerding JJ, Fieggen W, Bloemendal H. Cyanate formation in solutions of urea. I. Calculation of cyanate concentrations at different temperature and pH. *Biochim Biophys Acta* 1971;**243**:366-73.
2. Marier JR, Rose D. Determination of cyanate, a study of its accumulation in aqueous solutions of urea. *Anal Biochem* 1964;**7**:304-14.
3. Lippincott J, Apostol I. Carbamylation of cysteine: a potential artifact in peptide mapping of hemoglobins in the presence of urea. *Anal Biochem* 1999;**267**:57-64.
4. Sang-Hyuck Seo, Kyo-Cheol Mun, Sung-Bae Park, Hyun-Chul Kim. Effect of cyanate on the carbamylation and biological activity of superoxide dismutase. *Kor J Nephrol* 2000;**19**:1121-8.
5. Fluckiger R, Harmon W, Meier W, Gabbay KH. Hemoglobin carbamylation in uremia. *N Engl J Med* 1981;**304**:823-7.
6. Kraus LM, Jones MR, Kraus AP Jr. Essential carbamoyl-amino acids formed *in vivo* in patients with end-stage renal disease managed by continuous ambulatory peritoneal dialysis: isolation, identification, and quantitation. *J Lab Clin Med* 1998; **31**:425-31.
7. Roxborough HE, Millar CA. McEneny J. Young IS. Carbamylation inhibits the ferroxidase activity of caeruloplasmin. *Biochem Biophys Res Commun* 1995; **214**:1073-8.
8. Stim J, Shaykh M, Anwar F, Ansari A, Arruda JAL, Dunea G Factors determining hemoglobin carbamylation in renal failure. *Kidney Int* 1995;**48**:1605-10.
9. Balion CM, Draisey TF, Thibert RJ. Carbamylated hemoglobin and carbamylated plasma protein in hemodialyzed patients. *Kidney Int* 1998;**53**:488-95.
10. Horkko S, Savolainen MJ. Kervinen K. Kesaniemi YA. Carbamylation-induced alterations in low-density lipoprotein metabolism. *Kidney Int* 1992;**41**:1175-81.
11. Combet S, Ferrier ML, Van Landschoot M, Stoenoiu M, Moulin P, Miyata T, *et al*. Chronic uremia induces permeability changes, increased nitric oxide synthase expression, and structural modifications in the peritoneum. *J Am Soc Nephrol* 2001;**12**:2146-57.
12. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Anal Biochem* 1982;**126**:131-8.
13. Coles GA, Topley N. Long-term peritoneal membrane changes. *Adv Ren Replace Ther* 2000;**7**:289-301.
14. Williams JD, Craig KJ, Topley N, Von Ruhland C, Fallon M, Newman GR, *et al.* Morphologic changes in the peritoneal membrane of patients with renal disease. *J Am Soc Nephrol* 2002;**13**:470-9.
15. Plum J, Hermann S, Fusshöller A, Schoenicke G, Donner A, Röhrborn A, *et al.* Peritoneal sclerosis in peritoneal dialysis patients related to dialysis settings and peritoneal transport properties. *Kidney Int Suppl* 2001;**78**:S42-7.
16. Schwenger V, Zeier M, Henle T, Ritz E. Advanced glycation endproducts as uremic toxins. *Nahrung* 2001;**45**:172-6.
17. Williams JD, Craig KJ, Topley N, Williams GT. Peritoneal dialysis: changes to the structure of the peritoneal membrane and potential for biocompatible solutions. *Kidney Int Suppl* 2003;**84**:158-61.
18. Devuyst O, Topley N, Williams JD. Morphological and functional changes in the dialysed peritoneal cavity: impact of more biocompatible solutions. *Nephrol Dial Transplant* 2002;**17**:12-5.
19. Lameire N, Vanholder R, De Smet R. Uremic toxins and peritoneal dialysis. *Kidney Int Suppl* 2001;**78**:S292-7.