Correlations between Plasma Levels of Anionic Uremic Toxins and Clinical Parameters in Hemodialysis Patients

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When the kidney is seriously impaired, various uremic toxins (UTs) accumulate in the body, often exerting unfavorable effects on physiological functions and drug pharmacokinetics. To prevent this, it is important to determine plasma UT levels accurately in chronic kidney disease patients. Although attempts to predict plasma UT levels using biomarkers have been made, the correlation between UT levels and the markers is not yet fully understood. In this study, we assessed the correlations among plasma levels of indoxyl sulfate (IS), indoleacetic acid (IA), and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) in 20 hemodialysis patients and evaluated the relationship between the plasma levels of UTs and clinical parameters, such as serum creatinine (Scr), blood urea nitrogen, and estimated glomerular filtration rate (eGFR), with special focus on IS. There were no correlations among the plasma levels of the three UTs before and immediately after hemodialysis. However, a significant correlation was observed between plasma IS levels and Scr before hemodialysis ($r = 0.643, p = 0.002$), with the correlation becoming much stronger when using the data obtained immediately after hemodialysis ($r = 0.744, p < 0.001$). Further, plasma IS levels showed a significant negative correlation with eGFR ($r = -0.558, p = 0.011$). However, no correlations were observed for IA or CMPF. The results obtained from this study suggest that plasma IS levels can be predicted from Scr values, although the precise mechanism behind the correlation remains to be clarified.

Key words—anionic uremic toxin; indoxyl sulfate; plasma level; hemodialysis patient; serum creatinine

INTRODUCTION

When the kidney is seriously impaired, a variety of organic waste compounds accumulate in the body, often exerting unfavorable effects on the physiological functions of chronic kidney disease (CKD) patients. Such compounds are generally called “uremic toxins (UTs)”1–4. Recently, we evaluated the plasma levels of three typical anionic UTs, indoxyl sulfate (IS), indoleacetic acid (IA) and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF), in both hemodialysis (HD) patients and healthy subjects as these anionic UTs have been reported to interfere with the disposition of various drugs in vitro and in vivo5–7 and that clinically relevant interactions between these anionic UTs and drugs are assumed to be dependent on their plasma levels in patients. Consistent with the previous literature,8–11 the results obtained from the study demonstrated that the greatest difference in the mean plasma levels between HD patients and healthy subjects was for IS.12

Our recent study also showed that IS was capable of modulating the binding of pravastatin to albumin, indicating an interaction at albumin binding site II.12 As dyslipidemia is frequent in HD patients, hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors are often prescribed to them. Therefore, an elevation in the plasma IS level in HD patients receiving a HMG-CoA reductase inhibitor may trigger serious adverse effects such as rhabdomyolysis. Moreover, a number of studies have reported that IS is largely responsible for cardiovascular mortality in HD patients.3,13 In order to prevent such unfavorable events due to IS, it is important for clinicians and pharmacists to accurately identify plasma IS levels in HD patients. However, as highly sensitive and expensive assay equipment such as HPLC or LC/MS/MS is needed for determining plasma IS levels,8,9,14 direct analysis of IS in a patient’s plasma is not always easy in small and medium-sized medical institutions. Therefore, an easy way is required to roughly predict the plasma levels of UTs without using assay system.

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Possible alternatives are clinical parameters such as serum creatinine (Scr), blood urea nitrogen (BUN) or estimated glomerular filtration rate (eGFR).

To date, several papers have shown that plasma IS levels are significantly correlated with Scr or eGFR.15,16 Such observations appear somewhat curious because plasma IS levels and Scr are considered to vary independently in HD patients. The mechanism behind their correlation is not yet fully understood. Also, the correlations of CMPF and IA with clinical parameters are unclear. Moreover, although these three UTs are known to be transported by organic anion transporters (OATs) during tubular secretion in the kidney,7,17-19 the correlations among the plasma levels of these anionic UTs remain uncertain.

In this study, using the previous data obtained from 20 HD patients,12 we evaluated the correlations among the plasma levels of IS, IA and CMPF and the relationship between the plasma levels of these anionic UTs and three clinical parameters in HD patients, with special focus on the values obtained before and immediately after HD.

**MATERIALS AND METHODS**

**Materials** IS and CMPF were purchased from Cayman Chem. Co. (Ann Arbor, MI, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. IA was obtained from Wako Pure Chem. Ind. (Osaka). The chemical structures of three UTs used in this study are shown in Fig. 1. All other reagents were of the highest grade available.

**Blood Sampling from Hemodialysis Patients**

With the aid of a written outline, the purpose of this study and protocol for the protection of personal information were explained to outpatients undergoing HD at the Sapporo Higashi Tokushukai Hospital. Twenty HD patients (10 males and 10 females) agreed to donate blood before and immediately after HD. Hemodialysis conditions of 20 HD patients in this study were as follows: 3 h once a week (n=1), 4 h twice a week (n=1), 3 h three times a week (n=1), 4 h three times a week (n=14), 4.5 h three times a week (n=2), and 5 h three times a week (n=1).

Blood samples (approximately 4 mL) were drawn into blood collection tubes (Becton Dickinson Co., Franklin Lakes, NJ, USA) through the dialysis fluid circuit. The blood was immediately centrifuged at 2600 g for 5 min at 5°C and the plasma obtained was stored at −30°C until assay. This study was performed under the approval of the Committee of Sapporo Higashi Tokushukai Hospital and with cooperation of the staff of the Dialysis Section.

**Collection of Clinical Parameters** The age, BUN and Scr for each patient were obtained from the patient’s charts. The average eGFR, Scr, BUN, and serum albumin values of the male and female HD patients in this study are presented in Table 1. eGFR was calculated using the following equations:

- Body surface area (BSA) = weight0.425 × height0.725 × 0.007184
- Male eGFR = 194 × Scr−1.094 × age0.287 × BSA
- Female eGFR = 194 × Scr−1.094 × age0.287 × BSA × 0.739

**Determination of Three Anionic UTs in the Patients’ Plasma** Total plasma level of each UT was assayed as follows: plasma sample (50 μL) obtained from HD patient was mixed with equal volume of saline and 200 μL of methanol, stood for 10 min in iced water, and then centrifuged at 5350 g for 10 min at 5°C. IS, IA and CMPF in the resultant supernatant fluid was determined using a HPLC system (LC-10AS, Shimadzu, Kyoto) equipped with a UV detector (SPD-10A, Shimadzu). Cosmosil 5C18AR-II (5 μm, 4.6 mm i.d. × 150 mm, Nacalai Tesque, Kyoto) and Inertsil ODS-3 (5 μm, 4.6 mm i.d. × 250 mm, GL Sciences Inc., Tokyo) columns were used for IS and IA assay and for CMPF assay, respectively, at a column temperature of 50°C for IS and IA and 40°C for CMPF. The mobile phases used were 0.05 M KH2PO4 : CH3CN (95 : 5 for IS and 90 : 10 for IA) and 0.08 M acetate buffer (pH 4.5) : CH3CN : CH3COOH (65 : 35 : 0.5 for CMPF). Assay wavelength was 280 nm for IS, 282 nm for IA, and 261 nm for IS.
plasma. The minimal concentration of the standard solutions between two combinations. A significant correlation between plasma IS levels and Scr both before and immediately after HD (Fig. 3). The r and p values obtained from the data before HD were 0.643 and 0.001, indicating that the correlation was much stronger when using the data immediately after HD (Table 3). On the other hand, no significant correlation was observed between plasma IS levels and BUN immediately after HD. As shown in Fig. 5, reflecting the significant correlation between plasma IS levels with Scr, a significant negative correlation was observed between plasma IS levels and eGFR immediately after HD (r = −0.558, p = 0.011) and immediately after HD (r = −0.645, p = 0.002). And much better correlations were obtained when IS levels were plotted as log scale to Scr (before HD: r = 0.799, p < 0.001, immediately after HD: r = 0.829, p < 0.001) and eGFR (before HD: r = −0.863, p < 0.001, immediately after HD: r = −0.909, p < 0.001). However, no meaningful correlations were observed between IA or CMPF and the clinical parameters assessed in this study (Table 3).

**DISCUSSION**

Currently, more than 150 compounds are catego-
It is well known that the plasma levels of various UTs including IS are markedly elevated in end-stage kidney disease (ESKD) patients. However, the common mechanisms underlying the elevation of their plasma levels have not yet been fully addressed.

The observations obtained from the present study can be summarized as follows: 1) plasma IS levels did not correlate with those of either IA or CMPF (Fig. 2), 2) there was a significant positive correlation between plasma IA and CMPF levels before and immediately after hemodialysis. Each point represents the individual data from hemodialysis patients. $r$: correlation coefficient; $p$: significance of correlation.
Fig. 3. Correlations between Plasma IS Levels and Scr before (A) and Immediately after Hemodialysis (B)
Each point represents the individual data from hemodialysis patients. r: correlation coefficient; p: significance of correlation; line: linear regression curve.

Fig. 4. Correlations between Plasma IS Levels and BUN before (A) and Immediately after Hemodialysis (B)
Each point represents the individual data from hemodialysis patients. r: correlation coefficient; p: significance of correlation.

Table 3. Correlation Coefficients between Plasma Concentrations of UTs and Renal Functions

|        | IS     | IA     | CMPF   |
|--------|--------|--------|--------|
| Scr    | 0.643**| 0.744***| 0.128  |
| BUN    | 0.347  | 0.150  | 0.558* |
| eGFR   | 0.558* | 0.347  | 0.437  |

*p<0.05, **p<0.01, ***p<0.001, statistically significant.

between plasma IS levels and Scr, and the correlation was stronger when using Scr values obtained immediately after HD rather than those before HD (Fig. 3), and 3) plasma IS levels were significantly negatively correlated with eGFR (Fig. 5).

The correlation of the second and third observation became much better when IS levels was plotted in log scale. As eGFR is calculated using Scr and BSA, the third observation is thought to be reasonable on the basis of the second observation.

We did not determine the adsorption of IS, IA and CMPF to dialysis membrane directly. However, the fact that plasma levels of CMPF were almost the same before and immediately after HD (Table 2) and that the HD clearance of these three UTs was well correlated with their protein binding rate12 could imply the relatively small influence of membrane adsorption on the results in this study.

The source of both IS and IA is dietary tryptophan.1,16,21 It was, therefore, expected that there was some correlation between these two UTs. The lack of any correlation between them can be attributed in part to differences in their protein binding capacities. IA weakly binds to albumin whereas IS readily binds to the plasma protein.12 Accordingly,
IA but not IS is easily subject to removal from the plasma by HD, leading to a much lower accumulation of IA than IS in HD patients. The fact that the \( r \) and \( p \) values between IS and IA worsened after HD (Fig. 2) appears to support the above interpretation. Of course, it is likely that IS and IA are synthesized to different extents in each HD patient, even if dietary tryptophan is the common source of these two anionic UTs. In contrast to the case of IS and IA, the \( r \) and \( p \) values between IS and CMPF were almost identical before and immediately after HD (Fig. 2). Unlike IS and IA, CMPF is produced in the body as a metabolite of furan fatty acid, which originates from a phospholipid contained in roe and other foodstuffs.\(^{22}\) Thus, differences in eating habits may be a key factor in the lack of correlation between IS and CMPF.

Consistent with previous reports,\(^{15}\) the present study demonstrated a significant positive correlation between plasma IS levels and Scr. Surprisingly, however, the \( r \) and \( p \) values improved when using data obtained immediately after HD rather than data obtained before HD. According to our previous understanding, the renal excretory pathways of IS and creatinine differ markedly. In the kidney, a large part of IS in the plasma is secreted into urine preferentially mediated by organic anion transporter (OAT) 1 and OAT3.\(^{27}\) On the other hand, the excretory route of creatinine has been considered to be glomerular filtration mainly. For example, creatinine was measured as the non-protein-bound uremic retention solutes in the study regarding the effect of daily short dialysis on removal of uremic toxins from bloods and on their pre-dialysis serum levels.\(^{23}\) Also, creatinine showing the feature of water solubility and non-protein binding was used as a control in the study regarding kinetics of \( p \)-cresol, a lipophilic and protein-bound compound.\(^{24}\) However, several papers have suggested that creatinine is secreted into urine as a substrate of the organic cation transporter (OCT). Moreover, it was recently demonstrated that, although creatinine is a basic compound, it is transported by OAT1 in the tubular epithelium. Eisner et al., showed that OAT-mediated tubular secretion contributed to excretion of creatinine because significant reduction of secreted fraction of creatinine was observed when PAH coexisted.\(^{25}\) Moreover, Vallon et al.\(^{26}\) reported that OAT3 and possibly OAT1 contributed to renal secretion of creatinine, from the result that creatinine clearance in wild type mice was higher thanulin clearance and that such tendency was not observed in the OAT knockout mice. Therefore, the current understanding of the renal handling of creatinine is rather complicated, making the interpretation of the abovementioned second observation difficult. As mentioned above, the precursor of IS is tryptophan. On the other hand, the precursor of creatinine is creatine, which is synthesized from arginine, glycine and methionine.\(^{27}\) As these are amino acids contained in various foods, blood concentrations of IS and creatinine elevate by food intake and they accumulate in HD patients. Both IS and creatinine are excreted into urine as the metabolites of amino acids. Such aspects of IS and creatinine are involved in the observation in this study unexpectedly. At present, exact reason is unclear for important findings that the better correlation was obtained between IS and Scr or eGFR when using data immediately after HD. However, this study suggested that Scr and eGFR might be useful

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**Fig. 5.** Correlations between Plasma IS Levels and eGFR before (A) and Immediately after Hemodialysis (B)

Each point represents the individual data from hemodialysis patients. \( r \): correlation coefficient; \( p \): significance of correlation; line linear regression curve.
clinical parameters for predicting plasma IS levels, leading to the avoidance of unfavorable IS-related events.

In this study, we did not address the therapeutic agents administered to the present 20 HD patients. Therefore, a possibility cannot be excluded that some therapeutic agents modulated the plasma levels of these UTs via interactions such as displacement of their protein binding. Further study is required to assess it.

In conclusion, the present results suggested that the handling of UTs in HD patients is complex and that the plasma levels of IS, one of the most variable UTs in HD patients, is predictable by Scr or eGFR obtained immediately after HD. However, further study is required to clarify the mechanisms behind the significant correlation between plasma IS levels and Scr.

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Conflict of Interest The authors declare no conflicts of interest.

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