Original Article

Measuring serum total and free indoxyl sulfate and p-cresyl sulfate in chronic kidney disease using UPLC-MS/MS

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ABSTRACT

Chronic kidney disease (CKD) is a complex disorder that affects multiple organs and increases the risk of cardiovascular complications. CKD affects approximately 12% of the population in Taiwan. Loss of kidney function leads to accumulation of potentially toxic compounds such as indoxyl sulfate (IS) and p-cresyl sulfate (pCS), two protein-bound uremic solutes that can stimulate the progression of CKD. The aim of this study was to assess whether IS and pCS levels were correlated with CKD stage. We developed and validated a method for quantitating total and free IS and pCS in serum by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Serum samples were pretreated using protein precipitation with acetonitrile containing stable isotope-labeled IS and pCS as internal standards. After centrifugation, the supernatant was diluted and injected into a UPLC-MS/MS system. Analyte concentrations were calculated from the calibration curve and ion ratios between the analyte and the internal standard. The calibration curves were linear with a correlation coefficient of \( R^2 > 0.999 \); the analytical measurement range was 0.05–5 mg/L. The limit of quantitation of this assay was 0.05 mg/L for both analytes. The reference interval was \( <0.05–1.15 \text{ mg/L for total-form IS,} \quad <0.05–5.33 \text{ mg/L for total-form pCS,} \quad <0.05 \text{ mg/L for free-form IS, and} \quad <0.12 \text{ mg/L for free-form pCS.} \) A positive correlation was observed between analyte concentration and CKD stage. Our sensitive UPLC-MS/MS method for quantifying total and free-form IS and pCS in serum can be used to monitor the progression of CKD in clinical settings, identify patients at risk, and facilitate development of further therapies for this devastating disease.

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1. **Introduction**

Chronic kidney disease (CKD) is caused by metabolic abnormalities that lead to the accumulation of uremic toxins; these are low-molecular-weight solutes, protein-bound low-molecular-weight solutes, and middle molecules [1]. The retention of uremic toxins, especially those poorly removed by hemodialysis, likely contributes to cardiovascular and inflammatory complications. The two protein-bound uremic toxins that have been most extensively evaluated are indoxyl sulfate (IS) and p-cresyl sulfate (pCS), and their levels may be predictive of clinical outcomes [2–7]. Multiple studies have assessed the biological effects of these two toxins [8–12].

IS and pCS are the products of gut microbial metabolism of tryptophan and tyrosine (or phenylalanine), respectively. Both are competitively bound with a high affinity to albumin, and the bound fraction in serum is in the range of 85%–97% [5,8,13]. The free-form concentration of pCS has been reported to be associated with vascular calcification, arterial stiffness, and mortality risk in CKD and hemodialysis patients [5,6], while the free form of IS is thought to be an independent predictor of coronary restenosis in patients with coronary artery disease following stent implantation [14].

CKD is recognized as a global health issue, and early detection and treatment may prevent or delay its progression. Currently, the creatinine-based estimated glomerular filtration rate (eGFR) is recommended for routine kidney function assessment in clinical practice [15,16], but this measure is heavily affected by diet and exercise [17]. Abnormal eGFR actually indicates renal damage and cannot be used as an early biomarker of renal disease. Moreover, eGFR values cannot accurately reflect the concentration of retained uremic solutes that accumulate during the progressive stages of CKD. Therefore, measuring the production of IS and pCS when assessing kidney disease may provide a reliable monitoring indicator allowing timely intervention to preserve renal function.

The purpose of this study was to develop and validate a mass spectrometry (MS)-based method for clinical laboratory measurement of total and free serum IS and pCS and determine their concentrations in patients with varying stages of CKD.

2. **Materials and methods**

2.1. **Chemicals and reagents**

Liquid chromatography-MS-grade acetonitrile was purchased from JT Baker (Center Valley, PA, USA) and formic acid was obtained from Fluka (St. Louis, MO, USA). Amicon Ultra 0.5-mL centrifugal filters (MWCO 30 kDa) were purchased from Merck Millipore (Burlington, MA, USA). IS standard was purchased from Sigma–Aldrich (St. Louis, MO, USA) and pCS was purchased from AlsaChim (Illkirch-Graffenstaden, France). Indoxyl sulfate-d4 potassium salt (IS-d4) was purchased from Toronto Research Chemicals (North York, ON, Canada) and p-cresyl sulfate-d7 (pCS-d7) was synthesized by Professor Ren-Shen Lee of Chang Gung University (Taoyuan City, Taiwan).

2.2. **Instrumentation**

The ultra-performance liquid chromatography (UPLC)-MS/MS system consisted of a Waters Acquity Ultra Performance Liquid Chromatograph system coupled with a Waters Xevo TQ-S triple-quadrupole mass spectrometer (Milford, MA, USA), and equipped with a stepwave ion transfer optics unit. Masslynx software (Waters) was used for instrument control and data acquisition.

2.3. **Calibrators and quality control materials**

Standard stock solutions were prepared in water and internal standard stock solutions were prepared in acetonitrile at 10 mg/mL and stored at −80 °C. Working solutions of standards and internal standards were freshly prepared. Calibration curves were generated with six points with concentrations of 0, 0.2, 0.5, 1, 2, and 5 mg/L for IS and pCS. Quality control materials were prepared by spiking appropriate standard solution of IS and pCS in pooling patients’ serum (Table 1). Control 1–3 contained IS at 0.2, 2, and 4 mg/L and pCS at 0.4, 2, and 4 mg/L, respectively. Control 4–6 contained free-form IS at 0.20, 2.65, and 7.80 mg/L and free-form pCS at 0.25, 1.54, and 4.40 mg/L, respectively. Aliquots of the quality control materials and all stock and working solutions were stored at −80 °C.

2.4. **Sample preparation**

Serum samples were thawed at room temperature, and 50-μL aliquots of each sample were pipetted into tubes. Five hundred microliters of a precipitating reagent (acetonitrile) containing 0.2 mg/L of IS-d4 and pCS-d7 as internal standards were added to each tube. The samples were vortexed for 30 s, followed by centrifugation at 13,400×g for 15 min. The supernatant from each tube was transferred to an autosampler vial after 10-fold dilution with water, and 5 μL of each supernatant were injected into the UPLC-MS/MS system for total-form IS and pCS analysis. To quantitate the free form of IS and pCS, a 100-μL aliquot of serum was centrifuged for 30 min at 10,621×g at 37 °C with a 3000 MWCO filter (Merck Millipore). Next, a 50-μL aliquot of filtrate was pretreated in the same manner as the samples prepared for total-form analysis. The free form IS and pCS can be quantitated by using the same calibration curve simultaneously with total-form quantitation.

2.5. **Chromatography and MS conditions**

The samples were injected into an Acquity UPLC BEHC 18 column (2.1 × 100 mm, 1.7-μm particle size, Waters) at 30 °C. The mobile phase flow rate was 0.45 mL/min, and the injection volume was 5 μL. Mobile phase A consisted of 0.1% (v/v) formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The linear gradient was set as follows: 0–1 min, 80% A; 1.0–1.7 min, 80%–20% A; 1.7–1.8 min, 20%–5% A; 1.8–3.4 min, 5% A; 3.5 min, 80% A. The run time was 5.5 min per injection with baseline-resolved chromatographic separation. The analytes were quantified with a Waters Acquity UPLC Xevo TQ-S operating in negative electrospray ionization and multiple reaction monitoring.
mode. The capillary voltage was 1.5 kV, and the desolvation gas flow was set at 150 L/h; the source temperature was 150 °C. The precursor ions, product ions, cone voltage, and collision energies for each analyte and internal standard were determined by direct infusion of IS and pCS. The multiple reaction monitoring (MRM) transition was selected to be m/z 212.04 → 80.14, 212.04 → 132.05 for IS, m/z 216.04 → 80.14 for IS-d4, and m/z 186.98 → 107.03, 186.98 → 80.02 for pCS, m/z 194.04 → 114.04 for pCS-d7, and optimized using IntelliStart software (Waters).

2.6. Method validation

Method validation was conducted according to Clinical Laboratory Improvement Amendments (CLIA) guidelines. The matrix effect was evaluated in 12 serum samples from healthy volunteers by comparing the signal obtained from samples spiked with analyte post-extraction with the signal obtained by spiking analytes into a neat solution according to the procedure described in the Clinical & Laboratory Standards Institute (CLSI) document C50 [18]. The effect of signal interference was evaluated on specimens containing the commonly prescribed drugs and supplements, including acetaminophen, allopurinol, folic acid, furosemide, omeprazole, rifampin, and ciprofloxacin.

Calibration curves were established by plotting the peak area ratios of the analytes to labeled stable isotopes against the analytes’ concentrations with 1/X weighted linear regression ranging from 0 to 5 mg/L for both IS and pCS. The validation parameters included the lower limit of the measuring interval, linearity, dilution, imprecision, assay interference, carryover, and accuracy. The lower limit of the measuring interval was experimentally determined using six different concentrations of diluted samples. The lowest actual amount of analyte should be reliably detected when imprecision of 40 replicates over five runs is within 20%, average inaccuracy is within 15%, and an analyte signal-to-noise ratio is 10. To analyze the samples at concentrations above 5 mg/L, serum samples spiked with total IS and pCS at 10 mg/L were 10-fold and 20-fold diluted with water and then analyzed. Imprecision and deviation from the nominal concentration for eight replicates after dilution should be less than 15%. The linearity of the method was estimated using 11 equally spaced samples with duplicates according to the procedure described in CLSI document EP06AE [19]. Linearity was evaluated using the polynomial regression method. Twenty-day intra- and inter-day imprecision was determined by analyzing quality control materials and quantified using calibrators. The imprecision of each concentration should be less than 15%. Carryover was evaluated by analyzing a set of high-concentration (10 mg/L) and a set of low-concentration (0.4 mg/L) serum samples as described in CLSI protocol EP10-A3 [20]. Accuracy was evaluated by recovery: Three concentrations (0.5, 1, and 4 mg/L) of analytes spiked in distilled water and pooled serum were compared, and 10 replicates of these three samples were analyzed to calculate the recovery. The concentrations calculated were compared against the calibration curve of the spiked samples with either water or pooled serum matrix. The deviation of the determined concentration from the expected concentration (endogenous

| Table 1 — Summary of analytical precision performance. |
|-------------------------------------------------------|
| **Within-run**                                        |
| **Indoxyl sulfate (mg/L)**                            |
| Control 1: 0.19, Control 2: 0.01, Control 3: 0.94    |
| Standard deviation                                   |
| Control 1: 0.01, Control 2: 0.02, Control 3: 0.28    |
| Coefficient of variation (%)                         |
| Control 1: 6.4, Control 2: 19, Control 3: 3.9        |
| Counts                                               |
| Control 1: 20, Control 2: 20, Control 3: 20           |
| **Indoxyl sulfate (mg/L)**                            |
| Control 1: 0.19, Control 2: 0.01, Control 3: 0.94    |
| Standard deviation                                   |
| Control 1: 0.01, Control 2: 0.02, Control 3: 0.28    |
| Coefficient of variation (%)                         |
| Control 1: 6.4, Control 2: 19, Control 3: 3.9        |
| Counts                                               |
| Control 1: 20, Control 2: 20, Control 3: 20           |
| **Between-run**                                       |
| **Indoxyl sulfate (mg/L)**                            |
| Control 1: 0.2, Control 2: 0.02, Control 3: 0.36     |
| Standard deviation                                   |
| Control 1: 0.01, Control 2: 0.05, Control 3: 0.26    |
| Coefficient of variation (%)                         |
| Control 1: 6.4, Control 2: 19, Control 3: 3.9        |
| Counts                                               |
| Control 1: 20, Control 2: 20, Control 3: 20           |
| **Indoxyl sulfate (mg/L)**                            |
| Control 1: 0.2, Control 2: 0.02, Control 3: 0.36     |
| Standard deviation                                   |
| Control 1: 0.01, Control 2: 0.05, Control 3: 0.26    |
| Coefficient of variation (%)                         |
| Control 1: 6.4, Control 2: 19, Control 3: 3.9        |
| Counts                                               |
| Control 1: 20, Control 2: 20, Control 3: 20           |
concentration of analyte in the matrix, plus the known concentration of the spike) can be used to determine accuracy. To assess the recovery of an analyte, the ratio of the result obtained in an analysis of the spiked material to the actual quantity of the analyte present in, or added to, the matrix was calculated and expressed as a percentage. The allowable range was 85%–115%. Analyte stability was assessed using three serum samples at different concentrations under various storage conditions, including 4 °C for 7 days and −20 °C and −70 °C for 3 months.

2.7. Patients

The study was approved by the institutional review board of Chang Gung Memorial Hospital, Keelung, Taiwan (IRB no. 103–0343C, 103–0408C, and 107–0898C). We recruited 342 participants (both healthy volunteers and patients at various stages of CKD) between January 2012 and April 2017. Written informed consent was obtained from all patients. Whole blood was collected in a serum separator tube. Serum was separated from cells by centrifuging at 2000×g for 10 min within 2 h of collection and stored at −70 °C until analysis. Reference ranges for aspartate transaminase, alanine aminotransferase, creatinine, albumin, and blood urea nitrogen were determined using serum samples collected from 45 apparently healthy volunteers. We included 205 clinically stable kidney disease patients aged >20 years, who received regular follow-up at Chang Gung Memorial Hospital. Quality control materials were pooled from patients’ serum to generate six concentrations of samples for total-form and free-form analysis.

Renal function was estimated by the isotope dilution MS traceable modification of diet in renal disease four–variable equation [21]: eGFR (mL/min/1.73 m²) = 175 × Cr⁻¹·¹⁵⁴ × age⁻⁰·²⁰³ (× 0.742 if a female patient was being observed). Patients were divided into five groups according to the National Kidney Foundation’s Kidney Disease Outcomes Quality Initiative guidelines [22]: CKD stages 1, 2, 3, 4, and 5 were considered if eGFR values were >90 mL/min/1.73 m², 60–89 mL/min/1.73 m², 30–59 mL/min/1.73 m², 15–29 mL/min/1.73 m², and <15 mL/min/1.73 m², respectively. Pregnant women, patients undergoing dialysis therapy, and recipients of renal transplant were excluded from the study.

2.8. Statistical analysis

Microsoft Excel (Microsoft Corp., Redmond, WA, USA) was used to calculate the mean, median, and standard deviation. Normality of the data was assessed using the Shapiro–Wilk test. Reference intervals were based on the central 95% of the data, considering 95% confidence intervals. R software ver. 2.11.1 (The R Foundation for Statistical Computing, Vienna, Austria) was used.

3. Results

3.1. UPLC-MS/MS optimization

The determination of optimal settings and MS/MS transitions was obtained by direct infusion of 200 ng/mL of each analyte into the MS/MS detector. Table 2 lists the multiple reaction monitoring transitions, cone voltage, and collision energy for each analyte. To optimize the chromatographic mobile phase, different concentrations of formic acid were tested. We decided to use 0.1% formic acid as mobile phase A and acetonitrile containing 0.1% formic acid as mobile phase B. The IS and pCS retention times were approximately 1.10 and 1.57 min, respectively (Fig. 1). The runtime was 5.5 min per injection with baseline-resolved chromatographic separation. Fig. 1 shows representative ion chromatograms of control material with 2 mg/L of IS and pCS.

3.2. Method validation

The matrix effect was evaluated by signal-recovery spiking experiments with 12 patient samples. The endogenous IS and pCS concentration in different patients varied between 0.06 and 4.61 mg/L. Each sample was spiked with 1 mg/L of standard solution of IS or pCS. The mean recovery was 104.7% for IS and 101.1% for pCS. Values around 100% indicated that there was neither ion suppression nor ion enhancement of the analytes. The lower limit of the measuring interval in calibration curve standards was 0.05 mg/L for both analytes, and was determined from 40 replicates from six different samples over five runs. The linearity was evaluated by analyzing 11 equally spaced samples with two replicates each, and was 0.05–5.18 mg/L for IS and 0.05–5.12 mg/L for pCS, with R² > 0.999 for both analytes. Dilution factors of 10 and 20 were investigated for sample concentration at 10 mg/L. The results were in good agreement with imprecision less than 2.8% and bias less than 7.6%.

The method precision was determined by analyzing six quality control materials prepared by spiking standard solution in pooling patients’ serum. Control 1–3 are for total-form IS and pCS and control 4–6 are for free-form IS and pCS. Three concentrations were tested, and the coefficient of variation ranged from 1.1% to 6.4% within each run (n = 20) and 2.2%–10.6% between runs (n = 40; Table 1).

The accuracy of the analytical methods was verified by recovery, which was 102%–107% for IS and 108%–113% for pCS over a concentration range of 0.53–4.25 mg/L in both water and serum matrices. The analytes were stable at 4 °C for 7 days and at −20 °C and −70 °C for 3 months.

3.3. Clinical application

Total serum IS levels in healthy participants were ≤0.05–3.02 mg/L, and total serum pCS levels were

| Table 2 – MRM transitions and parameters for indoxyl sulfate (IS), p-cresyl sulfate (pCS), and internal standards. |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| 1st (quantifier) 2nd (qualifier) Cone (V) Collision energy (eV) |
| IS | 212.04 → 80.14 | 212.04 → 132.05 | 6 | 20/18 |
| IS-d4 | 216.04 → 80.14 | 6 | 20 |
| pCS | 186.98 → 107.03 | 186.98 → 80.02 | 4 | 20/16 |
| pCS-d7 | 194.04 → 114.04 | 8 | 18 |
The histograms show that distributions were significantly non-Gaussian, so we used non-parametric analysis. The reference interval was 0.05–1.15 mg/L for total-form IS and 0.05–5.33 mg/L for total-form pCS. Out of 45 healthy participants, 43 (96%) had non-detectable levels of free-form IS, and 38 (84%) had non-detectable levels of free-form pCS. The results were highly skewed and the reference range was estimated non-
parametrically. The reference interval was ≤0.05 mg/mL for free-form IS and ≤0.12 mg/mL for free-form pCS. Characteristics and analyte accumulation in CKD patients were positively correlated with disease stage (Table 3).

4. Discussion

Uremic toxins are normally excreted in urine via tubular anion organic transporters, but accumulate in CKD patients. IS and pCS are representative uremic toxins that can exert harmful effects. We found that serum IS and pCS levels in CKD patients were significantly higher than in healthy volunteers, and were positively correlated with disease severity. Inulin-based GFR is generally used as a measure of kidney function, but is labor-intensive and expensive [23], and therefore reserved for selected patients. The creatinine-based calculated eGFR has been recognized as the gold standard for assessing the progression of renal failure [16], but is known to be affected by diet and exercise [17] and to poorly predict the concentrations of uremic solutes [24]. It is therefore necessary to develop a reliable assay for monitoring serum levels of IS and pCS, as early biomarkers of renal and cardiovascular outcomes.

Accurate measurements of IS and pCS offer not only diagnostic and predictive significance but also a role in CKD treatment, by reducing the concentrations of these uremic toxins using carbonaceous adsorbents. AST-120 (Kremezin, Kureha Co., Tokyo, Japan) is a spherical active carbon widely prescribed in Japan, Korea, and Taiwan to prolong the time to initiation of dialysis and treat uremic symptoms [25,26]. AST-120 can reduce serum and urine levels of IS in advanced CKD by adsorbing indole in the intestine and stimulating its excretion into feces [27]. The ability to quantitate uremic toxin concentrations in serum would contribute to monitoring treatment outcomes.

LC-MS/MS is currently the primary tool for quantifying uremic toxin levels. This study focused on developing and validating a high-throughput assay for routine monitoring of free and total IS and pCS. To measure the free form, serum samples were ultrafiltrated with a membrane to remove the bound form and serum proteins; the total and free forms can be measured in the same run. The addition of internal standards to samples prior to analysis represents the most valuable method enhancement that UPLC-MS/MS offers over other detection techniques. Selecting a proper internal standard is important to achieving high precision and accuracy in UPLC-MS/MS-based assays. Structural analogs are often used when stable isotope-labeled compounds are not available. When structural analogs are used as internal standards for the quantitation, matrix-induced ion suppression or enhancement cannot be corrected. We used stable isotope-labeled compounds as internal standards to correct the matrix-induced ionization suppression/enhancement, enabling

Table 3 – Patient characteristics and serum concentrations of indoxyl sulfate (IS) and p-cresyl sulfate (pCS) by disease stage.

|               | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 | p-value |
|---------------|---------|---------|---------|---------|---------|---------|
| N             | 29      | 49      | 64      | 40      | 22      |         |
| Sex, M:F      | 4:25    | 32:17   | 40:24   | 24:16   | 12:10   |         |
| Age, years    | 62.2 ± 10.4 | 64.4 ± 7.4 | 66.4 ± 8.2 | 69.1 ± 10.9 | 68.35 ± 8.0 | 0.017   |
| total IS, mg/L| 1.03 ± 0.85 | 1.54 ± 1.11 | 2.22 ± 1.79 | 4.74 ± 4.34 | 18.21 ± 15.06 | <0.001  |
| total pCS, mg/L| 2.69 ± 4.34 | 4.42 ± 4.47 | 6.45 ± 7.12 | 16.10 ± 13.98 | 27.00 ± 17.66 | <0.001  |
| free IS, mg/L | 0.08 ± 0.06 | 0.11 ± 0.09 | 0.17 ± 0.13 | 0.49 ± 0.72 | 2.36 ± 2.64 | <0.001  |
| free pCS, mg/L| 0.15 ± 0.20 | 0.24 ± 0.29 | 0.36 ± 0.37 | 1.36 ± 2.58 | 2.38 ± 2.03 | <0.001  |

Data represent the mean ± standard deviation.
accurate quantification of IS and pCS. There are published quantitative methods for IS and pCS by LC-MS/MS with stable isotope-labeled standards [28, 29]. Both methods tend to require longer analysis time, and sample preparation is complex. We successfully developed and validated a simple, fast, accurate, and analytically robust method to quantify the free and total forms of IS and pCS in clinical application, but several limitations should be addressed. First, using formula-based eGFR to estimate CKD severity instead of inulin-based eGFR may underestimate actual renal function in some patients. Testing both healthy individuals and patients with varying stages of CKD minimized the possible bias and strengthened our study. A previous study has suggested that healthy aging is associated with IS and pCS serum levels [30]. Aging is associated with functional changes and may result in accumulation of uremic toxins. In the present study, we found that age was positively correlated with disease stage. Aging may contribute to IS and pCS accumulation and the consequent progression of disease; we therefore suggest that a baseline measurement be taken before a patient commences treatment.

CKD is a dangerous clinical condition because renal impairment may lead to the development of end-stage renal disease, significant comorbidities, and increased risk of mortality. CKD affects approximately 12% of the population in Taiwan, a country with the highest prevalence and third-highest incidence of end-stage renal disease [31]. Early detection and treatment of CKD can prevent or delay the progression of kidney disease and the resulting cardiovascular complications. Identifying accurate and meaningful biomarkers of disease is essential to early prediction and timely intervention. Serum IS and pCS represent emerging promising biomarkers of CKD and cardiovascular disease. In this study, we developed and validated a fast and sensitive UPLC-MS/MS method for quantitating the total and free forms of IS and pCS in serum. This assay can assist in identifying patients at risk, predict disease progression, and facilitate the development of further therapies for this devastating disease.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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REFERENCES

[1] Vanholder R, De Smet R, Glorieux G, Argilès A, Baumeister U, Brunet P, et al. Review on uremic toxins: classification, concentration, and interindividual variability. Kidney Int 2003;63:1934–43.
[2] Barreto FC, Barreto DV, Liabeuf S, Meert N, Glorieux G, Temmar M, et al. Serum indoxyl sulfate is associated with vascular disease and mortality in chronic kidney disease patients. Clin J Am Soc Nephrol 2009;4:1551–8.
[3] Wang CP, Lu LF, Yu TH, Hung WC, Chiu CA, Chung FM, et al. Serum levels of total p-cresylsulphate are associated with angiographic coronary atherosclerosis severity in stable angina patients with early stage of renal failure. Atherosclerosis 2010;211:579–83.
[4] Lin CJ, Pan CF, Chuang CK, Sun FJ, Wang DJ, Chen HH, et al. P-cresyl sulfate is a valuable predictor of clinical outcomes in pre-ESRD patients. BioMed Res Int 2014;2014:526932.
[5] Liabeuf S, Barreto DV, Barreto FC, Meert N, Glorieux G, Schepers E, et al. Free p-cresylsulphate is a predictor of mortality in patients at different stages of chronic kidney disease. Nephrol Dial Transplant 2010;25:1183–91.
[6] Wu IW, Hsu KH, Hsu HJ, Lee CC, Sun CY, Tsai CJ, et al. Serum free p-cresyl sulfate levels predict cardiovascular and all-cause mortality in elderly hemodialysis patients—a prospective cohort study. Nephrol Dial Transplant 2012;27:1169–75.
[7] Wu IW, Hsu KH, Lee CC, Sun CY, Hsu HJ, Tsai CJ, et al. p-Cresyl sulphonate and indoxyl sulphate predict progression of chronic kidney disease. Nephrol Dial Transplant 2011;26:938–47.
[8] Meijers BK, De Loor H, Bammens B, Verbeke K, Vanrenterghem Y, Evenepoel P. p-Cresyl sulphate and indoxyl sulphate in hemodialysis patients. Clin J Am Soc Nephrol 2009;4:1932–8.
[9] Sun CY, Hsu HH, Wu MS. p-Cresol sulphate and indoxyl sulphate induce similar cellular inflammatory gene expressions in cultured proximal renal tubular cells. Nephrol Dial Transplant 2013;28:70–8.
[10] Vanholder R, Schepers E, Pletinck A, Nagler EV, Glorieux G. The uremic toxicity of indoxyl sulfate and p-cresyl sulfate: a systematic review. J Am Soc Nephrol 2014;25:1897–907.
[11] Shiba T, Makino I, Sasaki T, Fukuhara Y, Kawakami K, Kato I, et al. p-Cresyl sulfate decreases peripheral B cells in mice with adenine-induced renal dysfunction. Toxicol Appl Pharmacol 2018;342:50–9.
[12] Zhang H, Chen J, Shen Z, Gu Y, Xu L, Hu J, et al. Indoxyl sulfate accelerates vascular smooth muscle cell calcification via microRNA-29b dependent regulation of Wnt/beta-catenin signaling. Toxicol Lett 2018;284:29–36.
[13] Bammens B, Evenepoel P, Keuleers H, Verbeke K, Vanrenterghem Y. Free serum concentrations of the protein-bound retention solute p-cresol predict mortality in hemodialysis patients. Kidney Int 2006;69:1081–7.
[14] Tsai ML, Hsieh IC, Hung CC, Chen CC. Serum free indoxyl sulfate associated with in-stent restenosis after coronary artery stentings. Cardiovasc Toxicol 2015;15:52–60.
[15] Isakova T, Nickolas TL, Denburg M, Yarlagadda S, Weiner DE, Gutierrez OM, et al. KDOQI US commentary on the 2017 KDIGO clinical practice guideline update for the diagnosis, evaluation, prevention, and treatment of chronic kidney disease-mineral and bone disorder (CKD-MBD). Am J Kidney Dis 2017;70:737–51.
[16] National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis 2002;39:51–266.
[17] Schwinsgshackl L, Hoffmann G. Comparison of high vs. normal/low protein diets on renal function in subjects without chronic kidney disease: a systematic review and meta-analysis. PloS One 2014;9:e97656.
[18] Clinical & Laboratory Standards Institute. C50-A mass spectrometry in the clinical laboratory: general principles and guidance; approved guideline. Clinical and Laboratory Standards Institute; 2007.
[19] Clinical & Laboratory Standards Institute. EP06-A evaluation of the linearity of quantitative measurement procedures: a statistical approach; approved guideline. Clinical and Laboratory Standards Institute; 2003.

[20] Clinical & Laboratory Standards Institute. EP10-A3-AMD preliminary evaluation of quantitative clinical laboratory measurement procedures; approved guideline. 3rd ed. Clinical and Laboratory Standards Institute; 2014.

[21] Levey AS, Coresh J, Greene T, Marsh J, Stevens LA, Kusek JW, et al. Expressing the Modification of Diet in Renal Disease Study equation for estimating glomerular filtration rate with standardized serum creatinine values. Clin Chem 2007;53:766–72.

[22] Hogg RJ, Furth S, Lemley KV, Portman R, Schwartz GJ, Coresh J, et al. National Kidney Foundation’s Kidney Disease Outcomes Quality Initiative clinical practice guidelines for chronic kidney disease in children and adolescents: evaluation, classification, and stratification. Pediatrics 2003;111:1416–21.

[23] Stevens LA, Coresh J, Greene T, Levey AS. Assessing kidney function–measured and estimated glomerular filtration rate. N Engl J Med 2006;354:2473–83.

[24] Snaauwaert E, Van Biesen W, Raes A, Holvoet E, Glorieux G, Van Hoeck K, et al. Accumulation of uremic toxins is reflected only partially by estimated GFR in paediatric patients with chronic kidney disease. Pediatr Nephrol 2018;33:315–23.

[25] Niwa T, Emoto Y, Maeda K, Uehara Y, Yamada N, Shibata M. Oral sorbent suppresses accumulation of albumin-bound indoxyl sulphate in serum of haemodialysis patients. Nephrol Dial Transplant 1991;6:105–9.

[26] Niwa T. Role of indoxyl sulfate in the progression of chronic kidney disease and cardiovascular disease: experimental and clinical effects of oral sorbent AST-120. Ther Apher Dial 2011;15:120–4.

[27] Wu IW, Hsu KH, Sun CY, Tsai CJ, Wu MS, Lee CC. Oral adsorbent AST-120 potentiates the effect of erythropoietin-stimulating agents on Stage 5 chronic kidney disease patients: a randomized crossover study. Nephrol Dial Transplant 2014;29:1719–27.

[28] Sato E, Saigusa D, Mishima E, Uchida T, Miura D, Morikawa-Ichinose T, et al. Impact of the oral adsorbent AST-120 on organ-specific accumulation of uremic toxins: LC-MS/MS and MS imaging techniques. Toxins 2018;10:19.

[29] Kanemitsu Y, Asaji K, Matsumoto Y, Tsukamoto H, Saigusa D, Mukawa C, et al. Simultaneous quantitative analysis of uremic toxins by LC-MS/MS with a reversed-phase/cation-exchange/anion-exchange tri-modal mixed-mode column. J Chromatogr B Analyt Technol Biomed Life Sci 2017;1068–1069:1–8.

[30] Wyczalkowska-Tomasik A, Czarkowska-Paczek B, Giebultowicz J, Wroczynski P, Paczek L. Age-dependent increase in serum levels of indoxyl sulphate and p-cresol sulphate is not related to their precursors: tryptophan and tyrosine. Geriatr Gerontol Int 2017;17:1022–6.

[31] USRDS 2017 annual data report. In: System USRD, editor. USRDS coordinating center; 2017. Available at: https://www.usrds.org/2017/view/Default.aspx. [Accessed 7 October 2018].