Simultaneous Analysis of Glucuronyl- and Sulpho-Conjugates of Intestinal Putrefactive Compounds in Human Urine by Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry

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Abstract
Glucuronyl- and sulpho-conjugates of intestinal putrefactive compounds (IPC) are considered kidney disease-associated toxic agents. Recently, several simultaneous methods of detection by liquid chromatography tandem mass spectrometry (LC-MS/MS) of the conjugated forms of glucuronyl- and sulpho-conjugates in blood serum were reported. To analyze these conjugated forms in urine, we modified and validated the LC-MS/MS detection methods. The range of the calibration curves were determined using phenyl glucuronide (PhG; 1.56-25.0 μM), indoxyl glucuronide (IndG; 1.25-20.0 μM), p-cresyl glucuronide (PCG; 0.625-10.0 μM), phenyl sulfate (PhS; 3.13-50.0 μM), indoxyl sulfate (IndS; 7.81-250 μM) and p-cresyl sulfate (PCS; 1.56-25.0 μM). Urine was diluted 2-4 fold to determine PhG, IndG and PCG concentrations, and 4-16 fold to determine PhS, IndS and PCS concentrations. We validated the newly-modified detection methods by a recovery test, and intra-day and inter-day repeatability. We analyzed six clinical urine samples, PCS and IndS, major toxic agents, which were mostly detected. Creatinine-corrected values of PCS and IndS were high in six conjugates. In summary, in the present work, the concentrations of six urinary conjugated forms of IPC were successfully measured by LC-MS/MS.

Keywords: Glucuronyl-conjugate; Putrefactive Compound; Sulpho-conjugate; UHPLC-MS/MS; Urine

1. Introduction
The hindgut microbiota produces many types of metabolites such as short-chain fatty acids, hydrogen, methane, hydrogen sulfide, ammonia, amines and phenols via anaerobic fermentation [1]. Short-chain fatty acids (SCFA) are the major and most useful metabolites produced during hindgut fermentation. SCFA are used as metabolic energy when they are absorbed by the host [2]. Moreover, during hindgut fermentation, intestinal putrefactive compounds (IPC) such as phenol, indole and p-cresol, which are considered toxic metabolites, are often produced and absorbed from the lumen into blood. Phenol, indole and p-cresol are almost entirely metabolized to glucuronyl- or sulpho-conjugates in the intestinal wall and liver [3].

The kidneys possess an excretory function of endogenous and exogenous substances. The conjugated forms of IPC are among those substances excreted in the urine by the kidneys. Chronic kidney disease (CKD), mainly caused by chronic nephritis and metabolic syndromes such as hypertension and diabetes, increases proteinuria and decreases kidney function. Recently, research on the toxicity of the conjugated forms of IPC has increased exponentially [4]. In particular, the relation of the gut microbiota and CKD has attracted attention [5]. For example, the conjugated forms of IPC were observed to accelerate CKD [6]. Among the conjugated forms of IPC, p-cresyl sulfate (PCS) and indoxyl sulfate (IndS) are representative and have been repeatedly associated with overall mortality, cardiovascular disease and progression of CKD [7]. Particularly, the progression of CKD caused by IndS is believed to be induced by gene
transcription associated with renal fibrosis [6]. IndS increases extracellular superoxide dismutase-sensitive O$_2$ production and intracellular hydroxyl radical production in mesangial cells [8]. In addition, showing a mechanism similar to that of IndS [9], PCS is also a toxic factor in CKD progression [10,11].

Rapid and accurate detection of the conjugated forms of IPC in urine and blood is likely to be useful for establishing the occurrence or degree of progression of CKD. Several simultaneous methods by liquid chromatography-tandem mass spectrometry (LC-MS/MS) of quantification of the conjugated forms of IPC in blood serum were reported [7,12]. However, those methods were designed for the analysis of human serum, and their detection sensitivity of the conjugated forms of IPC in human urine is still limited.

In the present study, we modified the previously described determination methods [7] to analyze human urine samples by using high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). In addition, we validated the newly-modified methods.

2. Materials and methods

2.1. Reagents

The standards used in the present study were as follows: Phenyl-β-D-glucuronide (PhG; Sigma-Aldrich Japan, Tokyo, Japan), indoxyl β-D-glucuronide cyclohexylammonium salt (IndG; Sigma-Aldrich Japan), p-cresol glucuronide, also known as p-cresyl glucuronide (PCG; Toronto Research Chemicals; Toronto, Canada), potassium phenyl sulfate (PhS; Tokyo Chemical Industry, Tokyo, Japan), indoxyl sulfate potassium salt (IndS; Sigma-Aldrich Japan), and potassium p-toly sulfmate (p-cresyl sulfmate: PCS; Tokyo Chemical Industry). The internal standards were as follows: Phenylacetyl-d$_5$ L-glutamine (internal standard for PhG, Sigma-Aldrich Japan), p-cresol sulfate-d$_7$; internal standard for IndG and PCG; Toronto Research Chemicals), and indoxyl-3a,4,5,6,7,7a-13C$_6$ sulfate potassium salt (indoxyl sulfate-d$_6$; internal standard for IndS; Sigma-Aldrich Japan).

Methanol and purified water of MS grade were purchased from FUJIFILM-Wako Pure Chemical Corporation (Osaka, Japan). Other reagents used in this study were purchased from Sigma-Aldrich Japan.

2.2. Preparation of stock solutions and working solution

The stock solution of all standards was separately prepared from Sigma-Aldrich Japan. The gradient procedure was as follows: start point at 3% methanol as solution B and a gradually increase to 16% in 1 min, then to 100% methanol delivered at a flow rate of 0.5 mL/min at 40 °C, was a VanGuard; particle size 1.7 μm; Waters). The mobile phase, 10% formic acid (4 µL) were then added, and the mixture internal standard solution (5 µL), methanol (300 µL) and internal standard solution (5 µL), methanol (300 µL) and 10% formic acid (4 µL) were then added, and the mixture was vortexed and centrifuged (15,000×g, 10 min, 0 °C). The supernatant was loaded into a centrifugal filter (Amicon® Ultra-0.5; Millipore, MA, USA), and centrifuged (14,000×g, 10 min, 4 °C). A flow-through solution were used to analyze the conjugated forms of IPC.

2.3. Samples of urine

Six urine samples were purchased from KAC Co. Ltd. (Kyoto, Japan). The age and sex of donors were 22–47 years and Western female/male: 3/3, respectively. All samples were collected from donors to receive informed consent with the ethical aspect by a commercial supplier (Clinical Trials Laboratory Services Ltd., London, UK). Urine samples were stored at -80 °C until use.

2.4. Preparation of blank urine sample

The blank urine sample was prepared from the aforementioned urine samples. All frozen samples were thawed to room temperature and equally mixed. To remove sulpho-conjugates, the mixed urine sample was transferred to Oasis WAX cartridges (Waters, Milford, MA, USA). To remove glucuronyl-conjugates, the filtrated urine was then transferred to Oasis Hydrophilic-Lipophilic cartridges (Waters). These cartridges were conditioned with purified water prior to usage. The double-filtnated urine was used as the blank urine sample.

2.5. Sample preparation

Thawed urine samples or working standard solution (100 µL) were transferred to 1.5 mL micro-tubes. Aliquots of the internal standard solution (5 µL), methanol (300 µL) and 10% formic acid (4 µL) were then added, and the mixture was vortexed and centrifuged (15,000×g, 10 min, 0 °C). The supernatant was loaded into a centrifugal filter (Amicon® Ultra-0.5; Millipore, MA, USA), and centrifuged (14,000×g, 10 min, 4 °C). A flow-through solution were used to analyze the conjugated forms of IPC.

2.6. Instruments

The analytical condition was adjusted as previously described [7], with some modifications. Briefly, an UHPLC device equipped with a binary solvent manager, an autosampler, a column heater, and tandem mass spectrometry (Acquity TQD System; Waters) was used to detect the conjugated forms of IPC. Chromatographic separation was conducted using an ACQUITY CSH Fluoro-Phenyl column 2.1×50 mm (particle size 1.7 μm; Waters) with a guard column (ACQUITY CSH Fluoro-Phenyl 2.1×5 mm VanGuard; particle size 1.7 μm; Waters). The mobile phase, delivered at a flow rate of 0.5 mL/min at 40 °C, was a gradient of 0.1% formic acid as solution A and MS grade methanol as solution B.

The gradient procedure was as follows: start point at 3% solution B and a gradually increase to 16% in 1 min, then to
80% in 3 min, to 95% in 30 s, maintained 95% for 1 min, then to 3% in 30 s, and maintained 3% for 2.5 min. Total run time of the method was 8.5 min.

Solutes were detected using a TQD System with a Z-spray ion interface. The system was controlled using Waters MassLynx mass spectrometry software (Waters). Ionization was carried out using an alternating electrospray negative ionization mode. The negative ion mode was set as follows: ion source temperature, 150 °C and capillary desolvation temperature, 500 °C. The multiple-reaction monitoring (MRM) transitions, cone voltage and collision energy were individually optimized for each compound and the optimal dwell time was experimentally determined for each component (Table 1). The injection volume was 5 µL.

### 2.7. Method validation

Validation was conducted to the guidelines for analytical procedures and methods validation for analytical procedures and methods validation by International Conference on Harmonisation (ICH) [13] and Food and Drug Administration (FDA) [14].

#### 2.7.1. Linearity of calibration curve

The working standard solutions were prepared from the stock solutions and diluted 2-, 4-, 8-, 16-, 32-, 64- or 128-fold with the blank urine sample. To evaluate the linearity of calibration curve, three replicates of working standard solutions were prepared and loaded for UHPLC-MS/MS analysis on a same day. The peak area values of each calibration standard were divided by the peak area of the respective internal standard and plotted afterward. Calibration curves were constructed with 5-6 plots using linear regression and the coefficient of determination was then calculated according to the ICH guideline [13]. To validate the linearity of calibration curve, values higher than 0.995 of the mean coefficient of determination ($r^2$) of three replicates were determined to be acceptable. The limit of quantitation (LOQ) concentration was determined by the criteria such as “the lowest values within 80-120% against theoretical value”.

#### 2.7.2. Intra-day and inter-day repeatability

Intra-day repeatability for accuracy and precision were determined by the values of three replicates of working solutions obtained in a day. Accuracy was defined as follows:

$$= \frac{\text{the mean measured value of 3 replicates}}{\text{the theoretical value}} \times 100.$$

Precision was defined as follows:

$$= \frac{\text{the standard deviation of 3 replicates}}{\text{the mean measured value}} \times 100.$$

Values of accuracy and precision were determined to be acceptable when they were 85 to 115%, and lower than 15%, respectively [at LOQ, 80 to 120% and lower than 20%, respectively].

Inter-day repeatability for accuracy were determined by the values of three replicates of working solutions obtained for consecutive 3 days. Inter-day repeatability for accuracy and precision was defined using the same calculation procedure as for intra-day repeatability.

#### 2.7.3. Carryover test

A carryover test was conducted as follows: the highest concentration of a given working standard solution (PhG, 50.0 µmol/L; IndG and PCG, 20.0 µmol/L; PhS, 200 µmol/L; IndS, 500 µmol/L; PCS, 50.0 µmol/L) was injected, followed by 3 times of an injection of the blank urine. Appearance of peaks of standard substances of 6 conjugates and internal standards were recorded during the loading of the blank urine.

#### 2.7.4. Validation of dilution

To validate the dilution, the aforementioned mixed urine was diluted 2-, 4-, 8- or 16-fold with blank urine. All samples were prepared in triplicate. Internal standards were added to both diluent, blank urine and non-diluted urine, performed sample preparation as mentioned chapter 2.5. and their peak areas were measured. Relative peak area ratios of diluents and non-diluted urine against the blank urine were recorded. A relative peak ratio was considered acceptable if the value was 85 to 115%. Next, the precision of the concentrations of the conjugated forms within the diluents was determined. In addition, concentration ratios of diluents against 2-fold diluents in glucuronyl-conjugates such as PhG, IndG and PCG and 4-fold diluents in sulpho-conjugates such as PhS, IndS and PCS were calculated. The values of the

### Table 1. Retention time and instrument settings for the analyzed compounds.

| Reference standards            | Retention time (min) | Q1 (m/z) | Q2 (m/z) | Dwell (s) | Cone voltage (V) | Collision energy (V) |
|--------------------------------|----------------------|----------|----------|-----------|------------------|---------------------|
| Phenyl-β-D-glucuronide (PhG)   | 2.0                  | 269.1    | 113.0    | 0.078     | 35               | 10                  |
| Phenylacetylglutamine-d5       | 2.0                  | 268.3    | 145.2    | 0.078     | 35               | 15                  |
| Indoxyl-β-D-glucuronide (IndG) | 2.5                  | 308.2    | 113.0    | 0.078     | 35               | 15                  |
| p-Cresyl glucuronide (PCG)     | 2.5                  | 283.2    | 112.9    | 0.078     | 35               | 10                  |
| Phenyl sulfate (PhS)           | 4.0                  | 172.8    | 93.1     | 0.061     | 35               | 40                  |
| Indoxyl sulfate (IndS)         | 4.2                  | 211.9    | 132.2    | 0.061     | 35               | 18                  |
| Indoxyl sulfate-d6             | 4.2                  | 217.9    | 138.3    | 0.061     | 35               | 18                  |
| p-Cresyl sulfate (PCS)         | 4.3                  | 186.9    | 107.2    | 0.061     | 35               | 50                  |
| p-Cresyl sulfate-d7            | 4.3                  | 194.0    | 114.2    | 0.061     | 35               | 20                  |
concentration ratio and precision were considered to be acceptable if these values were 85 to 115% and lower than 15%, respectively.

2.7.5. Recovery test

Three (low, middle and high) different concentrations from each standard substance (Table 2) were prepared. Six clinical urine samples (chapter 2.3.) were equally mixed and diluted 4-fold or 16-fold with blank urine. Ten µL of the standard solutions was added to 190 µL of urine diluted with blank urine at three levels (low, middle or high). They were named Solution X-4low, Solution X-4middle, Solution X-4high, Solution X-16low, Solution X-16middle, or Solution X-16high. One hundred and ninety µL of 4 or 16-fold diluted urine was diluted with 10 µL of blank urine and the resulting solutions were named Solution Y-4 or Solution Y-16. In addition, Solution Z-low, Solution Z-middle or Solution Z-high were prepared by mixing 190 µL of blank urine with 10 µL of each standard solution (Table 2). The recovery ratio of each substance was defined as follows: \( \text{recovery ratio} = \frac{\text{the concentration of 6 conjugates in Solution X} - \text{the concentration of 6 conjugates in Solution Y}}{\text{the concentration of 6 conjugates in Solution Z}} \times 100 \). A recovery test was considered successful at 70-110%. The recovery test was carried out in triplicate.

2.7.6. Freeze-thaw stability

Freeze-thaw stability was evaluated with the aforementioned diluted urine using in chapter 2.7.5. Nine volumes of 4-fold diluted urine and nine volumes of 16-fold diluted urine (120 µL) were prepared. Six volumes of each diluent were frozen at -80 °C and stored overnight. The remaining three volumes of each diluent were used as unfrozen control. On the next day, all frozen volumes were thawed to room temperature, and three volumes of each diluent were re-frozen at -80 °C. Three freeze-thaw cycle testing was carried out on re-frozen volumes. The remaining three volumes of each diluent were used as one-time freeze-thaw samples. Post-treated urine samples were analyzed by UHPLC-MS/MS and the peaks were detected. The freeze-thaw stability ratio was defined as follows: \( \text{freezing stability ratio} = \frac{\text{no freezing control}}{\text{freeze-thaw sample}} \times 100 \).

2.8. Measurement of clinical urine sample

Clinical validation of the six urine samples (chapter 2.3.)

Table 2. Concentrations of reference standards used in the recovery test.

| Tested standards                  | Low (µM) | Middk (µM) | High (µM) |
|----------------------------------|----------|------------|-----------|
| Phenyl-β-D-glucuronide (PhG)     | 3.13     | 6.25       | 12.5      |
| Indoxyl β-D-glucuronide (IndG)   | 1.25     | 2.50       | 5.00      |
| p-Cresyl glucuronide (PCG)       | 1.25     | 2.50       | 5.00      |
| Phenyl sulfate (PhS)             | 12.5     | 25.0       | 50.0      |
| Indoxyl sulfate (IndS)           | 31.3     | 62.5       | 125       |
| p-Cresyl sulfate (PCS)           | 3.13     | 6.25       | 12.5      |

Fig. 1. Representative chromatograms and peaks of the glucuronyl-or sulpho-conjugates of putrefaction compounds in the blank urine.

Fig. 2. Representative chromatograms and peaks of the glucuronyl-or sulpho-conjugates of putrefaction compounds in the standard solutions of phenyl glucuronide, indoxyl glucuronide, p-cresyl glucuronide, phenyl sulfate, indoxyl sulfate, p-cresyl sulfate, phenyl acetyl-d5 L-glutamine, p-cresyl sulfate-d7 and indoxyl-d6 sulfate.
was carried out. At first, four-fold diluents were used to measure the concentrations of the conjugated forms. In second, 2-fold and 16-fold diluents were measured if glucuronyl- or sulpho- conjugates concentrations, respectively, were over the range of the calibration curve.

The urinary concentration of creatinine (Cr) was also measured using a commercial colorimetric assay (Creatinine Microplate Assay; Oxford Biomedical Research, Oxford, MI, USA). The procedures were carried out as per the manufacturer’s instructions, and samples were analyzed in duplicate.

3. Results and discussion

3.1. Peak specificity

Representative chromatograms of the standards of 6 conjugates of IPC, internal standards, blank urine, 4-fold diluents of mixed urine and 16-fold diluents of mixed urine are shown in Figs. 1-4. While peaks of standards and internal standards were detected as shown in Fig. 2, no peaks of these standards (6 conjugates and internal standard) were detected in blank urine at the same retention time (Fig. 1) with an exception. An adjacent peak (2.3 min) close to peak of IndG (2.5 min) was detected in 4-fold diluents of mixed urine (Fig. 3). We did not suggest the substrate derived this adjacent peak. The unknown peak at 2.3 min was not detected in blank urine (Fig. 1), therefore unknown substrate could be removed by the solid phase extraction. Hence, this unknown substrate might be closely similar molecular formula and physical property of IndG. de Loor et al. [7] did reported no adjacent peaks close to IndG in serum samples; thus, it is likely that it may be a fact observed only in urine. Nonetheless, the peak of IndG was hardly detected in 16-fold diluents of mixed urine (Fig. 4), the concentration of IndG was relatively low in human urine.

We considered our method by column separation successfully detected the conjugated forms of IPC in urine and accurately discriminated them from endogenous components. It is particularly noted that use of blank urine was excellent to detect the conjugated forms of IPC successfully, because it was suppressed matrix effects on ionization in mass spectrometry.

3.2. Linearity of calibration curve

The validated range of the calibration curves of six conjugated forms is conducted as follows: PhG, 0.781-25.0 µmol/L; IndG, 1.25-20.0 µmol/L; PCG, 0.625-10.0 µmol/L; PhS, 3.13-50.0 µmol/L; IndS, 3.91-250 µmol/L; PCS, 1.56-25.0 µmol/L.

Although some compounds at lower concentrations were rejected, and the following LOQ were considered the minimum acceptable concentrations of the calibration curve: PhG=0.781 (98.1%), IndG=1.25 (92.6%), PCG=0.625 (111.6%), PhS=3.13 (91.1%), IndS=3.91 (82.7%) and PCS=1.56 (101.0%), respectively (data not shown). The mean coefficient of determination for the analyzed compounds was as follows: PhG, $r^2=0.9992$; IndG, $r^2=0.9969$; PCG, $r^2=0.9955$; PhS, $r^2=0.9996$; IndS, $r^2=0.9998$; PCS, $r^2=0.9984$. We considered that the linearity of the calibration curve of the six conjugated forms was acceptable.
3.3. Intra- and inter-day repeatability

The results of the intra-day repeatability test are shown in Table 3. Accuracy of PhG, IndG, PhS, IndS and PCS were in range of 92.3-103.0%, 82.0-106.5%, 89.6-106.9%, 86.4-102.8% and 93.6-112.7%, respectively. Accuracy of IndG in 1.25 µmol/L (82.0%) was in the range of LOQ criterion (80-120%), however IndS in 3.91 µmol/L (74.8%) is beyond the criterion. Therefore, we changed the LOQ concentration of IndS to 7.81 µmol/L. Precision was acceptable under the validated ranges. For example, precision of PhG, IndG, PhS, IndS and PCS was in range of 0.8-14.0%, 0.5-14.4%, 0.5-14.0%, 0.3-7.7%, 0.5-7.5%, and 0.9-9.0%, respectively.

The results of the inter-day repeatability are shown in Table 4. For example, accuracy of PhG, IndG, PhS, IndS and PCS were in range of 91.7-113.1%, 96.8-106.3%, 94.8-110.3%, 87.2-103.7%, 99.1-106.9% and 99.0-105.7%, respectively. Precision of PhG, IndG, PhS, IndS and PCS was in range of 0.8-14.0%, 0.5-14.4%, 0.9-17.8%, 0.5-4.7%, 0.4-5.1% and 1.3-7.7%, respectively. Precision of PhG in 1.56 µmol/L (16.6%) is beyond criterion (less than 15%), though in the range of criterion at LOQ (80-120%). Therefore, we changed the LOQ concentration of PhG to 1.56 µmol/L, though 0.781 µmol/L (10.4%) is in the range of criterion (less than 15%). Precision of PCG in 0.625 µmol/L (17.8%) was in the range of criterion at LOQ (80-120%). Based on the results of the intra-day and inter-day validation, to draw the calibration curve, we corrected the ranges: PhG, 1.56-25.0 µmol/L; IndG, 1.25-20.0 µmol/L; PCG, 0.625-10.0 µmol/L; PhS, 3.13-50 µmol/L; IndS, 7.81-250 µmol/L; PCS, 1.56-25.0 µmol/L, respectively (Table 5). The mean coefficient of determination for the analyzed compounds was as follows: PhG, \( r^2 = 0.9998 \); IndG, \( r^2 = 0.9969 \); PCG, \( r^2 = 0.9955 \); PhS, \( r^2 = 0.9996 \); IndS, \( r^2 = 0.9999 \); PCS, \( r^2 = 0.9984 \). We considered that the linearity of the calibration curve of the six conjugated forms was acceptable.

3.4. Carryover test

Carryover was not detected as peaks of standards of 6 conjugates and internal standards. The peaks did not appear in the first replicate of blank urine (data not shown).

3.5. Validation of dilutions

When compared with the peak area of blank urine, the relative peak areas of phenylacetyl-d5 L-glutamine were...
acceptable in 2-16 fold diluents (90.7-97.1%), while we rejected in undiluted urine (80.2%; less than 85%). In addition, compared with the peak area of blank urine, the relative peak areas of p-cresyl sulfate-d7 potassium were acceptable in 4-16 fold diluents (93.2-99.5%), while we rejected in undiluted urine (67.4%) and the 2-fold diluent (82.1%). Compared with the peak area of blank urine, the relative peak areas of indoxyl-3α,4,5,6,7,7α-13C6 sulfate potassium were acceptable in 2-16 fold diluents (103.6-111.4%), while we rejected in undiluted urine (115.3%; more than 115%). Thus, dilution of urine was agreed 2-16 fold to analyze glucuronyl-conjugates and 4-16 fold to analyze sulpho-conjugates.

The precision and concentration ratios of diluents are shown in Table 5. Precision of PhG was acceptable in 2- and 4-fold diluents (1.2 and 4.2%, respectively), while we rejected in 8- and 16-fold diluents (15.2 and 77.8%, respectively). Precision of IndG was acceptable in 2- and 4-fold diluents (13.2 and 5.7%, respectively), while we rejected in 8- and 16-fold diluents (33.4 and 52.8%, respectively). Precision of PCG was acceptable in all diluents (3.8-11.6%). Precision of PhS, IndS and PCS were also acceptable in all diluents.

Table 4. Calculated inter-day repeatability for accuracy and precision of the investigated compounds.

|          | Theoretical value (μmol/L) | Mean measured value | Standard deviation | Accuracy | Precision |
|----------|-----------------------------|---------------------|--------------------|----------|-----------|
| PhG      | 0.781                       | 0.736               | 0.077              | 94.2%    | 10.4%     |
|          | 1.56                        | 1.43                | 0.24               | 91.7%    | 16.6%     |
|          | 3.13                        | 3.54                | 0.50               | 113.1%   | 14.1%     |
|          | 6.25                        | 6.47                | 0.16               | 103.6%   | 2.4%      |
|          | 12.5                        | 12.8                | 0.6               | 103.2%   | 4.7%      |
|          | 25.0                        | 25.9                | 1.6               | 103.4%   | 6.4%      |
| IndG     | –                           | 1.25                | 0.15               | 106.3%   | 11.3%     |
|          | –                           | 2.50                | 0.08               | 96.8%    | 3.2%      |
|          | –                           | 5.00                | 0.09               | 99.1%    | 1.9%      |
|          | –                           | 10.0                | 0.4               | 100.5%   | 3.6%      |
| PCS      | –                           | 0.625               | 0.117              | 105.7%   | 17.8%     |
|          | –                           | 1.25                | 0.12               | 110.3%   | 8.6%      |
|          | –                           | 2.50                | 0.13               | 94.8%    | 5.6%      |
|          | –                           | 5.00                | 0.22               | 98.0%    | 4.5%      |
|          | –                           | 10.0                | 0.1               | 100.6%   | 0.9%      |

Table 5. Calibration range, linear regression equation, and coefficient of determination of the calibration curves of analyzed compounds.

| Tested standards  | Calibration range | Diluent numbers | Linear regression | Coefficient of determination |
|-------------------|-------------------|-----------------|------------------|-------------------------------|
| Phenyl-β-D-glucuronide (PhG) | 1.56-25.0         | 5               | y = 0.1356x + 0.0344 | 0.9998±0.0004               |
| Indoxyl β-D-glucuronide (IndG) | 1.25-20.0         | 5               | y = 0.0166x + 0.0230 | 0.9969±0.0016               |
| p-Cresyl glucuronide (PCG) | 0.625-10.0        | 5               | y = 0.1774x + 0.0182 | 0.9955±0.0062               |
| Phenyl sulfate (PhS) | 3.13-50.0         | 5               | y = 0.0314x + 0.0343 | 0.9996±0.0003               |
| Indoxyl sulfate (IndS) | 7.81-250          | 6               | y = 0.0629x + 0.0684 | 0.9999±0.0001               |
| p-Cresyl sulfate (PCS) | 1.56-25.0         | 5               | y = 0.0073x + 0.0011 | 0.9984±0.0009               |

Coefficient of determination was calculated from the results of 3 replicates. Parameter x in linear regression means "concentration (μmol/L)", and parameter y means "peak area / internal standard area".
diluents (1.7-2.7, 0.7-4.1 and 2.3-4.7%, respectively). The concentration ratio of PhG against the 2-fold diluent was acceptable in all other diluents (87.7-98.9%). The concentration ratio of IndG against the 2-fold diluent was acceptable in 4- and 8-fold diluents (94.9 and 104.4%, respectively), while we rejected in the 16-fold diluent (121.2%). The concentration ratio of PCG against the 2 diluent was acceptable in all other diluents (98.9-101.3%). The concentration ratio of PhS against the 4-fold diluent was acceptable in all other diluents (94.8-96.8%). The concentration ratio of IndS against the 4-fold diluent was acceptable in all other diluents (103.9-104.8%). The concentration ratio of PCS against the 4-fold diluent was acceptable in all other diluents (104.8-106.5%).

Our results suggested that appropriate dilution was needed to analyze the conjugated forms precisely. Four times dilution was acceptable to analyze all conjugated forms. Furthermore, consideration of some other dilution ratios was needed to include the range of calibration curve. Glucuronyl-conjugates were analyzed acceptable within 2 and 4-fold dilution, whereas sulpho-conjugates were analyzed acceptable within 4-16 fold dilution.

3.6. Recovery test

The recovery ratios of 3 different concentrations of the conjugated forms of IPC are shown in Table 7. The recovery ratios of PhG, IndG, PCG, PhG, IndG and PCS were in range of 95.2-104.5%, 90.0-103.5%, 89.2-99.5%, 86.6-94.2%, 91.0-94.8% and 86.4-96.1%, respectively. The results of the recovery test were acceptable, because all values were in range of criterion (85-110%).

3.7. Freeze-thaw stability

Following the single freeze-thaw cycle, the percentages of compounds in urine were as follows: PhG=102.3%, IndG=97.9%, PCG=105.7%, PhS=101.1%, IndS=112.4% and PCS=98.6%, and all ratios were within the ranges of criterion (85-115%). Thus, it was considered that the single freeze-thaw cycle test did not affect the concentrations of compounds in urine. Likewise, following 3 freeze-thaw cycles, the percentages of compounds in urine PhG=97.3%, IndG=98.9%, PCG=107.2%, PhS=102.1%, IndS=107.4% and PCS=107.0%, and all ratios were also within the range of criterion (85-115%). Again, we considered that 3-freeze-thaw cycle testing did not affect the concentrations of the conjugated forms of IPC in urine.

3.8. Detection in clinical urine

The results of the analysis of the urinary concentrations of the conjugated forms of IPC and Cr in the six clinical urine samples are shown in Table 8. The concentrations of Glucuronyl-conjugates were lower compared with those of sulpho-conjugates. PhG was detected in two positive-type urine samples. IndG was detected only in one urine sample from a 22-year-old female donor. Although PCG was detected in five urine samples, a sample from a 32-year-old male donor was not detected. The mean concentration of PCG-positive urine was 23.5 µmol/L. PhS was detected in two urine samples from PhG-positive subjects. The mean concentration of IndS detected in all six urine samples was 143 µmol/L. PCS was detected in five urine samples, however, a sample from a 32-year-old male donor was not detected. The mean concentration of PCS in positive urine was 227 µmol/L. Poesen et al. reported that a median of PCS concentrations was 50.7 µmol/L, and a median of PCG concentration was approximately 4 µmol/L in urine of the patients with CKD [15]. These values were in range of our results (Table 8), therefore the detection methods suggesting this study seemed valuable.

Cr was detected in six clinical urine samples. All conjugated forms were detected in urine (sample from a 22-year-old female) with the highest Cr concentration, but in urine (sample from a 32-year-old male) with the lowest Cr concentration only IndS was detected. The ratios of conjugated forms of IPC to Cr (IPC/Cr) are shown in Table 8. PCS/Cr and IndS/Cr were detected at high levels in all conjugated forms. Urine with the lowest concentrations of Cr

### Table 6. Results of the validation of dilutions.

| Reference standards          | 2-fold | 4-fold | 8-fold | 16-fold | 2-fold | 4-fold | 8-fold | 16-fold |
|------------------------------|--------|--------|--------|---------|--------|--------|--------|---------|
| Phenyl-β-D-glucuronide (PhG) | 1.2%   | 4.2%   | 15.2%  | 77.8%   | 100.0% | 96.9%  | 98.9%  | 87.7%   |
| Indoxyl-β-D-glucuronide (IndG) | 13.2% | 5.7%   | 33.4%  | 52.8%   | 100.0% | 94.9%  | 104.4% | 121.2%  |
| p-Cresyl glucuronide (PCG)   | 7.1%   | 11.6%  | 9.4%   | 3.8%    | 100.0% | 99.3%  | 101.3% | 98.9%   |
| Phenyl sulfate (PhS)         | –      | 2.5%   | 2.7%   | 1.7%    | –      | 100.0% | 96.8%  | 94.8%   |
| Indoxyl sulfate (IndS)       | –      | 0.7%   | 2.5%   | 4.1%    | –      | 100.0% | 104.8% | 103.9%  |
| p-Cresylsulfate (PCS)        | –      | 4.7%   | 2.3%   | 3.6%    | –      | 100.0% | 104.8% | 106.5%  |

### Table 7. Results of the recovery test.

| Reference standards          | Low conc. | Middle conc. | High conc. |
|------------------------------|-----------|--------------|------------|
| Phenyl-β-D-glucuronide (PhG) | 104.5%    | 103.9%       | 95.2%      |
| Indoxyl-β-D-glucuronide (IndG) | 103.5% | 99.8%        | 90.0%      |
| p-Cresyl glucuronide (PCG)   | 89.2%     | 99.0%        | 99.5%      |
| Phenyl sulfate (PhS)         | 91.6%     | 94.2%        | 86.6%      |
| Indoxyl sulfate (IndS)       | 94.8%     | 91.0%        | 92.5%      |
| p-Cresylsulfate (PCS)        | 86.4%     | 91.7%        | 96.1%      |

Acceptable range of recovery: 70-110%.
(226 mg/L) and IndS (71.1 µmol/L) had the highest IndS/Cr ratio (0.315 mmol/g) among all six urine samples. We believed that Cr correction may be important to determine the excretion of the conjugated forms of IPC in urine.

4. Conclusion

In the present study we demonstrated that our modified methodology of a previous procedure [7] to analyze the urinary concentration of the conjugated forms of IPC by UHPLC-MS/MS was successful. In addition, the validation of our newly-modified detection method proved the method was robust. It is worth noting that the use of blank urine was critical to detect the conjugated forms.

Although we attempted to analyze clinical urine samples by the newly-modified method, but we were unsuccessful as compounds were often undetected, even though PCS and IndS, typical toxic agents, were detected most of the time. Interestingly, the urinary concentrations of the conjugated forms of IPC seemed to depend on the concentration of Cr, suggesting that the conjugated forms of IPC and Cr should be simultaneously analyzed. For the present study, only the clinical urine samples from 6 donors were available, which were insufficient to establish a wider concentration range for each conjugate. We foresee that our modified method, which is noninvasive, will be beneficial and useful for the analysis of urine of both healthy subjects and CKD patients. We plan for the next study to improve the detection limit of the conjugates, as well as further investigation on age and sex differences and urine sampling time.

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