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Metabolomic analysis of uremic toxins by liquid chromatography/electrospray ionization-tandem mass spectrometry☆

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ABSTRACT

We applied the metabolomic analysis of comprehensive small-molecular metabolites using liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) and principal component analysis to identify uremic toxins accumulated in the serum of chronic renal failure (CRF) rats. CRF rats were produced by 5/6-nephrectomy. Indoxyl sulfate was demonstrated to be the first principal serum metabolite which differentiates CRF from normal, followed by phenyl sulfate, hippuric acid and p-cresyl sulfate. Then, we measured the serum levels of indoxyl sulfate, phenyl sulfate, hippuric acid and p-cresyl sulfate by the selected reaction monitoring (SRM) of LC/ESI-MS/MS, and demonstrated that these serum levels were markedly increased in CRF rats as compared with normal rats. As creatinine clearance decreased, the serum levels of the metabolites increased.

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1. Introduction

The uremic syndrome is considered to be caused by retention of metabolites in the body, due to kidney dysfunction, which are normally excreted into urine. The retention metabolites responsible for the uremic syndrome are called uremic toxins. Ninety compounds have been considered to be candidates of uremic toxins [1]. Sixty-eight have a molecular weight less than 500 Da, 12 exceed 12,000 Da, and 10 have a molecular weight between 500 and 12,000 Da. Twenty-five compounds are protein-bound.

Recently, the research on uremic toxins arouses interest, even if the problem is an old matter, thanks to the application of new analytical methodologies [2,3]. The development of soft ionization methods such as electrospray ionization (ESI) has enabled mass spectrometry (MS) analysis of heat-labile low-molecular-weight compounds and high-molecular-weight substances such as peptides and proteins as well. Tandem mass spectrometry (MS/MS) involves multiple steps of mass selection, with fragmentation occurring in between the stages, and permits identification of fragment structure by generating product ion spectrum of selected precursor ion of a compound. Liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) is especially useful to separate, identify and quantify a number of uremic retention metabolites simultaneously. The recent development of these analytical methods enabled the metabolome analysis of small-molecule metabolites in a biological sample.

In this study, we applied the metabolomic analysis of comprehensive small-molecular metabolites using LC/ESI-MS/MS to identify and quantify uremic toxins accumulated in the serum of chronic renal failure (CRF) rats.

2. Material and methods

2.1. Chemicals

Acetonitrile, methanol, formic acid and distilled water of high-performance liquid chromatography (HPLC) grade were obtained from Kishida Chemical (Osaka, Japan). Ammonium acetate of MS grade was obtained from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Indoxyl sulfate potassium salt was obtained from Sigma–Aldrich (St. Louis, MO, USA). Hippuric acid was obtained from Tokyo Chemical Industry (Tokyo, Japan). Sodium pentobarbital was obtained from Kyoritsu Seiyaku (Tokyo, Japan). 4,5,6,7-D_4-Indoxyl sulfate potassium salt, phenyl sulfate sodium salt and p-cresyl sulfate sodium salt were synthesized by Kureha (Tokyo, Japan).

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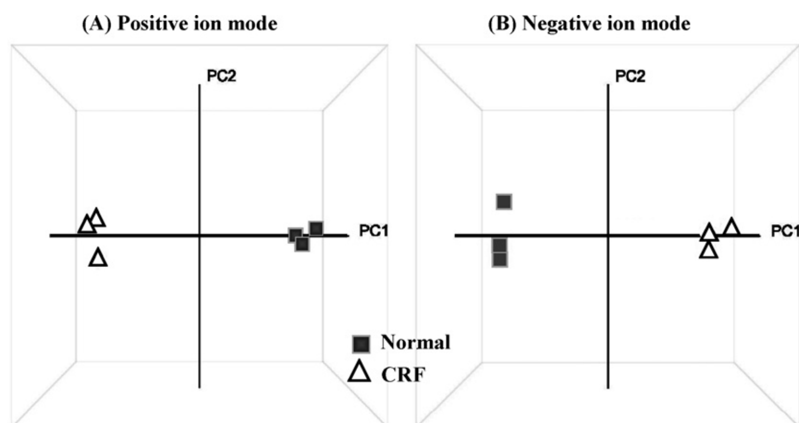

Fig. 1. The principal component PC_1 versus PC_2 score plot of LC/ESI-MS data in positive (A) and negative (B) ion modes of rat serum samples from normal and CRF groups.

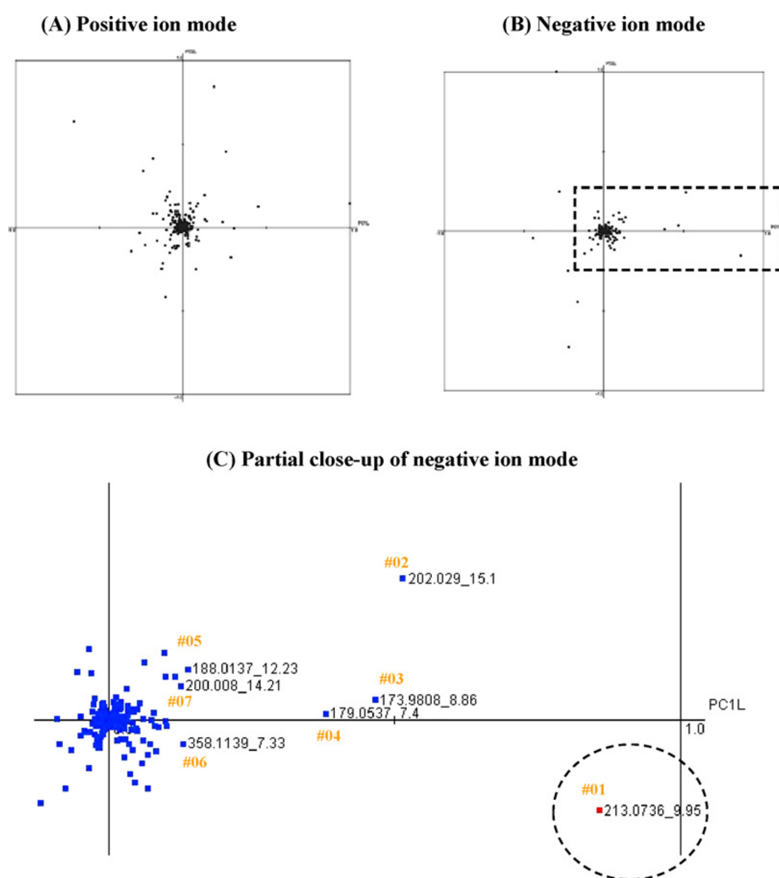

Fig. 2. Loading plot for PC_1 from principal component analysis of ion intensities from positive ion (A) and negative ion (B) LC/ESI-MS of serum from normal and CRF rats, and a partial close-up of the negative ion mode loading plot (C).

Table 1
Ranking list of metabolites with positive ions, which were enhanced in CRF serum as compared with normal serum.

	Ionized mass	Neutral mass	Retention time	PC1L	*P*-value	
#P01	121.07	120.06	4.33	−0.3930	1.0.E−04	
#P02	180.06	179.06	7.43	−0.1842	2.0.E−04	Hippuric acid
#P03	202.05	201.04	7.44	−0.1559	2.0.E−04	
#P04	121.07	120.06	5.49	−0.1417	1.7.E−03	
#P05	309.18	308.17	2.60	−0.1343	1.6.E−05	
#P06	166.08	165.08	2.55	−0.1077	1.3.E−02	
#P07	121.07	120.06	5.90	−0.1073	2.9.E−03	
#P08	143.05	142.05	2.35	−0.0941	2.6.E−05	
#P09	249.11	248.10	2.87	−0.0926	1.0.E−04	
#P10	194.08	193.07	8.62	−0.0765	2.6.E−02	

Table 2
Ranking list of metabolites with negative ions, which were enhanced in CRF serum as compared with normal serum.

	Ionized mass	Neutral mass	Retention time	PC1L	*P*-value	
#01	212.07	213.07	9.95	0.58300	0.0002	Indoxyl sulfate
#02	201.02	202.03	15.10	0.34980	0.0008	
#03	172.97	173.98	8.86	0.31690	3.03E−05	Phenyl sulfate
#04	178.05	179.05	7.40	0.25770	0.0001	Hippuric acid
#05	187.01	188.01	12.23	0.09440	0.0177	p-Cresyl sulfate
#06	357.11	358.11	7.33	0.08880	0.0001	Hippuric acid dimer
#07	199.00	200.01	14.21	0.08660	0.0005	
#08	191.01	192.01	1.79	0.07920	0.0056	
#09	281.51	282.51	44.52	0.07020	2.03E−07	
#10	194.93	195.93	1.87	0.06880	0.0002	

2.2. Sample preparation

Five-sixths of the normal kidney mass was removed from 6-week-old male Sprague–Dawley rats (Japan Charles River Laboratories, Tsukuba, Japan) to make animal models of CRF. In the first operation, two-third of the left kidney was removed. One week after the first operation, the right kidney was removed. These procedures were performed under anesthesia with sodium pentobarbital. Serum and urine levels of creatinine levels were measured using the Synchron CX3 analyzer (Beckman Coulter, Fullerton, CA, USA).

2.2.1. Identification of uremic toxins (experiment 1)

For identification of uremic toxins, three rats were selected 21 weeks after 5/6-nephrectomay, which showed similar levels of serum creatinine (normal: 0.54 mg/dL, ranging from 0.51 to 0.56 mg/dL, CRF: 1.62 mg/dL ranging from 1.59 to 1.68 mg/dL). Three normal rats at the same age were used for control. Serum was obtained by centrifuging the blood specimen for 2 min at 2800 × *g*. Samples were stored at −20 °C until analysis.

Serum samples (250 μL) were prepared by protein precipitation with acetonitrile (1 mL) followed by centrifugation at 20,600 × *g* for 10 min at 4 °C. Then, the supernatant (1.2 mL) was evaporated to dryness under nitrogen stream in a TurboVap (Caliper Life Sciences, Hopkinton, MA, USA) for 1 h at 37 °C. The dry sample was reconstituted with 200 μL of acetonitrile/0.5% formic acid solution (25:75, by volume).

2.2.2. Quantification of uremic toxins (experiment 2)

For quantification of uremic toxins, 11 rats were used 13 weeks after 5/6-nephrectomay, which showed serum creatinine levels ranging from mild to severe (normal: 0.50 mg/dL ranging from 0.45 to 0.60 mg/dL, CRF: 1.68 mg/dL ranging from 0.97 to 2.03 mg/dL). Ten normal rats at the same age were used for control. Serum was obtained by centrifuging the blood specimen for 2 min at 2800 × *g*. Samples were stored at −20 °C until analysis.

For analysis of serum samples, standard samples (0.01–5 mg/dL) were prepared to make calibration as follows: rat serum (20 μL), which was treated with active charcoal to remove endogenous metabolites, was spiked with 40 μL of a standard solution

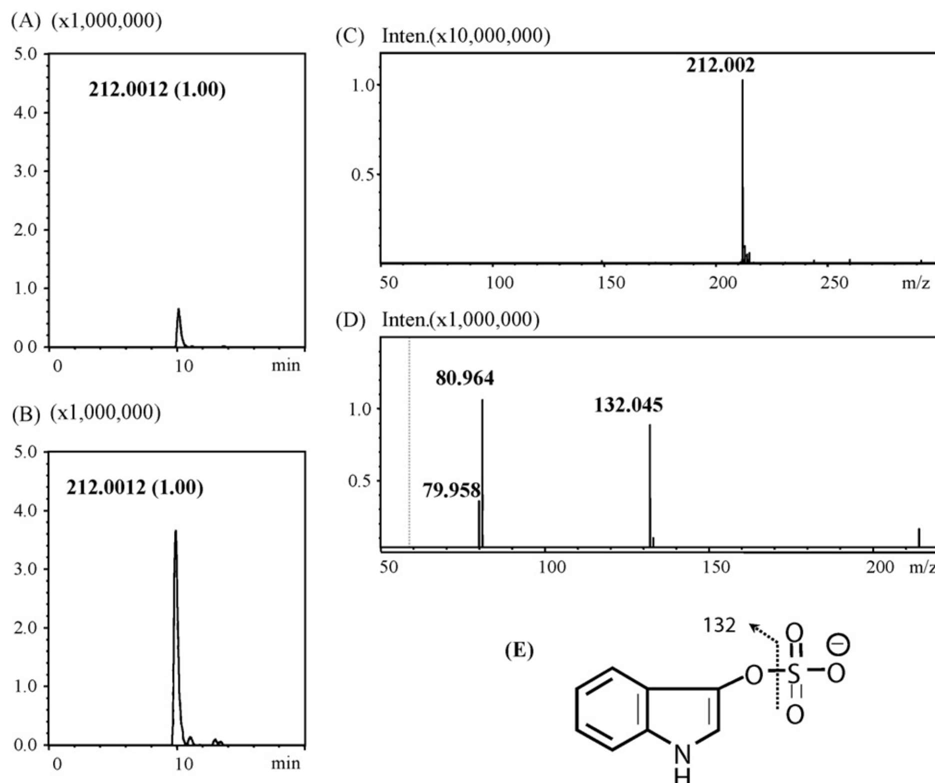

Fig. 3. Mass chromatograms of normal (A) and CRF (B), mass spectrum of the peak with retention time of 9.95 min (C), product ion spectrum of *m*/*z* 212 (D), and chemical structure and fragmentation pattern of indoxyl sulfate (E).

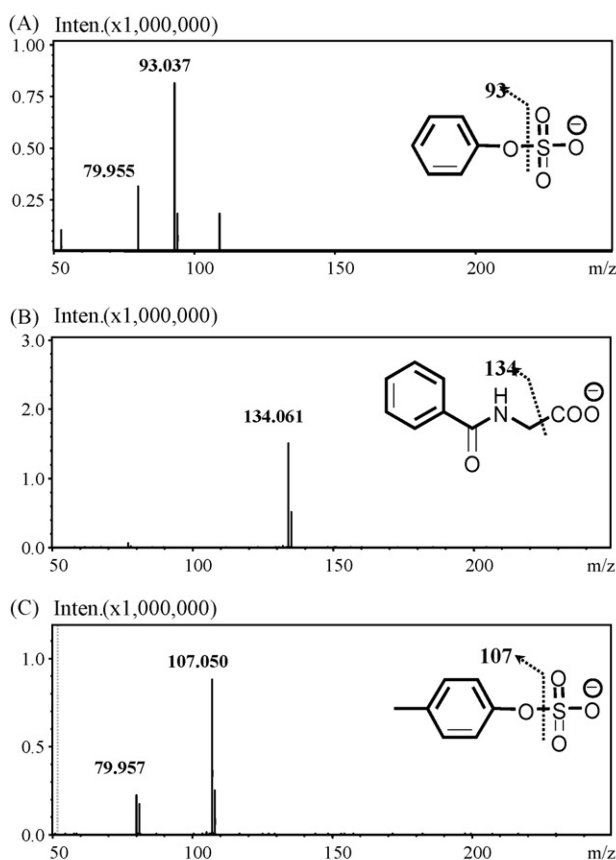

Fig. 4. Product ion spectra of m/z 172.97 (metabolite #03) with retention time of 8.86 min (A), m/z 178.05 (metabolite #04) with retention time of 7.40 min (B), m/z 187.01 (metabolite #05) with retention time of 12.23 min (C).

(0.0125–2.5 mg/dL). Then, serum sample was mixed with 40 μL of distilled water. Each mixture (50 μL) was transferred to a Sirocco 96 well-protein precipitation plate (Waters, Milford, MA, USA). The well had been prepared for protein precipitation with 200 μL of internal standard solution (100 ng/mL D_4-indoxyl sulfate in acetonitrile). After adding each mixture, the plate was vacuumed to collect the elution. Then, the elution (40 μL) was transferred to a microplate, and was mixed with 200 μL of 5 mmol/L ammonium acetate solution.

Standard solutions of indoxyl sulfate, phenyl sulfate, p-cresyl sulfate and hippuric acid were prepared at concentrations of 0.0125–25 mg/dL in distilled water.

2.3. LC/MS analysis

2.3.1. LC/ESI-MS/MS analysis for identification of uremic toxins (experiment 1)

HPLC analysis of a sample (10 μL) was separated by HPLC using gradient elution with a Prominence LC system (Shimadzu, Kyoto, Japan) on a Shim-pack VP-ODS (2.0 mm × 150 mm, 5 μm) column (Shimadzu, Kyoto, Japan) at 0.2 mL/min with the column maintained at 40 °C. The gradient solution consisted of solvent A (5 mmol/L ammonium acetate solution) and solvent B (acetonitrile). The elution solution was 2% B (A:B; 98:2, by volume) followed by a linear gradient up to 100% B over the next 45 min. After the elution solution was kept at 100% B for 10 min, it was returned to 2% B over the next 0.1 min, followed by 2% B for 10 min, making a total cycle time of 65 min/sample.

Mass spectra were acquired on a quadrupole ion trap time-of-flight mass spectrometer (LCMS-IT-TOF, Shimadzu, Kyoto, Japan) equipped with an ESI source at a mass resolution of 10,000. This instrument is based on the ability of a quadrupole ion trap to deliver MS^n capability and the TOF mass analyzer to support accurate mass measurements. The mass range is m/z 50–5000 in mass mode and m/z 50–3000 for MS^n experiments.

The parameters for sample analysis were as follows: mass range of m/z 50–1000 in MS and m/z 50–1000 in MS^n mode; ion source temperature of 200 °C, heated block temperature of 200 °C, ESI voltage (positive ion mode) of 4.5 kV, ESI voltage (negative ion mode) of −3.5 kV, ESI nebulization gas flow of 1.5 L/min, drying gas flow of 0.1 MPa, detector voltage of 1.65 kV, ion accumulation time of 30 ms. Automated data-dependent functions were set to acquire five scans for each precursor detected using the most intense ion signal as the trigger. Positive and negative ion modes were performed. Mass calibration was carried out using a trifluoroacetic acid sodium solution (2.5 mmol/L) from m/z 50 to 1000. Data acquisition and processing were performed using software LCMS solution 3.41.

Profiler software (Profiler M (AM) plus, Phenomenome Discoveries Inc., Saskatoon, Saskatchewan, Canada) was used to create data arrays of retention time, m/z and intensity data. The data arrays are tables of signal intensity and m/z value between the two groups of sample. Principal component analysis was accomplished with the Profiler software. Principal component analysis is an unsupervised statistical analysis that linearly reduces the dimensionality of data by compressing the total number of variables into a set of higher dimensional variables known as principal components, where each principal component consists of loadings from all the original set of variables and each principal component (PC) is orthogonal to all other principal components. PCs are determined so that PC_1 exhibits the greatest amount of the variation in the data, PC_2 exhibits the second greatest amount of the variation of the data, and so on.

2.3.2. Selected reaction monitoring (SRM) method of LC/ESI-MS/MS for quantification of uremic toxins (experiment 2)

HPLC analysis of a sample (10 μL) was performed using gradient elution with a LC-10Avp LC system (Shimadzu, Kyoto, Japan) on a Waters Atlantis dC18 (2.1 mm × 50 mm, 3 μm) column (Milford, MA, USA) with a guard column (10 mm C18) attached at 0.2 mL/min with the column maintained at 40 °C. The gradient solution consisted of solvent A (5 mmol/L ammonium acetate solution) and solvent B (methanol). The initial gradient condition was 20% B (A:B, 8:2, v/v) for 2 min followed by a linear gradient up to 95% B over the next 0.1 min. After the elution solution was kept at 95% B for 5 min, it was returned to 20% B over the next 0.1 min, followed by 20% B for 5 min, making a total cycle time of 12 min/sample.

SRM analysis was carried out using a triple quadrupole mass spectrometer (API4000, Applied Biosystems, Life Technologies, WayCarlsbad, CA, USA) equipped with an ESI source to quantify potential biomarkers of CRF found by principal component analysis of the experiment 1. The following mass parameters were employed in data acquisition: curtain gas of 10 psi, ion source gas 1 of 80 psi, ion source gas 2 of 85 psi, interface heater; on, collision gas of 5, ESI voltage (negative ion mode) of −4.2 kV, temperature ion source of 525 °C. Data acquisition and processing were carried out using software Analyst 1.4.2. Quantification of uremic toxins was performed as described in Section 2.2.2.

2.4. Formula prediction

To identify analytes in the experiment 1, the Formula Predictor software (Shimadzu, Kyoto, Japan) was used which predicts a candidate list based on MS and MS^2 data by taking into account a number of variables, such as isotopic profile analysis, accurate mass and mass resolution of the experimentally derived pseudo-molecular peaks and related fragment ion table. To sup-

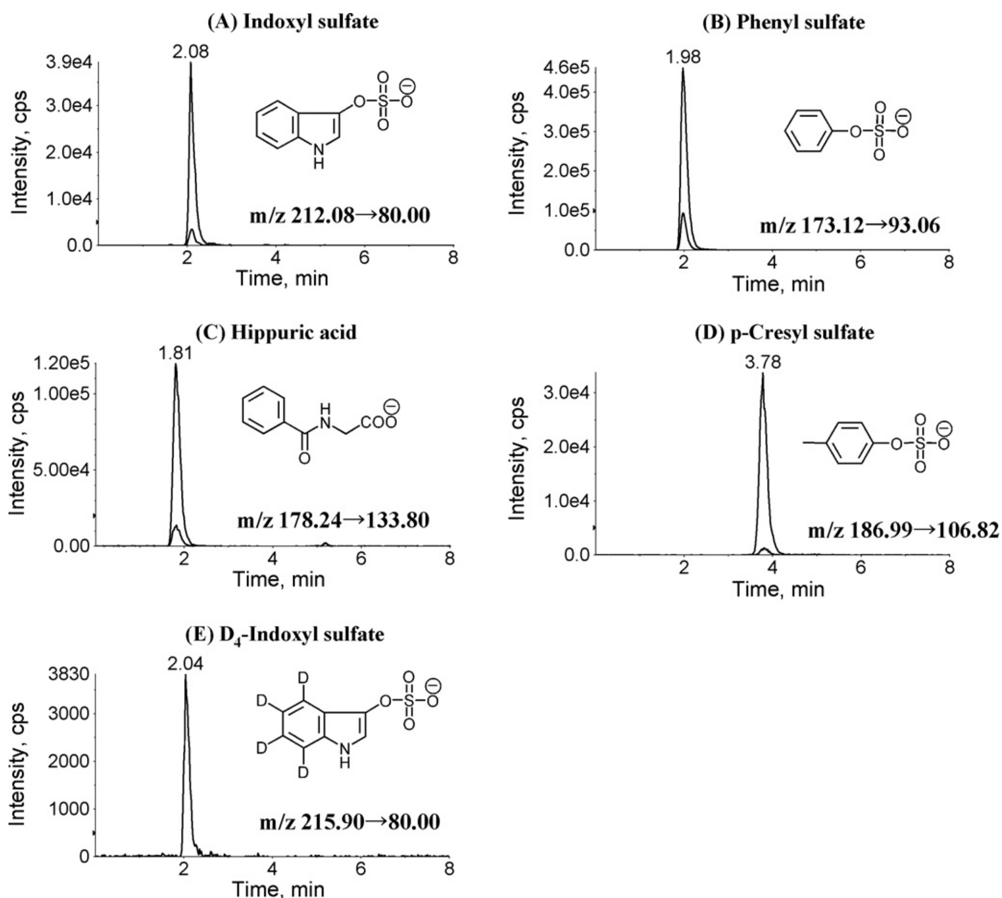

Fig. 5. SRM chromatograms of indoxyl sulfate (A), phenyl sulfate (B), hippuric acid (C), p-cresyl sulfate (D) and D_4-indoxyl sulfate (E). The peaks with lower and higher intensity at the same retention time represent normal and CRF rats, respectively.

port the metabolite identification, the following databases have been used: the uremic toxin database (http://www.nephro-leipzig.de/eutoxdb/index.php), and the mass bank (http://www.massbank.jp/) and KEGG (http://www.kegg.com/), and the human metabolite database (http://www.hmdb.ca/).

2.5. Statistics

Statistical significance between two groups for these metabolites was examined by using Student's *t*-test.

3. Results

3.1. Identification of uremic toxins (experiment 1)

A profiling software tool was used to create arrays of mass intensity and retention time pairs which consist of 7241 ions in positive ion mode and 7475 ions in negative ion mode in all the samples. There were 461 positive ions and 423 negative ions, of which peak intensity was statistically significantly (p value <0.05) higher in CRF rat serum than in normal rat serum. These ions were used for principal component analysis within a group.

Fig. 1 shows the PC_1 versus PC_2 score plots of positive (A) and negative (B) ion modes. The two groups could be easily distinguished by a line of PC_1. Fig. 2 shows loading plots of positive ion mode (A), negative ion mode (B), and a partial close-up of the negative ion mode (C) of rat serum samples from normal and CRF groups. There are a number of markers that were clearly associated with CRF. Table 1 shows ranking list of metabolites with positive ions, which were enhanced in CRF serum. Only hippuric acid was identified in the positive ion list.

Table 2 shows the ranking list of metabolites with negative ions, which were enhanced in CRF rat serum. As shown in a circle of Fig. 2C, the loading plot in PC_1 detected the ion (metabolite #01) at m/z 212 (neutral mass 213) with retention time of 9.95 min as the most prominently associated with CRF. Fig. 3 shows LC/MS mass chromatograms at m/z 212 with retention time of 9.95 min in serum samples of a normal (A) and a CRF (B) rat. The peak intensity of the ion at m/z 212 with retention time of 9.95 min was markedly increased in the CRF rat as compared with the normal rat. Tandem MS demonstrated that the precursor ion at m/z 212.00 (Fig. 3C) produced the product ions at m/z 132.05, 80.96 and 79.96 (Fig. 3D). The product ion at m/z 79.96 is characteristic of sulfate conjugation. The metabolite (#01) was identified as indoxyl sulfate by comparing with the authentic compound.

Fig. 4A shows the product ion spectrum of the metabolite (#03) at m/z 172.97 with retention time of 8.86 min. The product ion at m/z 79.96 is characteristic of sulfate conjugation. The product ion at m/z 93.04 was estimated to be phenol using the uremic toxin database. The metabolite (#03) was identified as phenyl sulfate. Fig. 4B shows the product ion spectrum of the metabolite (#04) at m/z 178.05 with retention time of 7.40 min. The metabolite (#04) was identified as hippuric acid using the mass bank. The metabolite (#06) is a dimer of the metabolite (#04). Fig. 4C shows the product ion spectrum of the metabolite (#05) at m/z 187.01 with retention time of 12.23 min. The metabolite (#05) was identified as p-cresyl sulfate. The identification of these metabolites was confirmed by comparing m/z, fragment ion patterns and retention times with those of authentic compounds. The metabo-

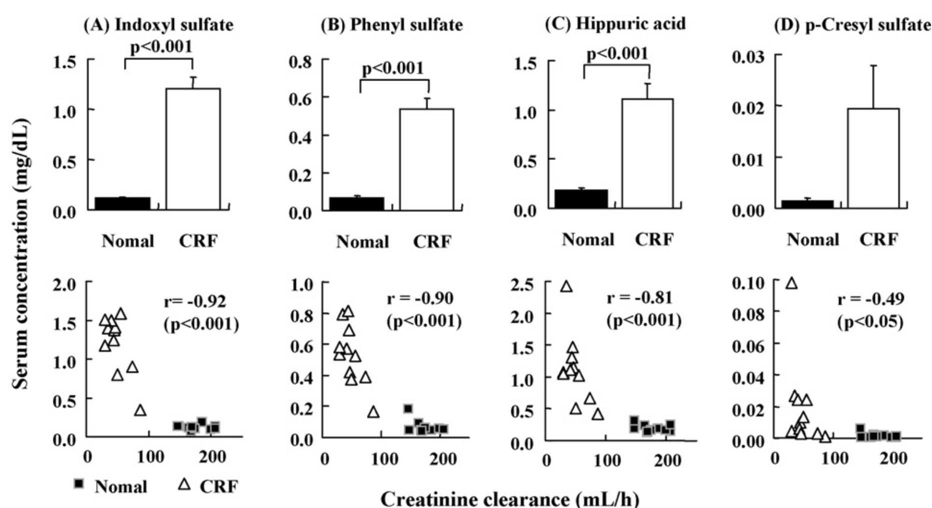

Fig. 6. Serum concentrations of indoxyl sulfate (A), phenyl sulfate (B), hippuric acid (C), p-cresyl sulfate (D), and their relation to creatinine clearance in normal and CRF rats. Error bars represent standard errors from the mean.

lite #02 was not yet identified, because its MS^n data could not be obtained.

3.2. Quantification of uremic toxins (experiment 2)

Indoxyl sulfate, phenyl sulfate, hippuric acid, and p-cresyl sulfate were quantified in the serum samples of normal and CRF rats using D_4-indoxyl sulfate as an internal standard by SRM mode of LC/ESI-MS/MS. Fig. 5 shows the SRM chromatograms of indoxyl sulfate, phenyl sulfate, hippuric acid, phenyl sulfate, p-cresyl sulfate and D_4-indoxyl sulfate. The peak intensities of these metabolites were greater in CRF rats than in normal rats.

Fig. 6 shows the serum levels of indoxyl sulfate, phenyl sulfate, hippuric acid, and p-cresyl sulfate, and their relation to creatinine clearance in normal and CRF rats. The serum level of indoxyl sulfate in CRF rats was significantly much higher than in normal rats. As creatinine clearance decreased, the serum level of indoxyl sulfate increased. These results indicated that indoxyl sulfate is a good biomarker of CRF. Similarly, serum levels of phenyl sulfate, hippuric acid, and p-cresyl sulfate in CRF rats were higher than in normal rats. These serum levels were well correlated with creatinine clearance, although the correlation is not so impressive for p-cresyl sulfate.

4. Discussion

This is the first study using the metabolomic analysis of uremic toxins by LC/ESI-MS/MS and principal component analysis, in which indoxyl sulfate was demonstrated to be the first principal metabolite which differentiates CRF from normal serum. Further, phenyl sulfate, hippuric acid and p-cresyl sulfate were listed in the top five metabolites which differentiate CRF from normal serum. Then, we measured the serum levels of indoxyl sulfate, phenyl sulfate, hippuric acid and p-cresyl sulfate by SRM of LC/ESI-MS/MS, and demonstrated that these serum levels were markedly increased in CRF rats as compared with normal rats. As creatinine clearance decreased, the serum levels increased. Creatinine clearance is an index of renal excretory function. These results suggest that indoxyl sulfate, phenyl sulfate, hippuric acid and p-cresyl sulfate were selected as principal uremic toxin candidates from about 7000 negative ions detected.

Indoxyl sulfate, hippuric acid and p-cresyl sulfate have been recognized to be accumulated in uremic serum as protein (albumin)-bound uremic retention solutes. Previously, we [4,5] measured serum levels of indoxyl sulfate by internal-surface reversed-phase HPLC, and confirmed its identification by using MS. The serum level of indoxyl sulfate is markedly increased in hemodialysis patients. Because of binding to serum albumin, indoxyl sulfate cannot be removed effectively by hemodialysis. Indoxyl sulfate is derived from dietary protein. A part of protein-derived tryptophan is metabolized into indole by tryptophanase in intestinal bacteria such as *Escherichia coli*. Indole is absorbed into the blood from the intestine, and is metabolized to indoxyl sulfate in the liver. Indoxyl sulfate shows various uremic toxicity such as inhibition of drug binding to albumin, nephrotoxicity (progression of chronic kidney disease) [6–10], vascular toxicity (aortic calcification, vascular smooth muscle cell proliferation, and endothelial dysfunction) [11–14], and osteoblastic dysfunction accompanied by skeletal resistance to parathyroid hormone. An oral sorbent AST-120 reduces the serum and urine levels of indoxyl sulfate in CRF rats and patients by adsorbing indole in the intestines, and consequently stimulating its excretion into feces [6,15–19].

Previously, we measured the serum levels of phenol and p-cresol in CRF patients using the selected ion monitoring (SIM) method of gas chromatography–mass spectrometry (GC/MS) [20]. The serum levels of phenol and p-cresol were higher in CRF patients than in normal subjects, and they existed in uremic serum mainly as sulfate esters [20]. Recently, de Loor et al. [21] demonstrated using GC/MS that most p-cresol in human serum is present as its sulfated metabolite, and a small proportion is glucuronidated. Unconjugated p-cresol is undetectably low in most cases. Martinez et al. [22] also found by using HPLC and MS that p-cresyl sulfate was accumulated in the plasma from hemodialysis patients, but no detectable unconjugated p-cresol. P-Cresyl sulfate and indoxyl sulfate were 94% and 93% bound to albumin, respectively. Thus, p-cresol circulates in the form of its sulfate conjugate, p-cresyl sulfate. p-Cresyl sulfate is poorly removed by hemodialysis because its clearance is limited by albumin binding. p-Cresyl sulfate shows a pro-inflammatory effect on leucocytes [23]. Phenyl sulfate and p-cresyl sulfate are the sulfate conjugates of phenol and p-cresol, which are produced by intestinal bacteria from tyrosine through 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid, respectively. Hippuric acid is the glycine conjugate of benzoic acid which is derived from phenylalanine.

In conclusion, the metabolomic analysis using LC/ESI-MS/MS and principal component analysis is useful to select and identify the most valuable serum metabolites that differentiate CRF from

normal. By using the method, indoxyl sulfate was found to be the most valuable metabolite, followed by phenyl sulfate, hippuric acid and p-cresyl sulfate.

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