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Metabolomic search for uremic toxins as indicators of the effect of an oral sorbent AST-120 by liquid chromatography/tandem mass spectrometry

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ABSTRACT

An oral sorbent AST-120 composed of spherical porous carbon particles has superior adsorption ability for certain small-molecular-weight organic compounds known to accumulate in patients with chronic renal failure (CRF). A metabolomic approach was applied to search for uremic toxins as possible indicators of the effect of AST-120. Serum metabolites in normal and CRF rats before and after administration of AST-120 for 3 days were analyzed by liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) and principal component analysis. Further, serum and urine levels of the indicators were quantified by selected reaction monitoring of LC/ESI-MS/MS. Indoxyl sulfate was the first principal serum metabolite, which could differentiate CRF from both normal and AST-120-administered CRF rats, followed by hippuric acid, phenyl sulfate and 4-ethylphenyl sulfate. CRF rats showed increased serum levels of indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate and *p*-cresyl sulfate. Administration of AST-120 for 3 days to the CRF rats reduced the serum and urine levels of these metabolites. In conclusion, indoxyl sulfate is the best indicator of the effect of AST-120 in CRF rats. Hippuric acid, phenyl sulfate and 4-ethylphenyl sulfate are suggested as the additional indicators. 4-Ethylphenyl sulfate is a newly identified uremic substance.

1. Introduction

The uremic syndrome is considered to be caused by retention of uremic toxins in the body, due to kidney dysfunction [1]. Godfrey et al. developed a multi-dimensional liquid chromatography/tandem mass spectrometry (LC/MS/MS) to identify known uremic analytes in a clinical hemodialysate sample. They discovered a selection of known uremic analytes and two novel compounds [2]. Rhee et al. applied LC/MS/MS-based metabolite profiling to survey >350 small molecules in patients with chronic renal failure (CRF), before and after hemodialysis. In addition to numerous previously identified uremic toxins, they identified several additional markers of CRF [3]. Thus, LC/MS/MS is especially useful to separate, identify and quantify a number of uremic toxins simultaneously [4,5]. The recent development of the analytical methods enabled the metabolome analysis of biological samples to search for uremic toxins as possible indicators of the effect of an oral sorbent (AST-120: Kremezin) in CRF.

The oral sorbent AST-120 composed of spherical porous carbon particles has superior adsorption ability for certain small-molecular-weight organic compounds known to accumulate in CRF patients. AST-120 is clinically used for the treatment of pre-dialysis CRF patients to slow the progression of CRF. AST-120 exerts its inhibitory effect on progression of CRF by adsorbing uremic toxins and/or their precursors in intestines. For example, AST-120 reduces the serum levels of indoxyl sulfate, a uremic toxin, accumulating in uremic rats and patients by adsorbing its precursor, indole, in the intestines, and consequently stimulating its excretion into feces [6–9]. A multicenter, randomized, double-blind, placebo-controlled, dose-ranging study has demonstrated that AST-120 decreased serum indoxyl sulfate levels in a dose-dependent fashion in patients with CRF [10]. AST-120 reduces oxidative stress in the kidney, cardiovascular system and bone in CRF by removing uremic toxins such as indoxyl sulfate that induces oxidative stress in renal proximal tubular cells, glomerular mesangial cells, vascular endothelial cells, vascular smooth muscle cells, cardiac myocytes, and osteoblasts [11]. Thus, AST-120 suppresses the progression of not only CRF [6–9] but also cardiovascular disease [12,13] and renal osteodystrophy [14].

A metabolomic approach using liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-

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MS/MS) and principal component analysis (PCA) was applied to search for uremic toxins as possible indicators of the effect of AST-120. Further, to confirm that their serum levels were increased in CRF rats, and that administration of AST-120 reduced their levels, their serum as well as urine levels were quantified by selected reaction monitoring (SRM) method of LC/ESI-MS/MS.

2. Materials 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 Avocado (Alfa Aesar, Ward Hills, MA, USA). Hippuric acid was obtained from Tokyo Chemical Industry (Tokyo, Japan). *N*-Benzoyl-D_5-glycine (D_5-hippuric acid) was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Sodium pentobarbital was obtained from Kyoritsu Seiyaku (Tokyo, Japan). 4,5,6,7-D_4-Indoxyl sulfate potassium salt, phenyl sulfate sodium salt, *p*-cresyl sulfate sodium salt and 4-ethylphenyl sulfate sodium salt were synthesized by Kureha (Tokyo, Japan).

2.2. Animal model of CRF

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 thirds 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. All animal studies were performed under the institutional guide for care and use of laboratory animals (Biomedical Research Laboratories, Kureha, Tokyo, Japan).

For Experiment 1, CRF rats ($n = 7$; serum creatinine ranging from 1.32 to 1.77 mg/dL), fifteen weeks after 5/6-nephrectomy, were administered with the chow containing 5% AST-120 for 3 days. Serum samples were taken before and after administration of AST-120. Serum samples before the administration were referred to CRF, and serum samples after the administration were referred to CRF + AST-120. Serum samples from normal rats ($n = 7$; serum creatinine ranging from 0.32 to 0.49 mg/dL) at the same age were referred to normal.

For Experiment 2, CRF rats ($n = 16$; serum creatinine ranging from 0.82 to 2.53 mg/dL), 18 weeks after 5/6-nephrectomay, were administered with the chow containing 5% AST-120 for 3 days. Normal rats at the same age, ($n = 8$, serum creatinine ranging from 0.27 to 0.34 mg/dL), were used for control.

The different samples were used in these two experiments. For Experiment 1, the diversity of renal function of the CRF rats was minimized to extract the possible indicators of the effect of AST-120. For Experiment 2, the renal function of the CRF rats was diversified to study the correlation between creatinine clearance and serum levels of the possible indicators.

2.3. Identification of indicators of the effect of AST-120 (Experiment 1)

To identify the possible indicators of the effect of AST-120, serum metabolites were analyzed by LC/ESI-MS/MS. Then, PCA of the LC/MS data was used to extract the principal components from the detected metabolites to differentiate CRF rats from both AST-120-treated CRF rats and normal rats.

Serum samples (350 μL) were prepared by protein precipitation with acetonitrile (1400 μL) followed by centrifugation at 20,600 × *g* for 10 min at 4 °C. Then, the supernatant (1610 μL) 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 105 μL of acetonitrile/0.5% formic acid solution (25:75, by volume).

HPLC analysis of a sample (1 μL) was performed using gradient elution with a Prominence LC system (Shimadzu, Kyoto, Japan) on a Shim-pack VP-ODS column (2.0 mm × 150 mm, 5 μm) (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 9.9 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. The parameters for sample analysis were as follows: mass range of *m/z* 50–1000 in MS mode and *m/z* 50–1000 in MS^n mode, ion source temperature of 200 °C, heated block temperature of 200 °C, ESI voltage of −3.5 kV, ESI nebulisation gas flow of 1.5 L/min, drying gas flow of 0.1 kPa, 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. Data acquisition and processing were performed using software LCMS solution 3.41 (Shimadzu, Kyoto, Japan).

Profiler software 2.6 (Profiler M (AM) plus, Phenomenome Discoveries, Saskatoon, Saskatchewan, Canada) was used to create data arrays of retention time, *m/z* and intensity data of MS mode. The data arrays were tables of signal intensity and *m/z* value between the three groups of sample. PCA was accomplished with the Profiler software.

To identify metabolites, the Formula Predictor software (Shimadzu, Kyoto, Japan) was used. Identification of metabolites was confirmed by comparing MS and MS/MS (product ion) spectra, and retention times with those of authentic compounds.

2.4. Quantification of indicators of the effect of AST-120 (Experiment 2)

To validate the possible indicators of the effect of AST-120 identified in Experiment 1, their serum and urine levels were quantified by SRM of LC/ESI-MS/MS.

Rat serum or urine sample (20 μL) was spiked with 40 μL of distilled water. Each mixture (50 μL) was transferred to a Sirocco 96 well-protein precipitation plate (Waters, Milford, MA, USA), in which the well had been prepared for protein precipitation with 200 μL of internal standard solution (for serum: 100 ng/mL D_4-indoxyl sulfate and D_5-hippuric acid in acetonitrile; for urine: 1000 ng/mL D_4-indoxyl sulfate and D_5-hippuric acid 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 5 mmol/L ammonium acetate solution (for serum, 120 μL; for urine, 200 μL).

For calibration, 20 μL of rat normal serum (50 mL of the serum was pretreated with 2.5 g of active charcoal to remove endogenous metabolites), or 20 μL of saline for analysis of urine was spiked with 40 μL of a standard work solution to give final concentrations of the metabolites. The following procedures are the same as described above.

HPLC analysis of a sample (1 μL or 10 μL) was performed using gradient elution at 0.2 mL/min with a LC-10Avp LC system (Shi-

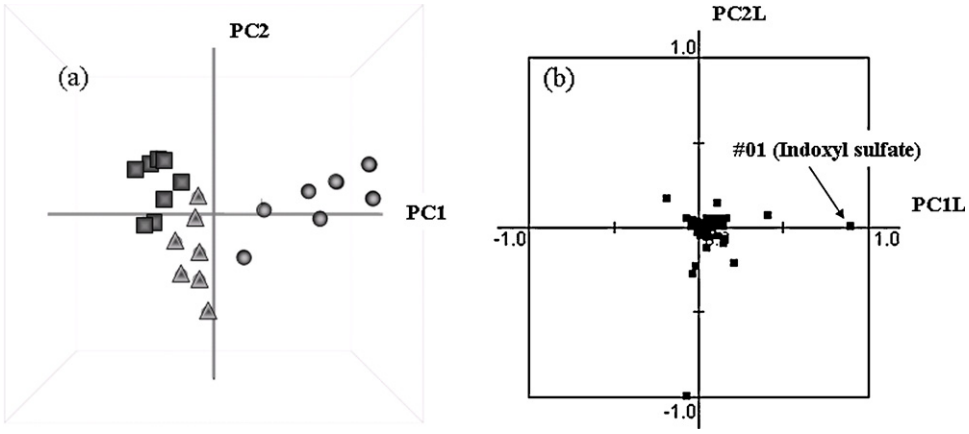

Fig. 1. Score plot of PC1 versus PC2 (a) and loading plot of PC1 (b) from the principal component analysis (PCA) of LC/MS data obtained from the serum samples of 3 rat groups (normal ■, CRF ●, and CRF + AST-120 ▲).

madzu, Kyoto, Japan) on an Atlantis dC18 column (2.1 mm × 50 mm, 3 μm) (Waters, Milford, MA, USA) maintained at 40 °C with a guard column (10 mm). 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, by volume) 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 4.8 min, making a total cycle time of 12 min/sample.

SRM method of LC/ESI-MS/MS was carried out using a triple quadrupole mass spectrometer (API4000, AB SCIEX, WayCarlsbad, CA, USA) equipped with an ESI source to quantify the indicators of the effect of AST-120 found by PCA of the Experiment 1. The mass ion source parameters were as follows: ion source temperature of 700 °C, ESI voltage of −4.0 kV, curtain gas of 10 psi, ion source gas 1 of 60 psi, ion source gas 2 of 80 psi, collision gas of 4, and interface heater, on.

Data acquisition and processing were carried out using software Analyst 1.5.1. The correlation coefficients of the calibration lines for quantification of indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate were 0.9985, 0.9995, 0.9953, 0.9990, and 0.9961, respectively. D_4-indoxyl sulfate was used as an internal standard for quantification of indoxyl sulfate, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate, and D_5-hippuric acid for quantification of hippuric acid.

2.5. Statistics

The Mann–Whitney *U*-test and the Wilcoxon signed-rank test were performed with JMP 8.0.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Identification of indicators of the effect of AST-120 (Experiment 1)

Profiler software automatically picked up 4680 peaks with retention time between 2 min and 30 min according to the peak intensity from full scan MS data of 21 samples (normal, $n = 7$; CRF, $n = 7$; CRF + AST-120, $n = 7$), and created data array of retention time, *m/z*, and the peak intensity data of MS mode. Subsequently, 252 peaks were selected for PCA according to the *p*-value ranking among 3 groups.

Fig. 1(a) shows the PC1 versus PC2 score plot. Normal, CRF and CRF + AST-120 groups could be distinguished by a line of PC1. Fig. 2 shows the loading plot. Table 1 shows the ranking list of top 10 indicators of the effect of AST-120 obtained from PCA (PC1L). These indicators with the PC1L values close to 1.0 could be separated from most endogenous peaks with PC1L values close to 0. The metabolite #01 at *m/z* 212.00 with retention time of 9.90 min was ranked as the top with the PC1L value (0.780) most close to 1.0, which was most prominently associated with CRF rats. The peak intensity of the metabolite #01 was significantly increased in the CRF serum as compared with the normal serum, and was significantly decreased by administration of AST-120. For this reason, the metabolite #01 is the first principal indicator of the effect of AST-120 in CRF rats.

The peak intensities of the metabolites (#02 to #10) were also significantly increased in the CRF serum as compared with the normal serum, and were significantly reduced by administration of AST-120. Thus, these metabolites (#02 to #10) also could be additional indicators of the effect of AST-120 in CRF rats. MS and MS/MS spectra of the metabolites (#01, #03, #05, #08, and #44), and authentic compounds (indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate) are shown in Fig. 2. The product ions of the metabolites #01, #05, and #08 at *m/z* 79.96 are characteristic of sulfate conjugates. The metabolite #01 was identified as indoxyl sulfate by comparing MS and MS/MS spectra, and retention time with the authentic compound. Further, by comparing with the authentic compounds, the metabolites #03, #05, and #08 were identified as hippuric acid, phenyl sulfate, and 4-ethylphenyl sulfate, respectively. *p*-Cresyl sulfate was barely detected by intentional search to be the metabolite #44 with low ion peak intensity. Indoleacetic acid could not be extracted by the Profiler software for PCA.

3.2. Quantification of indicators of the effect of AST-120 (Experiment 2)

To validate these metabolites detected in Experiment 1 for the indicators, we measured the serum and urine levels of indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate using the SRM method of LC/ESI-MS/MS. SRM chromatograms of the metabolites in CRF rat serum are shown in Fig. 3.

Table 2 shows that serum and urine levels of indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate in normal, CRF and CRF + AST-120 rats. The serum levels of these metabolites were significantly increased in CRF rats as compared with normal rats. The urinary excretion of indoxyl sulfate was significantly increased in CRF rats as compared with normal rats. However, the urinary excretion of phenyl sulfate, hippuric acid, 4-ethylphenyl sulfate, and *p*-cresyl sulfate was not increased in CRF rats. Administration of AST-120 for 3 days to CRF rats significantly

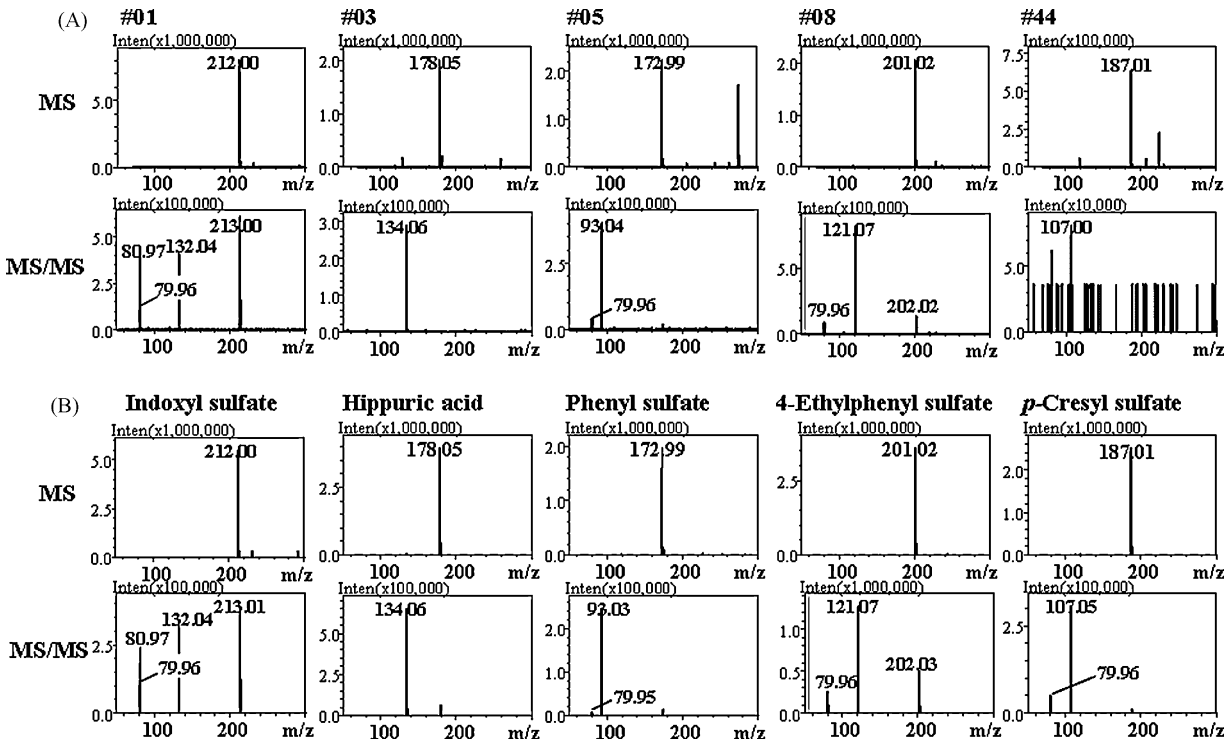

Fig. 2. MS and MS/MS (production) spectra of the possible indicators (A: #01, #03, #05, #08, and #44) obtained from CRF rat serum, and of authentic compounds (B: indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate). The metabolites #01, #03, #05, #08, and #44 were identified as indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate, respectively, by comparing MS and MS/MS spectra, and retention times with the authentic compounds.

Table 1
Ranking list of possible indicators of the effect of AST-120 obtained from LC/MS and PCA.

No.	Retention time (min)	Ionized mass (*m/z*)	PC1L	Formula	Calculated exact mass	
#01	9.90	212.00	0.780	$C_8H_7NO_4S$	213.0096	Indoxyl sulfate
#02	7.56	242.01	0.358			
#03	7.19	178.05	0.188	$CgHgNO_3$	179.0582	Hippuric acid
#04	12.28	417.11	0.148			
#05	8.76	172.99	0.135	$C_6H_6O_4S$	173.9987	Phenyl sulfate
#06	10.82	242.01	0.134			
#07	7.20	357.10	0.130			Hippuric acid (dimeric ion)
#08	15.20	201.02	0.125	$C_8H_{10}O_4S$	202.0300	4-Ethylphenyl sulfate
#09	7.89	188.98	0.098			
#10	15.74	321.04	0.097			
#44	12.14	187.01	0.020	$C_7H_8O_4S$	188.0143	*p*-Cresy sulfate

Table 2
Serum and urine levels of possible indicators of the effect of AST-120 in normal, CRF, and CRF + AST-120 rats.

Metabolite	Normal ($n = 8$)	CRF ($n = 16$)	CRF + AST-120 ($n = 16$)
Serum (mg/dL)			
Indoxyl sulfate	0.060 ± 0.010***	0.340 ± 0.065	0.140 ± 0.023***
Hippuric acid	0.180 ± 0.014***	0.690 ± 0.106	0.386 ± 0.037***
Phenyl sulfate	0.041 ± 0.004***	0.146 ± 0.023	0.076 ± 0.012***
4-Ethylphenyl sulfate	0.033 ± 0.004***	0.150 ± 0.025	0.018 ± 0.003***
p-Cresyl sulfate	0.002 ± 0.000***	0.019 ± 0.006	0.005 ± 0.001***
Urine (mg/day)			
Indoxyl sulfate	1.89 ± 0.13*	2.65 ± 0.22	1.08 ± 0.09***
Hippuric acid	31.9 ± 1.3	32.3 ± 0.9	16.7 ± 0.7***
Phenyl sulfate	1.59 ± 0.06	1.54 ± 0.09	0.646 ± 0.043***
4-Ethylphenyl sulfate	0.506 ± 0.085	0.563 ± 0.036	0.061 ± 0.006***
p-Cresyl sulfate	0.089 ± 0.017	0.129 ± 0.019	0.029 ± 0.004***

Mean ± SE.
* $p < 0.05$.
*** $p < 0.001$ versus CRF.

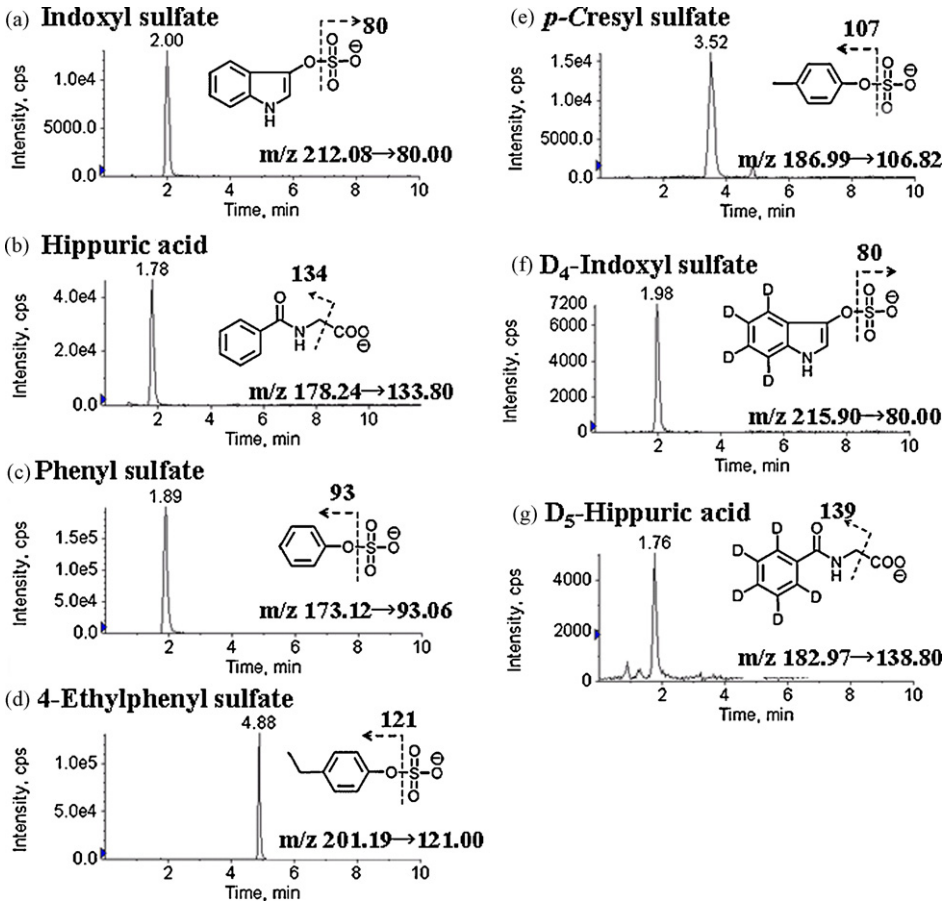

Fig. 3. SRM chromatograms of indoxyl sulfate (a), hippuric acid (b), phenyl sulfate (c), 4-ethylphenyl sulfate (d), and *p*-cresyl sulfate (e) in CRF rat serum, and of internal standards; D_4-indoxyl sulfate (f) and D_5-hippuric acid (g).

reduced both the serum and urine levels of indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate.

Fig. 4 shows correlation between creatinine clearance and serum levels of indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate in normal and CRF rats. As creatinine clearance decreased, serum levels of these metabolites increased. Creatinine clearance is an index of renal excretory function.

4. Discussion

In this study, we have demonstrated that indoxyl sulfate was extracted as the first principal indicator of the effect of AST-120. Hippuric acid, phenyl sulfate, and 4-ethylphenyl sulfate were the additional indicators. 4-Ethylphenyl sulfate was identified as a new uremic substance accumulating in CRF rat serum.

Metabolic profiling with LC/ESI-MS/MS has been previously used for the analysis of uremic substances accumulating in CRF [2,3]. However, these studies did not use PCA method to extract most characteristic uremic toxins for CRF. In the present study, we used PCA to extract the most principal metabolites as possible indicators of the effect of AST-120.

The serum levels of indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate and *p*-cresyl sulfate were significantly increased in CRF rats, due to reduced renal clearance of these metabolites. The urinary excretion of indoxyl sulfate was increased in CRF rats, probably because of increased generation due to impaired intestinal assimilation of protein [15]. The administration of AST-120 to CRF rats significantly reduced both the serum and urine levels of indoxyl sulfate, hippuric acid, phenyl sulfate, 4-ethylphenyl sulfate, and *p*-cresyl sulfate, by adsorbing their precursors such as indole, benzoic acid, phenol, 4-ethylphenol, and

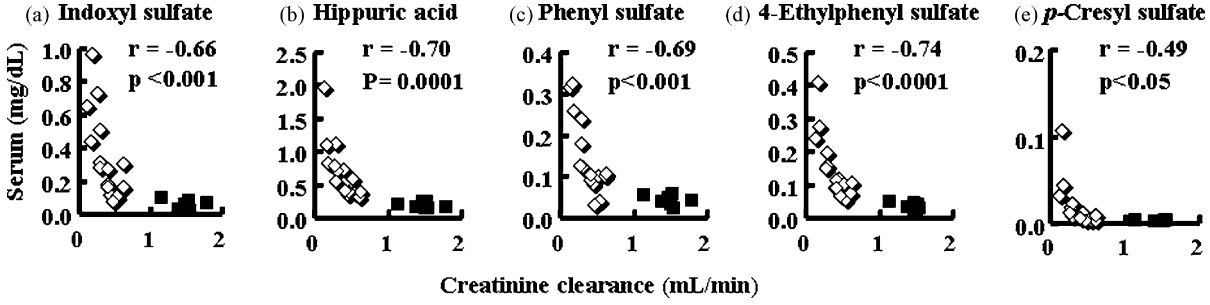

Fig. 4. Correlation between creatinine clearance and the serum levels of indoxyl sulfate (a), hippuric acid (b), phenyl sulfate (c), 4-ethylphenyl sulfate (d), and *p*-cresyl sulfate (e) in normal and CRF rats.

p-cresol, respectively, in the intestines, and consequently reducing the production of these metabolites.

The present study supports the notion that indoxyl sulfate seems to be the best indicator of the effect of AST-120. Indoxyl sulfate is a nephro-vascular toxin [16–27]. The administration of indoxyl sulfate to CRF rats promoted the progression of CRF, accompanied by increased gene expression of transforming growth factor (TGF)-β1, tissue inhibitor of metalloproteinase (TIMP)-1 and proα1(I)collagen [16–18]. Organic anion transporters (OAT1 and OAT3) play an important role in the transcellular transport of indoxyl sulphate and the induction of its nephrotoxicity [19]. The administration of indoxyl sulfate to hypertensive rats promoted aortic calcification and aortic wall thickening [23]. A recent clinical study has demonstrated that indoxyl sulfate may play a significant role in the vascular disease and higher mortality observed in CRF patients [26]. Indoxyl sulfate has pro-fibrotic and pro-hypertrophic effects on cardiac fibroblasts and myocytes, and pro-inflammatory effects, indicating that indoxyl sulfate might play an important role in adverse cardiac remodeling [27]. Further, indoxyl sulfate is a potent endogenous agonist for the human aryl hydrocarbon receptor, and its prolonged activation by indoxyl sulfate may contribute to toxicity observed in kidney dialysis patients [28].

Indoxyl sulfate, *p*-cresyl sulfate, hippuric acid and indoleacetic acid are the well-known protein-bound uremic toxins [1,29,30]. We searched for *p*-cresyl sulfate in the peaks detected by LC/MS, and barely found it as the metabolite #44 with low ion peak intensity due to its low serum level. However, the serum level of *p*-cresyl sulfate was significantly increased in CRF rats as compared with normal rats, and the administration of AST-120 significantly reduced its serum and urine levels. Recently, it has been demonstrated using gas chromatography/mass spectrometry and HPLC with fluorescence detection that *p*-cresyl sulfate is accumulated in the plasma from hemodialysis patients, but no detectable unconjugated *p*-cresol, and that *p*-cresol is an artifact produced from *p*-cresyl sulfate during sample preparation [31,32]. Hippuric acid is produced by glycine conjugation of benzoic acid derived from phenylalanine, and is primarily eliminated from the plasma via the kidney by active tubular secretion via OAT1 [33]. Indoleacetic acid could not be extracted by the Profiler software for PCA. Phenyl sulfate, a sulfation metabolite of phenol, is produced by decarboxylation of 4-hydroxybenzoic acid which is derived from tyrosine. The protein binding of phenyl sulfate has not yet been reported.

Rat metabolism is not necessarily the same as that of humans, and the present data should not necessarily be extrapolated to the human condition. For example, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid could not be detected in CRF rat serum, although it is one of major protein-bound uremic toxins in human CRF serum.

In conclusion, indoxyl sulfate may be the best indicator of the effect of AST-120 in CRF rats. Hippuric acid, phenyl sulfate and 4-ethylphenyl sulfate are suggested as the additional indicators. 4-Ethylphenyl sulfate was identified as a new uremic substance.1711

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