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Two rapid, accurate liquid chromatography tandem mass spectrometry methods for the quantification of seven uremic toxins: an application for describing their accumulation kinetic profile in a context of acute kidney injury

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Abstract

Acute kidney injury (AKI) is a frequent, serious complication in critically ill patients. Even if renal replacement therapy is rapidly initiated, AKI may lead to the acute accumulation of metabolic waste products called uremic toxins (UTs). Although the accumulation and effects of UTs have been extensively described in the setting of chronic kidney disease (CKD), few data are available for AKI. A rapid, sensitive, specific method with simple sample preparation is required to facilitate routine blood monitoring of UTs in a context of acute accumulation.

We have developed and validated two fast liquid chromatography tandem mass spectrometry methods for the quantification of seven UTs in human serum. The first method (in negative ionization mode) enables the quantification of five UTs (hippuric acid (HA), indoxyl sulfate (IxS), para-cresyl sulfate (pCS), para-cresyl glucuronide (pCG), 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF)). The second method (in positive ionization mode) enables the quantification of two UTs (indole-3-acetic acid (IAA), and trimethylamine N-oxide (TMAO)). Sample preparation consisted of the deproteinization of a small volume of serum (50 μ L). The run-times required to assay all the UTs in negative and positive ionization modes were only 2.5 and 2 minutes, respectively. In order to obtain a reliable, toxin-free matrix for the preparation of calibration standards and quality controls, serum was pretreated with activated charcoal. We used these methods to determine the time course of UT accumulation in eight patients who developed an AKI after cardiac surgery.

The calibration curves ranged from 0.1 to 100 μ g mL⁻¹ for all the UTs (except for IAA: 0.5 to 100 μ g mL⁻¹), and the correlation coefficients were above 0.999 for all. The methods were reproducible, repeatable, and accurate, with all coefficients of variation and biases below 15%. The highest concentrations measured in patients with AKI were lower than those reported in CKD stages 4 and 5 but higher than those observed in patients with no impairment of renal function (particularly for IxS and pCS). Our results also highlighted low accumulation of the other toxins (IAA, HA, TMAO, pCG, and CMPF). The UT concentrations did not rise earlier than that of creatinine; although the return to baseline took longer than for

creatinine for some compounds. Lastly, assessment of the time course of UT accumulation as a prognostic marker for AKI (particularly for pCS and IxS) appears to be promising and should be continued in a larger number of patients.

Keywords

Uremic toxin, para-cresyl sulfate, indoxyl sulfate, acute kidney injury, liquid chromatography tandem mass spectrometry

Abbreviations

AKI: acute renal injury

C_{max}: maximal concentration

CMPF: 3-carboxy-4-methyl-5-propyl-2-furanpropionate

CKD: chronic kidney disease

HA: hippuric acid

IAA: indole-3-acetic acid

ICU: intensive care unit

IxS: indoxyl sulfate

LLOQ: lower limit of quantification

LOD: limit of detection

LOQ: limit of quantification

ME: matrix effect

MRM: multiple reaction monitoring

pCS: para-cresyl sulfate

pCG: para-cresyl glucuronide

QC: quality control

RRT: renal replacement therapy

SAPS II: Simplified Acute Physiology Score II

TMAO: trimethylamine N-oxide

T_{max}: time to maximum concentration

UT: uremic toxin

1. Introduction

Acute kidney injury (AKI) is a frequent, serious illness in patients admitted to the intensive care unit (ICU). It significantly increases morbidity and mortality, with a death rate of between 50% and 80% [1]. Several situations (such as septic shock, or cardiac surgery with extracorporeal circulation) are frequently associated with the development of AKI in the ICU (in nearly 30% and 40% of cases, respectively) [2]. Patients with more severe AKI require renal replacement therapy (RRT) and have a higher number of failing organs and higher severity scores (the Simplified Acute Physiology Score (SAPS II) or the Acute Physiologic Assessment and Chronic Health Evaluation Score II) and a longer length of hospital stay, which partly explains the excess mortality in these patients [3]. However, the greater disease severity in patients with RRT may not be the only reason for such a poor prognosis [4] [5]. Indeed, a decrease in the kidneys' excretion capacity typically leads to the accumulation of metabolic waste products called uremic toxins (UTs), which may contribute to this excess mortality— even after RRT has been initiated.

Uremic toxins correspond to a group of heterogeneous compounds classified according to their molecular weight and their ability to bind to plasma proteins. Thus, one can distinguish between small (<500 Da) hydrophilic molecules that are easily eliminated by dialysis (e.g. trimethylamine-N-oxide [TMAO]), middle molecules (>500 Da), and strongly protein-bound compounds (such as 3-carboxy-4-methyl-5-propyl-2-furanpropionate [CMPF], hippuric acid [HA], indole-3-acetic acid [IAA], indoxyl sulfate [IxS], and p-cresyl sulfate [pCS]) [6]. The accumulation of UTs has mainly been studied in the context of chronic kidney disease (CKD); blood UT concentrations rise progressively as CKD progresses [7]. The accumulation of UTs is closely related to their toxicokinetics, and notably their production by gut microbiota and their elimination by the kidneys. Since UTs come from the amino acids and their derivatives in dietary proteins (IxS from tryptophan, p-CS from tyrosine, and TMAO from choline and L-carnitine), a person's diet and gut microbiota have fundamental roles in toxin production and thus contribute to toxin accumulation [8]. For example, a diet rich in seafood

and fish (which contain both TMAO precursors and a considerable amount of free TMAO) resulted in a high TMAO blood concentration [9]. Furthermore, many UT are strongly bound to plasma proteins and so cannot be removed by conventional dialysis techniques. These strongly bound UTs (e.g. lxS, pCS, and IAA) are the most toxic and lead to the progression of cardiovascular, renal and neurologic complications [7,10–12].

In contrast to the CKD setting, very few studies have evaluated the accumulation and toxicity of UTs in a context of AKI. A recent study of patients having developed AKI in hospital found that the lxS concentration may prognostic value [13]. The group of patients with an lxS concentration greater than $2.74 \mu\text{g mL}^{-1}$ (the median lxS concentration in the cohort) had a significantly shorter survival time than patients with a below-median lxS concentration. On the same lines, a study of 194 patients admitted to the ICU for sepsis found that those who subsequently developed AKI presented higher lxS and pCS levels than patients without AKI did. Furthermore, UT concentrations were lower in patients with a favorable evolution of their AKI [14]. In both studies, the UT concentrations measured during the episode of AKI were lower than those measured in patients with stage 4 or 5 CKD [15–18]. In both studies, however, the concentrations were only measured at baseline (*i.e.*, when AKI was diagnosed) and several days after the onset of the AKI episode (4 and 7 days in the studies by Wang *et al* [13] and Veldeman *et al* [14], respectively). However, there are no published data on the time course of UTs accumulation in the context of AKI, the peak concentration (C_{max}), the time to C_{max} (T_{max}), and the time to return to basal values. Hence, there is a lack of knowledge about the optimal time point for assaying UTs in a context of AKI. Furthermore, the accumulation of other UTs involved in the development of cardiovascular complications (such as IAA, CMPF, and TMAO) has not previously been investigated.

Hence, a rapid, sensitive, specific analytical technique with a simple sample preparation method is required for the routine monitoring of blood UT levels in patients with AKI. In this context, we have developed two high performance liquid chromatography tandem mass spectrometry (LC-MS-MS) methods for the determination of seven UTs in negative ionization

mode (CMPF, HA, IxS, pCS, and p-cresyl glucuronide [pCG]) or positive ionization mode (IAA and TMAO). We have used these methods to describe the accumulation kinetics of UTs in the serum of eight patients who developed functional AKI after cardiac surgery. These are preliminary data for exploring the value of UTs as potential AKI biomarkers.

2. Materials and methods

2.1 Reagents

Hippuric acid, IAA, IxS, TMAO, and d9-TMAO (isotopic purity: >98% deuterated forms (d1-d9), chemical purity: >98% TMAO) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). The charcoal dextran coated used to prepare the matrix blanks was also supplied by Sigma Aldrich. The pCS was from TCI chemicals (Paris, France). The CMPF and its deuterated internal standard (IS) (d5-CMPF, isotopic purity: >99% deuterated forms (d1-d5), chemical purity: >98% CMPF) were from Bertin bioreagent (Montigny-le-Bretonneux, France). Para-cresyl glucuronide, [$^{13}\text{C}_6$]-1H-IAA (isotopic purity: >99% ^{13}C , chemical purity: >95% IAA), d4-pCS (isotopic purity: >99% deuterated forms (d1-d4), chemical purity: >98% pCS) and [$^{13}\text{C}_6$]-IxS (isotopic purity: >99% ^{13}C , chemical purity: >98% IxS) were purchased from Alsachim (Illkirch-Graffenstaden, France). Acetonitrile, methanol and formic acid were LC-MS grade and were all obtained from VWR (Val-de-Reuil, France). Drug-free human serum from volunteers was provided by the French National Blood Service (Lille, France).

2.2 Solutions

Hippuric acid, CMPF, IAA, IxS, pCS, pCG and TMAO stock solutions (all 10 mg mL⁻¹) were prepared in either a methanol/water (50/50 V/V) mixture (for IAA, IxS, pCS, and pCG), in methanol (for HA and CMPF) or in water (for TMAO) and stored at - 80°C. The UT stock solutions were diluted in water to obtain intermediate stock solutions containing all the toxins at 0.01, 0.1 and 1 mg mL⁻¹, which were used to spike blank matrix and thus to obtain calibration standards or quality control (QC) samples.

Each IS powder was reconstituted at 1 mg mL⁻¹ by adding an appropriate volume of methanol (for [$^{13}\text{C}_6$]-IAA, [$^{13}\text{C}_6$]-IxS, and d4-pCS), methanol/water mixture (50/50 V/V, for d5-CMPF) or water (for d9-TMAO), and then kept at - 80°C until use. These solutions were used to prepare a final acetonitrile deproteinization solution containing all the ISs at 500 ng mL⁻¹.

2.3 Apparatus – LC-MS-MS analysis

All samples were analyzed on a Prominence HPLC system (Shimadzu®, Marne-la-Vallée, France) coupled to a 3200 QTRAP™ tandem mass spectrometer (Sciex®, Les Ulis, France). The chromatographic chain consisted of three LC-20A binary pumps, a DGU-20A5 degasser, a CMB-20A control module, a CTO-20AC column oven, and a SIL-20AC XR auto sampler. The tandem mass spectrometer was equipped with a Turbo V™ ion source operating in electrospray ionization (ESI) mode.

After undergoing the same preparation steps, the sample was injected twice (injection volume: 15 µL) into the chromatographic system for analysis in negative mode (for CMPF, HA, IxS, pCS, and pCG) or positive mode (for TMAO and IAA). The negative and positive mode acquisitions lasted 2.5 and 2 minutes, respectively. In both acquisition methods, chromatographic separation was performed at 40°C on an ultra PFP Propyl column (5 µm, 50 x 2.1 mm, Restek, Lisses, France) with an ultra PFP Propyl precolumn (5 µm, Restek). The column was eluted with a gradient of acetonitrile with 0.1% formic acid (mobile phase B) and ultra-pure water with 0.1% formic acid (mobile phase A) delivered at a flow rate of 0.8 mL/min. Before acquisition in negative mode, the gradient of solvent was as follows: 0 min: 40% mobile phase B (B); 0-1 min: 40-80% B; 1-2 min: 80% B; 2-2.2 min: 80-40% B; 2.2-2.5 min: 40% B. Before acquisition in positive mode, elution was performed under the following conditions: 0-0.5 min: 10% B; 0.5-0.7 min: 10-80%; 0.7-1.5 min: 80%; 1.5-1.7 min: 80-10%; 1.7-2 min: 10%.

Data were acquired in multiple reaction monitoring (MRM) mode after ionization in negative or positive ESI modes. The source parameters were set as follows: ESI voltage, -4500 V in negative mode and 4500 V in positive mode; ion source temperature, 350°C; heater gas, 70 psi; nebulizer gas, 40 psi; curtain gas, 30 psi. Spectrometer settings (*i.e.* MRM transitions, declustering potential, entrance potential, collision energy, and collision cell exit potential) were optimized for each analyte (Table 1). The structures of the seven UTs' product ions and their respective ISs are shown in supplemental figure A. Data were acquired and processed with Analyst™ software 1.6.2 version (Sciex).

2.4 Sample preparation

2.4.1 Calibration standards and QC preparation

Since UTs are endogenous compounds, it was not possible to directly obtain a toxin-free (*i.e.* blank) serum matrix. However, a toxin-free blank matrix was essential for assaying UTs (i) in patients with no impairment in renal function (*i.e.* with low concentrations of UTs) or (ii) in patients who developed acute or chronic renal failure (*i.e.* with high concentrations of UTs). In order to obtain a toxin-free matrix, the serum was first placed in contact with activated charcoal overnight at 4°C. The serum was then centrifuged at 2200 rpm for 15 minutes several times until the activated charcoal had been completely removed. The blank serum matrix thus obtained was spiked with appropriate volumes of aqueous solutions containing a mixture of UTs at 0.01, 0.1 and 1 mg mL⁻¹, in order to obtain calibration standards at 0.1, 0.5, 1, 5, 10, 50 and 100 µg mL⁻¹ and QC samples at 0.5, 2.5, 25 and 75 µg mL⁻¹ for CMPF, HA, IxS, pCG, PCS and TMAO, or at 1.5, 2.5, 25 and 75 µg mL⁻¹ for IAA.

2.4.2 Assay of the total amount of UTs

Fifty microliters of calibration standards, QC samples or patient samples were collected following homogenization of the sample. Serum proteins were removed by adding 200 µL of an iced acetonitrile solution containing the 5 ISs at 500 ng mL⁻¹. The samples were vortexed and centrifuged at 16500 x g for 15 minutes. After centrifugation, 50 µL of the supernatant was diluted 20x in ultra-pure water (total volume: 1000µL) before transfer to a vial for injection into the chromatographic system.

2.5 Method validation

The novel methods were fully validated according to the guidelines issued by the European Medicine Agency (EMA) [19] and the Scientific Working Group for Forensic Toxicology (SWGTOX) [20].

2.5.1 Selectivity and carryover

Selectivity: five human serum samples stripped with activated charcoal (*i.e.* blank matrix) and spiked samples at the limit of quantification (LOQ) were tested to investigate whether

endogenous components from the matrix or the sample interfered with determination of the UTs. Assay selectivity was defined as evidence of non-interference with the UTs' retention times and MRM transitions.

Carryover: intersample contamination was evaluated by analyzing three high calibration standards and then three blank matrix samples five times, without any rinse sequences.

2.5.2 Calibration

Six calibration curves were analyzed in separate runs. Each curve contained seven calibration standards (at 0.1, 0.5, 1, 5, 10, 50 and 100 $\mu\text{g mL}^{-1}$, in addition to a zero sample) covering the concentrations measured in patients with normal or impaired renal function. For CMPF, IAA, IxS, pCS and TMAO, the calibration model described the correlation between the peak area ratio of the analyte to the IS and the analyte concentration in the sample. For HA and pCG, the calibration model described the correlation between the peak area of the analyte and the analyte concentration in the sample. The back-calculated concentrations and the mean accuracy values were established for each calibration standard. The back-calculated concentrations had to be $\pm 15\%$ of the nominal value ($\pm 20\%$ for the lower limit of quantification (LLOQ)).

2.5.3 Limit of detection (LOD) and limit of quantification

The LOD was calculated as the concentration that gave a signal-to-noise ratio of 3. The LLOQ was the lowest concentration calculated with satisfactory accuracy (a range of 80% to 120%) and precision (a coefficient of variation (CV) of $\pm 20\%$). In all cases, the signal was at least five times as intense as the signal from a sample blank.

2.5.4 Accuracy and precision

Within-run accuracy (expressed as a percentage of the nominal value) and precision (expressed as the CV) were determined by analyzing six QC sera per level in a single run. The between-run accuracy and precision were evaluated for 18 QC sera per level, on 3 days. An accuracy of 85% to 115% of the nominal values and a precision with a CV of $\pm 15\%$ were required.

2.5.5 The matrix effect (ME)

The ME was evaluated by comparing the mean peak areas of the spiked UTs and ISs after extraction at 25 $\mu\text{g mL}^{-1}$ or 75 $\mu\text{g mL}^{-1}$ in five batches of serum blank matrix (previously cleaned with activated charcoal) with the respective mean peak areas obtained in neat solutions spiked at the same concentrations. For CMPF, IxS, pCS, IAA and TMAO, the ME was normalized by calculating the ME ratios for the molecule and the corresponding IS. The CV was calculated for each ME or IS-normalized ME and had to be below 15%. For CMPF, IxS, pCS, IAA and TMAO, we evaluated the impact of serum pretreatment with activated charcoal on the ISs' area under the curve (AUC). We compared the mean peak area for the IS in the samples used for the calibration curves with those in the patients' serum samples.

2.5.6 Stability

Stability was evaluated in order to choose the preparation and storage conditions that would not affect the UT concentrations. Stability tests were conducted on three QC levels, with a triplicate analysis for each level. The mean signal from QC samples analyzed immediately upon preparation was used as a reference and was compared with mean signal obtained in four storage conditions: at between 4 and 8°C for 6 hours and for 24 hours; at -20°C for 1 month; and after three freeze-thaw cycles.

2.6 Use of the methods to determine serum UT kinetic profiles in patients with AKI.

The patients included in the present study were all adults hospitalized in the ICU at Amiens-Picardie University Medical Center (Amiens, France) for additional treatment after cardiac surgery. The patients had developed AKI, starting from Kidney Disease Improving Global Outcomes stage 1 - defined as an elevation of the serum creatinine concentration by at least 1.5 times the baseline level within 72 hours of admission to the ICU) [21]. The following clinical and laboratory data were collected during patient follow-up: age, gender, weight, height, mean arterial pressure, length of stay, length of invasive mechanical ventilation, duration of extracorporeal circulation, the SAPS II on ICU entry, death, serum creatinine and urea concentrations (baseline and peak values), proteinemia, albuminemia, serum C-reactive

protein, red blood cell count, hemoglobin, hematocrit, white blood cell count, neutrophilic granulocyte count and platelet count. Nephrotoxic drugs (aminoglycosides, and glycopeptides) and catecholamines (norepinephrine, dobutamine, and epinephrine) were also screened for during the stay. Using our methods and the samples taken to measure serum creatinine levels, UTs were measured daily throughout the patient's stay in hospital. In line with French legislation on retrospective, non-interventional studies, approval by an investigational review board was not required. The study was nevertheless registered with the French National Data Protection Commission (Commission nationale de l'informatique et des libertés, Paris, France; reference: PI2020_843_0008). We used GraphPad Prism software (version 7, GraphPad Software Inc. San Diego, CA, USA) to plot the serum samples' UT concentrations and creatinine concentrations against time.

3. Results

3.1 The LC-MS-MS quantification of UTs

Figures 1a and 1b show representative chromatograms obtained for UTs (HA, IxS, pCS, pCG and CMPF) after acquisition in MRM mode and negative-mode ionization of a standard calibration sample at $10\ \mu\text{g mL}^{-1}$ (Figure 1a) and of a sample blank (Figure 1b). Chromatograms obtained after positive-mode ionization are shown in Figure 1c (standard calibration sample at $10\ \mu\text{g mL}^{-1}$) and Figure 1d (sample blank). Injections of sample blank did not reveal any analytical interference with UT transitions (Figures 1b and d).

3.2 Method validation

3.2.1 Selectivity and carryover

The analysis of five blank serum matrices previously cleaned with activated charcoal and analyzed in the presence or absence of ISs did not reveal any interference with any of the UTs. The inter-sample carryover never exceeded 1%. Therefore, no particular precautions were subsequently taken after the analysis of a high-concentration sample.

3.2.2 Calibration, LOD and LOQ

A quadratic regression (for HA and pCG) with $1/x$ weighting best described the correlation between the peak area of the analyte and the analyte concentration in the sample (supplemental Table A). A linear regression (for CMPF, IAA, IxS, pCS, and TMAO) with $1/x$ weighting best described the correlation between the peak area ratio of the analyte to the IS and the analyte concentration in the sample (supplemental Table A). The calibration range was established from 0.1 to $100\ \mu\text{g mL}^{-1}$ for all UTs (except for IAA: 0.5 to $100\ \mu\text{g mL}^{-1}$), and the correlation coefficient was above 0.999 for all curves (supplemental Table A). The mean measured concentrations, CVs, and bias for each standard calibration, and the LOD and LLOQ for each UT are given in Table 2. The calculated concentrations did not exceed $100 \pm 15\%$ of the nominal value, and the CVs were below 15% for all calibration standards.

3.2.3 Accuracy and precision

Within-run and between-run accuracy and precision were determined for each QC sample level (Table 3). The mean calculated concentrations were within $100 \pm 15\%$ of the nominal values for each QC level. The within-run and between-run CVs did not exceed 15% (Table 3).

3.2.4 Matrix effect

The matrix effect was negligible for all UTs except HA (supplemental Table B). Our results showed that pretreatment with activated charcoal did not have a significant effect on the ISS' AUC (data not shown).

3.2.5 Stability

Stability was assessed by calculating the percentage loss of concentration for a sample stored in the test condition vs. a reference sample prepared immediately before analysis. In serum, all UTs were found to be stable after 24 hours at between 4 and 8°C (Table 4). With the exception of TMAO, the UTs were also stable after storage for 1 month at -20°C (Table 4). At least one control level of IAA, HA, IxS or pCG did not meet the acceptance criteria for stability (percentage loss of concentration <15%) after three freeze-thaw cycles (Table 4).

3.3 Application of the methods to serum samples from patients with AKI

The characteristics of the eight patients included in the study are summarized in Table 5 and Supplemental Table C. The time courses of IAA, IxS, pCS, HA, TMAO, pCG, and CMPF were determined for all included patients. Figure 2 shows representative profiles for IAA, IxS, pCS, HA, and TMAO obtained from one of the study participants. The observed changes in IxS, pCS, HA, and TMAO concentrations were similar to that of serum creatinine. The T_{\max} values for the UTs were no earlier than that of creatinine. In the patient in Figure 2, the UT concentrations returned to baseline levels on much the same timescale as creatinine did. For IxS and pCS, creatinine-like accumulation and elimination profiles were observed in most patients, although there was marked inter-patient variability (Supplemental Figure B). In some patients, however (e.g. patients #2, #5 and #8 for pCS in Supplemental Figure B), the

return to baseline took longer than for creatinine. We did not detect CMPF or pCG in 59.48% and 58.17% of samples, respectively. Lastly, TMAO and HA did not accumulate markedly in most patients (data not shown).

4. Discussion

We have developed two rapid, accurate methods for the determination of seven UTs (CMPF, HA, IAA, IxS, pCS, pCG, and TMAO) associated with morbidity and mortality (especially renal and cardiovascular toxicity) in patients with CKD. These novel techniques were successfully used to monitor UT concentrations in patients developing AKI - suggesting that it may be of value for studying pCS and IxS as potential blood biomarkers of AKI.

Firstly, our LC-MS-MS methods' levels of analytical performance appeared to be suitable for clinical application. Indeed, the speed of the technique will be a fundamental for evaluating UT accumulation in the context of AKI. The assay method combines rapid sample preparation (a simple deproteinization step only) and the assay of five UTs (CMPF, HA, IxS, pCG and pCS) in only 2.5 minutes in negative ionization mode and two additional UTs (IAA and TMAO) in only 2 minutes in positive ionization mode. Our analytical run time is shorter than that described for other LC-MS-MS methods (respectively 15, 16 and 20 minutes with the methods developed by Boelaert et al. [17], Zhang et al. [22] and Kanemitsu et al. [23]). As the sample volume required for analysis is very small (50 µl), no additional samples will be required to perform routine UT monitoring in ICU patients. These low volumes are also compatible with the determination of UTs in basic research on small animals. The matrix used for the preparation of calibration standards and QC samples was serum that had been treated with activated charcoal. We did not observe an effect of serum pretreatment with activated charcoal on the AUC for the ISs. The use of this type of UT-free matrix avoids the use of an unsatisfactory standard addition method for the determination of UT levels in healthy subjects, where concentrations are close to those of a non-overloaded blank. During the validation of our analytical method, we found a significant matrix effect for HA. However, the relative matrix effect (expressed as the CV of the mean peak area of the HA spiked into extracts from five batches of serum) was negligible (<15%) [24]. Thus, the use of spiked blank serum matrix to prepare calibration standards enabled us to quantify HA in our patients. Nevertheless, we recommend the use of a stable isotope-labeled analogue of HA,

since it would exhibit the same matrix effect as HA in all serum batches. In our stability study, the storage conditions tested (6 hours or 24 hours at between 4 and 8 °C, 1 month at -20 °C, and three freeze-thaw cycles) did not affect the concentrations of most UTs. The exception was TMAO, which did not prove to be stable after 1 month at -20°C. Accordingly, we recommend preparing fresh calibration standards and QC samples for TMAO and analyzing patient samples immediately. Given that IAA, HA, IxS and pCG were found not to be stable after three freeze-thaw cycles, we recommend aliquoting suitable volumes of samples (calibration standards, QC samples and patient samples) and thus avoiding freezing-thawing.

Our techniques were successfully used to monitor UT concentrations in eight patients who had developed AKI following cardiac surgery. There are very few published studies on the accumulation of UTs and their effects in the context of AKI. Indeed, most of the work in this field concerned patients with CKD, in whom elevated UT levels are associated with poor outcomes [7,10–12]. The results of animal studies have suggested that the acute accumulation of UTs leads to pneumotoxicity [25] and neurotoxicity [26]. Very recently, two research groups reported on the accumulation of two UTs (IxS and pCS) in AKI by making to single-point concentration measurements (i.e. the first before AKI and the second 4 or 7 days after the diagnosis of AKI); the concentrations or the change in concentrations were correlated with the outcome of the AKI [13,14].

Our description of the serum kinetic profiles for seven UTs highlighted the value of studying IxS and pCS in the context of AKI. Overall, the pCS and IxS concentrations measured in our study ($9.3 \mu\text{g mL}^{-1}$ and $1.5 \mu\text{g mL}^{-1}$ for the mean peak concentrations, respectively) were lower than those observed in CKD stages 4 and 5 [15–18] but much higher than those measured in patients with normal renal function [17,26,27]. Our results also revealed that other UTs (IAA, HA, TMAO, and particularly pCG and CMPF) do not accumulate greatly. Indeed, pCG and CMPF were not detected in the majority of the samples. These results are in line with Mabuchi and Nakahashi's report on serum CMPF concentrations in patients who had developed acute or chronic renal function impairment [28]. The researchers suggested

that because CMPF accumulated only in the context of CKD, it was a potential marker of chronic renal impairment. Furthermore, pCG and CMPF are usually undetectable [17,29] or close to the LOQ [17] in patients with normal renal function. Even though our method's LOQ was similar to those described in the literature [16,17,30], it was nevertheless probably not low enough to detect an accumulation in the context of AKI. Given the preliminary nature of our results, the small number of patients, the marked variability in the time course of UT accumulation and the method's lack of sensitivity for CMPF and pCG, it is difficult to state definitively whether or not monitoring IAA, HA, TMAO, pCG, and CMPF is of value in an AKI setting. A study of a larger number of patients is required before firm conclusions can be drawn. *In vitro* and *in vivo* investigations have shown that CMPF, TMAO, HA and IAA are all substrates of organic anion transporters 1 and 3 (OAT1 and OAT3), although OAT1 mainly transports HA and IAA and OAT3 mainly transports CMPF and TMAO [31,32]. Thus, CMPF, TMAO, HA and IAA have much the same renal tubular secretion mechanism as IxS and pCS [32]. The disparities in accumulation might therefore be due to the toxins' differing affinities for these transporters, although there are currently no published data on this subject.

Interestingly, we found that the serum UT concentrations in our patients with cardiac surgery-associated AKI did not peak earlier than the creatinine concentration. Furthermore, the time interval between T_{\max} and the return to baseline values differed from one UT to another (respectively 39.8 h, 95.2 h, 12.8 h and 24.8 for IxS, pCS, IAA and HA) and from one patient to another. Nevertheless, the time to return to baseline values for pCS and IxS was similar to or even shorter to that observed for creatinine (114.3 h) – in contrast to Wang et al.'s recent results [13]. In the latter study, the IxS concentrations at day 7 (D7) of the AKI were always higher than those measured at D0 (i.e. when AKI had been diagnosed). However, the IxS concentrations at D7 showed high inter-individual variability. Furthermore, measurements at D7 were not available for all the cohort participants. Given that IxS is an UT secreted mainly by renal proximal tubular cells [33], this difference could be explained by the presence or absence of tubular necrosis. The results of Veldeman *et al.*'s study [14] also underline the

value of studying the elimination time course of pCS and IxS. A decrease in pCS and IxS concentrations was observed only in the group of patients with a favorable AKI outcome. Thus, a time-course assessment of UT elimination might provide insight into the mechanism of AKI and the severity of the associated lesions.

The identification of novel, early, specific biomarkers of renal impairment might enable the timely, appropriate management of AKI and so is currently an important research focus. Indeed, plasma creatinine is a far from ideal diagnostic biomarker of AKI because it is a late biomarker; in AKI, the first site of injury corresponds to the kidney's tubular cells [34]. Other promising urine and blood biomarkers (such as cystatin C, neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, interleukin-18 and liver-type fatty acid binding protein) [34,35] have been shown to be superior to plasma creatinine in (i) predicting the occurrence of AKI, (ii) discriminating between acute prerenal failure and tubular injury, and (iii) predicting mortality or the need for dialysis. However, there is still a need to define clear cut-off concentration thresholds with diagnostic and prognostic value in AKI. Furthermore, most of the markers have been validated in a urine matrix, which is an obvious limitation in the context of AKI. Lastly, the commercial kits needed to perform these assays are expensive and not routinely available. In this context, we consider that the assessment of IxS and pCS as prognostic markers for AKI is valuable and should be continued.

5. Conclusion

We have developed rapid, sensitive, accurate, specific methods for assaying seven UTs in a clinical context, such as AKI in critically ill patients. The simplicity of sample preparation and the low sample volume requirement are well suited to routine blood UT monitoring in a context of acute accumulation. The time courses of UT accumulation and elimination observed in eight patients who developed AKI within 72 hours of cardiac surgery highlighted the value of measuring serum pCS and IxS concentrations. In this context, we found that IAA, TMAO, CMPF, pCG and HA do not accumulate greatly. Our results emphasize the

value of seeking to assess the toxic effects of UT accumulation (and particularly pCS and IxS accumulation) in an AKI setting.

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Table 1: Spectrometer settings

Compound	ESI mode	m/z Q1	m/z Q3	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
TMAO	positive	76.1	58	31	12	10	23	4
		76.1	59	31	12	10	15	4
d9-TMAO	positive	85.1	66.1	46	11.5	10	27	4
		85.1	68	46	11.5	10	17	4
IAA	positive	176.2	130.1	31	10.5	12	19	4
		176.2	103.1	31	10.5	12	39	4
[¹³ C ₆]-IAA	positive	182.2	136	11	7	12	47	4
		182.2	109.1	11	7	12	19	4
CMPF	negative	238.8	195	-40	-7	-14	-12	-2
		238.8	151	-40	-7	-14	-24	0
d5-CMPF	negative	244	200	-35	-9	-14	-12	-2
		244	156	-35	-9	-14	-22	0
HA	negative	177.9	133.7	-35	-7.5	-12	-12	0
		177.9	76.8	-35	-7.5	-12	-24	0
pCS	negative	186.8	106.8	-40	-2.5	-12	-30	0
		186.8	79.9	-40	-2.5	-12	-30	0
d4-pCS	negative	190.8	111	-30	-2.5	-14	-30	0
		190.8	79.9	-30	-2.5	-14	-30	0
IxS	negative	211.9	79.9	-55	-2.5	-10	-34	0
		211.9	132	-55	-2.5	-10	-24	0
[¹³ C ₆]-IxS	negative	217.7	79.7	-65	-9.5	-12	-34	0
		217.7	138	-65	-9.5	-12	-24	0
pCG	negative	283	106.7	-50	-5.5	-16	-40	0
		283	112.7	-50	-5.5	-16	-18	0

Abbreviations: CE, collision energy; CEP, cell entrance potential; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate; CXP, collision cell exit potential; DP, declustering potential; EP, entrance potential; HA, hippuric acid; IAA, indole-3-acetic acid; IxS, indoxyl sulfate; pCG, para-cresyl glucuronide; pCS, para-cresyl sulfate; TMAO, trimethylamine N-oxide

Table 2: Calibration, LOD and LOQ

Nominal standards concentrations ($\mu\text{g mL}^{-1}$)		0.1	0.5	1	5	10	50	100	Calibration curve r^2	LOD ($\mu\text{g mL}^{-1}$)	LLOQ ($\mu\text{g mL}^{-1}$)
TMAO	Mean \pm SD measured concentrations	0.09 \pm 0.01	0.50 \pm 0.01	1.0 \pm 0.03	5.1 \pm 0.10	10.3 \pm 0.16	49.3 \pm 0.31	100.3 \pm 0.52	0.9999 \pm 0.00005	0.02	0.1
	CV (%)	5.85	2.58	2.74	1.89	1.57	0.64	0.51	0.01		
	Bias (%)	-8.17	0.90	4.27	1.30	2.62	-1.50	0.33	-0.01		
IAA	Mean \pm SD measured concentrations	N/A	0.49 \pm 0.07	1.0 \pm 0.14	5.3 \pm 0.46	10.2 \pm 1.04	49.7 \pm 2.05	100.1 \pm 0.80	0.9991 \pm 0.0007	0.05	0.5
	CV (%)	N/A	14.18	14.55	8.58	10.17	2.05	0.80	0.07		
	Bias (%)	N/A	-1.67	-0.52	6.76	1.94	-0.70	0.05	-0.09		
HA	Mean \pm SD measured concentrations	0.09 \pm 0.01	0.52 \pm 0.03	1.0 \pm 0.10	5.1 \pm 0.39	10.5 \pm 0.42	48.4 \pm 1.37	101.5 \pm 1.38	0.9994 \pm 0.0006	0.04	0.1
	CV (%)	14.01	5.28	9.45	7.66	4.01	2.84	1.36	0.06		
	Bias (%)	-7.54	3.43	2.62	2.57	5.02	-3.30	1.50	-0.08		
pCS	Mean \pm SD measured concentrations	0.10 \pm 0.00	0.47 \pm 0.01	1.0 \pm 0.05	5.2 \pm 0.18	10.0 \pm 0.24	49.7 \pm 0.59	100.0 \pm 0.04	0.9999 \pm 0.00007	0.006	0.1
	CV (%)	2.78	1.92	4.74	3.53	2.37	1.18	0.04	0.01		
	Bias (%)	-0.63	-5.20	2.77	3.77	-0.32	-0.67	-0.02	-0.01		
IxS	Mean \pm SD measured concentrations	0.09 \pm 0.01	0.50 \pm 0.04	1.0 \pm 0.11	4.9 \pm 0.40	10.1 \pm 0.84	50.0 \pm 1.24	100.0 \pm 0.54	0.9996 \pm 0.0004	0.04	0.1
	CV (%)	13.70	7.07	10.51	8.24	8.31	2.47	0.54	0.04		
	Bias (%)	-9.02	-0.90	3.62	-1.97	0.52	0.03	-0.02	-0.04		
CMPF	Mean \pm SD measured concentrations	0.11 \pm 0.01	0.49 \pm 0.07	0.9 \pm 0.11	5.0 \pm 0.53	10.2 \pm 0.49	49.7 \pm 0.46	100.0 \pm 0.12	0.9997 \pm 0.0002	0.03	0.1
	CV (%)	9.97	13.62	11.17	10.58	4.74	0.93	0.12	0.02		
	Bias (%)	5.27	-2.27	-5.53	0.83	2.40	-0.70	-0.05	-0.03		
pCG	Mean \pm SD measured concentrations	0.10 \pm 0.01	0.50 \pm 0.06	1.1 \pm 0.07	5.0 \pm 0.36	10.4 \pm 0.88	48.9 \pm 1.83	101.0 \pm 1.59	0.9993 \pm 0.0012	0.02	0.1
	CV (%)	14.83	11.27	6.47	7.18	8.44	3.75	1.57	0.12		
	Bias (%)	-4.80	-1.00	7.22	0.53	4.07	-2.23	0.95	-0.07		

Abbreviations: CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate; CV, coefficient of variation; HA, hippuric acid; IAA, indole-3-acetic acid; IxS, indoxyl sulfate; LOD, limit of detection; LLOQ, lower limit of quantification; pCG, para-cresyl glucuronide; pCS, para-cresyl sulfate; SD, standard deviation; TMAO, trimethylamine N-oxide

Table 3: Within-run and between-run accuracy and precision of the LC-MS-MS methods

Compound	Nominal concentration ($\mu\text{g mL}^{-1}$)	Within-run precision and accuracy			Between-run precision and accuracy		
		Mean \pm SD measured concentration ($\mu\text{g mL}^{-1}$) (n=6)	CV (%)	Bias (%)	Mean \pm SD measured concentration ($\mu\text{g mL}^{-1}$) (n=18)	CV (%)	Bias (%)
TMAO	0.5	0.52 ± 0.011	2.13	4.07	0.57 ± 0.036	6.37	4.07
	2.5	2.72 ± 0.046	1.69	8.93	2.75 ± 0.045	1.62	10.02
	25	25.23 ± 0.151	0.60	0.93	25.49 ± 0.513	2.01	1.96
	75	77.07 ± 1.117	1.45	2.76	82.39 ± 4.342	5.27	9.86
IAA	1.5	1.42 ± 0.199	14.02	-5.20	1.44 ± 0.166	11.59	-4.20
	2.5	2.73 ± 0.193	7.09	9.00	2.71 ± 0.171	6.28	8.58
	25	24.55 ± 0.999	4.07	-1.80	24.34 ± 1.098	4.51	-2.62
	75	84.32 ± 5.870	6.96	12.42	84.08 ± 4.950	5.89	12.10
HA	0.5	0.49 ± 0.073	14.96	-2.60	0.49 ± 0.067	13.64	-1.24
	2.5	2.54 ± 0.283	11.18	1.47	2.63 ± 0.332	12.62	5.38
	25	24.05 ± 1.218	5.06	-3.80	23.87 ± 0.976	4.09	-4.51
	75	79.47 ± 2.169	2.73	5.96	81.23 ± 3.579	4.41	8.31
pCS	0.5	0.51 ± 0.055	10.66	2.67	0.48 ± 0.062	12.92	-4.48
	2.5	2.86 ± 0.087	3.03	14.48	2.74 ± 0.151	5.51	9.73
	25	27.27 ± 0.812	2.98	9.07	25.74 ± 1.572	6.10	2.98
	75	82.75 ± 2.500	3.02	10.33	84.47 ± 5.270	6.23	12.62
IxS	0.5	0.52 ± 0.020	3.85	3.80	0.53 ± 0.045	8.47	6.10
	2.5	2.25 ± 0.154	6.82	-9.84	2.55 ± 0.362	14.23	-9.84
	25	22.96 ± 2.464	10.73	-8.16	21.37 ± 2.270	10.62	-14.49
	75	67.28 ± 5.205	7.74	-10.29	63.91 ± 9.290	14.54	-14.79
CMPF	0.5	0.48 ± 0.018	3.68	-4.83	0.48 ± 0.047	9.87	-3.82
	2.5	2.48 ± 0.084	3.41	-0.67	2.86 ± 0.394	13.76	14.51
	25	23.52 ± 0.801	3.41	-5.93	23.90 ± 2.607	10.91	-4.38
	75	68.57 ± 2.805	4.09	-8.58	75.02 ± 6.139	8.18	0.03
pCG	0.5	0.56 ± 0.059	10.54	11.53	0.50 ± 0.064	12.78	0.67
	2.5	2.87 ± 0.317	11.02	14.93	2.84 ± 0.311	10.97	13.47
	25	26.32 ± 1.622	6.16	5.27	26.21 ± 1.877	7.16	4.84
	75	81.83 ± 6.309	7.71	9.11	85.99 ± 5.786	6.73	14.65

Abbreviations: CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate; CV, coefficient of variation; HA, hippuric acid; IAA, indole-3-acetic acid; IxS, indoxyl sulfate; pCG, para-cresyl glucuronide; pCS, para-cresyl sulfate; SD, standard deviation; TMAO, trimethylamine N-oxide

Table 4: Stability

Compound	Nominal concentration ($\mu\text{g mL}^{-1}$)	Percentage loss of concentration (%)			
		After 6 hours at 4°C	After 24 hours at 4°C	After 1 month at -20°C	After 3 freeze-thaw cycles
TMAO	2.5	1.12	2.31	23.7	7.04
	25	3.42	1.46	27.7	2.95
	75	1.23	4.11	19.5	2.23
IAA	2.5	7.73	1.68	6.08	23.09
	25	3.13	6.87	9.52	13.20
	75	1.41	2.23	3.23	6.19
HA	2.5	11.22	8.50	7.54	8.60
	25	3.81	1.30	0.42	19.48
	75	0.69	10.13	5.52	18.82
pCS	2.5	9.97	2.62	4.41	4.80
	25	5.65	5.99	8.48	3.33
	75	7.65	4.60	10.37	8.05
IxS	2.5	4.08	9.48	2.64	25.57
	25	6.32	3.23	2.73	20.86
	75	2.83	0.80	2.49	26.13
CMPF	2.5	4.57	12.00	1.94	9.21
	25	0.18	8.54	5.72	0.30
	75	4.82	12.09	12.85	1.69
pCG	2.5	0.39	7.04	3.36	1.62
	25	1.36	12.17	10.91	11.28
	75	2.17	10.25	1.78	17.30

Abbreviations: CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate; HA, hippuric acid; IAA, indole-3-acetic acid; IxS, indoxyl sulfate; pCG, para-cresyl glucuronide; pCS, para-cresyl sulfate; TMAO, trimethylamine N-oxide

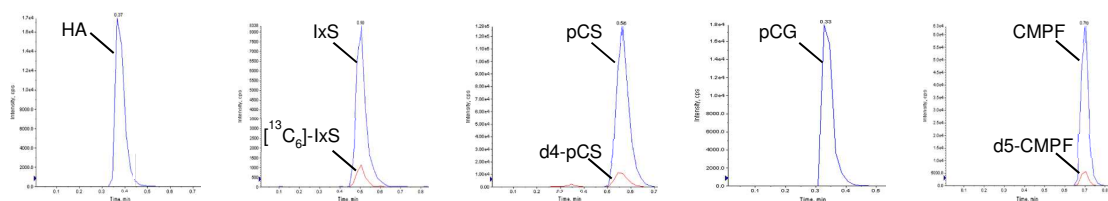
Table 5: Demographic characteristics and key UT pharmacokinetic parameters for the eight patients included in the study

Demographic characteristics	
Age (yrs), m (min - max)	67.75 (42 - 83)
Male, n (%)	7 (87.5)
BMI (kg m ⁻²), m (min - max)	28.60 (22.9 - 39.1)
UTs	
Creatinine	
Basal concentration (μmol L ⁻¹), m (min - max)	98.875 (70 - 171)
Peak concentration (μmol L ⁻¹), m (min - max)	254.25 (107 - 493)
Time to creatinine peak concentration (h), m (min - max)	64.25 (34 - 137)
Time to return to baseline concentration (h), m (min - max)	114.25 (23 - 181)
IxS	
Basal concentration (μg mL ⁻¹), m (min - max)	0.56 (0.23 - 0.97)
Peak concentration (μg mL ⁻¹), m (min - max)	1.52 (0.35 - 2.62)
Time to IxS peak concentration (h), m (min - max)	63.50 (23 - 174)
Time to return to baseline concentration (h), m (min - max)	39.83 (7 - 87)
pCS	
Basal concentration (μg mL ⁻¹), m (min - max)	3.30 (1.24 - 8.75)
Peak concentration (μg mL ⁻¹), m (min - max)	9.33 (4.30 - 16.00)
Time to pCS peak concentration (h), m (min - max)	73.26 (23 - 213)
Time to return to baseline concentration (h), m (min - max)	95.20 (27 - 148)
IAA	
Basal concentration (μg mL ⁻¹), m (min - max)	0.57 (0.31 - 1.31)
Peak concentration (μg mL ⁻¹), m (min - max)	0.97 (0.60 - 1.80)
Time to IAA peak concentration (h), m (min - max)	60.00 (2 - 160)
Time to return to baseline concentration (h), m (min - max)	12.80 (7 - 144)
HA	
Basal concentration (μg mL ⁻¹), m (min - max)	1.27 (0.34 - 2.09)
Peak concentration (μg mL ⁻¹), m (min - max)	2.85 (1.99 - 4.50)
Time to HA peak concentration (h), m (min - max)	49.80 (19 - 96)
Time to return to baseline concentration (h), m (min - max)	24.75 (7 - 38)
TMAO	
Basal concentration (μg mL ⁻¹), m (min - max)	0.19 (0.11 - 0.32)
Peak concentration (μg mL ⁻¹), m (min - max)	0.68 (0.59 - 1.15)
Time to TMAO peak concentration (h), m (min - max)	100.80 (39 - 223)
Time to return to baseline concentration (h), m (min - max)	73.00 (15 - 194)

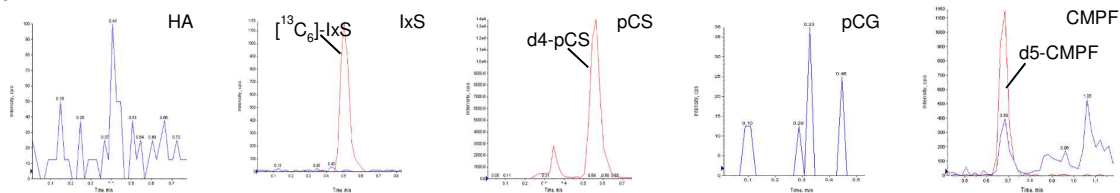
Abbreviations: BMI, body mass index; h, hours; HA, hippuric acid; IAA, indol-3-acetic acid; IxS, indoxyl sulfate; m, mean; min, minimum; max, maximum; pCS, para-cresyl sulfate; TMAO, trimethylamine N-oxide; UTs, uremic toxins; yrs, years

Figure 1

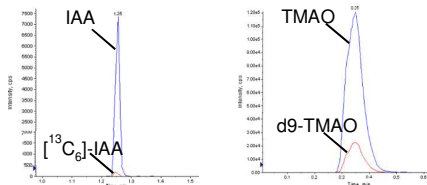
a



b



c



d

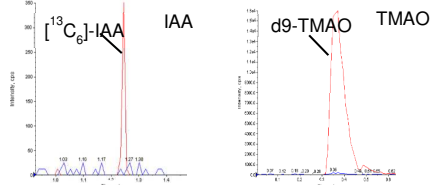


Figure 2

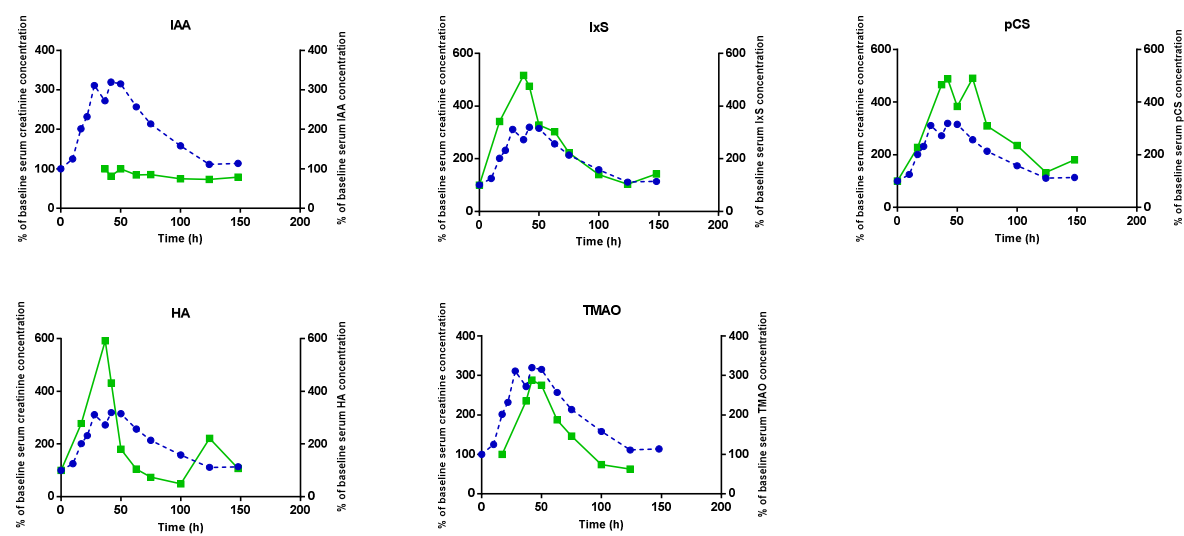


Figure legends

Figure 1: representative chromatograms of UTs after acquisition in MRM mode with negative-mode ionization for (a) HA, IxS, pCS, pCG and CMPF at 10 $\mu\text{g mL}^{-1}$ and (b) for a sample blank. Representative chromatograms of UTs after acquisition in MRM mode with positive-mode ionization for (c) IAA and TMAO at 10 $\mu\text{g mL}^{-1}$ and (d) a sample blank. Abbreviations: HA, hippuric acid; IxS, indoxyl sulfate; pCS, para-cresyl sulfate; pCG, para-cresyl glucuronide; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate; IAA, indole-3-acetic acid; TMAO, trimethylamine N-oxide.

Figure 2: Representative serum UT concentrations (in green) and serum creatinine concentrations (in blue) versus time, after admission of a patient to the Thoracic and Vascular Surgical ICU at Amiens University Medical Center for further treatment after cardiac surgery. Values for creatinine and UT concentrations are expressed as a percentage of the corresponding baseline concentration on day 1 of the stay in the ICU. The CMPF and pCG concentrations were below the LOQ for all measurements. Abbreviations: IAA, indole-3-acetic acid; IxS, indoxyl sulfate; pCS, para-cresyl sulfate; HA, hippuric acid; TMAO, trimethylamine N-oxide; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate; pCG, para-cresyl glucuronide.