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Development of a LC–MS/MS analytical method of 15 compounds related to renal function for a prognostic method of progression risk in patients with diabetic kidney disease

Ryota Kujirai [a], Yotaro Matsumoto [a,*], Mizuki Abe [a], Kodai Hiramoto [a], Takumi Watanabe [a], Chitose Suzuki [b], Takafumi Toyohara [b,c], Takaaki Abe [b,c,d], Yoshihisa Tomioka [a]

[a] Laboratory of Oncology, Pharmacy Practice and Sciences, Tohoku University Graduate School of Pharmaceutical Sciences, 6–3, Aoba, Aramaki, Aoba-ku, Sendai 980–8578, Japan
[b] Division of Nephrology and Hypertension, Tohoku University Hospital, 1–1, Seiryo-machi, Aoba-ku, Sendai 980–8574, Japan
[c] Division of Medical Science, Tohoku University Graduate School of Biomedical Engineering, 6–6–12, Aoba, Aramaki, Aoba-ku, Sendai 980–8579, Japan
[d] Department of Clinical Biology and Hormonal Regulation, Tohoku University Graduate School of Medicine, 2–1, Seiryo-machi, Aoba-ku, Sendai, 980–8575, Japan

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ABSTRACT

Diabetic kidney disease (DKD) onset and progression is a major cause of end-stage renal failure in diabetic patients, however, no practical method has been reported to predict the progression rate of renal function decline. Nine serum compounds are reported to associate with prognosis in type 1 diabetes patients; however, quantitative analytical methods for these compounds lacks. Herein, we developed a simultaneous quantitative method for 15 compounds, including Niewczas's nine biomarker candidates, associated with renal function and its prognosis in kidney disease patients to achieve a prognostic method of renal function decline in DKD patients. This report describes the development and validation of a LC–MS/MS analytical method for 15 compounds of biomarker candidates using human plasma, serum, and urine as sample matrices. The analytes are N-acetyl-L-alanine, N6-acetyl-L-lysine, N-acetyl-L-serine, N-acetyl-L-threonine, phenyl sulfate, pseudouridine, N6-threonylcarbamoyladenosine, tryptophan 2-C-mannoside, tyrosine O-sulfate, creatinine, p-cresol sulfate, 4-ethylphenyl sulfate, indoxyl sulfate, N1-methyladenosine, and trimethylamine N-oxide. The Capcell Pak ADME-HR column was compared to several general columns and selected as the most suitable column for the simultaneous analysis of all 15 compounds. The proposed method was validated for selectivity, accuracy, precision, stability, dilution integrity, and parallelism. This report describes the suitability of the calibration ranges established and the actual sample concentrations of serum and urine from type 2 diabetic patients, as well as new findings on the unknown analyte levels of several compounds in these samples. The proposed method can be used to aid the development of prognostic methods for renal function decline in patients with DKD.

1. Introduction

Diabetic kidney disease (DKD) is a chronic complication of type 1 diabetes (T1D) that develops in approximately one-third of T1D patients [1,2]. The progressive decline of renal function in patients with DKD leads to end-stage renal disease (ESRD), increasing the risk of cardiovascular disease and death [1,3]. The rate of renal function decline in DKD is heterogeneous, ranging from non-progressive to very rapid [4,5]. Krolewski et al. [6] reported four renal function decline rate categories in the Joslin T1D ESRD Cohort: very fast (>15 mL/min/1.73 m^3 of annual eGFR decline), fast (>10 mL), moderate (>5 mL), and slow (≤5 mL). Because patients with very fast and fast renal function decline rates require intervention within 10 years of the start of decline until the onset of ESRD, clinicians must be able to predict the progression rate of renal function and ESRD risk. To the best of our knowledge, however, there are no practical and effective methods for predicting this risk. In typical diabetic nephropathy, the urinary albumin-to-creatinine ratio (UACR) has been used as a predictor of renal function decline [4,7]. However, patients with renal function decline without albuminuria have limited the suitability of UACR as a predictive biomarker [4,6]. Several reviews [8–10] have reported candidate biomarkers that are correlated with prospective renal function decline and ESRD progression in patients

* Corresponding author.
E-mail address: yotaro.matsumoto.a5@tohoku.ac.jp (Y. Matsumoto).

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with DKD from T1D or type 2 diabetes (T2D). However, these biomarkers do not have sufficient predictive power to meet clinical requirements, and the discovery of a novel and effective biomarker is required.

Niewczas et al. [11] discovered nine serum metabolites positively correlated with annual eGFR decline in patients with DKD and T1D. These metabolites are as follows: N-acetyl-L-alanine (acAla), N6-acetyl-L-lysine (ac^{6}Lys), N-acetyl-L-serine (acSer), N-acetyl-L-threonine (acThr), phenyl sulfate (PS), pseudouridine (psiU), N6-threonylcarbamoyladenosine (t^{6}A), tryptophan 2-C-mannoside (cManTrp), and tyrosine O-sulfate (TyrS). However, the accuracy of the predictive equation generated by logistic regression analysis using these metabolites was inadequate (C-index = 0.75 at maximum). One of the reasons for the low predictive power is that the quantitation method of Niewczas's study was only a comparison of peak area. Accurate quantitative methods are required to demonstrate the predictive power of these nine metabolites; however, to the best of our knowledge, validated quantitative methods for these metabolites have not been well reported. Although analytical methods for psiU and acThr have been reported by Freed et al. [12] and those for ac^{6}Lys have been reported by Gessner et al. [13], they have not been reported comprehensively enough. Therefore, the development of a new quantitative analytical method for candidate biomarker compounds is necessary for the discovery of clinically effective biomarkers and the development of prognostic methods.

For biomarker discovery, high-throughput quantitative methods are required, which have a large number of candidate analytes. This necessity arise from the high volume of clinical samples requiring analysis, as a large number of candidate analytes increases hit rate, and multivariate discriminants are more accurate than univariate ones [14]. To develop a quantitative method for candidate biomarkers found by Niewczas et al., we added six additional analytes that were previously focused on a previous study [15]: creatinine (Cre), p-cresol sulfate (pCS), 4-ethylphenyl sulfate (4EPS), indoxyl sulfate (IS), N1-methyladenosine (m1A), and trimethylamine N-oxide (TMAO). These compounds are known as uremic toxins, organic waste compounds that influence the progression of chronic kidney disease (CKD) and cardiovascular disease (CVD) [16]. In addition, Cre is a key variable in GFR, has a well-known association with renal function, and plays the role of a correction factor in urine sample analysis. We also reported the potential role of m1A as biomarkers of CKD progression [17]. Furthermore, Rysz et al. [18] reviewed the association of TMAO, pCS, and IS with the risk of renal function progression and CVD and death. Thus, these uremic toxins can also be candidate biomarkers for prognosis prediction. This report describes the development and validation of a liquid chromatography–tandem mass spectrometry (LC–MS/MS) quantitative method of 15 candidate predictive biomarker compounds in advance of research to develop a prognostic method of renal function decline in patients with DKD with T1D.

2. Materials and methods

2.1. Reagents

The reagents were obtained as follows: Cre and psiU from Fujifilm Wako Chemicals (Osaka, Japan); m^{1}A and acAla from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); TMAO, PS potassium salt, pCS potassium salt, and ac^{6}Lys from Tokyo Chemical Industry (Tokyo, Japan); IS potassium salt and [^{2}H$_3$]-Cre from Cayman Chemical Company (Ann Arbor, MI, USA); [^{2}H$_9$]-TMAO from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA); 4EPS potassium salt, [^{2}H$_4$]-IS potassium salt, acThr, cManTrp, [^{2}H$_4$]-cManTrp, and [^{13}C$_1$,^{15}N$_2$]-psiU from Toronto Research Chemicals (North York, ON, Canada); and acSer from Watanabe Chemical Industries (Hiroshima, Japan). [^{2}H$_5$]-m^{1}A, [^{13}C$_6$]-PS sodium salt, [^{2}H$_4$]-pCS potassium salt, [^{2}H$_4$]-4EPS potassium salt, [^{2}H$_3$]-acAla, [^{2}H$_3$]-acSer, [^{13}C$_4$,^{15}N$_1$]-acThr, [^{2}H$_3$]-ac^{6}Lys, t^{6}A, [^{13}C$_4$,^{15}N$_1$]-t^{6}A, TyrS sodium salt, and [^{2}H$_4$]-TyrS sodium salt were synthesized and characterized in our laboratory. Liquid chromatography/mass spectrometry (LC/MS)-grade methanol (MeOH), 2-propanol, and acetonitrile (MeCN) were obtained from Kanto Chemical Company (Tokyo, Japan), while LC/MS-grade ammonium formate (NH_4OFo), formic acid (FoOH), ammonium acetate (NH_4OAc), and acetic acid (AcOH) were purchased from Fujifilm Wako Chemicals (Osaka, Japan). Ultrapure-grade water was prepared in-house using a PureLab flex obtained from Organo Corporation (Tokyo, Japan). Human plasma, serum, and urine samples for method validation were obtained from Biopredic International (Rennes, France), Clinical Trials Laboratory Services Limited (Allentown, PA, USA), and BioIVT (Westbury, NY, USA).

2.2. Preparation of stock solutions and calibration standards

All reference standards of the 15 analytes and their corresponding stable isotope-labeled internal standards (SILISs) (Fig. S1) were accurately weighed and dissolved separately with water as stock solutions. Primary standard solutions were prepared by mixing the stock solutions according to the calibration ranges listed in Table 1. SILIS solutions were prepared with the compositions listed in Tables S1 and S2. The primary standard solutions and SILIS solutions were prepared for blood and urine analysis, respectively. All solutions were stored at − 80 °C. Working standard solutions were prepared by stepwise dilution of the primary standard solutions with water. QC samples were prepared by spiking

Table 1
Analyte names, internal standards, and calibration ranges.

Analyte name	Internal standard	Calibration range (μM)	
		Plasma and serum	Urine
N-acetyl-L-alanine (acAla)	[^{2}H$_3$]-acAla	0.4–200	2–1000
N6-acetyl-L-lysine (ac^{6}Lys)	[^{2}H$_3$]-ac^{6}Lys	0.01–5	0.2–100
N-acetyl-L-serine (acSer)	[^{2}H$_3$]-acSer	0.1–50	1–500
N-acetyl-L-threonine (acThr)	[^{13}C$_4$,^{15}N$_1$]-acThr	0.1–50	1–500
Phenyl sulfate (PS)	[^{13}C$_6$]-PS	0.04–200	4–2000
Pseudouridine (psiU)	[^{13}C$_1$,^{15}N$_2$]-psiU	0.2–100	4–2000
N6-threonylcarbamoyladenosine (t^{6}A)	[^{13}C$_4$,^{15}N$_1$]-t^{6}A	0.02–10	0.2–100
Tryptophan 2-C-mannoside (cManTrp)	[^{2}H$_4$]-cManTrp	0.02–10	0.4–200
Tyrosine O-sulfate (TyrS)	[^{2}H$_4$]-TyrS	0.04–20	2–1000
Creatinine (Cre)	[^{2}H$_3$]-Cre	2–1000	100–50000
p-Cresol sulfate (pCS)	[^{2}H$_4$]-pCS	0.2–1000	10–5000
4-Ethylphneyl sulfate (4EPS)	[^{2}H$_4$]− 4EPS	0.004–20	0.04–20
Indoxyl sulfate (IS)	[^{2}H$_4$]-IS	0.4–200	4–2000
N1-methyladenosine (m^{1}A)	[^{2}H$_5$]-m^{1}A	0.004–2	0.2–100
Trimethylamine N-oxide (TMAO)	[^{2}H$_9$]-TMAO	0.1–500	20–10000

pooled human plasma, serum, or urine with the working standard solutions at the following four concentration levels: 3/4 of the upper limit of quantification (ULOQ) (high QC), 1/20 of the ULOQ (middle QC), 2.5 times the lower limit of quantification (LLOQ) (low QC), LLOQ (LLOQ QC), and 10 times the ULOQ (super QC).

2.3. Sample processing

A 10-μL aliquot of the working standard solution or study sample of plasma or serum was mixed with 10 μL of SILIS solution, 20 μL of water, and 100 μL of MeCN–FoOH (100:0.1, v/v). The sample mixture was homogenized for 10 min in an ultrasonic bath. After centrifugation at 20,400 g for 10 min at 4 °C, 100 μL of the supernatant was collected and evaporated to dryness using a centrifugal concentrator for 30 min at 40 °C. The dried sample was reconstituted with 20 μL of mobile phase A solution, and a 5-μL aliquot of the sample was injected into LC–MS/MS. When preparing a urine sample, the amount of supernatant collected after centrifugation was decreased from 100 μL to 20 μL.

2.4. LC–MS/MS system

LC–MS/MS analysis was performed on an ACQUITY UPLC system (Waters, Milford, MA, USA) coupled to a TSQ Endura triple quadrupole mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was performed on a Capcell Pak ADME-HR column (2.1 mm i.d. × 100 mm, 2 μm; Osaka Soda, Osaka, Japan) at 40 °C. Mobile phase A was NH_4OFo (2.5 mM)–FoOH (100:0.1, v/v), while mobile phase B was MeOH. The flow rate was 0.4 mL/min. An elution gradient was used as follows: initial elution with 0% B, followed by a linear gradient to 20% B from 1.0 to 2.0 min, to 100% B from 2.0 to 4.0 min, and 100% B until 6.0 min to wash the column. Thereafter, the column was re-equilibrated with 0% B for 1.5 min. The flow rate was increased to 0.6 mL/min between 4.4 and 6.0 min. A post-column addition of 2-propanol was used at a flow rate of 0.6 mL/min from 0.5 to 4.4 min. The total run time for each sample analysis was 7.5 min. The mass spectrometer was equipped with an electrospray ionization source. Electrospray ionization was performed in both positive and negative ion modes, and the spray voltage was 1000 V for both modes. The vaporizer temperature and ion transfer tube temperature were 300 °C and 250 °C, respectively. Nitrogen gas was used for nebulization and desolvation, and argon gas was used as the collision gas. The gas conditions were as follows: a sheath gas pressure of 60 arbitrary units, auxiliary gas pressure of 20 arbitrary units, and collision gas pressure of 2.0 mTorr. The mass spectrometer was operated in the selected reaction monitoring mode. The mass transition, RF lens voltage, and collision energy conditions are presented in Table 2. To increase the sensitivity, the number of analytes that were scanned simultaneously was limited to 10 by shifting the scan time for each analyte. LC–MS/MS control, data acquisition, and data processing were performed using Xcalibur.

2.5. Comparison of analytical columns

The retention of 15 compounds was compared on five columns: ACQUITY UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm, Waters), ACQUITY UPLC HSS T3 (2.1 mm × 50 mm, 1.8 μm, Waters), ACQUITY UPLC BEH Amide (2.1 mm × 50 mm, 1.7 μm, Waters), Scherzo SS-C18 (2.0 mm × 50 mm, 3 μm, Imtakt, Kyoto, Japan), and the Capcell Pak ADME-HR. The following solvents were combined and used as mobile phases: water containing 0.1–0.2% acid (FoOH or AcOH), 1–10 mM salt (NH_4OFo or NH_4OAc), or buffers combining these acids and salts as phase A, and MeOH or MeCN as phase B. For the Scherzo SS-C18 column, the salts were also added to mobile phase B, and salt concentration was optimized for good elution on the ion-exchange system. The dead times (t_0) were calculated from the column volume, flow rate, and no-column elution times, which were measured using Eq. (1). The no-column elution times derived from instruments were 0.15 min for the Scherzo SS-C18 and 0.07 min for the other columns. This difference in no-column elution times was owing to the use of alternate instrument for the Scherzo SS-C18 column, namely a Nanospace si-2 HPLC system (Osaka Soda) with a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific).

$$t_0 = \frac{0.5 \times column\ length \times (internal\ diameter)^2}{flow\ rate} + non\text{-}column\ elution\ time \quad (1)$$

2.6. Calibration and method validation

Calibration and method validation were performed following the 2018 FDA guidance [19]. Because all analytes in this study were endogenous compounds, simple water was used as a surrogate matrix. The validation procedure was partially modified, and parallelism was demonstrated. Calibration was performed using 9 or 12 concentration points of the reference standard; the calibration ranges are displayed in Table 1. Regression models were tested in sequence from a linear function to a logarithmic quadratic function, and the simplest fitting model was selected.

The method was validated in terms of selectivity, accuracy and precision (A&P), stability, dilution integrity, and parallelism. The validation criteria were also set following the 2018 FDA guidance. Selectivity was evaluated by analyzing the single-donor plasma, serum, and urine samples (six individuals each). Almost all reference standard

Table 2
Instrument parameters for liquid chromatography–tandem mass spectrometry analysis.

Analyte	Retention time (min)	Mass transition [qualifier ion]		Polarity	Collision (eV)	RF lens (V)
		Reference standard	Internal standard			
acAla	2.54	130.1–88.0 [–*]	133.1–89.0	–	10	70
ac^6Lys	1.42	189.1–126.1 [84.1]	192.1–129.1	+	10	80
acSer	1.18	146.1–116.1 [74.1]	149.1–119.1	–	10	70
acThr	2.13	160.1–98.1 [74.1]	165.1–101.1	–	15	60
PS	3.24	173.0–93.0 [80.0]	179.0–99.1	–	20	90
psiU	1.38	243.1–153.0 [140.1]	246.1–156.0	–	15	100
t^6A	3.45	413.2–136.1 [281.1]	418.2–136.1	+	30	110
cManTrp	3.06	367.1–230.1 [245.1]	371.2–234.1	+	20	120
TyrS	1.80	260.0–180.1 [199.1]	264.0–184.1	–	20	140
Cre	0.84	114.1–44.0 [72.1]	117.1–47.0	+	20	80
pCS	3.63	187.0–107.1 [80.0]	191.0–111.1	–	20	100
4EPS	3.90	201.0–121.1 [80.0]	205.0–125.1	–	20	100
IS	3.32	212.0–80.0 [104.1]	216.0–80.0	–	20	100
m^1A	1.95	282.1–150.1 [133.1]	287.1–155.1	+	20	120
TMAO	0.84	76.1–58.1 [59.0]	85.1–66.1	+	20	80

* No quantifier of acAla contributing to evaluating selectivity was detected.

compounds were endogenous in the blood and urine samples; therefore, their selectivity was evaluated by the relative error of the quantifier/qualifier ratio (Q/q) between the unspiked authentic matrix and the spiked surrogate matrix of approximately the same concentration. A&P was evaluated with the four QC levels of high, middle, low, and LLOQ. An independent A&P run included five replicates per QC level and was repeated three times. The accuracy was determined by the following equation (Eq. (1)), while the endogenous concentration was determined by analyzing the level of the unspiked authentic matrix sample five times. The precision was evaluated by the coefficient of variance (CV) of five replicates on the first run for intra-assay precision and 15 replicates over three runs for inter-assay precision. The stability of the analytes under the expected sample storage and handling conditions was evaluated for the high and low QC samples and the primary standard solutions. The tested conditions were room temperature for 6 h before sample processing (bench-top stability), 4 °C in an autosampler for 72 h (autosampler stability), five freeze–thaw cycles from − 80 °C to room temperature (freeze–thaw stability), and − 80 °C for 365–371 days (long-term stability). The dilution integrity was evaluated by the relative error calculated by the following equation (Eq. (2)). The super QC sample was diluted at a 20-fold rate in this test. Parallelism for the range above the endogenous concentration was evaluated by spiking the working standard solutions into the authentic matrix samples. The evaluation index was the spike recovery (Eq. (3)), which was calculated for each concentration compartment: 0.3–1, 1–3,., and 300–1000 times the endogenous concentration. The acceptance threshold of the 0.3–1-fold compartment was within 20% error, while the acceptance thresholds of the other compartments were within 15% error. Parallelism for the range below the endogenous concentration was evaluated by diluting the authentic matrix sample with the surrogate matrix at 3-, 10-, 30-, and 100-fold dilution rates. The evaluation index was the recovery for dilution linearity (Eq. (4)), which was calculated for each dilution rate.

$$Accuracy = \frac{After\ spiked\ concentration}{Exogenous\ spike\ concentration + Endogenous\ concentration} \quad (2)$$

$$Relative\ error_{Dilution\ integrity} = \frac{20 \times After\ spiked\ and\ diluted\ concentration}{Exogenous\ spike\ concentration + Endogenous\ concentration} - 1 \quad (3)$$

$$Spike\ recovery = \frac{After\ spiked\ concentration - Endogenous\ concentration}{Exogenous\ spike\ concentration} \times 100 \quad (4)$$

$$Recovery_{Dilution\ linearity} = \frac{After\ diluted\ concentration \times Dilution\ rate}{Endogenous\ concentration\ before\ dilution} \times 100 \quad (5)$$

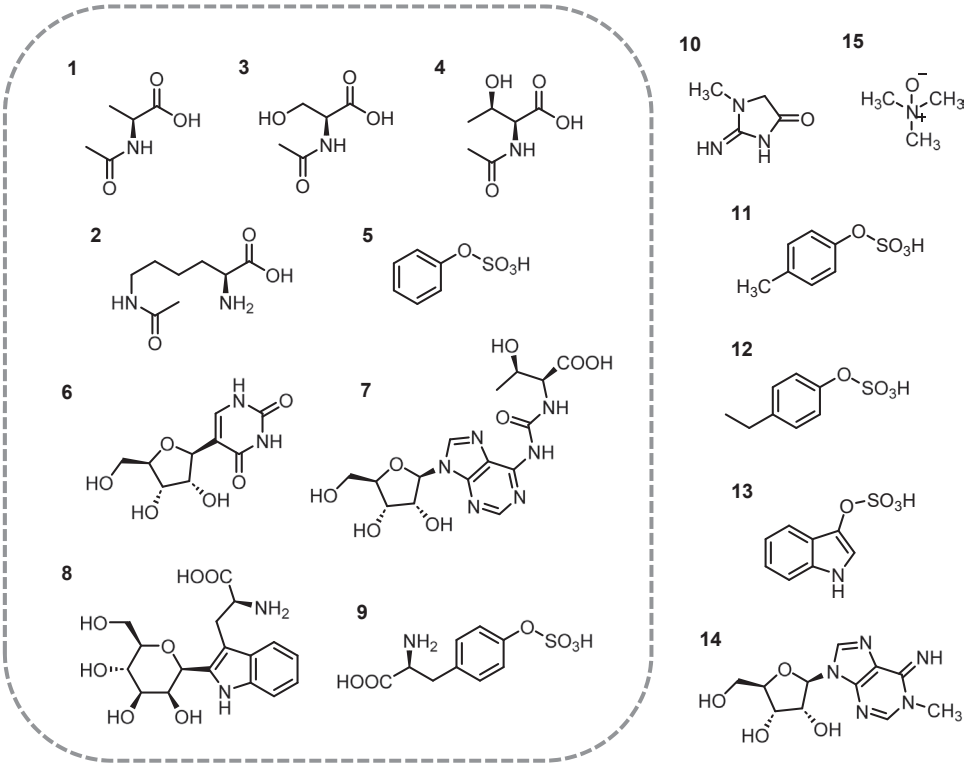

Fig. 1. Structure of 15 analytes. The compound numbers refer to the following analytes: (1) acAla, (2) ac^{6}Lys, (3) acSer, (4) acThr, (5) PS, (6) psiU, (7) t^{6}A, (8) cManTrp, (9) TyrS, (10) Cre, (11) pCS, (12) 4EPS, (13) IS, (14) m^{1}A, and (15) TMAO. The analytes surrounded by the dashed line are candidate biomarker compounds discovered by Niewczas et al. [11].

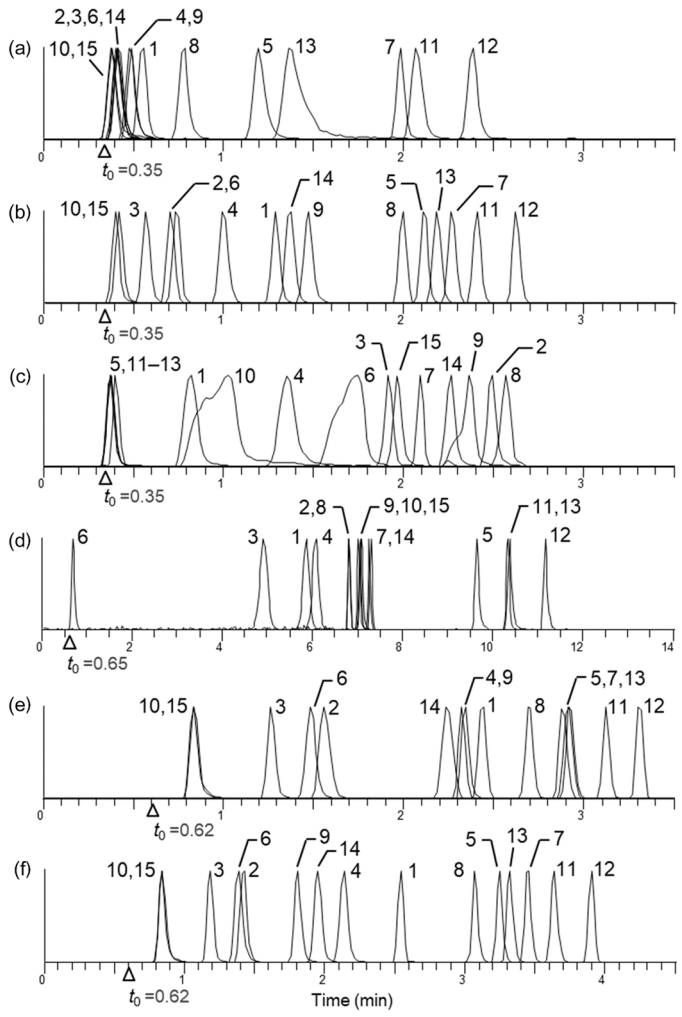

Fig. 2. MS/MS chromatograms obtained on five analytical columns.The peak numbers on the chromatograms refer to the following analytes: (1) acAla, (2) ac^{6}Lys, (3) acSer, (4) acThr, (5) PS, (6) psiU, (7) t^{6}A, (8) cManTrp, (9) TyrS, (10) Cre, (11) pCS, (12) 4EPS, (13) IS, (14) m^{1}A, and (15) TMAO. (**a**)–(**e**) In (a)– (c) and (e), the retention of the analytes on several analytical columns was tested under the condition of isocratic elution for 1 min followed by gradient elution for 2 min. Similarly, in (d), isocratic elution was performed for 4 min, and gradient elution was performed for 10 min. The gradient elution condition was a linear change of the mobile phase from a weak solvent for isocratic elution to a strong solvent. The analytical column, weak solvent, and strong solvent were as follows: (a) BEH C18 column, MeOH–H_2O–FoOH (10:90:0.1, v/v/v), MeOH–FoOH (100:0.1, v/v); (b) HSS T3 column, H_2O–FoOH (100:0.1, v/v), MeOH–FoOH (100:0.1, v/v); (c) BEH Amide column, MeCN–NH_4OFo (10 mM)–FoOH (95:5:0.01, v/v/v), MeCN–NH_4OFo (10 mM)–FoOH (50:50:0.1, v/v/v); (d) SS-C18 column, H_2O–acetatic acid (100:0.2, v/v), MeCN–ammonium acetate (200 mM) (60:40, v/v); (e) ADME-HR column, H_2O–FoOH (100:0.1, v/v), MeOH–FoOH (100:0.1, v/v). (**f**) MS/MS chromatograms of 15 analytes on the ADME-HR column under the optimized LC condition.

2.7. Clinical application of the proposed method

Forty-five patients with T2D, partially including DKD, treated at the outpatient clinic of Tohoku University Hospital, were recruited. The study protocol was approved by the ethics committee of Tohoku University (Reference number: 2022–1–823). All patients provided written informed consent. Patients' plasma and urine samples were analyzed as previously described and remeasured with dilution as needed.

3. Results

3.1. Chromatographic separation

The retention of the 15 analytes (Fig. 1) was tested with the BEH C18, the HSS T3, the BEH Amide, the Scherzo SS-C18, and the Capcell Pak ADME-HR columns, as illustrated in Fig. 2a–e. The MS/MS chromatograms are illustrated for the best retention conditions in acidic to neutral combinations of water and methanol or acetonitrile. In reversed-phase mode chromatography with the BEH C18 column, the five analytes PS, pCS, IS, 4EPS, and t^{6}A were retained, whereas the other 10 analytes were co-eluted at the dead time (t_0). Although using the HSS T3 column and starting the elution with 100% aqueous phase improved the retention of eight analytes, Cre and TMAO were not retained. Hydrophilic interaction liquid chromatography (HILIC) with the BEH Amide column did not retain four analytes PS, pCS, IS, and 4EPS. In addition, this column made the waveforms of Cre and psiU very broad. The Scherzo SS-C18 column, which was used in a previous study [15], retained 14 analytes; however, psiU was eluted at t_0. Finally, the Capcell Pak ADME-HR column retained all analytes with retention factors above 0.35 (t_0 = 0.62 min and retention time of Cre = 0.84 min) and exhibited good separation from each isobar in the biological samples. Therefore, the Capcell Pak ADME-HR column was selected for the simultaneous quantification of the 15 analytes in this study.

3.2. LC–MS/MS optimization

The optimal liquid chromatography mobile-phase condition was determined from various aqueous solutions with acid and/or salt as mobile phase A and MeOH or MeCN as mobile phase B. To increase the sensitivity, the post-column infusion method of 2-propanol was selected. The ionization conditions were optimized based on the analyte peak areas, especially for acSer, acThr, cManTrp, and t^{6}A, which had low levels in the blood samples and/or weak ionization efficiency. The RF lens voltage, mass transition, and collision energy were optimized for each compound, as displayed in Table 2. On Cre and its SILIS, the third optimal mass transition was selected to avoid detection saturation at a high concentration. IS had inseparable isobars at the optimal mass transition (212.0 −132.1) in the blood sample; therefore, the second optimal mass transition (212.0 −80.0) was selected.

3.3. Calibration and method validation

3.3.1. Calibration

Because all analytes were endogenous in the authentic matrix of plasma, serum, and urine, the surrogate matrix approach [20] was selected in this study. In the surrogate matrix approach, a surrogate matrix that is free of endogenous analytes (e.g., buffers, dialyzed serum) is used for the preparation of a calibration curve. In this study, simple water was used as a surrogate matrix. The calibration ranges were determined considering the respective analyte levels in blood and urine and the sensitivity of the instrument. Whereas calibration curves with nine points were prepared for most analytes, for TMAO, PS, pCS, and 4EPS in plasma and serum, calibration curves with 12 points were prepared to cover the broader analyte levels, as illustrated in Table 1. Examples of regression equations and coefficients of determination are presented in Tables S1 and S2. pCS and ac^{6}Lys were fitted to a linear regression model with a weighting of 1/concentration. TMAO could not be fitted to a simpler model than a logarithmic quadratic model, while the other analytes could not be fitted to a simple linear model (data not shown). The coefficients of determination were above 0.99 for all analytes. These calibration curves met the acceptance criteria for calibration curves, sensitivity, and carryover (Table S3) following the FDA guidance.

Table 3
Summarized results of method validation.

Item	Range of evaluation results over 15 analytes			Criterion
	Plasma and serum	Urine	Unit*2	
Selectivity	≤ 14.1	≤ 14.1	%RE of Q/q	≤ 15.0
of SILIS	≤ 2.6	≤ 3.4	%Peak area	≤ 5.0
Accuracy	85.4–113.3	89.6–110.0	%	85.0–115.0
of LLOQ	94.6–112.3	93.5–114.2	%	80.0–120.0
Intra-assay precision	≤ 5.2	≤ 8.3	%CV	≤ 15.0
of LLOQ	≤ 5.3	≤ 8.6	%CV	≤ 20.0
Inter-assay precision	≤ 12.1	≤ 10.1	%CV	≤ 15.0
of LLOQ	≤ 16.1	≤ 16.1	%CV	≤ 20.0
Stability				
Bench-top	− 13.9–8.6	− 6.8–6.9	%RE	− 15.0–15.0
Long-term	− 8.6–14.9	− 10.9–13.6	%RE	− 15.0–15.0
Autosampler	− 11.5–8.0	− 13.9–14.3	%RE	− 15.0–15.0
Freeze–thaw	− 3.8–5.5	− 5.1–6.9	%RE	− 15.0–15.0
Dilution integrity	− 10.2–8.3	− 10.0–4.4	%RE	− 15.0–15.0
	≤ 2.2	≤ 8.7	%CV	≤ 15.0
Parallelism				
Spike recovery	87.2–113.4	85.1–114.7	%	85.0–115.0
(acThr)	81.4–97.5	85.1–92.0	%	85.0–115.0
0.3–1 × *1	89.9–115.5	83.9–111.6	%	80.0–120.0
(acThr)*1	79.1 and 80.8	93.9	%	80.0–120.0
Dilution linearity	85.8–114.7	85.7–109.8	%	85.0–115.0

Note: The stated range includes the results of 15 analytes with up to three-point concentrations. *1This was the sample spiked 0.3–1 times the endogenous concentration. The error of spike recovery calculated in Eq. (3) tends to be larger in this group, so the criterion was set 5% wider. *2RE is relative error.

3.3.2. Selectivity

The selectivity was evaluated and met the acceptance criteria, as illustrated in Table 3 and S4. The selectivity of endogenous analytes cannot be evaluated by the standard procedure that verifies the absence of interference in a blank sample of a biological matrix. To evaluate the selectivity of endogenous analytes, a product ion called the qualifier, which is set differently from the product ion for quantitation (called the quantifier), is often used [21–23]. The precursor ion of an analyte compound should dissociate into products of the same composition independent of the sample matrix type. Thus, the little error of the Q/q between biological samples of the authentic matrix and calibration standard samples in the surrogate matrix suggests no interference from other compounds with the analyte peak. The identity of 14 endogenous analytes (excluding acAla) was confirmed on the error of the Q/q between the authentic matrix sample and spiked sample of the surrogate matrix. The error of the Q/q of the 14 analytes was within 14.1% as an absolute value. AcAla did not have a suitable qualifier with a sufficiently strong peak intensity for this test. The 15 analytes (including acAla) were also confirmed for the waveform of ion peaks, as illustrated in Fig. S2. The selectivity of the internal standards was evaluated by the standard procedure. Interference in the biological samples of the authentic matrix was less than 2.6% (plasma and serum) and less than 3.4% (urine) compared to the samples spiked with SILIS solution.

3.3.3. A&P

The validation results of A&P are presented in Table 3 and S5–S7. The accuracy was evaluated by the recovery, as expressed in Eq. (1), and was 85.4%− 113.3% (plasma and serum) and 89.6%− 114.2% (urine). The precision included the intra-assay precision (n = 5) and intra-assay precision (n = 15). The intra-assay precision was less than 5.3% (plasma and serum) and less than 8.6% (urine), whereas the inter-assay precision was less than 16.1%. Excluding the LLOQ, the inter-assay precision was less than 12.1% (plasma and serum) and less than 10.1% (urine).

3.3.4. Stability

All the analytes met the stability acceptance criteria and were sufficiently stable under each of the conditions expected in actual operation (Table 3 and S8–S11). The validated conditions were as follows: bench-top at room temperature for 6 h before sample processing, standby in an autosampler for 3 d after sample processing, five freeze–thaw cycles, and storage in a deep freezer set to − 80 °C for 12 months. The changes in analyte levels in these conditions were as follows: − 13.9–8.6%, − 10.9–14.9%, − 14.9–14.3%, and − 5.1–6.9%, respectively.

3.3.5. Dilution integrity

For adaptation to highly concentrated samples that exceeded the calibration ranges, the dilution integrity was evaluated at a 20-fold dilution rate using super QC samples, which were authentic matrix samples spiked with standard solution of 10 times higher concentration than ULOQ, and met the acceptance criteria (Table 3 and S12). The pre-dilution analyte levels calculated from the measured post-dilution levels had errors of − 10.2–8.3% (plasma and serum) and − 10.0–4.4% (urine). The CV of dilution was less than 2.2% (plasma and serum) and less than 8.7% (urine).

3.3.6. Parallelism

In the surrogate matrix approach, a surrogate matrix that does not contain endogenous analytes is used to create the calibration curve. In general, the identity of the slopes of the calibration curves prepared with

Table 4
Serum and urine levels of the 15 analytes in patients with T2D.

Analyte	Serum level (μM)			Urine level (μmol/mmol creatinine)		
	Mean	SD	n of BLQ	Mean	SD	n of BLQ
acAla	1.74	0.55	0	0.69	0.77	18 (40%)
ac^{6}Lys	0.31	0.17	0	3.75	1.67	0
acSer	0.90	0.54	0	1.82	1.09	1 (2%)
acThr	0.58	0.33	0	9.66	3.97	0
PS	8.0	12.6	1 (2%)	43.3	54.8	3 (7%)
psiU	4.06	3.05	0	62.6	13.4	0
t^{6}A	0.053	0.038	0	1.33	0.30	0
cManTrp	0.26	0.23	0	3.82	1.12	0
TyrS	1.25	0.92	0	21.2	6.7	0
Cre	92.6	52.1	0	3335	2340	0
pCS	32.2	35.7	5 (11%)	149	141	5 (11%)
4EPS	1.22	1.61	1 (2%)	2.07	2.77	0
IS	8.4	7.4	0	85.2	56.3	0
m^{1}A	0.12	0.03	0	5.49	1.53	0
TMAO	10.5	12.8	0	197	167	0

The urine level of Cre is described as a simple concentration with a unit of "μM". The BLQ in the table mean the levels below the limit of quantitation or such samples.

a surrogate matrix and an authentic matrix is evaluated for parallelism [19,24]. In this study, however, the calibration curves for the 13 analytes were prepared using a logarithmic regression model, making it difficult to simply compare the slopes. Therefore, instead, the identity of each point of the calibration standard concentration was validated using spike recovery for the concentration range from the endogenous level to the ULOQ of each analyte. Parallelism based on spike recovery was successfully demonstrated for 14 analytes (excluding acThr), as illustrated in Table 3 and S13–S15. As calculated by Eq. (3), the spike recoveries, excluding acThr, were 83.9–111.6% in the sample spiked with the standard of 0.3–1 times the LLOQ and 85.1–114.7% in the other ranges. The spike recovery of acThr was 79.1–93.9% and 81.4–97.5%, respectively, which was less than 5% below the acceptance criteria at two points of the plasma matrix and one point of the serum matrix for the lower concentrations. In addition, the dilution linearity of the authentic matrix samples was evaluated to validate the parallelism for a concentration range lower than the endogenous level. Parallelism based on dilution linearity was successfully demonstrated for all analytes, as displayed in Table 3 and S13–S15. The recovery of the diluted authentic matrix samples, as displayed in Eq. (4), was 85.7–114.7% in all matrices.

3.3.7. Clinical application of the proposed method

To verify the applicability of the proposed quantification method to samples from real patients, serum and urine samples were obtained from 45 patients with diabetes mellitus including DKD. For convenience in obtaining a sufficient number of samples, the diabetes mellitus of all patients was T2D. The clinical characteristics of the patients are presented in Table S16. In summary, 57% of the patients were male with a mean age of 70 ± 11 years. Their renal function was as follows: 78% of the patients had an eGFR of 30 − 90 mL/min/1.73 m^3, and approximately half the number had microalbuminuria. The levels of the 15 analytes in the serum and urine samples of these patients were quantified, as presented in Table 4. The calibration ranges of the proposed method covered the analyte levels of almost all samples, except for some analytes. In 2–11% of the samples, the serum levels of PS, pCS, and 4EPS and the urine levels of PS, pCS, and acSer were below the LLOQ. Most notably, urinary acAla levels in 40% of the samples were below the LLOQ.

4. Discussion

The proposed analytical method simultaneously quantified 15 water-soluble analytes possessing a wide range of physicochemical properties in plasma, serum, and urine samples. Basically, reversed-phase, HILIC, and ion-exchange mode solid phases are first selected for analytical columns and BEH C18 and HSS T3, BEH Amide, and Scherzo SS-C18 columns were compared from each mode, respectively; however, these columns failed to retain all 15 compounds. BEH C18 and HSS T3 did not retain weak hydrophobic compounds, such as Cre and TMAO; conversely, BEH Amide did not retain the four sulfate compounds that have no functional groups to form hydrogen bonds with the solid phase of the column. In general, no retention of analytes in analytical columns should be avoided because it causes weak sensitivity of ion detection from ion suppression and poor column separation with isobars. In a previous report [15], Cre, TMAO, and the four sulfate compounds were co-retained by the Scherzo SS-C18 column, which operated in a mixed mode consisting of the reversed-phase mode and both ion-exchange modes. However, the Scherzo SS-C18 column did not retain psiU, even under the lowest elution power condition, suggesting that psiU has weak hydrophobicity and its basicity is too weak to respond to ionic bonding with the solid phase of the column. Therefore, we expected that a reversed-phase column with stronger polar retention than the HSS T3 column, or a mixed-mode column with HILIC mode and ion-exchange mode was suitable for retaining all 15 compounds. Herein, the ADME-HR columns were selected as solid phases that met the former requirement. Solid phases with high carbon numbers provide a greater retention of high hydrophobic compounds, whereas those with low carbon numbers favor the retention of polar compounds. The retention properties vary similarly depending on the ratio of surface modification of silica gel. In both cases, the hydrophobicity and surface polarity of reversed-phase columns exhibit a trade-off relation. ADME-HR columns have a basket-shaped adamantyl ethyl group comprising ten carbon atoms, which accentuates the silica gel surface and moderately increases both hydrophobicity and surface polarity compared to solid phases featuring linear alkyl groups of the same carbon number [25]. Thus, ADME-HR columns offer to a unique combination of hydrophobic interactions inherent in reversed-phase columns while achieving enhanced retention for polar compounds. This property enabled ADME-HR column to retain highly polar compounds such as Cre and TMAO, which were not retained by the HSS T3 column, all without sacrificing its ability to interact with hydrophobic compounds such as PS. The proposed analytical method was validated following 2018 FDA guidance [19]. Because the analytes were endogenous, the Q/q was used to validate the selectivity, and the procedure for validating the accuracy was modified. For the use of a surrogate matrix of water, the parallelism between the surrogate matrix and the authentic matrix was verified.

The following discusses problems in the development and validation of the proposed analytical method. First, the calibration range of acAla for urine (2–1000 μM) did not cover the urinary levels. As one of the reasons for this, acAla had constant high background noise (Fig. S2h), resulting in a higher LLOQ than the concentration range of the biological samples. This problem was difficult to solve because acAla did not have an alternative product ion with sufficient peak intensity. This lack of alternative product ion also made validation of the selectivity using the Q/q of acAla difficult. Second, acThr slightly failed the acceptance criteria in the validation of parallelism, with errors of less than 20%. This may have been caused by the LLOQ of acThr, which was close to the limit of detection, and/or the isobars of close retention time (Fig. S2j). These problems are difficult to solve by simple modifications; therefore, further improvement of the proposed method requires a machine with a higher detection sensitivity and/or better analytical column.

The validated analytical method was applied to serum and urine samples from 45 patients with T2D partially including DKD, and the analyte levels in these samples were reported. They included unreported accurate levels of acAla, ac^6Lys, acSer, acThr, t^6A, and TyrS. However, some analyte levels in serum and urine, particularly acAla in urine, were below the calibration range. The following discusses the comprehensiveness of the analyte levels in the clinical samples. The patients' renal function based on their GFR categories was mostly G2 (60–90 mL/min/1.73 m^2) and G3 (30–60 mL/min/1.73 m^2), covering the renal failure range from normal to moderate. However, the clinical samples did not adequately cover the low renal function of G4–5 (less than 30 mL/min/1.73 m^2). Although verification in these samples was inadequate, unpredictable analyte levels in these samples could be accurately quantified by sample dilution because the concentration of the 15 analytes is inversely correlated with renal function [13,15] and would be higher in G4–5 samples than in G2–3 samples.

5. Conclusions

This report describes the development of an LC–MS/MS quantitation system for 15 metabolites that have the potential to predict the rate of renal function decline in DKD patients caused by T1D. The proposed system was evaluated in terms of selectivity, accuracy, precision, stability, dilution integrity, and parallelism in plasma, serum, and urine matrices following 2018 FDA guidance [19] and was successfully validated for almost all combinations of matrix types and analytes. Good coverage of the calibration curves with clinical sample concentrations was also demonstrated. The proposed system can be widely applied not only for the development of a prediction method for the progression of renal function decline in DKD but also for a mechanistic study of renal diseases and prediction of renal function decline in diseases other than T1D.

CRediT authorship contribution statement

Ryota Kujirai: Methodology, Formal analysis, Investigation, Validation, Data curation, Project administration, Writing – Original draft preparation. **Yotaro Matsumoto:** Methodology, Investigation, Data Curation, Writing – Review & Editing. **Mizuki Abe:** Investigation, Validation, Data curation. **Kodai Hiramoto and Takumi Watanabe:** Investigation. **Chitose Suzuki and Takafumi Toyohara:** Data Curation. **Takaaki Abe:** Conceptualization, Resources, Funding acquisition, Writing - Review & Editing. **Yoshihisa Tomioka:** Supervision, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpbao.2023.100021.

References

[1] R.Z. Alicic, M.T. Rooney, K.R. Tuttle, Diabetic kidney disease: challenges, progress, and possibilities, Clin. J. Am. Soc. Nephrol. 12 (2017) 2032–2045, https://doi.org/10.2215/CJN.11491116.

[2] V. Harjutsalo, P.-H. Groop, Epidemiology and risk factors for diabetic kidney disease, Adv. Chronic Kidney Dis. 21 (2014) 260–266, https://doi.org/10.1053/j.ackd.2014.03.009.

[3] H.-J. Anders, T.B. Huber, B. Isermann, M. Schiffer, CKD in diabetes: diabetic kidney disease versus nondiabetic kidney disease, Nat. Rev. Nephrol. 14 (2018) 361–377, https://doi.org/10.1038/s41581-018-0001-y.

[4] A.S. Krolewski, Progressive renal decline: the new paradigm of diabetic nephropathy in type 1 diabetes, Diabetes Care 38 (2015) 954–962, https://doi.org/10.2337/dc15-0184.

[5] G. Penno, E. Russo, M. Garofolo, G. Daniele, D. Lucchesi, L. Giusti, V.S. Bornez, C. Bianchi, A. Dardano, R. Miccoli, S. Del Prato, Evidence for two distinct phenotypes of chronic kidney disease in individuals with type 1 diabetes mellitus, Diabetologia 60 (2017) 1102–1113, https://doi.org/10.1007/s00125-017-4251-1.

[6] A.S. Krolewski, J. Skupien, P. Rossing, J.H. Warram, Fast renal decline to end-stage renal disease: an unrecognized feature of nephropathy in diabetes, Kidney Int 91 (2017) 1300–1311, https://doi.org/10.1016/j.kint.2016.10.046.

[7] H.-H. Parving, B. Oxenbøll, P.A. Svendsen, J.S. Christiansen, A.R. Andersen, Early detection of patients at risk of developing diabetic nephropathy. A longitudinal study of urinary albumin excretion, Acta Endocrinol. 100 (1982) 550–555, https://doi.org/10.1530/acta.0.1000550.

[8] F. Barutta, S. Bellini, S. Canepa, M. Durazzo, G. Gruden, Novel biomarkers of diabetic kidney disease: current status and potential clinical application, Acta Diabetol. 58 (2021) 819–830, https://doi.org/10.1007/s00592-020-01656-9.

[9] W.R. Zhang, C.R. Parikh, Biomarkers of acute and chronic kidney disease, Annu. Rev. Physiol. 81 (2019) 309–333, https://doi.org/10.1146/annurev-physiol-020518-114605.

[10] P.R. Pereira, D.F. Carrageta, P.F. Oliveira, A. Rodrigues, M.G. Alves, M. P. Monteiro, Metabolomics as a tool for the early diagnosis and prognosis of diabetic kidney disease, Med. Res. Rev. 42 (2022) 1518–1544, https://doi.org/10.1002/med.21883.

[11] M.A. Niewczas, A.V. Mathew, S. Croall, J. Byun, M. Major, V.S. Sabisetti, A. Smiles, J.V. Bonventre, S. Pennathur, A.S. Krolewski, Circulating modified metabolites and a risk of ESRD in patients with type 1 diabetes and chronic kidney disease, Diabetes Care 40 (2017) 383–390, https://doi.org/10.2337/dc16-0173.

[12] T.A. Freed, J. Coresh, L.A. Inker, D.R. Toal, R. Perichon, J. Chen, K.D. Goodman, Q. Zhang, J.K. Conner, D.M. Hauser, K.E.T. Vroom, M.L. Oyaski, J.E. Wulff, G. Eiríksdóttir, V. Gudnason, V.E. Torres, L.A. Ford, A.S. Levey, Validation of a metabolite panel for a more accurate estimation of glomerular filtration rate using quantitative LC-MS/MS, Clin. Chem. 65 (2019) 406–418, https://doi.org/10.1373/clinchem.2018.288092.

[13] A. Gessner, M. Mieth, D. Auge, A. Chafai, F. Müller, M.F. Fromm, R. Maas, Establishment of reference values for the lysine acetylation marker N^{ε}-acetyllysine in small volume human plasma samples by a multi-target LC-MS/MS method, Amino Acid. 51 (2019) 1259–1271, https://doi.org/10.1007/s00726-019-02765-8.

[14] M. Zou, P.-J. Zhang, X.-Y. Wen, L. Chen, Y.-P. Tian, Y. Wang, A novel mixed integer programming for multi-biomarker panel identification by distinguishing malignant from benign colorectal tumors, Methods 83 (2015) 3–17, https://doi.org/10.1016/j.ymeth.2015.05.011.

[15] Y. Kanemitsu, K. Asaji, Y. Matsumoto, H. Tsukamoto, D. Saigusa, C. Mukawa, T. Tachikawa, T. Abe, Y. Tomioka, Simultaneous quantitative analysis of uremic toxins by LC–MS/MS with a reversed-phase/cation-exchange/anion-exchange trimodal mixed-mode column, J. Chromatogr. B 1068–1069 (2017) 1–8, https://doi.org/10.1016/j.jchromb.2017.10.009.

[16] Y.J. Lim, N.A. Sidor, N.C. Tonial, A. Che, B.L. Urquhart, Uremic toxins in the progression of chronic kidney disease and cardiovascular disease: mechanisms and therapeutic targets, Toxins (Basel) 13 (2021) 142, https://doi.org/10.3390/toxins13020142.

[17] E. Mishima, C. Inoue, D. Saigusa, R. Inoue, K. Ito, Y. Suzuki, D. Jinno, Y. Tsukui, Y. Akamatsu, M. Araki, K. Araki, R. Shimizu, H. Shinke, T. Suzuki, Y. Takeuchi, H. Shima, Y. Akiyama, T. Toyohara, C. Suzuki, Y. Saiki, T. Tominaga, S. Miyagi, N. Kawagishi, T. Soga, T. Ohkubo, K. Yamamura, Y. Imai, S. Masuda, V. Sabbisetti, T. Ichimura, D.B. Mount, J.V. Bonventre, S. Ito, Y. Tomioka, K. Itoh, T. Abe, Conformational change in transfer RNA is an early indicator of acute cellular damage, J. Am. Soc. Nephrol. 25 (2014) 2316–2326, https://doi.org/10.1681/ASN.2013091001.

[18] J. Rysz, B. Franczyk, J. Ławiński, R. Olszewski, A. Ciałkowska-Rysz, A. Gluba-Brzózka, The Impact of CKD on uremic toxins and gut microbiota, Toxins (Basel) 13 (2021) 252, https://doi.org/10.3390/toxins13040252.

[19] U.S. FDA, Bioanalysis Method Validation Guidance for Industry, https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf, 2018 (Accessed 11 June 2023).

[20] R. Thakare, Y.S. Chhonker, N. Gautam, J.A. Alamoudi, Y. Alnouti, Quantitative analysis of endogenous compounds, J. Pharm. Biomed. Anal. 128 (2016) 426–437, https://doi.org/10.1016/j.jpba.2016.06.017.

[21] G. Falascina, L.B. Bindels, V. Di Marzo, A. Cutignano, Validation of a fast and sensitive UPLC-MS/MS quantitative method for N-acyl taurine analysis in biological samples, J. Pharm. Biomed. Anal. 226 (2023), 115252, https://doi.org/10.1016/j.jpba.2023.115252.

[22] N. Muthmann, P. Špaček, D. Reichert, M. van Dülmen, A. Rentmeister, Quantification of mRNA cap-modifications by means of LC-QqQ-MS, Methods 203 (2022) 196–206, https://doi.org/10.1016/j.ymeth.2021.05.018.

[23] N. Liu, E. Tengstand, K.O. Boernsen, S. Bek, F. Hsieh, Validation of a multiplexed LC–MS/MS clinical assay to quantify insulin-like growth factor-binding proteins in human serum and its application in a clinical study, Toxicol. Appl. Pharmacol. 371 (2019) 74–83, https://doi.org/10.1016/j.taap.2019.03.024.

[24] B.R. Jones, G.A. Schultz, J.A. Eckstein, B.L. Ackermann, Surrogate matrix and surrogate analyte approaches for definitive quantitation of endogenous biomolecules, Bioanalysis 4 (2012) 2343–2356, https://doi.org/10.4155/bio.12.200.

[25] M. Taniguchi, T. Minatani, H. Miyazaki, H. Tsuchihashi, K. Zaitsu, A highly sensitive quantification method for 12 plant toxins in human serum using liquid chromatography tandem mass spectrometry with a quick solid-phase extraction technique, J. Pharm. Biomed. Anal. 192 (2021), 113676, https://doi.org/10.1016/j.jpba.2020.113676.