

THE APPLICATIONS OF MASS SPECTROMETRY FOR IDENTIFYING MODERN BIOCHEMICAL MARKERS OF NEPHROPATHIES

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Abstract

Mass spectrometry (MS) was introduced into clinical laboratories, and first applied to the evaluation of children at risk of inborn errors of metabolism.

For the analysis of macromolecules and in particular for proteins, glycoconjugates and polynucleotides, a major milestone was achieved with the development in 1987 of matrix assisted laser desorption ionization by Karas and Hillenkamp and in 1988 of electrospray ionization by J. Fenn. Over the past decade progress in mass spectrometry and its hyphenation with separation techniques has made these tools essential in life sciences.

The use of MS is, however, not yet routine in many fields where it could influence clinical decisions. While medical research using MS is expanding, few applications have become part of the standard “bedside” practice. This is partly because the transition of MS from a research tool to a reliable clinical diagnostic platform requires rigorous standardization, spectral quality control and assurance, standard operating procedures for robotic and automatic sample application, and standardized controls to ensure generation of highly reproducible spectra. Reliable identification of protein expression patterns and associated protein biomarkers that differentiate affected kidneys from healthy ones or that distinguish different stages of a disease has now started to become feasible.

Rezumat

Spectrometria de masă este o metodă extrem de utilă în laboratoarele clinice, fiind inițial introdusă în Medicină pentru evaluarea riscului de boli metabolice majore în cazul nou-născuților (identificarea compușilor organici în urina copiilor diagnosticați cu acidurie prin gaz-cromatografie cuplată cu spectrometrie de masă).

În ultimii ani această metodă a revoluționat cercetările științifice, devenind o tehnologie indispensabilă în momentul actual pentru studiul complex al unui mare număr de afecțiuni foarte grave. Un exemplu în acest sens sunt nefropatiile. În acest caz spectrometria de masă permite identificarea unui număr important de biomarkeri serici sau urinari pentru diferențierea biochimică și clinică a diferitelor tipuri de nefropatii, precum și aprecierea gravității acestora.

Keywords: nephropathies, mass spectrometry

Introduction

The first clinical applications of mass spectrometry (MS) date back to 1966, when the use of gas chromatography coupled to mass spectrometry (GC–MS) for identification of organic acidurias in children was reported. Twenty years later tandem mass spectrometry (MS/MS) was introduced into clinical laboratories, and first applied to the evaluation of children at risk of inborn errors of metabolism. Using stable-isotope-labeled (i.e., not radioactive) internal standards, the method was further enhanced. These standards are identical to the native analytes, except that their molecular masses are slightly different. Addition of these isotopes at known concentration to the sample before analysis serves as a positive control, helping identification and quantification of the analytes. For the analysis of macromolecules and in particular for proteins, glycoconjugates and polynucleotides, a major milestone was achieved with the development in 1987 of matrix assisted laser desorption ionization and in 1988 of electrospray ionization. Over the past decade progress in mass spectrometry and its hyphenation with separation techniques has made these tools essential in life sciences [2,3].

The use of MS is, however, not yet routine in many fields where it could influence clinical decisions. While medical research using MS is expanding, few applications have become part of the standard “bedside” practice. This is partly because the transition of MS from a research tool to a reliable clinical diagnostic platform requires rigorous standardization, spectral quality control and assurance, standard operating procedures for robotic and automatic sample application, and standardized controls to ensure generation of highly reproducible spectra. Reliable identification of protein expression patterns and associated protein biomarkers that differentiate diseased kidneys from healthy ones or that distinguish different stages of a disease has now started to become feasible [27].

Mass spectrometry screening of peptide and protein as candidate biomarkers in various nephropathy

Using LC-MALDI-TOF (liquid chromatography- matrix-assisted laser desorption with time of flight), Klein & all [18] identified three peptides that decreased in the urine of patients with early renal function decline (fragments of $\alpha 1(\text{IV})$ and $\alpha 1(\text{V})$ collagens and tenascin-X) and three peptides that increased (fragments of inositol pentakisphosphate 2-kinase, zona occludens 3, and FAT tumor suppressor 2). In renal biopsies from patients with early nephropathy from type 1 diabetes, they observed increased expression of inositol pentakisphosphate 2-kinase, which was

present in granule-like cytoplasmic structures. These results indicated that urinary peptide fragments reflected changes in expression of intact protein in the kidney, suggesting new potential mediators of diabetic nephropathy and candidate biomarkers for progressive renal function decline [18].

In an interesting study concerning the finding of new biomarkers of early diabetic nephropathy (DN), Kienlle & all [9] used LC-ESI MS/MS to detect three peptides differently expressed in the serum of patients with DN. The three peptides were the whole fibrinopeptide A and two of its fragments, respectively. The two fragments were under-expressed in diabetic patients, while fibrinopeptide A was over-expressed, suggesting that anomalous turnover of fibrinopeptide A could be involved in the pathogenesis of DN [9].

New biomarkers for detection of renal cell carcinoma (RCC) were assessed from serum protein using SELDI-TOF MS (surface- enhanced laser desorption/ionization with time of flight) [7]. Beijnen and his research group found 15 peaks significantly different ($p < 0.01$) between RCC and healthy controls (HC). Although two serum protein profiles comprising 5 protein peaks were found that could separate RCC from HC, the sensitivity and specificity is not sufficient to recommend large scale use. Upon structural identification and quantitative validation, however, these proteins might prove suitable markers in the follow up of RCC patients [7]. In order to identify the proteomic differences between renal cell carcinoma (RCC) and benign renal tumors, Zhang [30] analyzed 168 serum samples from 65 RCC patients, 34 patients with benign renal tumors, and 69 healthy persons using SELDI-TOF mass spectrometry technology to detect protein biomarkers. Two peaks with molecular masses corresponding to $m/z = 3887.11$ and 11079.8 were detected that are potentially useful for the diagnosis or screening of RCC. It was found that these two peaks can be used, not only to distinguish the RCC vs. healthy cases, but also to distinguish RCC from benign renal tumors. In combination with computer tomograph (CT) scanning, the authors hope to improve the sensitivity and specificity of the diagnosis of renal tumors [30].

Ultrastructural alterations of podocytes are closely associated with loss of glomerular filtration function. Kerjaschki and his group [17]., explored changes at the proteome level that paralleled the disturbances of podocyte architecture in the early stages of puromycin aminonucleoside (PA) nephrosis *in vivo*. Using two-dimensional fluorescence difference gel electrophoresis and MALDI-TOF mass spectrometry combined with postsource decay fragment ion analysis and high-energy collision-induced dissociation (CID) tandem mass spectrometry, 23 differentially expressed

protein spots, corresponding to 16 glomerular proteins that are involved in various cellular functions, were unambiguously identified. The majority of these proteins were primarily related to fatty acid metabolism and redox regulation. Key enzymes of the mitochondrial beta-oxidation pathway and antioxidant enzymes were consistently down-regulated in PA nephrosis. These results indicate that a disrupted fatty acid metabolism in concert with an impaired antioxidant defense mechanism in podocytes may play a role in the early stages of PA-induced lesions in podocytes [17].

The urinary excreted peptides, indicative of a pathogenetic mechanism leading to tubular dysfunction in the patients with renal Fanconi syndrome (FS) (characterized by renal glucosuria, loss of electrolytes, bicarbonate, lactate, generalized hyperaminoaciduria and low-molecular-weight proteinuria) were studied using capillary electrophoresis coupled with mass spectrometry (CE-MS)[6]. The marker peptides constituting the proteome pattern are fragments derived from osteopontin, uromodulin and collagen alpha-1. CE-MS used to diagnose FS in paediatric patients, might be a future tool for the non-invasive diagnosis of FS. The reduced amount of the marker proteins osteopontin and uromodulin indicates loss of function of tubular excretion in all patients suffering from FS regardless of the underlying cause. Moreover, other six different fragments of the collagen alpha-1 (I) chain were either elevated or reduced in the urine. This indicates a change of proteases in collagen degradation as observed in interstitial fibrosis. These changes were prominent irrespectively of the stages of FS. This indicates fibrosis as an early starting pathogenetic reason for the development of renal insufficiency in FS patients [6]. The isolation and identification of proteins contained in the urine of the children with systemic lupus erythematosus was achieved using SELDI or MALDI-TOF mass spectrometry [22]. The proteins contained in the lupus nephritis (LN) urine were transferrin (Tf), ceruloplasmin (Cp), alpha1-acid-glycoprotein (AGP), lipocalin-type prostaglandin-D synthetase (L-PGDS), albumin, and albumin-related fragments (PS). Serial plasma and urine samples were also analyzed using immunonephelometry or ELISA in 98 children with SLE and 30 controls with juvenile idiopathic arthritis. All urinary proteins were significantly higher with active vs. inactive LN or in patients without LN indicating that these PS proteins are biomarkers of LN activity and may help anticipate the future course of LN [22].

Urine samples from diabetic, nephropathic, diabetic-nephropathic patients as well as healthy subjects were analyzed by MALDI-TOF mass spectrometry in order to establish evidence of some possible differences in the peptide profile related to the pathological states [14]. Multivariate

analysis suggested the possibility of a distinction among the considered groups of patients. Some differences have been observed, in particular, in the relative abundances of three peptidic ions at m/z =1912, 1219 and 2049. Sequence study was carried out by MALDI-TOF/TOF to individualise their possible origin. By this approach, the peptide ion at m/z =1912 was found to originate from uromodulin, and its lower expression in the case of nephropathy can be well related to the pathological condition. Peptidic ions at m/z =2049 and 1219 originate from the collagen alpha-1(I) chain precursor and from the collagen alpha-5 (IV) chain precursor, respectively, and, in the study case their different expressions can be related to the pathologies under investigation [14].

The levels of retinol-binding protein 4 (RBP4) are tightly regulated under healthy circumstances. The kidney, the main site of RBP4 catabolism, contributes to an elevation of RBP4 levels during chronic kidney disease (CKD) whereas during chronic liver disease (CLD) RBP4 levels decrease. RBP4 and his isoforms from plasma patients suffering with CDL, CKD and healthy control, were analysed by polyacrylamide gel electrophoresis (PAGE), ELISA, immunoprecipitation and finally with MALDI-TOF-MS by Schweigert and his group [8]. RBP4 isoforms and levels were highly increased in CKD patients compared to controls whereas in CLD patients RBP4 isoforms were not different from controls. In addition, in hepatic dysfunction RBP4 levels were decreased whereas the amount of isoforms was not affected. The occurrence of RBP4 isoforms is not influenced by liver function but seems to be strongly related to kidney function and may therefore be important in investigating kidney function and related disorders [8].

In IgA nephropathy, IgA1 molecules with incompletely galactosylated *O*-linked glycans in the hinge region (HR) are present in mesangial immunodeposits and in circulating immune complexes. The Gal deficiency in IgA1 proteins can occur randomly or preferentially at specific sites. To develop experimental approaches to address this question, scientists have analyzed by FT-ICR mass spectrometry the synthetic IgA1 hinge region and hinge region from a naturally Gal-deficient IgA1 myeloma protein. To localize sites of *O*-glycan attachment, synthetic IgA1 HR glycopeptides and HR from a naturally Gal-deficient polymeric IgA1 myeloma protein were analyzed by electron capture dissociation (ECD) and activated ion-electron capture dissociation. Multiple sites of *O*-glycan attachment (including sites of Gal deficiency) in myeloma IgA1 HR glycoforms were identified. The results represent the first direct identification of multiple sites of *O*-glycan attachment in IgA1 hinge region by mass spectrometry, thereby enabling future characterization at the

molecular level of aberrant glycosylation of IgA1 in diseases such as IgA nephropathy.

The nephrotic syndrome is characterized by massive urinary loss of major plasma proteins. Starting from this point, Ghiggeri and his group analyse the urine and plasma of 23 patients with different idiopathic nephritic syndromes (10 steroid-sensitive minimal-change nephropathy, seven steroid-resistant focal segmental glomerulosclerosis (FSGS), and six membranous glomerulonephritis) [1]. Combining two-dimensional electrophoresis in soft gel, Western blot, and MALDI-TOF mass spectrometry, 72 urinary components corresponding to fragments of albumin and/or of α 1 antitrypsin were found. Several (21 of 72) urinary albumin fragments also were detected in plasma, although in lower concentration, suggesting a preferential excretion. The bulk of components with low molecular weight were detected only in urine, suggesting an *in situ* formation. A final but not secondary point was the characterization of albumin adducts that harbor both the COOH and NH₂ terminal parts of the protein, suggesting the formation of new covalent chemical groups. Altogether, these new findings reveal unexpected structural and functional aspects of proteinuria that may play a key role in pathogenesis and should be extended to other renal diseases [1]. In order to diagnose the type of glomerular diseases that cause the nephrotic syndrome, proteomic methods and informatic analysis were used to identify patterns of characteristic urine proteins [26]. Patients with focal segmental glomerulosclerosis (FSGS), lupus nephritis, membranous nephropathy, or diabetic nephropathy were analyzed. Urine proteins were separated by two-dimensional electrophoresis, the spots were cut from the gel, and 20 of them were identified using a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF-MS) mass spectrometer, an ABI 4700 MALDI-TOF/TOF mass spectrometer or a Finnigan LTQ linear ion trap MS. 11 plasma proteins were identified: orosomucoid, transferrin, α -1 microglobulin, zinc α -2 glycoprotein, α -1 antitrypsin, complement factor B, haptoglobin, transthyretin, plasma retinol binding protein, albumin, and hemopexin. The experimental data showed that diseases that cause nephrotic syndrome change glomerular protein permeability in characteristic patterns. The fingerprint of urine protein charge forms identifies the glomerular disease. The identified proteins are candidate biomarkers that can be tested in assays that are more amenable to clinical testing [26].

Urine samples from patients with type 2 diabetes (DM) without nephropathy and without microalbuminuria (DM-WNP), patients with DM and macro or microalbuminuria (DM-NP), patients with proteinuria due to

nondiabetic renal disease and healthy controls were analyzed using surface-enhancement laser desorption-ionization time-of-flight (SELDI-TOF) mass spectrometry and strong anionic exchange (SAX2) protein arrays [4]. To purify and isolate urine proteins, anionic exchange, reversed phase fractionation and gel electrophoresis were used. A protein with $m/z = 6188$ (identified as a processed form of ubiquitin) was strongly released in the urine of healthy controls, patients with proteinuria due to nondiabetic disease, and DM-WNP in contrast to DM-NP patients. Another $m/z = 14\ 766$ protein (identified as UbA52, an ubiquitin ribosomal fusion protein) was selectively excreted in the urine of DM-NP patients, whereas the protein with $m/z = 11\ 774$ (identified as $\beta 2$ -microglobulin) was significantly excreted by patients with proteinuria and DM-NP. The release of high amounts of UbA52 in urine of DM-NP patients could serve as a diagnostic marker, whereas the lack of the short form of ubiquitin raises interesting questions about the pathophysiology [4].

Mass spectrometry in detection and quantification of lipids and other metabolites

An ultra performance liquid chromatography-time of flight-mass spectrometry (UPLC-TOF-MS) system was employed to distinguish the global serum profiles of 8 diabetic nephropathy (DN) patients, 33 type 2 diabetes mellitus (T2DM) patients and 25 healthy volunteers, and tried to find potential biomarkers [31]. Principal component analysis (PCA) was used for group differentiation and marker selection. Several compounds were tentatively identified based on accurate mass, isotopic pattern and MS/MS information. In addition, significant changes in the serum level of leucine, dihydrosphingosine and phytosphingosine were noted, indicating the perturbation of amino acid metabolism and phospholipid metabolism in diabetic diseases, with implications in clinical diagnosis and treatment. [31]

Hara & all recently proposed serum sulfatides as a novel biomarker for cardiovascular disease in patients with end-stage renal failure (ESRF), based on the possible antithrombotic properties of this molecule [15]. Using high-throughput analysis combined with MALDI-TOF mass spectrometry, they measured the levels of sulfatides in the sera, liver, small intestines and kidneys in protein overload nephropathy mice. As the disease progressed, the levels of sulfatides in sera, livers and small intestines decreased to approximately 60% of the original levels. On the contrary, those in kidneys increased by approximately 1.4-fold. These results indicate that kidney dysfunction affects the levels of sulfatides in lipoprotein-producing organs, such as livers and small intestines, and lowers the levels of sulfatides in the sera [15]. Using high-performance liquid

chromatography with ultraviolet detection coupled with mass spectrometry (HPLC-UV/MS/MS), Luo and his research group [29] developed a sensitive and specific method for simultaneous determination of 21 compounds related to the diabetic nephropathy (DN) in a single analysis in human plasma. With the case and control study, they found five potential biomarkers of DN, including adenosine, inosine, uric acid, xanthine and creatinine [29]. It was reported that Hcy was related to the development of kidney disease, but it remains unknown whether Hcy is an independent biomarker for diabetic nephropathy [12].

A high-performance liquid chromatography-electrospray tandem mass spectrometric (HPLC-ESI-MS/MS) method was established for simultaneous quantitation of Cysteine (Cys), total homocysteine (tHcy), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), cystathionine (Cysta), methionine (Met), glutathione (GSH) and cysteinylglycine (Cys-gly) in plasma with N-(2-mercaptopropionyl)-glycine (MPG) as internal standard. The method had simple pretreatment (without derivatization), the chromatograms showed better separation of the eight amino thiols, the analytic time was 20 min. The method was also successfully applied in the case-control study of patients with diabetes mellitus (DM) and diabetic nephropathy (DN). The method allowed to quantitate the concentrations of amino thiols in the human plasma. SAH and SAM were suggested as better potential biomarkers of DM and DN. [12]

Mass spectrometry detection and quantification of Advanced Glycation End (AGE) products in uremia

The term protein glycation summarizes non-enzymatic reactions between amino groups of proteins and carbonyl group of sugars or other metabolite of sugars, leading to early glycation products and advanced glycation ends (AGEs) (Figure 1). Protein glycation is involved in the progression of not only diabetes mellitus, but also uremia and atherosclerosis. MS has been successfully used to structural analysis of glycated proteins and peptides.

Scientists applied MALDI-TOF-MS to the structural investigation of glycation products. The analyses of plasma proteins from uremic or diabetic patients allowed a precise determination of the average number of sugar residues attached to serum albumin or immunoglobulins of each patient. The glycation rate of hemoglobin, isolated from diabetic blood and of β 2-microglobulin (β 2m) isolated from amyloid plaques of uremic patients was determined. Whereas the measurement of the intact proteins allows the determination of the average number of sugar residues attached to proteins, peptide mapping prior to MALDI-TOF-MS can reveal the exact structures

of the glycation products and the glycation site. The molecular mass changes of serum albumin of uremic patients were studied by MALDI-MS. AGE-modified peptides are thought to bind and modify plasma proteins. Monitoring the consequent increase in molecular mass of serum albumin may be used in surveillance of the clinical management of uremia. As a result of clinical trials significant increase in the molecular mass of albumin in uremia was found. The molecular mass of albumin *in vivo* was indicative of a minimal and not a high extent of glycation. Protein glycation adducts, early glycation adducts, such as Nε-fructosyl-lysine, and AGEs are considered to be uremic toxins. Glycation products are found in plasma and tissue proteins (glycation adduct residues), in peptides (glycation adduct peptide residues), and glycated amino acids (glycation free adducts).

The latter two analyte groups may arise from proteolysis of glycated proteins or by glycation of peptides and amino acids.

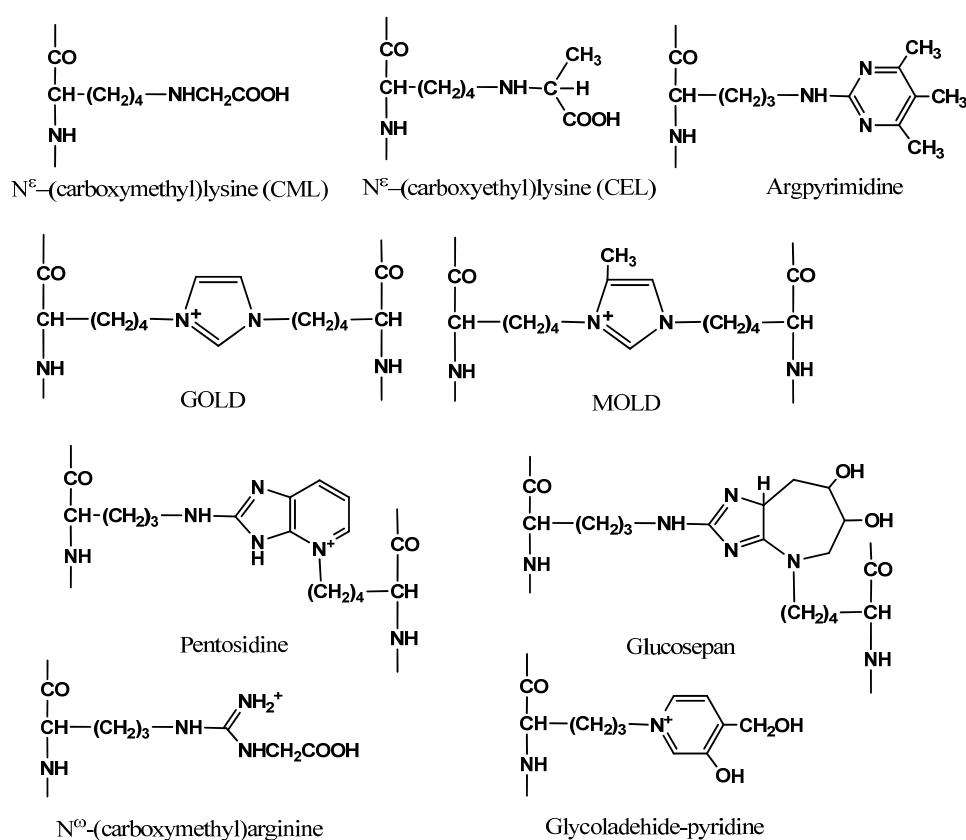


Figure 1

Chemical structures of AGE products
(GOLD=glyoxal lysine dimer, MOLD=methylglyoxal lysine dimer)

Dawnay [25] measured the concentrations of the early glycation adduct fructosyl-lysine and 12 AGEs by LC-MS/MS. Underivatized analytes were detected free in physiological fluids and in enzymic hydrolysates of cellular and extracellular proteins. Hydroimidazolones were the most important glycation biomarkers quantitatively, whereas monolysyl adducts such as N^ε-(carboxymethyl)lysine and N^ε-(carboxyethyl)lysine were found in moderate amounts. Bis(lysyl)imidazolium cross-links and pentosidine were found in lowest amounts. Low levels of free advanced glycation endproducts were found in blood plasma and levels were 10-100-fold higher in urine. Advanced glycation endproduct residues were increased in blood plasma and at sites of vascular complications development in experimental diabetes, renal glomeruli, retina and peripheral nerve. In clinical uremia, the concentrations of plasma protein advanced glycation endproduct residues increased 1-7-fold and free adduct concentrations increased up to 50-fold. Increased levels of these AGEs were associated with vascular complications in uremia [25]. Thornalley demonstrated by LC-MS/MS analysis of plasma, urine, and dialysate samples of uremic patients, that glycation free adducts accumulate in the plasma of uremic patients, and are eliminated in the peritoneal dialysate. LC-MS/MS has been used to quantify concentrations, extents of protein modification (multiple glycation adducts, protein oxidation and nitration adducts) excretion rates of glycation adducts (the major form), and clearances in uremia [24].

Mironova and Niwa [21] characterized the molecular heterogeneity and AGEs modification of β 2m extracted from the amyloid tissue of a hemodialysis patient, by using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS), Western blotting and N-terminal amino acid sequence analysis. Amyloid β 2m was composed of full-length β 2m, truncated β 2m and dimer β 2m. Truncated β 2m and dimer β 2m were modified with AGEs such as imidazolone and N^ε-(carboxymethyl)lysine, and showed fluorescence characteristic of AGEs. Truncated β 2m species were formed by cleavage between amino acid residues of Pro6/Ile7, Gln8/Val9 and Val9/Tyr10. Heterogeneous dimer β 2m species showed the molecular masses to m/z = 22591 and 22675, which resulted from crosslinking between truncated β 2m [21]. It was investigated whether the use of polyglucose solution (icodextrin) for the long dwell in peritoneal dialysis patients (40 patients during a 4-month study period) would result in a reduction in plasma and dialysate levels of N^ε-(carboxymethyl)lysine, one of AGEs. N^ε-(Carboxymethyl)lysine was measured by stable isotope dilution MS/MS. N^ε-(Carboxymethyl)lysine

levels in plasma increased significantly in patients treated with icodextrin, and the same held true for N^ε-(carboxymethyl)lysine levels in dialysate. Thus, contrary to expectation, plasma and dialysate levels of N^ε-(carboxymethyl)lysine increased in patients treated using icodextrin for the long dwell [20]. Perturbation of interactions between cells and the extracellular matrix (ECM) of renal glomeruli may contribute to characteristic histopathological lesions found in the kidneys of patients with diabetic nephropathy. The mechanism by which the diabetic conditions may affect cell-ECM interactions is unknown. One hypothesis suggests a role of glucose in direct modification of ECM. It has been demonstrated that carbonyl compound methylglyoxal (MGO) completely inhibits endothelial cell adhesion to recombinant α_3 noncollagenous 1 domain of type IV collagen mediated via a short collagenous region containing RGD (Arg-Gly-Asp) sequence as well as binding of purified $\alpha_v\beta_3$ integrin to this protein. Specific MGO adducts of the arginine residue were detected using liquid chromatography-tandem mass spectrometry (LC-MS/MS). MGO strongly inhibited adhesion of renal glomerular cells, podocytes, and mesangial cells to native collagen IV and laminin-1 as well as binding of collagen IV to its major receptor in glomerular cells, $\alpha_1\beta_1$ integrin. In contrast, modification of these proteins by glucose had no effect on cell adhesion. Pyridoxamine, a promising drug for treatment of diabetic nephropathy, protected cell adhesion and integrin binding from inhibition by MGO. These results suggest that in diabetes, perturbation of integrin-mediated cell matrix interactions occurs via the modification of critical arginine residues in renal ECM by reactive carbonyl compounds. This mechanism may contribute to the development of new biomarkers in diabetic nephropathy.

Detection and quantification of uremic toxins by mass spectrometry

Mass spectrometry (MS) has been successfully applied, for the identification and quantification of uremic toxins and uremia-associated modified proteins. Uremic toxins include low-molecular-weight compounds (e.g., indoxyl sulfate, p-cresol sulfate, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, asymmetric dimethylarginine), middle-molecular-weight peptides, and proteins modified with advanced glycation and oxidation. These uremic toxins are considered to be involved in a variety of symptoms which may appear in patients with stage 5 chronic kidney disease. Based on MS analysis of these uremic toxins, the pathogenesis of the uremic symptoms will be elucidated to prevent and manage the symptoms [20].

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was applied to the direct analysis of melamine cyanurate (MC)[23]. The three commonly used MALDI matrixes, namely, alpha-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2,5-dihydroxybenzoic acid (DHB), were able to desorb/ionize melamine from MC upon laser irradiation, with CHCA showing the highest detection sensitivity in the positive mode. Only DHB and SA were able to desorb/ionize cyanuric acid from MC in the negative mode but with remarkably lower sensitivity. The method is able to detect melamine unambiguously from a small amount of MC (down to 12.5 microg) spiked into urine and was successfully applied for the rapid and sensitive detection of melamine in urine stones/residues of the samples collected from patients clinically confirmed of having kidney stones associated with the consumption of melamine-tainted food products. The analytical time of the method for high-throughput analysis from the time of sample treatment to analysis is less than 7 minutes per sample, with sensitive detection of the presence of melamine in the urine stones/residues of the patient samples [23].

Aristolochic acids (AAs) are substances responsible for Chinese herbs nephropathy [11]. Recently, strong indications have been presented, which dietary poisoning with AA is responsible for endemic Balkan nephropathy (EBN), an enigmatic renal disease that affects rural population living in some countries in Southeastern Europe. A mouse model was applied to follow the effects of two forms of aristolochic acids, AAI and AAI. SDS-PAGE and SELDI-TOF mass spectrometry were used to evaluate changes in the urine of treated animals. These two methods are demonstrated to be comparable. The use of SELDI-TOF MS for rapid analysis of a large number of samples and the combination of this method with nano-LC-ESI MS/MS for protein identification were demonstrated [11].

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