Applications of LC-based metabolomics in chronic kidney diseases

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Abstract: Metabolomics, as an omics science in systems biology, is the global unbiased analysis of all the endogenous small-molecule metabolites within a biological system under a given set of conditions. Either individually or grouped as a metabolomic profile, the detection of metabolites is carried out in cells, tissues, or bio-fluids by different analytical approaches. Metabolomics offers the potential for a holistic approach to clinical medicine while improving disease diagnosis and our understanding of the pathological mechanisms. Chronic kidney diseases (CKDs) are a major challenge to public health. They include the primary chronic glomerulonephritis (IgA nephropathy), secondary chronic renal injury (diabetic nephropathy) and the chronic renal failure (end-stage kidney disease with and without undergoing replacement therapies). The root causes for disease onset remain poorly understood and no cures are available. In this review, the role of metabolomics is explored in gaining mechanistic insight into CKDs including animal models and clinical studies, and in the search for novel biomarkers. Particular challenges in the field are presented and placed within the context of the future of the applications of metabolomics approaches to the study of CKDs. A future hope for the metabolomic approach is the identification of biomarkers that are able to highlight individuals likely to suffer from CKDs and enable early diagnosis of the disease or the identification of those at risk.

赵英永，医学博士，西北大学生命科学学院副教授。主要从事真菌类利尿中药的提取分离与结构鉴定、定性定量分析、利尿及治疗慢性肾病活性成分筛选、药代动力学、代谢组学等研究。先后承担国家自然科学基金、陕西省社发攻关计划项目、陕西省国际合作项目等10余项；获全国百篇优秀博士学位论文提名奖、陕西省青年科技奖、施维雅青年药理学奖等；发表SCI论文65篇，累计影响因子为141.24；编著《猪苓的化学成分及其应用》等；申请国家发明专利4项。

Key words: chronic kidney diseases; metabolomics; liquid chromatography; animal model; clinical study

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Chronic kidney diseases (CKDs) are becoming a worldwide public health concern. CKDs are defined as kidney damage or a glomerular filtration rate (GFR) < 60 ml/min/1.73 m^2 for three months or more, irrespective of the cause[1]. According to the Kidney Disease Outcomes Quality Initiative, five stages of CKDs exist: stage 1, kidney damage (pathological abnormalities or markers of damage, including abnormalities in blood or urine tests or in imaging studies) with normal or raised GFR (≥90 ml per min per 1.73 m^2); stage 2, GFR 60 – 89 ml with evidence of kidney damage; stage 3, GRF 30 – 59 ml; stage 4, GFR 15 – 29 ml; and stage 5, end-stage renal failure; GFR < 15 ml per min per 1.73 m^2[2]. Progressive CKDs can lead to end-stage renal diseases (ESRDs) that require dialysis. CKDs have complex pathogenesis, involving the interplay of genetic and environmental factors through the early appearance of renal hypertrophy, the progressive accumulation of the extracellular matrix in the glomerulusand tubulointerstitium, and, consequently, glomerulosclerosis, tubulointerstitial fibrosis and tubular atrophy. The later three are the major morphological features of CKDs[3].

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Current treatment decisions which have remained unchanged for several decades, for CKDs make diagnostic use of a limited set of serum and urine biochemical markers, kidney histopathology and clinical manifestations of the disease. Serum creatinine (Scr), a very cheap and stable compound in routine clinical use, is the most common clinical biomarker of renal dysfunction. However, there are several limitations to its use[4]. In the short term, Scr and blood urea nitrogen (BUN) showed poor sensitivity and specificity to the detection of renal injury[5]. Monitoring renal function is hampered by a limitation of the sensitivity and specificity of a chosen biomarker. Knowledge of the complex molecular and pathophysiologic mechanisms leading to kidney disease remains limited, in part because conventional research tools have hampered investigators by restricting their focus to a single or relatively few potential markers at a time.

Metabolomics, also known as metabonomics[6] or metabolic profiling[7], is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification"[6,8-9]. Metabolomics is a non-targeted quantitative analysis of bio-fluids and tissue for low molecular mass organic endogenous metabolites. These representative small molecules found within a system cover a broad range of small molecules, such as glucose, cholesterol, adenosine triphosphate, biogenic amine neurotransmitters, and lipid signaling molecules. Metabolomics has evolved into a valuable tool in systems biology and has permeated into diverse areas, such as toxicity evaluation,

disease diagnosis, drug discovery, epidemiology, pharmacology, plant biology, human nutrition and environmental science. From bacteria to humans, examples of this principle are accruing at a rapid pace that has been made possible by remarkable recent developments in analytical chemistry, such as high-field nuclear magnetic resonance (NMR) and mass spectrometry (MS) platforms for small molecule separation, detection, and characterization, together with the availability of multivariate data analysis methods that are able to simplify the huge data matrices generated by metabolomic experiments. Here, we provide an overview of liquid chromatography (LC)-based metabolomic approaches applied to the study of CKDs. The purpose of the present review is to summarize current LC-based metabolomic applications in CKDs and to provide an overview of the contribution of metabolomics to CKDs research, through which such approaches have advanced our understanding of CKDs.

1 LC-based metabolomic analytical technology

There are many types of mass analyzers available for interfacing with LC, such as triple-quadrupoles, time-of-flight (TOF) and ion traps[10-13]. Quadrupole (Q) mass spectrometers remain the instrument of the most widespread use today. Quadrupole instruments have a high-linear dynamic range and are capable of analyzing an *m/z* range of 50 -4000.

Recently, the novel Q-TOF $Ms^{Elevated\ Energy}$ (MS^E) technique has been proven a powerful and reliable analytical approach for *in vivo* metabolite identification studies[14-18]. In 2005, Wrona *et al*[14] introduced the MS^E technique, in which two scanning functions are simultaneously used for data collection. A variety of data-processing algorithms can be used to extract metabolite information from these data[15]. In other words, MS^E can provide parallel alternating scans for acquisition at either low collision energies to obtain precursor ion information or high collision energies to obtain full-scan accurate mass fragment, precursor ion and neutral loss information. Q-TOF MS^E involves a simultaneous acquisition by alternating between high and low collision energies during a single chromatographic run. This ability is of major importance, as it offers the structural information required for the identification of unknown biomarkers in the context of untargeted analyses[19]. Ultra performance LC coupled with Q (UPLC Q)-TOF/MS/MS^E allowed for significant improvements in resolution, analysis speed, detection sensitivity and reduction of solvent waste using a short column packed with 1.7 -1.8 μm porous particles. Particle sizes achieve lower plate heights over a wider range of higher linear velocities, resulting in better resolution and sensitivity, as well as reduced analysis time[20-22].

2 Metabolomics for CKDs

2.1 Metabolomics in animal model research

UPLC-based metabolomics has been used to study kidney diseases for the past few years[23]. A series of experimental studies have been conducted on CKDs rodent models to investigate the metabolic profiles of serum, urine, feces or tissues, and these results have led to new insights into the development of CKDs[23]. The adenine-induced CKDs model has the advantage of being more similar to the development of human chronic renal injury than to genetic models, and, as a result, these models mirror the progression of renal injury after a prolonged period of development[24]. Adenine-induced chronic renal failure (CRF) rats demonstrate different serum UPLC-based metabolite concentrations in comparison with normal control rats[25]. Although the phosphatidylcholine (PC)(16:0/18:2), lysoPC(18:1), creatinine, lysoPC(17:0) and lysoPC (16:0) concentrations were higher in adenine-induced CRF rats than in normal control rats, the concentrations of dihydrosphingosine, tryptophan, ceramides (18:0/16:0), ceramides(18:0/14:0), L-acetylcarnitine and phytosphingosine were significantly lower in adenine-induced CRF rats. Furthermore, adenine-induced CRF could be predicted according to various metabolite levels, specifically those of creatinine, tryptophan, lysoPC(18:1), lysoPC (17:0), lysoPC(16:0), ceramides(18:0/16:0) and ceramides(18:0/14:0), and these metabolite levels could also be used to discriminate between CRF and normal animals. Additionally, the urinary metabolic profiles of adenine-induced CRF rats were significantly different from those of normal control rats[26]. Adenine-induced CRF rats were characterized by increases in phytosphingosine, adrenosterone, tryptophan, 2, 8-dihydroxyadenine, creatinine, and dihydrosphingosine, together with decreases in N-acetylleucine, 3-O-methyldopa, ethyl-N_2-acetyl-L-arginate, dopamine, phenylalanine and kynurenic acid in urine[26]. The altered metabolites demonstrated perturbations of amino acids, phospholipids and creatinine metabolism in the CRF rats. These results provide evidence for the complex perturbation of amino acids, phospholipids and creatinine metabolism in CKDs. It was reported that changes in the fecal metabolite profile, such as chenodeoxycholic acid, palmitic acid, phytosphingosine, monoacylglyceride (MG)(24:1/0:0/0:0), 12-hydroxy-3-oxocholadienic acid, lysophosphatidylethanolamin (lysoPE) (18:2/0:0), lysoPE(16:0/0:0) and 7-ketolithocholic acid, could be used as early biomarkers for adenine-induced CRF rats[27]. Kidney metabolomics based on the UPLC Q-TOF/HSMS with the MS^E data collection technique was calculated to explore the excretion pattern of low molecular mass metabolites in the adenine-induced CRF rats. The results showed that the most important CRF-related metabolites were polyunsaturated fatty acids, indoxyl sulfate and *p*-cresyl sulfate. Indoxyl sulfate and *p*-cresyl sulfate (uremic toxins) were significantly increased in CRF rats[28]. Furthermore, the above-mentioned UPLC-based metabolomics method was applied to therapeutic effect of ergosta-4, 6, 8 (14), 22-tetraen-3-one (ergone). The results showed that some biomarkers were completely reversed by ergone[29-31]. In addition, the results also showed that some biomarkers were completely reversed by the surface layer of *Poria cocos*[32-34].

The UPLC-MS metabolomic approach was also employed to characterize the metabolic profile of plasma, urine and kidney tissue extracts from rats treated with

Morning Glory seed (MGS)[35-37]. The metabolic changes suggested the involvement of specific pathways in the MGS-induced nephrotoxicity rats. The formation of lysophosphatidylcholines was accelerated, while the biosynthesis of phenylalanine was decreased[35]. Significant differences in the urine levels of amino acids, citric acid, creatinine, cholic acid and 5-methyltetrahydrofolate were observed in the MGS-induced nephrotoxicity rats[36]. Metabolomics has also been used to identify biomarkers to discriminate the MGS-induced model rats from the control rats at the second, sixth and tenth weeks before serious organic damage of kidney was found at tenth week by histopathological methods[37]. A metabolomic approach based on UPLC-MS was used to study the nephrotoxicity of rhizoma alismatis in rats. Thirteen metabolite biomarkers were detected in the urine. The metabolomic method combined with principal component analysis discriminated the treated rats from the control rats on d 60, 120, and 180 after treatment, before serious organic damage to the kidney was apparent on d 180 with histopathology[38].

One study utilized LC-MS/MS to identify uremic toxins that accumulated in the serum of 5/6 nephrectomy rats[39]. Indoxyl sulfate was demonstrated to be the first principal serum metabolite that differentiated CKDs rats from normal rats, followed by phenyl sulfate, hippuric acid and *p*-cresyl sulfate. Indoxyl sulfate stimulates the progression of CKDs by increasing the renal expression of transforming growth factor-β_1 (TGF-β_1), a tissue inhibitor of metalloproteinase-1 and proa1 (Ⅰ) collagen. The serum levels of uremic toxins were found to be markedly increased in CKDs rats as compared with normal rats[39]. Also, the LC-MS/MS method was applied to search for metabolites as possible indicators of the therapeutic effect of an oral sorbent, AST-120, which is used clinically for CKDs patients to delay the progression of CKDs[40]. Indoxyl sulfate was the best indicator of the therapeutic effect of AST-120 in CKDs rats, while hippuric acid, phenyl sulfate and 4-ethylphenyl sulfate were suggested as the additional indicators. LC-based studies also revealed serum variations of IgA nephropathy rats by oral immune and bovine serum albumin injections. It was found that the expression of intercellular adhesion molecule(ICAM)-1 in the glomeruli had a significant correlation with proteinuria in mouse IgA nephropathy. The association between plasma phospholipids and the expression of ICAM-1 in the glomeruli of IgA nephropathy suggested that phosphatidylserin (PS) (18∶0/18∶0), PS (18∶0/22∶5) and phosphatidylinositol (PI) (18∶0/20∶4) were biomarkers of IgA nephropathy[41]. One study investigated the wild-type and organic anion transporter-1 (Oat1) knockout mice-identified metabolites, including ones that had not been previously linked to Oat1-mediated transport. These compounds included indoxyl sulfate derivatives from the phaseⅡ metabolism of enteric gut precursors, which accumulate in CKDs, as well as pantothenic acid, 4-pyridoxic acid, urate, and metabolites in the tryptophan and nucleoside pathways. The concentrations of indoxyl sulfate, kynurenine and xanthurenic acid were elevated in the plasma[42]. A urinary metabolomics method based on UPLC-MS was developed and applied in Kidney-Yang Deficiency Syndrome, induced by a high dose of hydrocortisone and the therapeutic effects of *Rhizoma Drynariae*. Some significantly changed metabolites, such as phenylalanine, phenylacetylglycine, N_2-succinyl-L-ornithine, L-proline, creatinine, hippurate and citrate, were identified. These biochemical changes were found to be related to disturbances in the energy metabolism, amino acid metabolism and gut microflora, which shed light on the Kidney-Yang Deficiency Syndrome and the therapeutic mechanism of *Rhizoma Drynariae*[43].

Diabetic kidney disease is a common microvascular complication of diabetes mellitus that is associated with progressive loss of kidney function, systemic endocrine and cardiovascular complications, and premature death. The elucidation of metabolic profiling in diabetic nephropathy (DN) rats contributes much to understanding the pathogenesis of DN. [^{1}H] NMR-based metabolomics combined with HPLC measurements was used to quantitatively analyze the metabolic changes in urine and kidney tissues from streptozotocin-induced diabetic rats. The 8-week diabetic rats showed lower levels of creatine and dimethylamine, but higher levels of ascorbate, succinate, lactate, citrate, allantoin, 2-ketoglutarate and 3-hydrobutyrate in the urine samples. Moreover, the 8-week diabetic rats displayed lower levels of succinate, creatine, *myo*-inositol, alanine, lactate and ATP and higher levels of 3-hydrobutyrate and glucose in the kidney extracts. The observed metabolic changes imply enhanced pathways for either lipid or ketone body synthesis and decreased pathways for either the tricarboxylic acid cycle or glycolysis in DN rats. The results indicated that the energy metabolic changes are associated with the pathogenic process of DN[44]. Based on a combined UPLC-TOF/MS and GC-TOF/MS data acquiring platform, a metabolomic approach was applied to renal cortex samples from streptozocin-induced diabetic rats, which were treated with fosinopril. Some significantly changed metabolites, such as amino acids, carbohydrates, polyols, lysophospholipids and glucuronides, were identified. An increase in intrarenal organic toxins, including uremic toxins, glucuronides and glucotocixity-associated metabolites, was highly correlated with such diabetic kidney injuries as 24 h urinary protein levels and tubulointerstitial injury indices[45].

2.2 Metabolomics in clinical research

UPLC-MS technique has been used to study the serum or plasma metabolites of patients with CRF. It was reported that changes in the serum metabolite profile, such as in the levels of tryptophan, phenylalanine, lysoPCs, creatinine or kynurenine, could be used as early biomarkers for CRF patients. The serum concentrations of lysoPC (18∶0), creatinine, phenylalanine and kynurenine were higher in CRF patients than in healthy subjects. However, the serum concentrations of lysoPC (16∶0), lysoPC (18∶1) and tryptophan were lower in CRF patients than in the healthy subjects[46]. These results show that CRF has specific amino acid and phospholipid metabolic abnormality. A LC-MS/MS method was successfully applied to plasma metabolite profiling to survey > 350 small molecules in ESRDs patients, before and after hemodialysis. At base-

line, the increased levels of polar analytes and decreased levels of lipid analytes characterized the uremic plasma. Several metabolites in the plasma were identified as potential biomarkers, including dicarboxylic acids (adipate, malonate, methylmalonate, and maleate), biogenic amines, nucleotide derivatives, phenols, and sphingomyelins. The result showed a decrease in triacylglycerols (lower-molecular-weight) and an increase in several triacylglycerols (intermediate-molecular-weight) in ESRDs. These observations suggest a disturbed triglyceride catabolism and/or β-oxidation in uremic patients[47]. Other investigators developed an UPLC-MS approach to analyze the plasma samples of 10 patients with ESRDs who were being treated with hemodialysis, as well as in 16 healthy subjects. 1-Methylinosine was found to be an effective candidate biomarker to estimate an adequate dose of hemodialysis[48].

Lipid abnormalities are common in patients with kidney diseases. The plasma phospholipid metabolic profiles of chronic glomerulonephritis patients were investigated using LC/MS from 18 chronic glomerulonephritis patients, 17 CRF patients without renal replacement therapy and 18 healthy controls. The results showed that primary chronic glomerulonephritis and CRF had phospholipid metabolic abnormalities. Nineteen phospholipid species (C18∶0 – C18∶2, C18∶0 – C18∶1, C18∶0 – C18∶0, C18∶1 – C20∶4 *etc.*) were identified as possible biomarkers in plasma samples of chronic glomerulonephritis and CRF. This result suggests that phospholipids can be used as potential biomarkers of the progression in primary chronic glomerulonephritis[49]. In addition, other investigators determined the plasma and erythrocyte lipid profiles of patients with CRF during 30 months of hemodialysis. They found that the plasma and erythrocyte membrane levels of triglycerides were increased in CRF patients compared to healthy subjects. Plasma polyunsaturared fatty acids decreased, whereas palmitic and monounsaturated acids increased in CRF patients[50].

Oxylipin profiles in serum from IgA nephropathy patients supplemented with either fish oil or corn oil placebo were analyzed by LC-MS. A few of these metabolites were drivers of separation as assessed by multivariate analysis of fish oil patients pre- *vs* post-supplementation, including 17, 18-dihydroxyeicosatrienoic acid, prostaglandin D_3, prostaglandin E_3, resolvin E_1, 12-hydroxyeicosapentaenoic acid, and 10(11)-epoxydocosapentaenoic acid. In patients whose proteinuria improved, plasma total oxylipins as well as several hydroxyoctadecadienoic acids, hydroxyeicosatetraenoic acids, and leukotriene B_4 metabolites were among the metabolites that were significantly lower than in patients whose proteinuria either did not improve or worsen. These data support the involvement of oxylipins in the inflammatory component of IgA nephropathy as well as the potential use of oxylipin profiles as biomarkers and for assessing responsiveness to ω-3 fatty acid supplementation in IgA nephropathy patients[51].

The investigators performed comprehensive UPLC Q-TOF/MS analysis of urine in 73 nephrolithiatic children and 74 healthy children. The level of hypoxanthine was the most significant metabolite in nephrolithiasis patients. However, the levels of proline and 5C-aglycone were only barely detected in nephrolithiasis patients but represented remarkable metabolites in the healthy controls[52].

DN is one of the major complications of diabetes mellitus and has become the most prevalent cause of ESRDs across the world. A UPLC-TOF/MS method was employed to discriminate between the global serum profiles of eight DN patients, 33 type 2 diabetes mellitus patients and 25 healthy volunteers. The serum levels of leucine, dihydrosphingosine and phytosphingosine were observed, indicating perturbations of amino acid and phospholipid metabolism in diabetic diseases[53]. LC-MS method was established to discover the urinary biomarkers that differentiate the progressive form of albuminuria from the non-progressive one in humans. The discriminating metabolites included acyl-carnitines, acyl-glycines and metabolites related to the tryptophan metabolism[54].

3 Conclusion

Metabolomics is a novel discipline encompassing comprehensive metabolite evaluation, pattern recognition, and statistical analyses. Biomarkers are widely used in clinical medicine for prognostic or predictive interpretation of disease status. The application of metabolomics in CKDs studies has rapidly developed in the past five years and provided researchers with the opportunity to gain new insights into metabolic profiling and pathophysiological mechanisms. However, the use of metabolomics approaches in the study of CKDs is still in its initial phase and lags behind applications in other diseases. To date, LC-based metabolomics techniques have been applied to CKDs including glomerulonephritis, IgA nephropathy, CRF, ESRDs, diabetic nephropathy and nephrolithiasis in serum, plasma, urine, tissue and faeces. These researches have testified the power of metabolomics techniques to classify and potentially diagnose patients suffering from multiple CKDs. Many benefits have been shown from the use of metabolomics to identify biomarkers of CKDs. In particular, metabolomic approaches have the potential of diagnosing CKDs with better accuracy than traditional diagnostic methods. Furthermore, metabolomic methods have identified many metabolisms and metabolic pathways on CKDs.

Metabolomics used to study CKDs has produced some promising results and its future applications can mainly be in two areas. The first is to identify and describe the shifts in metabolite components and metabolism associated with different CKDs. Initial aims of metabolomics should concentrate on obtaining as much information as possible to provide an overview of specific-disease biochemistry. This should be done using both untargeted and targeted metabolic-profiling techniques, because untargeted metabolic-profiling often lacks the sensitivity of global metabolomics methods. Accordingly, in order to examine the complete underlying metabolic picture, there is a need for targeted metabolic-profiling methods to be performed in parallel to detect low intensity metabolites. The second area to which metabolomics can contribute to improved

disease management is improving our understanding of the metabolic mechanisms of disease pathology and how this underlying metabolic phenotype responds to therapeutic intervention.

The next decade should see some beneficial developments in the field of metabolomics. It is expected that the use of metabolomics techniques will become more routine, both in general and in applications to CKDs. One of the greatest challenges will continue to be how to obtain comprehensive coverage of the metabolome. While it is not expected that a single analytical platform will develop in the near future to address this need, the ability of MS to acquire a greater portion of the metabolome will increase. These efforts will most likely include multi-dimensional chromatography equipped with the ability to analyze several stationary phases in a single analysis. These complex multistationary phase instruments in combination with extremely high pressures, long analytical columns and potentialy microfluidics will provide the resolution necessary to chromatographically separate a metabolome. These instrument configurations will require high-resolution MS with very fast scanning rates and the ability to perform multiple MS/MS or MSn experiments without a significant loss in signal. Lastly, it is important to integrate the results of metabolomic assessments with other genomics, proteomics and transcriptomics technologies so that the entire spectrum of the CKDs can be characterized. Accordingly, multiple challenges remain to the widespread application of metabolomic methods, but the future is very bright. The field of metabolomics, as well as its application to the study of CKDs, will continue to grow.

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基于液相色谱的代谢组学的慢性肾病研究进展

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摘要：代谢组学属于系统生物学的一部分，是在某个特定环境下研究生物体系受刺激或扰动前后内源性小分子代谢产物图谱及其动态变化。代谢组学是采用现代不同分析技术测定生物体液、细胞提取物、细胞培养液或组织中代谢物的变化。代谢组学研究将为临床用药、疾病诊断和病理机制研究提供一个整体的方法。慢性肾脏病是常见病和多发病之一，其包括早期的慢性肾小球性肾炎（IgA 肾病）、后续的慢性肾损伤（糖尿病性肾病）、慢性肾衰竭（肾移植前后的终末期肾病）等。目前慢性肾病的病理机制和治疗尚未完全明确，本文概括液相色谱-代谢组学技术应用于阐明慢性肾病（动物实验和临床应用）的生物化学作用机制研究，以便寻找新的生物标志物，提出代谢组学应用于慢性肾病面临的挑战，希望代谢组学能鉴定的生物标志物应用于慢性肾病的早期诊断及相关治疗研究。

关键词：慢性肾病；代谢组学；液相色谱；动物模型；临床研究

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