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Review

UPLC-based metabonomic applications for discovering biomarkers of diseases in clinical chemistry

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ABSTRACT

Objectives: Metabonomics is a powerful and promising analytic tool that allows assessment of global low-molecular-weight metabolites in biological systems. It has a great potential for identifying useful biomarkers for early diagnosis, prognosis and assessment of therapeutic interventions in clinical practice. The aim of this review is to provide a brief summary of the recent advances in UPLC-based metabonomic approach for biomarker discovery in a variety of diseases, and to discuss their significance in clinical chemistry.

Design and methods: All the available information on UPLC-based metabonomic applications for discovering biomarkers of diseases were collected via a library and electronic search (using Web of Science, Pubmed, ScienceDirect, Springer, Google Scholar, etc.).

Results: Metabonomics has been used in clinical chemistry to identify and evaluate potential biomarkers and therapeutic targets in various diseases affecting the liver (hepatocarcinoma and liver cirrhosis), lung (lung cancer and pneumonia), gastrointestinal tract (colorectal cancer) and urogenital tract (prostate cancer, ovarian cancer and chronic kidney disease), as well as metabolic diseases (diabetes) and neuropsychiatric disorders (Alzheimer's disease and schizophrenia), etc.

Conclusions: The information provided highlights the potential value of determination of endogenous low-molecular-weight metabolites and the advantages and potential drawbacks of the application of UPLC-based metabonomics in clinical setting.

Contents

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Introduction

Metabonomics is a powerful new technology that allows assessment of global low-molecular-weight metabolites in biological systems and holds great potential in biomarker discovery. Analysis of the key metabolites in the body fluids has become an important part of the diagnosis, prognosis, and assessment of therapeutic interventions in clinical application [1]. This review is intended to provide an overview of the main applications of ultra-performance liquid chromatography (UPLC) in metabonomics and the current utility of the UPLC-based metabonomics in the fields of oncology, metabolic, neuropsychiatric, cardiovascular, infectious, and other diseases. Especial emphasis is placed on the potential use of endogenous low-molecular-weight metabolites in clinical chemistry.

Metabonomics

Metabonomics is defined as the "quantitative measurement of the dynamic multi-parametric metabolic responses of living systems to pathophysiological stimuli or genetic modifications" [2]. It is used to characterize the biochemical patterns of the endogenous metabolites in cells, body fluids or tissues for physiological evaluation, disease diagnosis and disease prognosis [3]. In contrast to classical biochemical approaches that often focus on a single metabolite, metabonomics reveals a collection of molecules which covers a broad range of small molecules such as lipids, amino acids, sterols, nucleic acids, peptides, organic acids, carbohydrates and vitamins and as such provides a comprehensive overview of the impact of the pathophysiological processes or pharmacological interventions of interest on metabolism and metabolic dynamics.

Metabonomic analytical technologies

Various analytical techniques are used in metabonomics which can be classified into two categories: I—nuclear magnetic resonance (NMR) and II—mass spectrometry (MS) [4]. Although other spectral approaches including Fourier transform ion cyclotron resonance, Raman and ultraviolet spectrum are employed for metabonomics studies, they are generally less sensitive than MS [5]. An increasing number of publications have described metabonomics using analytical techniques including ^{1}H NMR, gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (UPLC–MS) [6]. ^{1}H NMR, which is as one of the first methods used for metabonomics, represents a rapid, non-destructive and highly robust technology that provide highly informative structural information [7]. ^{1}H NMR is often used without any pre-separation process and unlike chromatography it does not require development. However, as each metabolite participates in the ^{1}H NMR spectra, the deconvolution of the signals is often quite tedious [8]. GC–MS based metabonomics can resolve hundreds of metabolite peaks, with metabolite identification performed by matching the fragmentation ion spectra and retention indices to the established database. The use of GC–MS is limited to the analysis of thermally stable, volatile and relatively non-polar components. Components' volatility can be increased using derivatization, which is laborious and increases annotation complexity [9]. UPLC–MS can analyze volatile, non-volatile and polar compounds, over a wider mass range than GC–MS and does not require sample derivatization [6]. However, UPLC–MS based metabonomics is hindered by the lack of established spectral database. Among the analytical techniques in metabonomics research, it is generally accepted that LC–MS is superior to NMR in terms of selectivity and sensitivity, while ^{1}H NMR and GC–MS based metabonomics are characterized by high reproducibility. Therefore use of UPLC–MS combined with ^{1}H NMR and GC–MS can provide a superior approach to study metabonomics.

UPLC–MS technique

The recently introduced UPLC technique is considered to be suitable for metabonomics, especially for large-scale untargeted metabonomics due to its high sensitivity in detecting metabolites [6]. UPLC operates with 1.7 μm chromatographic particles and a fluid system capable of operating at pressures in the 6000–15000 psi range, providing an increased chromatographic selectivity compared with conventional high performance liquid chromatography (HPLC) which uses larger particles [10]. Due to a reduction of peak width, there will also be a greater S/N ratio and an increasing sensitivity compared with the conventional HPLC. This can provide better peak resolution and higher sensitivity and speed for complex mixture separation. Because of the superior UPLC resolution, the problem of ion suppression is greatly reduced [10].

Mass spectrometry$^{\text{Elevated Energy}}$ (MS^{E}) technique was first applied to metabonomics by Plumb and co-workers [11]. Two scanning functions are simultaneously used for data collection. In the first function, Q1 is scanned from m/z 50–1000, and Q2 (collision cell) uses a normal low collision energy that provides for the transmission of intact ions through the cell collisions. These ions are then pushed into the TOF analyzer and detected with high resolution and mass accuracy. The second scan function also scans Q1 over the same mass range; however, Q2 has a high collision energy that fragments all of the ions transmitted through Q1. The resulting ions are again detected in the TOF analyzer. In this way, two mass chromatograms are generated, one with information on the intact molecules from the first function, and the other with the fragmented ion information from the second function. A variety of data-processing algorithms can be used to extract metabolite information from these data [12,13]. 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 small neutral molecules.

MS^E involves a simultaneous acquisition through 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 the unknown biomarkers in the context of untargeted analyses. Recently, the MS^E technique has proved to be a powerful tool for the identification of trace components of complex mixtures and for confirming their presence [14–19].

Data analysis of the UPLC-based metabonomics

The detection of biomarkers includes the use of various methods for taking the acquired mass values, retention time, and peak intensity and performing pattern recognition by the multivariate statistics including principal component analysis (PCA), orthogonal partial least squares-discriminant analysis (OPLS-DA), and partial least squares-discriminant analysis (PLS-DA). PCA is the most commonly used statistical method in metabonomics. PCA is an unsupervised multivariate data analysis method which provides a comprehensive view of the clustering trend for the multi-dimensional data. PCA can visualize correlated variations in more than two dimensions. This method represents data in the form of a linear combination of scores containing information on the tested samples and loadings containing information on the variables. The advantage of PCA is that the results are intuitively understandable owing to the graphical representation [20]. However, PCA is limited by the fact that it is not based on a statistical analytical model [21]. PLS-DA has gained wide applications in metabonomics and bioinformatics. PLS-DA is a PLS regression of a set Y of binary variables representing the kinds of a categorical variable on a set X of predictor variables [22]. It is a compromise between the usual discriminant analysis and a discriminant analysis on the significant principal components of the predictor variables. This method is suitable for processing plenty of predictors [22]. The OPLS-DA method is an extension of the PLS-DA method which integrates an orthogonal signal correction filter to distinguish variations that are suited to predict a quantitative response from variations that are orthogonal to prediction. OPLS-DA was shown to be a powerful tool for the analysis of qualitative data structures. The OPLS-DA score plot revealed good fitness and high predictability of the OPLS-DA model with high statistical values of R^2 and Q^2[23]. OPLS-DA method was used as a complement to the PLS-DA to discriminate two or more groups using multivariate data [24,25]. OPLS-DA model is calculated between the multivariate data and a response variable that only contains class information. The advantage of OPLS-DA compared to PLS-DA is that a single component is used as a predictor for the class, while the other variables describe the variation orthogonal to the first predictive component. UPLC-MS based metabonomics produces large amounts of raw data. The handling of such complex data sets manually is practically impossible. Hence several software tools and methods have been developed for processing and advanced statistical analysis of the raw data. A number of software tools from MS manufacturers and researchers have been developed to process metabonomics MS data. The software packages are linked to the corresponding analytical platform such as MarkerLynx from Waters to process the raw data. MS software packages apply special algorithms that filter and bin the raw data and then assign as a pair of retention time and m/z ratio. The software next aligns and normalizes the features found in the sample set, finally producing a large data matrix, which is then subjected to PCA, OPLS-DA, PLS-DA and other statistical analysis tools. In a comprehensive review article, Katajamaa and Oresic have described in detail the data processing methods for the MS-based metabonomics [26]. The final goal is to identify ions of interest on which the investigations can focus as a possible source of biomarker information. Biomarker identification employs a range of mass spectral techniques including MS, MS/MS, MS^E, isotope patterns and neutral losses, and searches in HMDB, Chemspider and KEGG. A sample workflow of UPLC–QTOF/MS is shown in Fig. 1.

UPLC-based metabonomics and biomarker discovery in clinical chemistry

Clinical chemistry deals with any analysis performed on the body fluids for a medical purpose including disease diagnosis, prognosis and treatment. Nowadays, most clinical tests still use the old method including single biomarker test, histopathology and immunohistochemistry. Current test methods are usually neither specific nor sensitive for a particular disease, and traditional biomarkers only change significantly after substantial disease injury or dysfunction has occurred. For example, serum creatinine (Scr) is the most commonly used biomarker of renal function. However, Scr concentrations may not change until a significant amount of renal function has been lost, meaning that renal injury is already present or occurs before Scr is elevated. In addition, the amount of tubular secretion of creatinine results in overestimation of renal function at lower glomerular filtration rates. Moreover, inter-individual differences in the body's muscle mass significantly alter Scr independent of renal function. Therefore, novel and more sensitive biomarkers are urgently needed for early detection and diagnosis of the disease. The UPLC-based metabonomic approach is now increasingly considered as a novel diagnostic approach in clinical studies including liver, lung, gastrointestinal, urogenital and other diseases. Table 1 displays UPLC-based metabonomic applications for discovering biomarkers of various diseases in clinical chemistry.

Hepatocarcinoma (HCC), liver cirrhosis and chronic liver diseases

HCC

Late diagnosis of HCC is one of the primary reasons for poor survival of patients. Identification of sensitive and specific biomarkers is of great importance in early diagnosis of HCC. Ressom et al. studied serum metabolites in HCC patients and cirrhotic controls. They found increased sphingosine-1-phosphate and LPC (17:0) and decreased glycochenodeoxycholic acid (GCDCA) 3-sulfate, glycocholic acid (GCA), glycodeoxycholic acid (GDCA), taurocholic acid (TCA), and taurochenodeoxycholate which are involved in bile acid biosynthesis and cholesterol metabolism in HCC patients compared to patients with liver cirrhosis [27]. Another study identified serum 1-methyladenosine as a characteristic metabolite in HCC [28]. Serum and urinary metabonomics were performed on patients with HCC and benign liver tumor as well as healthy controls. 43 serum metabolites and 31 urinary metabolites involved in bile acids, free fatty acids, glycolysis, and methionine metabolism as well as urea cycle were identified in HCC patients. Bile acids, histidine and inosine were markedly elevated in HCC patients. However, liver cirrhosis and hepatitis were associated with alterations of several bile acids including GCDCA, GCA, TCA and chenodeoxycholic acid (CDCA). The HCC patients with α fetoprotein were successfully differentiated from healthy controls using metabolite biomarkers [29]. In addition, UPLC–QTOF/MS and UPLC–MS/MS approaches were used for qualitative and quantitative analyses of serum biomarkers for patients with HCC. The results indicated that patients with HCC had decreased LPCs, increased long-chain and decreased medium-chain acylcarnitines, and increased aromatic and decreased branched-chain amino acid [30]. UPLC–QTOF/MS and UPLC triple quadrupole linear ion trap MS approaches were performed on qualitative and quantitative comparisons of metabolite levels in sera of HCC patients and cirrhosis patients from Egypt [31]. The metabolites including GCA, GDCA, 3β,6β-dihydroxy-5β-cholan-24-oic acid, oleoyl carnitine and Phe-Phe were identified by UPLC–QTOF/MS. UPLC triple quadrupole linear ion trap MS-based quantitation confirmed significant differences between HCC and cirrhotic controls

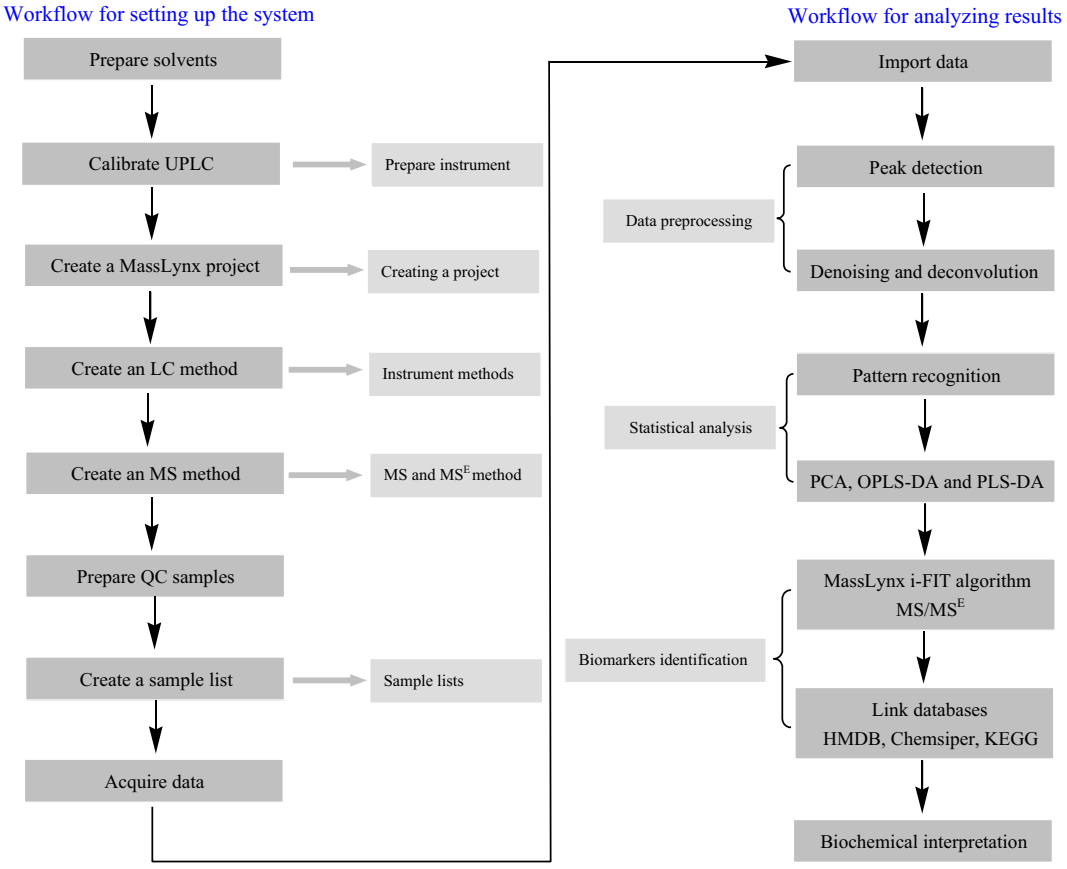

Fig. 1. A typical example workflow of metabolomics using UPLC Q-TOF/MS with MS^E data collection technique as research tools for discovering and quantifying metabolites in complex mixtures.

in the metabolite levels of bile acid metabolites, long chain carnitines and small peptide.

To discriminate HCC from liver cirrhosis, UPLC-based metabonomics has been conducted to characterize serum profiles from HCC patients, liver cirrhosis patients and healthy subjects. Metabolic profiles were capable of discriminating not only HCC patients from the controls but also HCC from liver cirrhosis. Thirteen biomarkers were identified and suggested that there were significant disturbances of organic acids, phospholipids, fatty acids, bile acids and gut flora metabolism in HCC patients. Canavaninosuccinate was first identified as a metabolite that is significantly reduced in liver cirrhosis and increased in HCC. In addition, GCDCA was suggested to be an important indicator for HCC diagnosis and disease prognosis [32]. Chronic liver diseases including chronic hepatitis B and hepatic cirrhosis are the major risk factors for development of HCC. The differential diagnosis between chronic liver diseases and HCC is a challenge. Serum metabonomics showed that long-chain acylcarnitines accumulated, whereas free carnitine, medium and short-chain acylcarnitines decreased with the severity of the non-malignant liver diseases, accompanied by the corresponding alterations of the enzyme activities. However, the magnitude of the changes was smaller in HCC than in hepatic cirrhosis, possibly due to the differences in energy metabolism in the tumor cells [33].

Urinary metabolome of patients suffering from HCC was studied using UPLC–MS (Fig. 2) and 21 metabolites were considered as potential biomarkers. Urinary metabolites related to arginine and proline metabolism, alanine and aspartate metabolism, lysine degradation, fatty acid oxidation, nicotinate and nicotinamide metabolism were significantly changed in HCC patients[34]. UPLC-based urinary metabonomics was used to explore common and specific metabolites in HCC patients with hepatitis B virus (HBV) or hepatitis C virus (HCV) infections. Increased arachidonic acid and decreased lysophosphatidylcholines (LPCs) were observed in the HCC and cirrhosis patients compared with the healthy control, which may partly contribute to chronic inflammation and the initiation and progression of the malignant hepatoma. Decreased ratios of polyunsaturated to saturated LPCs in patients with HCC compared with chronic liver disease patients with HBV or HCV infection and healthy controls further demonstrated the profound influence of the malignant liver tumor independent of viral infection. Significant increases in serum endocannabinoids, anandamide and palmitylethanolamide, were found in the HCC compared with the healthy control and in HCC patients with HCV compared with corresponding patients with chronic liver disease. Endocannabinoids anandamide and palmitylethanolamide showed better sensitivity and specificity as potential biomarkers to distinguish the HCC from cirrhosis associated with HCV infection [35]. The UPLC metabonomics was also used to identify and measure the metabolic profile of GCA in HCC patients. HCC patients had increased urinary GCA which was associated with changes in primary bile acid biosynthesis, secondary bile acid biosynthesis and bile secretion [36]. UPLC-based fecal metabonomics were performed on the liver cirrhosis and HCC patients and healthy volunteers. CDCA dimeride, urobilin, urobilinogen, 7-ketolithocholic acid, LPC(18:0) and LPC(16:0) were considered as potential biomarkers with a strong increase in LPCs and a dramatic decrease in bile acids and bile pigments in patients with liver cirrhosis and HCC compared with the healthy volunteers [37]. In addition, UPLC and linear trap quadrupole-Orbitrap XL-MS platform were used to analyze endogenous metabolites in the homogenate of central tumor tissue, adjacent tissue, and distant tissue obtained from 10 HBV-related HCC patients [38]. Five metabolites quinaldic acid, β-sitosterol, arachidyl carnitine, oleamide and tetradecanal were observed for the first time. Nine metabolite lysophosphatidylethanolamines,

Table 1
UPLC-based metabolomic applications for discovering biomarkers of diseases in clinical chemistry.

Experimental conditions	Application	Biological medium	Reference
UPLC–QTOF/MS UPLC–QqQLIT/MS UPLC–QqQ/MS	HCC with liver cirrhosis	Serum	[27]
UPLC–MS	HCC	Serum	[28]
UPLC–QTOF/MS GC–TOF/MS	HCC	Serum, Urine	[29]
UPLC–QTOF/MS UPLC–MS/MS	HCC	Serum	[30]
UPLC–QTOF/MS UPLC–QqQLIT/MS	HCC	Serum	[31]
UPLC–QTOF/MS	HCC from liver cirrhosis	Serum	[32]
RRLC–QTOF/MS	HCC and CLD	Serum	[33]
UPLC–QTOF/MS	HCC	Urine	[34]
UPLC–QTOF/MS HPLC–TQ/MS	HCC with HBV or HCV	Serum	[35]
UPLC–QTOF/HDMS/MS^E	HCC	Urine	[36]
UPLC–QTOF/MS	HCC and liver cirrhosis	Feces	[37]
UPLC–LTQ Orbitrap XL-MS	HCC	Liver tissue	[38]
UPLC–QTOF/MS UPLC–TQ/MS GC–TOF/MS	HCC	Plasma	[41]
UPLC–QTOF/MS GC–TOF/MS	Hepatitis B cirrhosis	Urine	[42]
UPLC–QTOF/MS	Acute and chronic liver failure	Plasma	[43]
UPLC–QTOF/MS	Liver cirrhosis	Serum	[44]
UPLC–QTOF/HDMS/MS^E	HBV	Urine	[45]
UPLC–QTOF/MS	Primary biliary cirrhosis	Serum	[46]
UPLC-QTOF/MS	Liver transplantation	Bile	[47]
UPLC–QTOF/MS	Lung cancer	Plasma	[53]
UPLC–QTOF/MS	Lung cancer	Plasma	[54]
RRLC–QTOF/MS	Lung cancer	Urine	[55]
UPLC–HILIC-QTOF/MS	Lung cancer	Plasma	[58]
UPLC–Orbitrap MS GC–MS	Lung cancer	Serum, Plasma	[59]
UPLC–QTOF/MS	Pneumonia	Plasma, Urine	[60]
UPLC–QTOF/MS	Colorectal cancer	Urine	[64]
UPLC–QTOF/MS GC–TOF/MS	Colorectal cancer	Serum	[65]
UPLC–QTOF/MS GC–TOFMS	Colorectal cancer	Serum	[66]
UPLC–QTOF/MS SPE-HPLC	Colorectal cancer	Urine	[67]
UPLC–QTOF/MS	Colorectal cancer	Colon	[68]
UPLC–QTOF/MS	Intestinal fistulas	Urine	[69]
UPLC–LTQ/MS GC–MS	Prostate cancer	Plasma	[70]
UPLC–QTOF/MS	Ovarian cancer	Serum	[72]
UPLC–QTOF/MS	Ovarian cancer	Plasma	[73]
UPLC–QTOF/MS	Chronic renal failure	Serum	[74]
UPLC–QTOF/MS	Acute kidney injury	Urine	[75]
UPLC–QTOF/MS	Renal nephrolithiasis	Urine	[78]
UPLC–QTOF/MS GC × GC–TOF/MS	Autoimmune diabetes	Serum	[80]
UPLC–MS/MS ^{1}H NMR	Type 1 diabetes	Plasma	[81]
UPLC–QTOF/MS	Type 2 diabetes	Serum	[85]
UPLC–QTOF/MS ^{1}H NMR	Type 2 diabetes	Serum	[86]
UPLC–QTOF/MS GC–MS ^{1}H NMR	Type 2 diabetes	Serum	[87]
HILIC/UHPLC–MS RP/UHPLC–MS	Alzheimer's disease	Cerebrospinal fluid	[94]
HILIC/UHPLC–MS	Alzheimer's disease	CSF, Plasma	[95]
UPLC–QTOF/MS	Cerebral infarction	Serum	[99]
UPLC–QTOF/MS	Acute cerebral infarction	Plasma	[101]
UPLC–QTOF/MS GC × GC–TOF/MS	Schizophrenia	Serum	[107]
UPLC–QTOF/MS ^{1}H NMR	Schizophrenia	Plasma	[111]

glycerophosphocholine, LPCs, CDCA glycine conjugate and L-phenylalanine had been reported as serum metabolite biomarkers for HCC diagnosis in previous research [39–41].

Urinary metabonomics using GC–TOF/MS and UPLC–QTOF/MS was carried out to study post-hepatitis B cirrhosis patients. The study showed significant changes in α-hydroxyhippurate, tyrosine-betaxanthin, 3-hydroxyisovalerate, canavaninosuccinate, estrone, and glycoursodeoxycholate among cirrhotic patients reflecting disturbance of amino acid, bile acids, hormonal and intestinal microbial metabolism [42].

Liver cirrhosis and chronic liver diseases

Liver failure induced by HBV is a severe disease with a high mortality rate. Plasma was employed to investigate metabonomics in acute-on-chronic liver failure patients. LPCs, primary fatty acid amides and conjugated bile acids were identified. LPCs and conjugated bile acids were found to be associated with survival whereas primary fatty acid amides represented risk factors [43]. Serum metabonomics was analyzed from control subjects and patients with alcoholic cirrhosis or HBV-induced cirrhosis. Decreased serum LPCs and increased serum GCA, GCDCA, hypoxanthine and stearamide were observed in cirrhosis patients and are considered common biomarkers for hepatic cirrhosis. Oleamide and myristamide were increased in patients with alcoholic cirrhosis but were decreased in those with HBV-induced cirrhosis. These could be specific biomarkers for differential diagnosis between alcohol- and HBV-induced hepatic cirrhosis [44]. Eleven urinary metabolites were identified potential predictors of progression of HBV-related liver disease. The biotin sulfone, 5-oxo-heneicosanoic acid, D-glucosaminide and 2-methylhippuric acid were effective for the diagnosis of human HBV [45].

Primary biliary cirrhosis and primary sclerosing cholangitis are two cholestatic diseases characterized by hepatic accumulation of bile acids. Serum metabonomics was carried out to explore patients with primary biliary cirrhosis, with severe pruritus, and without pruritus and healthy controls. More than 400 serum metabolites were identified from patients with primary biliary cirrhosis. The metabolic profile of patients with primary biliary cirrhosis and pruritus was characterized by a significant change in the lipid metabolites, particularly in the ceramides, sphingomyelins and LPCs [46]. Bile flow restoration is a crucial step in the recovery process following liver transplantation. UPLC metabonomics has been conducted to monitor total bile fingerprint during human liver transplantation. Ten major conjugated bile acid salts were measured and significantly increased TCA and taurochenodeoxycholic acid (TCDCA) were observed after transplantation. Bile acid ratios in the donor liver at the pre-transplant and post-transplant stage may be important and that profiling of secreted bile after transplantation may aid clinical assessment and progress post-transplantation [47].

It has been reported that the abnormal bile acids and lysophospholipids are associated with liver cirrhosis and hepatitis [48]. Conjugated bile acids can bind lipids, cholesterol and fat-soluble vitamins. GDCA was reported to play an important role in the detoxification of lipophilic compounds [49]. Decreased serum bile acids have been related to the accumulation of toxic and even carcinogenic bile acid in liver that may be caused by the alteration in the bile acid transport pathway [50]. GCDCA has been reported as an inducer of apoptosis in human hepatocyte [51]. Abnormality of bile acid biosynthesis leads to development and progression of liver cancer [52].

Lung cancer and pneumonia

Lung cancer

Lung cancer is one of the most common cancers in the world, but reliable clinical biomarkers that can help to diagnose and assess prognosis of the disease at an early stage are lacking and urgently needed. UPLC-based metabonomics was used to find potential biomarkers by

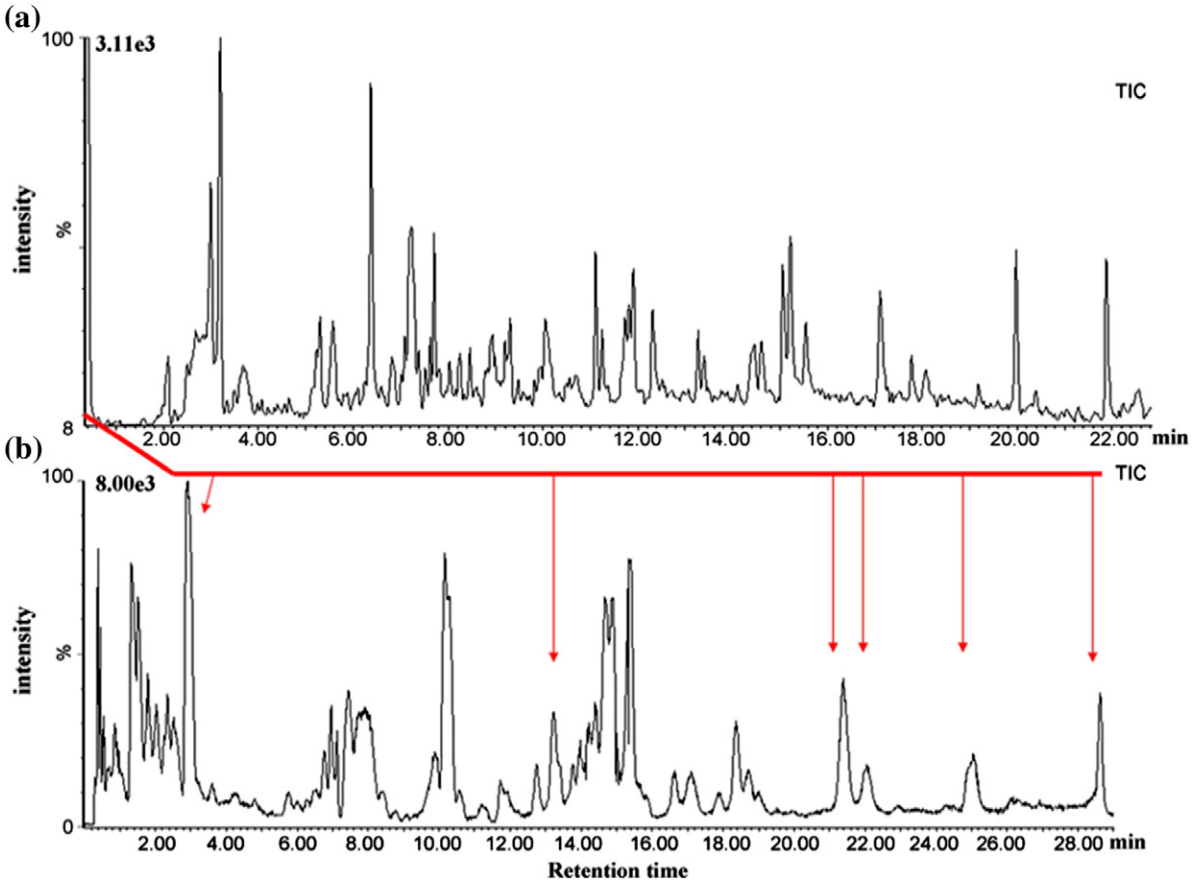

Fig. 2. Typical urinary total ion chromatograms separated on (a) reversed-phase liquid chromatography and (b) hydrophilic interaction chromatography from patients with liver cancer. Several metabolites marked with an arrow were not retained on reversed-phase column but well separated on a hydrophilic interaction column.

comparing serum samples from lung cancer patients with healthy volunteers. LPC(16:0), isomer of LPC(16:0), LPC(18:0), LPC(18:1) and LPC(18:2) were identified as specific biomarkers [53,54]. Decreased LPCs may be explained by Lands' cycle pathway. In lung cancer, cell proliferation is accompanied by a high metabolic state, and abnormal LPCAT1 results in the reduction of LPCs. Other investigators developed a rapid resolution liquid chromatography–mass spectrometry (RRLC–MS) for global metabolic profiling of the urine in lung cancer patients. Eleven potential biomarkers including amino acids, nucleosides and indole were identified. The study revealed elevated amino acid and nucleoside metabolism as well as protein degradation in patients with lung cancer [55]. Previous studies indicated increased urinary aromatic amino acids that might be caused by derangement of protein metabolism in cancer patients [56]. Indoxyl is metabolic end-products of the tryptophan's metabolite indole, both of which have been implicated as etiological factors in development and growth of cancer. Significant variations of modified nucleosides have been demonstrated in various types of cancer due to the regulated cell turnover rate, activity of modifying enzymes, and RNA/DNA modifications [57]. In addition, highly polar metabolites were also compared in the plasma from lung cancer patients and healthy volunteers. Nineteen metabolites showed a significant difference between lung cancer patients and healthy controls. This method was also applied to the effect of radiotherapy on highly polar metabolites. Nineteen metabolites were altered at different points during the course of radiotherapy [58]. Serum and plasma metabonomics were developed and tested in patients with small-cell lung cancer. Plasma glycerophosphocholines, erythritol, creatinine, hexadecanoic acid and glutamine were associated with life expectancy and response to the clinical management in small-cell lung cancer patients [59].

Pneumonia

Pneumonia remains the leading cause of death in young children worldwide. Increased plasma uric acid, hypoxanthine and glutamic acid and decreased L-tryptophan and adenosine-5′-diphosphate were observed in pneumonia patients. This was associated with decreased urinary uric acid and L-histidine in these patients [60]. Based on the previous studies, the identified metabolites are important for the host's response to infection through antioxidant, inflammatory, and antimicrobial pathways and energy metabolism [61–63].

Gastrointestinal diseases

Colorectal cancer (CRC) is the third most common cancer worldwide, and its prognosis if not detected at early stages is poor. Both targeted and untargeted metabonomics have been used to identify biomarkers of CRC. UPLC–MS was applied to explore urinary metabolic profile in patients with CRC undergoing colorectal resection. The study showed a significant increase in two compounds with molecular weights of 283 and 234 in patients before surgery compared with healthy volunteers. The levels of these metabolites significantly decreased after the surgical resection of the tumor [64].

GC–MS and UPLC–MS-based metabonomics developed and applied on the serum from CRC patients revealed perturbation of glycolysis, arginine and proline metabolism, fatty acid metabolism and oleamide metabolism and its association with CRC morbidity [65]. Tricarboxylic acid cycle, urea cycle, glutamine, fatty acids, and gut flora metabolism were disturbed [66], which are consistent with previous findings [67]. Other studies showed that identified metabolites form CRC patients are related to glutamine metabolism, fatty acid oxidation, nucleotide biosynthesis and protein metabolism [68]. UPLC–MS method was

developed and validated for the targeted profiling of eight relevant eicosanoids and the major metabolic precursor, arachidonic acid in the human colon. Arachidonic acid, prostaglandin E2, prostacyclin and 12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid were found to be significantly different between cancerous and normal mucosae [69]. The previous studies also showed that eicosanoids such as prostaglandins, leukotrienes, thromboxanes and hydroxy eicosatetraenoic acids play an important role in promoting inflammation in CRC patients.

Urogenital diseases

Prostate cancer

UPLC-based metabonomics has been applied to identify biomarkers for non-invasive diagnosis and prognosis, and aggressiveness of urogenital cancers. One of the major biomarker discovery studies in this field was the unbiased metabonomics performed using plasma, tissue and urine from patients with biopsy proven prostate cancer and biopsy negative controls [70]. More than 1126 metabolites were identified to be related to prostate cancer using two complementary GC–MS and UPLC–MS methods. The tissue metabonomics was able to distinguish normal prostate, localized prostate cancer, and metastatic prostate cancer. For 628 tissue metabolites, sixty metabolites were found in localized and/or metastatic tumors but not in normal prostate. Significantly increased sarcosine, uracil, kynurenine, glycerol-3-phosphate, leucine and proline levels were observed from benign to clinically localized prostate cancer and metastatic prostate cancer. Sarcosine was highly increased in tissues during prostate cancer progression to metastasis. Sarcosine was identified as a potential candidate for early disease detection and marker of aggressiveness of prostate cancer. However, monitoring of prostatic tissue sarcosine is of limited interest since histological examination of the available tissue is a more powerful tool for the diagnosis and the prognosis of cancer. For this reason the authors focused on urine sarcosine. They found that sarcosine was detectable in the urine at trace levels and had a modest but significant predictive value for prostate cancer diagnosis and disease progression. In addition to being a biomarker of prostate cancer, Sarcosine serves as a cytokine that contributes to the progression of the disease. In fact using molecular approaches and targeting metabolic pathways, the authors demonstrated the role of sarcosine in modulating cancer cell invasion and migration, making it a potential target for development of novel therapeutic interventions. Another study demonstrated that lipid metabolism and insulin resistance were decreased in this condition [71].

Ovarian cancer

The UPLC-based untargeted metabonomics has been conducted to identify and validate novel metabolic biomarkers for the epithelial ovarian cancer (EOC) and benign ovarian tumors (BOT). The study revealed increased 27-nor-5β-cholestane-3,7,12,24,25 pentol glucuronide (CPG), phenylalanine, GCA, propionylcarnitine, Phe-Phe and LPC(18:2) levels in EOC compared to the BOT specimens and as such could be considered as potential biomarkers. In particular tissue CPG level was significantly higher in EOC tissue compared with BOT tissue and increased CPG level was found in early stages of EOC and in all of its three histological types. For this reason CPG was considered to be complementary to CA125 and a relevant biomarker for detection of early stages of EOC [72]. UPLC–MS metabonomics has also been applied to differentiate between EOC and BOT. Decreased plasma L-tryptophan, LPC(18:3), LPC(14:0) and 2-piperidinone were observed in EOC patients when compared to the BOT patients. Tryptophan and LPCs have been suspected to participate in cancer progression, and 2-piperidinone might be a novel biomarker for EOC [73]. Accelerated L-tryptophan degradation has been observed in the blood of EOC patients when compared to the healthy controls [74]. A similar phenomenon has been observed in other malignant tumors. The abnormal levels of LPCs are due to binding and activation by the specific cell-surface G protein-coupled receptors, which initiate cell growth, proliferation, and survival pathways [75].

Chronic kidney disease (CKD)

UPLC-based metabonomics was employed to investigate the serum from chronic renal failure patients. Increased LPC(18:0), phenylalanine and kynurenine and decreased LPC(16:0), LPC(18:1) and tryptophan were observed in chronic renal failure patients [76]. UPLC-based metabonomics was developed to analyze the plasma samples from end-stage renal disease patients. 1-Methylinosine was found to be an effective candidate biomarker to estimate adequacy of a hemodialysis regimen [77]. One study reported that urinary hypoxanthine was the most significant metabolite in children with nephrolithiasis. However, other investigator demonstrated that proline and 5C-aglycone were barely detected in the urine of these patients but were abundant in the healthy controls [78]. Based on the ^{1}H NMR and UPLC–MS/MS techniques, urine metabolome was analyzed from 15 patients with CKD and 15 healthy controls to find a classification pattern clearly indicative of CKD. Seven urinary metabolites glutamate, 5-oxoproline, guanidoacetate, taurine, phenylacetylglutamine, trimethylamine N-oxide and citrate differed between CKD and non-CKD urine samples [79].

Metabolic diseases

Type 1 diabetic (T1D)

Insulin is as a major postprandial hormone with profound effects on carbohydrate, lipid, and protein metabolism. Serum metabonomics indicated that specific metabolic disturbances precede β-cell autoimmunity in humans and can be used to identify T1D children [80]. These findings suggest alternative metabolism-related pathways as therapeutic targets to prevent diabetes. Another UPLC-based metabonomic study has revealed significant perturbations in plasma amino acids and amino acid metabolites during insulin deprivation in T1D. Several known metabolic pathways are perturbed in acute insulin deprivation T1D [81]. Plasma branched chain amino acids are increased in untreated T1D [82] and have been attributed to the breakdown of muscle protein and release of amino acids in the circulation [83] and liver [84].

Type 2 diabetes (T2DM)

T2DM and its attendant complications have emerged as a major public health problem worldwide. T2DM is a typical metabolic disorder characterized by insulin resistance and relative deficiency of insulin production. Fatty acid metabolism and free fatty acid levels are markedly altered in diabetic patients [85]. Increased plasma acylcarnitines and tryptophan and decreased plasma LPC(16:0), LPC(18:0), LPC(18:2) and phenylalanine were reported in treated T2DM patients [86]. Another study has shown changes in plasma amino acid and perturbation of metabolic pathways linked to 3-indoxyl sulfate, glycerophospholipids, free fatty acids and interaction with the bile acids in diabetic patients [87]. Fatty acids can improve insulin secretion in the basal or glucose stimulated states and fatty acids are essential for stimulus-secretion coupling in β-cells [88]. However long-term elevation of free fatty acids can induce or aggravate insulin resistance and contribute to the development and progression of type 2 diabetes [89]. Extensive studies have shown that high level of circulating free fatty acids can inhibit insulin receptor substrates (IRSs) function [90,91].

Neuropsychiatric diseases

Alzheimer's disease (AD)

AD is a neurodegenerative disorder which is characterized by progressive loss of cognitive functions and is the most common cause of dementia among older people. The results of the UPLC-based metabonomic studies in AD have been summarized in several published

reviews [92,93]. UPLC–MS metabonomics used to investigate cerebrospinal fluid (CSF) has revealed significant metabolic differences in subjects during the course of AD progression [94]. Mild cognitive impairment (MCI) is considered as a transition phase between normal aging and AD and its presence increases the risk of developing AD. UPLC–MS was applied to plasma and CSF from patients with different AD severity. Plasma lysine and CSF Krebs cycle were significantly affected in individuals with MCI compared to those with normal cognitive function. Cholesterol and sphingolipids metabolisms were altered in both CSF and plasma of AD patients. Other metabolic pathways including energy metabolism, Krebs cycle, mitochondrial function, neurotransmitter and amino acid metabolism and lipid biosynthesis were disturbed in MCI and AD patients. Plasma polyamine and lysine, and tryptophan metabolism and aminoacyl-tRNA biosynthesis and CSF cortisone and prostaglandin 2 biosynthesis and metabolism could discriminate between different groups [95]. Abnormal neuronal networks and neurotransmitter systems are one of the main dysfunctions in AD. Many studies have demonstrated that synaptic malfunction and synaptic loss occur prior to the development of amyloid β-plaques and neurofibrillary tangles [96]. These alterations of synaptic function are directly related to deterioration of synaptic strength and synaptic plasticity [96]. Acetylcholine, dopamine, serotonin and noradrenalin are primarily affected in AD with subsequent loss of the associated neurons [97]. Lysine, is a strictly ketogenic amino acid that is required for the synthesis of L-carnitine. L-Carnitine is the sole transporter of fatty acids to mitochondria for energy production and carnitine level has been shown to be lower in CSF of MIC-AD and AD patients than in CSF from non-AD subjects [98].

Stroke

Cerebral infarction is an acute neurological disorder which has serious consequences. Serum metabonomics was obtained from cerebral infarction patients and healthy controls. Folic acid, cysteine, S-adenosyl homocysteine and oxidized glutathione were identified as potential biomarkers of cerebral infarction [99]. These biomarkers are associated with the conjoined activated one-carbon and the folate cycle which are involved in protein and DNA stabilization, synthesis of various molecules, and protection against toxins and reactive oxygen metabolites [100].

UPLC-based metabonomics was employed to investigate non-dampness-phlegm and dampness-phlegm patients. LPC(18:2) and LPC(20:3) were lower in dampness-phlegm than in non-dampness-phlegm stroke pattern. However, increased LPC(18:0) and LPC(16:0) were observed in dampness-phlegm pattern [101]. The results demonstrated that plasma LPCs with polyunsaturated fatty acid were associated with dampness-phlegm pattern and suggested that variation of plasma lipid profiles could serve as potential biomarker for diagnosis of dampness-phlegm pattern [102]. Previous reports suggested the possibility of relationships between plasma LPC levels and dampness-phlegm pattern. It was known that dampness-phlegm pattern was related to obesity and hyperlipidemia [103,104], and some metabonomic analyses showed that plasma LPC levels were also associated with obesity [105,106].

Schizophrenia

UPLC–MS and two-dimensional GC–MS serum metabonomics were applied to schizophrenia patients who had significantly higher lipid and amino acid levels compared with the health controls [107]. Previous studies demonstrated that metabolic abnormalities of schizophrenia were related to glucoregulatory processes and proline metabolism [108–110]. A combined UPLC–MS and ^{1}H NMR-based metabonomics was used to study patients with new-onset neuroleptic-naive schizophrenia before and after a 6-week risperidone monotherapy. A group of healthy control individuals served as controls. The study revealed a disturbance in neurotransmitters and their metabolites together with 32 identified biomarkers that underpin pathways involved in neurotransmitters, amino acids, glucose, lipids, and energy metabolism, as well as antioxidant defense system, bowel microflora and endocrine system in schizophrenic patients. Bonferroni analysis of the data showed that among these metabolites pregnanediol, citrate and α-ketoglutarate were significantly associated with symptomatology of schizophrenia and may be useful biomarkers for monitoring therapeutic efficacy [111].

Concluding remarks and perspectives

Metabonomics is a potent and promising new approach that allows the assessment of global low-molecular-weight endogenous and exogenous metabolites in a biological system and which shows great potential in investigation of physiological status, discovery of biomarkers, identification of metabolic pathways, and diagnosis of diseases and assessment of drug therapy and safety. The use of UPLC–QTOF/MS with a MS^E data collection technique has progressed and is now very popular because it is versatile, sensitive and provides comprehensive information about low-molecular-weight metabolites. The aim of this review was to introduce UPLC-based metabonomics and to present and discuss its key applications focusing on disease biomarkers in clinical chemistry. The clinical chemistry of application of metabonomics for study of the above-mentioned diseases has great potential for disease diagnosis and new biomarker discovery. Analysis of the key endogenous metabolites in the body fluids has become an important part of improving the diagnosis, prognosis, and therapy of human diseases. Metabonomics could help to discover early biochemical changes of disease and understand the mechanism of disease occurrence and progression on the metabolic level and provide information for the identification of early and differential metabolic markers. The clinical application of metabonomics could provide comprehensive information and improve the feasibility of high-throughput patient screening for diagnosis of disease status or risk evaluation. Indeed, identification of clinically relevant metabolites that may be regarded as potential new biomarkers will also help with the evaluation of prognosis and contribute to the development of new therapeutic strategies.

The metabolome is characterized by a large diversity of chemical structures requiring diverse analytical platforms (^{1}H NMR, UPLC, GC, MS, etc.) to reach its comprehensive coverage. Despite recent technological and conceptual improvements, metabonomics appears to be still in its infancy and sample preparation, acquisition of metabolic profiling, metabolite detection, statistical analyses and biomarker identification is a bottleneck in itself. How to accelerate metabonomics studies is, therefore, a challenging issue. The published papers in the field of clinical metabonomics have remained in the discovery phase and most of the identified potential biomarkers have not been adopted in routine clinical practice. Due to the complexity and various factorial interactions of diseases, the situation seems difficult in the field of clinical chemistry for which multiplexed targeted approaches provide the clinician with information on a limited number of metabolites by using MS/MS analysis performed on triple quadruple MS. Furthermore, recent improvements in UPLC–QTOF/MS/MS^E have improved the efficacy of global approaches by facilitating the identification of metabolites of interest thanks to high-resolution and accurate mass measurements, which Q-TOF/MS/MS^E can simultaneously acquire MS and MS/MS (MS^E) data of all the metabolites through alternating between high and low collision energies during a single chromatographic run [112–120].

Despite significant advances there are several limitations of current technology including lack of a single method for extensive analysis of the entire metabolome, limited spectral libraries and databases, and disadvantages of current metabonomic software for data processing and biomarker extraction. Further research is needed before finding a reasonable method for metabolite analysis that can replace or complement the traditional and non-specific diagnostic method or technology in clinical chemistry. Future technological development combined with more robust data analysis and bioinformatic tools will help to overcome

the current limitations and fully integrate small molecule biochemistry with systems biology. Because metabonomics is complementary to genomics, transcriptomics and proteomics, full integration of four omics technologies will ultimately improve personalized molecular diagnosis and disease treatment. Further improvements in the sensitivity and selectivity of analytical techniques and routine use of novel methods are certain to find novel targets in the future.

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