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A REVIEW ON METABOLOME ANALYSIS

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ABSTRACT
Metabolomics is a scientific study of various chemical processes involving metabolites, the small molecule intermediates and products of metabolism. Metabolic profiling that can give an instantaneous snapshot of the physiology of that cell, and thus, metabolomics provides a direct "functional read out of the physiological state" of an organism. Metabolomics offers a platform for the comparative analysis of metabolites that reflect the dynamic processes underlying cellular homeostasis, there are numerous analytical platforms that have been used for metabolomic applications, such as NMR, Fourier transform infrared spectroscopy (FT-IR) and MS coupled to separation techniques, including NMR, GC-MS, LC-MS, FT-MS and UPLC-MS. Whilst NMR spectroscopy is particularly appropriate for the analysis of bulk metabolites and GC-MS to the analysis of volatile organic compounds and derivatised primary metabolites, LC-MS is highly applicable to the analysis of a wide range of semi-polar compounds including so many secondary metabolites of interest. Since LC-MS can avoid chemical derivatization, it is a widely used instrument. MS-based metabolomics offers high selectivity and sensitivity for the identification and quantification of metabolites, therefore, parallel application of several techniques, for example GC-MS, LC-MS or NMR, is desirable to study the global metabolome. In this review the application of each hyphenated technique along with their strengths and limitations are discussed.

KEYWORDS: Metabolomics, Metabolome, Nuclear magnetic resonance (NMR), Mass spectrometry (MS), Liquid chromatography mass spectrometry (LC–MS), capillary electrophoresis mass spectrometry (CE-MS).

INTRODUCTION
The metabolome encompasses all low molecular weight metabolites that are produced by cells during metabolism, and provides a direct functional read out of cellular activity and physiological status. Metabolomics is an emerging discipline that aims to profile all low molecular weight metabolites present in biological samples.[1] It is concerned with both targeted and non-targeted analysis of endogenous and exogenous small molecule metabolites (<1500Da), and presents a promising tool for biomarker discovery.[2,3] It is being used in assessing responses to environmental stress, comparing mutants, drug discovery, toxicology, nutrition, studying global effects of genetic manipulation, cancer, comparing different growth stages, diabetes and natural product discovery.[4-6] Metabolomics is a global metabolic profiling framework which utilizes high resolution analytics (typically NMR and MS) together with chemo metric statistical tools such as principal component analysis (PCA) and partial least squares (PLS), to derive an integrated picture of both endogenous and xenobiotic metabolism. The small molecules, including peptides, amino acids, nucleic acids, carbo hydrates, organic acids, vitamins, polyphenols, alkaloid and inorganic species act as small-molecule biomarkers that represent the functional phenotype in a cell, tissue or organism.[7] Separation and identification of these small molecules is made possible by the technological advances in metabolomics.

The innovational technologies, including accurate measurement of high-resolution MS, NMR, CE, HPLC and UPLC technology, can accomplish detection of metabolites within a few minutes. Metabolites are biological characteristics that are objectively measured and evaluated as indicators of normal biological and pathological processes or pharmacological responses to a therapeutic intervention, widely used in clinical practice for clinical diagnosis.[8-10] Metabolomics has been applied to define metabolites related to prognosis or diagnosis of diseases and could provide greater pathophysiological understanding of disease. Application of the cutting edge and analytical technologies to the measurement of various metabolites and their characteristic changes in the metabolite concentrations under defined conditions have helped illuminate the effects of perturbations in pathways of interest.[11] Owing to the complexity of metabolites and the large number of

metabolites there in, advanced and high throughput separation techniques have been coupled to high resolution MS, but not exclusively, to make these measurements. Thus technological developments are the driving force behind advances in scientific knowledge.

These techniques include NMR, GC-MS, and LC-MS, etc. with these techniques, some favourable outcomes have been gained.[12-16] However, every technique has its advantages and drawbacks no existing analytical technique can be versatile. NMR has many advantages, but the sensitivity of NMR is relatively poor compared with MS methods, and concentrations of potential biomarkers may be below the detection limit. GC-MS requires sample derivatization to create volatile compounds. Some Non-volatile compounds that do not derivatize a large or thermo-labile compounds that will not be observed in the GC-MS analysis. The recent introduction of UPLC, employing porous particles with internal diameters smaller than 2mm, in combination with MS, results in higher peak capacity, improved resolution and increased sensitivity compared to the conventional HPLC columns, therefore making it them more even suitable for a metabolomics approach. Multi-analysis techniques can partially overcome the short comings of single-analysis techniques.[17] In data acquisition platforms, NMR, GC-MS, LC/MS^n are the prevalent techniques although at present, none of them is a perfect technique that can meet all the requirements of metabolomics for measuring all metabolites. Hyphenated analysis techniques are very suitable for metabolomics samples analysis, especially by reversed phase separation technology, as the sample can be injected directly into the column without the need for any pre-treatment, and the plasma protein can also be simply removed for analysis. In this review we present the latest developments of the above mentioned techniques applied in the field of metabolomics in particular, the strengths and limitations as well as some new trends in the development are discussed with selective illustrative examples.

NMR Based Metabolomics

As one of the most common spectroscopic analytical techniques, NMR can uniquely identify and simultaneously quantify a wide range of organic compounds in the micro-molar range. NMR has been introduced to the emerging field of metabolomics where it can provide un biased information about metabolite profiles. NMR-based metabolomics is able to provide a 'holistic view' of the metabolites under certain conditions, and thus is well-suited and advantageous for metabolomic studies.[18] NMR is straight forward and largely automated and non-destructive, so samples can continue to further analysis. It has been extensively used for metabolite fingerprinting, profiling and metabolic flux analysis. The major limitation of NMR for comprehensive metabolite profiling is its relatively low sensitivity, making it in appropriate for the analysis of large numbers of low-abundance metabolites. Conventionally, within the field of metabolomics of bio fluids, NMR has been the technique of choice, due to its ability to measure intact biomaterials non-destructively as well as the rich structural information that can be obtained. Hence, extensive research and significant improvements have been performed using NMR to measure populations of low-molecular-weight metabolites in biological samples.

High-resolution NMR could provide an ideal mechanism for the profiling of metabolites with in bio fluids or tissue extracts. Although it has many advantages, the sensitivity of NMR is relatively poor compared with MS methods, and concentrations of potential biomarkers may be below the detection limit. A number of bio fluids such as blood, urine, cerebrospinal fluid, cell culture media and many others can be obtained at a high sampling frequency with minimal invasion, permitting detailed characterisation of dynamic metabolic events.[19] NMR can provide detailed information regarding the structural transformation of a compound as a consequence of metabolism in drug discovery and development.[20]

Compared with other techniques, NMR-based metabolomics is becoming a useful tool in the study of body fluids and has a strong potential to be particularly useful for the non-invasive diagnosis of diseases that are very common and pose significant public health problems. Successful studies have shown that NMR spectroscopy as a particularly information-rich method offers unique opportunities for improving the structural and functional characterization of metabolomes, which will be essential for advancing the understanding of many biological processes.[21] Recently, there has been much interest in the use of high-throughput NMR techniques for the detection of biomarkers. From the perspective of drug discovery, each of these metabolites could fulfil a number of useful functions disease bio marker, surrogate marker of drug delivery, surrogate marker of drug efficacy and so on. Indeed, NMR is non-selective so that all the low molecular weight compounds are detected simultaneously in a single run, and provides rich structural information which is an important asset to characterize components of complex mixtures.[22] Urine and serum samples after chronic cysteamine treatment were analyzed by NMR-based metabolomics combined with multivariate statistics.[23]

MS Based Metabolomics

MS is gaining increasing interest in high-throughput metabolomics, often coupled with other techniques such as chromatography-MS techniques. MS has been extensively developed in the past few decades and holds a distinguished position in qualification and separation science. Recent advances in MS based metabolomics have created the potential to measure the levels of hundreds of metabolites that are the end products of cellular regulatory processes. Due to the high sensitivity and therefore wide range of various covered metabolites, MS has become the important technique in choice of

many metabolomics studies. Its utility derives from its wide dynamic range, reproducible quantitative analysis, and the ability to analyze bio fluids with extreme molecular complexity.[24] The aims of developing MS for metabolomics range from understanding the structural characterization of important metabolites to biomarker discovery. MS can be used to analyse biological samples either by direct-injection or following chromatographic separation. Recent developments and improvements in mass accuracy have dramatically expanded the range of metabolites that can be analysed by MS and have improved the accuracy of compound identification.

Direct-injection MS can provide a very rapid technique for the analysis of a large number of metabolites, and thus is extensively used for metabolic finger printing. However, direct injection of biological samples into MS has some draw backs including co-suppression and low ionization efficiencies. In this case, to avoid these problems and to decrease the complexity of the sample mixture, MS is often used as a key hyphenated technique in metabolomics. Recent research has established MS as a key technique of choice in metabolomics because of its high sensitivity and wide range of covered metabolites. MS analytical tools with in metabolomics can profile the impact of time, stress, nutritional status, and environmental perturbation on hundreds of metabolites simultaneously resulting in massive, complex data sets in a global or targeted manner. The high sensitivity and resolution of MS allows for the detection and quantification of thousands of metabolites. Motivated by the success of MS in metabolomics, the analytical community has initiated efforts towards MS-based metabolomics to investigate metabolic biomarkers. MS-based analysis of the metabolome facilitates the reconstruction of metabolic networks, discovery and functional annotation of bio markers. Multiple analytical techniques, used in a complementary manner, are required to achieve high coverage of the metabolome that is composed of a vast number of small-molecule metabolites that exist over a wide dynamic range in biological samples.[25,26]

LC-MS Based Metabolomics

The advancement of both HPLC and MS has contributed significantly to metabolomics analysis. MS and HPLC are commonly used for compound characterization and obtaining structural information in the field of metabolomics, these two analytical techniques are often combined to characterize unknown endogenous or exogenous metabolites present in complex biological samples. With HPLC coupled to MS, there is no need to derivatize compounds prior to analysis. HPLC separations are better suited for the analysis of labile and non- volatile polar and nonpolar compounds in their native form. Recently, LC-MS techniques have been developed employing a soft ionization approach, making MS more robust for daily use. Furthermore, it should be noted that LC-MS can provide a list of m/z values, retention times and an estimation of relative abundances of identified metabolites that are not actually identified. Overall, resolution-high and reproducible LC-MS measurement sets depending upon the basis for the subsequent data processing and multivariate data analysis. Large-scale metabolomic technologies based on LC-MS are increasingly gaining attention for their use in the diagnosis of human disease.[27]

Development of robust, sensitive, and reproducible diagnostic tests for understanding diseases is a world-wide control program. Due to its sensitivity and quantitative reproducibility, LC-MS based metabolomics is a powerful approach to this problem. An LC-MS metabolomics-based diagnostic provides an essential tool and has the potential to monitor the progression of onchocerciasis.[28] LC-MS based non targeted metabolomics has been thoroughly tested, validated, and applied to screen/identify and validate novel metabolic biomarkers for epithelial ovarian cancer; six key-metabolites were considered as potential biomarker candidates, ready for early stage detection.[29] In a study, an LC-MS method was successfully applied for metabolomics analysis of hydrophilic metabolites in a wide range of biological samples.[30] Classification separation for metabolites from different tissues was globally analysed by PCA, PLS-DA and HCA bio statistical methods. As a result, a total of 112 hydrophilic metabolites were detected within 8 min of running time to obtain a metabolite profile of the biological samples. Recently, an LC-MS method for targeted multiple reaction monitoring has become a useful tool for the analysis of hundreds of polar metabolites in a complex sample.[31] Targeted bile acid analysis using LC-MS metabolomics demonstrated increased levels of conjugated or unconjugated bile acids and may allow the distinction of different types of hepatobiliary toxicity[32] (Aihua Zhang et al., 2012).

By combining MS with liquid chromatography, molecular identification and quantification of polar, less-polar, and neutral metabolites can be achieved, even when they are present at relatively low concentrations levels and in a complex matrix. There have been major difficulties in coupling LC with MS e.g., the incompatibility caused by the liquid flow rate into the high vacuum of the mass spectrometer, the ionization of non-volatile and thermally labile analytes, and the incompatibility caused by the non-volatile mobile phase used in the chromatographic separation. Technical solutions have been provided by the recent introduction of the powerful soft ionization techniques thermo spray, APCI, and especially electro spray (Abian, 1999; Niessen, 1999), and by the modification of the liquid-chromatography methods. Thermo spray allows the ionization of moderately polar compounds (polyphenol, terpenoids) in the mass range 200–800Da, whereas ESI and APCI are suitable for a broader range of metabolites (Wolfender et al., 1995). Electrospray is the most widely method used for sensitive analysis of polar and ionic compounds. APCI can be used for the analysis of less-

polar and neutral chemical species. Generally APCI and ESI produce only molecular ions, but the multi charge ionization mechanism of electrospray can extend the mass range of the instrument to provide a mass range of greater than 80,000 Da to permit the accurate mass determination of macromolecules such as intact proteins. The impact of those innovations has been felt notably in pharmaceutical research, and more recently in the area of metabolome analysis.

LC-MS for Target Analysis

By providing a unique combination of resolving power, sensitivity, and specificity, LC-MS and particularly LC-MS^n offer the possibility to identify target metabolites in a very complex mixture. Actually, LC-MS^n is a tool used more frequently for the identification of metabolites in crude plant extracts to provide not only molecular mass information, but also structure information that is deduced from fragmentation patterns obtained by collision-induced dissociation. The advantage of the LC-MS is the ability to determine the selected metabolites in a very few minutes from only a small amount of material and therefore with a simple sample preparation. For LC-MS analysis, the sample preparation, chromatographic separation and MS methods are developed simultaneously. Because of its compatibility with ESI, the most frequently used LC method is reversed-phase HPLC separations with a volatile mobile phase. Special strategies have been developed for the analysis of plant metabolites. For example, an LC-APCI-MS method was developed and optimized for the analysis of chlorophylls (Airs & Keely, 2000), and enhanced sensitivity was achieved by one-line demetallation of the metabolites through the column-post introduction of formic acid into the eluent flow. In the report of Mellon et al.(2002), glucosinolates in crude plant extracts were analyzed by LC-MS in the negative-ion mode.

The sensitivity and the selectivity of a programmed cone-voltage electrospray LC-MS method were found to be better than a LC-MS-MS (triple quadrupole) method. The detection limits were in then grange. In another report, the differentiation of C-glycosidic flavonoid isomers was evaluated by quadrupole-TOF tandem-MS and ion trap multiple-stage MS at different collision-induced dissociation energy values (Waridel et al., 2001). Isomers were discriminated by the two approaches, but the specificity of multiple-stage experiments may be an advantage for the analysis of a mixture that contained co-eluting metabolites. LC-ESI-MS^n has been applied to analyze metabolites in several different matrices identification and determination of nucleosides in rat brain micro dialysates (Zhu et al., 2001); semi-quantification of monophosphate nucleotides in wine (Aussenac et al., 2001); quantification of metabolites involved in purine and pyrimidine metabolism defects in urine (Ito et al., 2000); and quantification of underivatized amino acids in blood (Qu et al., 2002). Finally, there are several examples on targeted analysis for medical diagnosis, using LC-MS (Yu, Cui, & Davis, 1999; Ito et al., 2000).

GC-MS Based Metabolomics

Currently, as a core analytical method for metabolomics, GC-MS has been used as a platform in non-targeted analysis, especially for hydrophilic metabolites.[33] Generally, GC-MS-based metabolomics requires a high-throughput technology to handle a volume large of samples which provides accurate peak identification through the retention standard times and spectra mass. The GC-MS that has been widely used for various metabolomics which can provide an efficient and reproducible analysis. For separation on the GC column, GC-MS requires a derivatization reaction to create volatile compounds. Non-volatile compounds are not derivatized and will not be detected in the GC-MS analysis. This limits the applicability to metabolomics. Using this approach, the volatile metabolites can be directly separated and quantified by GC-MS, and it is also possible to simultaneously profile several hundreds of compounds including organic acids, most amino acids, sugars, sugar alcohols, aromatic amines and fatty acids. For others, chemical derivatization is required to make them amenable to GC-MS analysis. Ma et al. explored the alteration of endogenous metabolites and identified potential biomarkers using a metabolomics approach with GC-MS in a rat model of estrogen-deficiency-induced obesity.[34] The series of potential biomarkers identified in the present study provided fingerprints of rat metabolomics changes during obesity and an overview of multiple metabolic pathways during the progression of obesity which involving the types of glucose metabolism, lipid metabolism, and amino acid metabolism. Kuhara et al. devised a more rapid and accurate diagnosis of citrin deficiency patients using the GC-MS urine metabolome.[35] The results show that, together with GC-MS, non-invasive urine metabolomics provides a more reliable and rapid chemical diagnosis of citrin deficiency (Xiuan Wang et al., 2012).

Usually, GC-MS analysis is performed on single-quadrupole mass spectrometers, which provide nominal-mass information. Recently more about the introduction of GC-TOFMS systems which offered an attractive supplement to quadrupole instruments and provided greater accuracy mass. TOF instruments also provide high scan speeds that are compatible with ultrafast GC-MS (Davis, Makarov, & Hughes, 1999), and the potential to profile complex mixtures in less time. Similarly, the GC coupled to multistage MS-MS [e.g., MS-MS or MS^n (triple-quadrupole and ion traps)] instruments that enabled the acquisition of very detailed fragmentation information, a higher level of specificity molecular, and selectivity higher (Nielsen et al., 2003). Nevertheless, quadrupole mass analyzers still present some extra advantages in metabolite analysis such as higher repeatability and large dynamic range for quantitative analysis. Quadrupole MS spectra can be easily compared with commercial or inter-laboratories

databases, and according to Nielsen et al. (2003), it is possible to improve detection sensitivity from the ng-level to the pg-level through the scanning for a selected number of characteristic ions [selected ion monitoring (SIM)], which is very useful for quantitative target analysis. However, the limitation of GC-MS is that the samples must be volatile to be separated on a GC column. Most occurring naturally metabolites that are not sufficiently volatile that to be analyzed directly on a GC system. Derivatization of the metabolites is, therefore, required, and this step adds time to the analysis as well as causes more complex sample-handling and an increased variance in the analysis. Furthermore, heat-labile compounds cannot be analyzed, and identification of unknown derivatized compounds can be difficult because they are chemically modified.

CE-MS Based Metabolomics

CE-MS is a powerful and promising separation technique for charged metabolites, offering high-analyte resolution, providing information mainly on polar or ionic compounds in biological fluids.[36] CE-MS, as an analytical platform, has made significant contributions in advancing metabolomics research. CE-MS represents a promising hyphenated micro separation platform in metabolomics, since the majority of primary metabolites are intrinsically polar.[37] Metabolites are first separated by CE based on charge and size, and selectively that are detected by using MS by ions monitoring over a large range of m/z values. CE-MS provides numerous key advantages over other separation techniques. One of the significant advantages of CE-MS is a short analysis time and very small sample requirement with injection volumes ranging from 1 to 20 nL. This feature makes CE-MS a very promising analytical technique for high-throughput metabolomics. Thus, CE-MS has been used for both targeted and non-targeted analysis of metabolites, including analysis of inorganic ions, organic acids, amino acids, nucleotides and nucleosides, vitamins, thiols, carbohydrates and peptides. Metabolome analysis of human HT29 colon cancer cells was investigated.[38] CE-MS analysis time was less than 20 min per sample and allowed the simultaneous and reproducible analysis of more than 80 metabolites that are detected in a single run with a minimum consumption of reagents and sample. Using CE-MS, Sato et al. analyzed the dynamic changes in the level of 56 basic metabolites in plant foliage at hourly intervals over a 24 hr period.[39] Adenine nucleosides and nicotin amide coenzymes were regulated by phosphorylation and de phosphorylation. It facilitates an understanding of large-scale interactions among components in biological systems. We conclude that CE-MS is a valid approach for targeted profiling of metabolites (Ying Han et al., 2012).

CE-MS for Target Analysis

In medical laboratories, the metabolite analysis routinely performed by GC-MS analysis takes several hours. However, CE-MS offers the possibility to overcome that drawback. Many efforts have been done to develop a fast and reliable method for screening patients, because these disorders, resulting of genetic defects, cause often the accumulation of a specific metabolite in serum or urine and ultimately organ damage. For instance, CEMS has been successfully applied for the diagnosis of various human metabolic disorders, with an analysis of only fifteen minutes (He et al., 1999; Vuorensola et al., 2001). Vuorensola et al. (2001) used CE-MS to analyze catecholamines that differ only by one amino group. Compared to LC-MS analysis, one major drawback was the high concentration limit of detection because of the very low injection volume used in CE.

In that study, the limits of detection obtained after optimization (0.19– 0.63mg/L) were sufficient to analyze catecholamines in concentrated urine samples. He et al. (1999) also analyzed ‘‘diagnostic metabolites’’ in urine by CE-MS. Elgstoen et al. (2001) have also used direct CEMS-MS (triple quadrupole) to analyze the urine of patients with different metabolic disorders. Specific transitions that were characteristic for each metabolite have been determined by MRM, and were used to identify diagnostic metabolites that were not sufficiently separated. Compared to CE-DAD (Elgstoen & Jellum, 1997), compounds that absorb light in the UV range and that do not absorb could both be detected by CE-MS.

The specific analysis of related chemical species is probably technically easier than analyzing all cell metabolites, and can be a comprehensive approach to characterize the whole pool of cellular metabolites. In the pharmaceutical area, attention is focused on plant secondary metabolites, which have potentially a therapeutic value. Unger et al. (1997) have presented different CE-MS methods to analyze more than 30 alkaloids without any pre-treatment with the advantage to overcome the problems that are normally associated with the derivatization procedures that are required for GC-MS analysis. Even if qualitative and quantitative analysis of alkaloids can be performed with HPLC, CE provides, in a shorter time, higher resolution and efficiency than LC (Tomas-Barberan, 1995).

Accordingly, CE-MS has been used to analyze four different classes of alkaloids i.e., mono terpenoid indole alkaloids, proto berberines/ benzo phenanthridines, beta carboline alkaloids and iso quinolines with only minor changes of the instrumental conditions. Almost allalkaloids within. one class were separated by CE, and were identified with electrophoretic mobility and m/z. Bianco et al. (2003) evaluated the glycol alkaloids (GA) content of wild-type potato plants and genetically modified virus Y-resistant potato plants with non-aqueous CE coupled with ESI-MS (Bianco et al., 2002). The improvement of potato plants resistance to virus infections can trigger changes in the levels of natural toxicants, like GA, those changes can be problematic in plants used as food. Wild-type potato plants were

transformed, and subsequently their GA content was characterized and quantified (Driedger et al., 2000).

The GA levels were, in both cases, inferior to the limit value recommended for food safety. Analysis of amino acids is valuable for many different applications such as biochemical studies, medical diagnosis, food technology, and biotechnology. Many methods have been developed for analysis of amino acids by GC, GC-MS, HPLC, and CE, but those methods usually require the chemical derivatization of the amino acids. That step can be avoided with CE-MS. Soga & Heiger (2000) have described a CE-ESI-MS method for the determination of 19 standard un derivatized amino acids in 17 min. All of the amino acids were not electrophoretically separated, but were selectively detected by MS in the positive-ion mode. The limits of detection (concentration) for alkaline amino acids such as Arg, His, and Lys were between 0.3 and 1.1mmol/L, where as it was 6 and11mmol/L for the acidic amino acids glutamate and aspartate, respectively.

The sensitivity of the CE-ESI-MS method is dramatically increased compared to a CE method within direct UV detection (Soga & Ross, 1999), but is inferior to an HPLC method that used derivatization. Schultz & Moini (2003) have analyzed in 35 min 20 underivatized amino acids and their D/L enantiomers by CE-ESI-MS. With a limit of detection in the femtomol range for amino acids, the method had more sensitivity hanthose proposed by Soga & Heiger (2000). It is probably because of the utilization of a sheath less CE-MS interface that delivered a few nL/min (Moini, 2001) instead of the sheath-flow interface that delivered a few mL/min. The method has been applied for the analysis of blood with minimal sample preparation, and thereby used for the diagnosis of metabolic diseases like phenyl keton urea and tyro sinemia by identifying increased levels of phenylalanine and tyrosine, respectively, in blood from sick infants compared to healthy infants. Low molecular mass organic acids also play an important role in biochemistry and food science, as well as in environmental science. Those compounds have also been studied by CE-MS to avoid any metabolite derivatization (contrary to GC analysis) and to improve the metabolite detection in comparison to CE-UV analysis. Johnson et al. (1999) have analyzed by CE-MS within 35min a mixture of succinic, maleic, malonic, and glutaric acids. An electro osmotic flow modifier (2mMdiethylenetriamine) was introduced into the CE mobile phase to cause the electro osmotic flow and the anions to move in the same direction.

UPLC-MS Based Metabolomics

UPLC-MS technology is a powerful technique in bio molecular research and can also be used to quantify the activity of signalling and metabolic pathways in a multiplex and comprehensive manner. The recent introduction of UPLC, employing porous particles with internal diameters smaller than 2 mm, in conjuction with MS, results in higher peak capacity, enhanced specificity and high-throughput capabilities compared to the conventional HPLC columns, making therefore it is even more suitable for a metabolomics approach. Because the optimum linear velocity has a broader range, UPLC also allows a more rapid analysis without loss of resolution. The combination of UPLC with MS detection covers a number of polar metabolites and thus enlarges the number of detected analytes. In view of the recent developments in the separation sciences, the advent of UPLC and MS based technology has shown ever improving resolution of metabolite species and precision of mass measurements. Q-TOFMS is coupled with UPLC for the analysis and identification of trace components in complex mixtures, as a powerful means to make accurate mass measurement levels of less than 5 ppm, with effective resolution. Given the power of the technology, the UPLC-MS technique represents a promising hyphenated micro separation platform in metabolomics, since the majority of primary metabolites are intrinsically polar. Recently, this technology has rapidly been accepted by the analytical community and hyphenated UPLC-MS has been used for coupling to MS in metabolomic studies to provide a complementary tool widely applied to various fields. Metabolomics includes the changes in the concentrations of endogenous metabolites, that may reflect various disease states as well as systemic responses to environmental, therapeutic, or genetic interventions. To investigate the effect of acupuncture on acute gouty arthritis and search for its mechanism, a metabolomics method was developed and endogenous metabolites were analyzed.[40,41] The plasma samples, when injected onto a reverse-phase 50 ×2.1 mm Acquity 1.7-µm C18 column using a UPLC coupled with Q-TOF-MS in positive and negative modes, yielded a data matrix with a total of 2600 features, showing the power of this technology. It indicates that UPLCMS-based metabolomics can be used as a potential tool for the investigation of the biological effect of acupuncture on acute gouty arthritis. Chaihu-Shu-Gan-San (CSGS), a traditional Chinese medicine (TCM) formula, has been effectively used for the treatment of depression in the clinic.[42] Metabolomics based on UPLC-Q-TOF-MS was used to profile the metabolic fingerprints of urine obtained from a chronic variable stress induced depression model in rats with and without CSGS treatment.

This study showed that the urinary UPLC-QTOF-MS approach was a powerful tool to study the efficacy and mechanism of complex TCM prescriptions. A urinary metabolomics method based on UPLC-MS was developed to study characteristics of 'Kidney-Yang Deficiency syndrome' and the therapeutic effects of Rhizoma Drynariae.[43] The biochemical changes are related to the disturbance in energy metabolism, amino acid metabolism and gut microflora, which are helpful to further understand 'Kidney-Yang Deficiency syndrome' and the therapeutic mechanism of Rhizoma Drynariae. UPLC-MS-based analytical methods were used to pathophysiologically characterize cholestasis.[44] More

than 250 metabolites were detected in both plasma and urine.

UPLC has been used for assessing the holistic efficacy and synergistic effects of TCM,[45-48] for analyzing toxicity,[49,50] severe childhood pneumonia,[51] non alcoholic fatty liver disease,[52] diabetes,[53-55] colorectalcarcinoma[56] and Alzheimer's disease,[57] and for nutritional studies[58] (Ying Hang et al., 2012).

CONCLUSION

Metabolomics is a rapidly growing field of study that aims to measure of all the possible detectable metabolites within a biological sample in order to achieve a global view of the state of the system. monitoring hundreds or more metabolites at a given time requires high-end techniques. Most of the analytical techniques useful for these purposes use GC or HPLC/UPLC separation molecules along with a fast and accurate MS. This review highlights the importance and benefits of the role of integrated tools in metabolomic research. The recent development of a range of integrated analytical platforms in metabolomics is an absolute strategy and will provide sensitive and reproducible detection of thousands of metabolites in a biofluid sample.

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