Chapter

Metabolomics

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Abstract

The aim of this chapter is to make a brief understanding on Metabolomics identification, extraction, and analysis techniques. As the name suggests, Metabolomics is the study of metabolites present in the body fluid (blood, plasma, urine, and saliva) or body parts (muscles, bone, tissue, and cells). These might be known metabolites or unknown metabolites. The metabolites can be endogenous (present in the body) or exogenous (formed by consuming external medicinal product). The molecular mass of these metabolites is usually lower (50–1500 Dalton) than the proteins and macromolecules. These metabolites can be extracted using various techniques such as solid phase extraction, liquid-liquid extraction, or simple protein precipitation. Extracted sample of metabolite then can be analyzed qualitatively or quantitatively using numerous analytical techniques such as high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC–MS/MS), dry blood spot (DBS), infrared (IR) spectroscopy, ultraviolet visible (UV) spectroscopy, nuclear magnetic resonance (NMR), ELISA, and chemiluminescence. Sensitivity of detection is the key factor, among many others, to decide which technique would be suitable for analysis. Liquid chromatography mass spectrometry (LC–MS/MS) is the latest and most sensitive technique among all the available methodology till date that has been extensively and exclusively used in current scenario.

Keywords: known metabolite, unknown metabolite, extraction, LC–MS/MS analysis, HPLC analysis, NMR analysis

1. Introduction

Current development, demand, and innovation in the field of science have made a tremendous effort to reduce the load of chemical/biologics in human body from gram to microgram level. Now scientists are evaluating how harmful is the effect of these chemicals (drugs) when consumed by any route in human body. Recent FDA/other regulatory agency examples are the presence of five to seven nitrosamine impurities (metabolites) (e.g., NDMA family) in sartan (e.g., valsartan, telmisartan, etc.) or ranitidine [1]. Lower therapeutic dose would be one of the solutions to reduce the level of these metabolites to an acceptable level in human matrix.

The metabolome is a close counterpart to the genome, the transcriptome, and the proteome. Together these four 'omes' constitute the building blocks of systems biology. Metabolomics is a newly emerging field of research concerned with the high-throughput identification and quantification of the small molecule metabolites in the metabolome. The metabolome can be defined as the complete complement of all small molecule (<1500 Da) metabolites found in a specific cell, organ, or organism. Metabolites are small molecules that are chemically transformed during metabolism and can provide a functional readout of the

cellular state. Metabolites, unlike genes and proteins, serve as direct signatures of biochemical activity and are much easier to correlate with phenotype. One of the challenges of systems biology and functional genomics is to integrate proteomic, transcriptomic, and metabolomic information to give a more complete picture of living organisms. While mRNA gene expression data and proteomic analyses do not tell the whole story of what might be happening in a cell, metabolic profiling can give an instantaneous snapshot of the physiology of that cell [2–6]. Pictorial diagram of all the omics interrelated to each other has been shown in **Figures 1** and **2**.

The metabolites formed in the body can be generated either by Phase 1 or Phase 2 metabolic pathway [7]. The representation of these pathways is shown in **Figure 3**.

Regulatory guidance and agencies are emphasizing on the need of identification and analysis of each possible molecule separately and in combination with the intended matrix to generalize the pros and cons of that particular drug before human use [8–11].

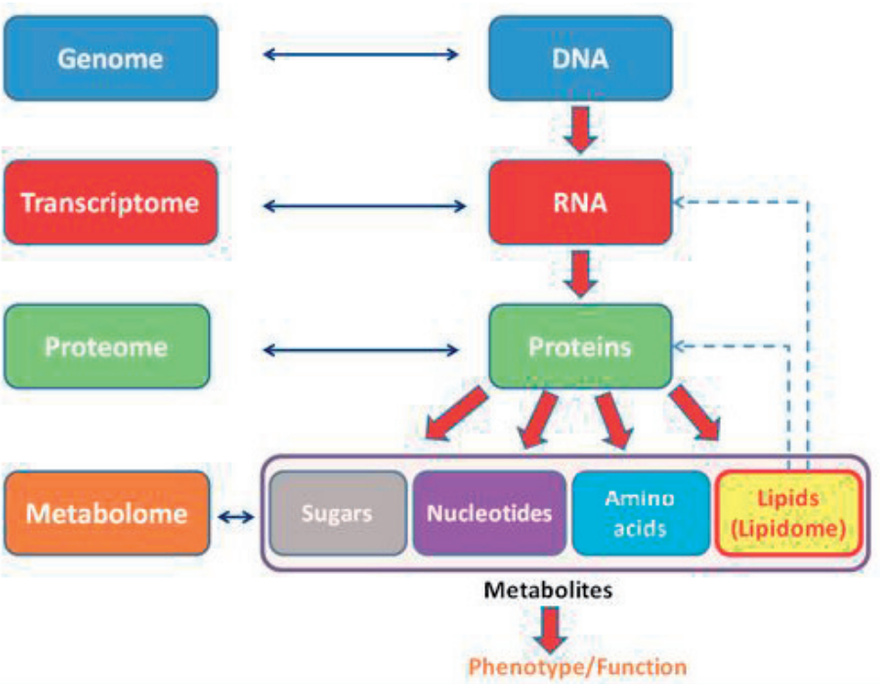

Figure 1.
The "omics" cascade.

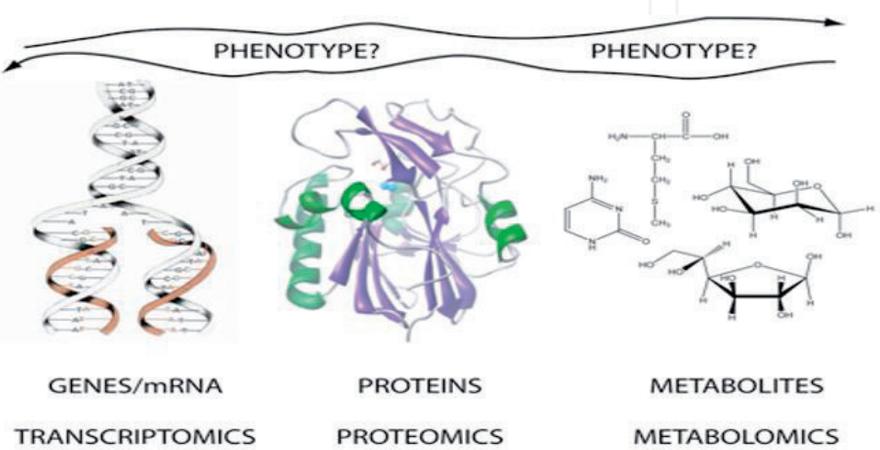

Figure 2.
Genes to phenotype.

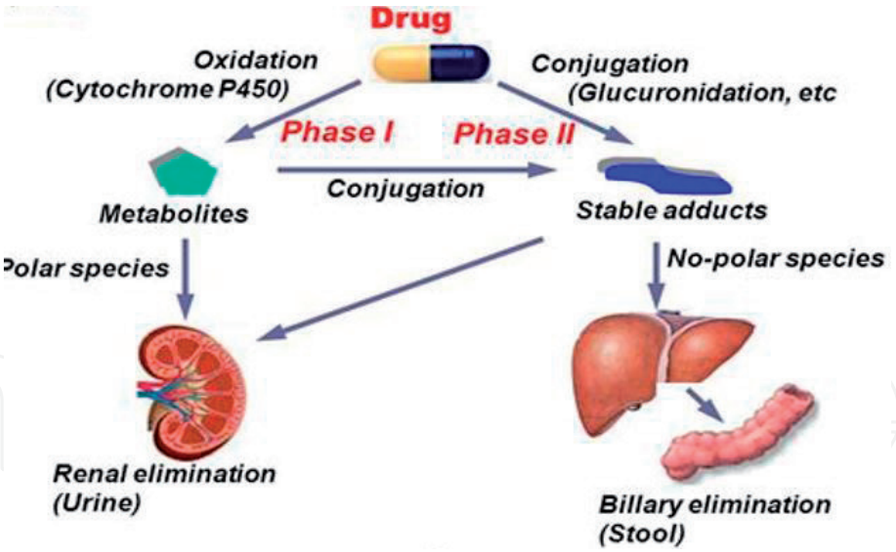

Figure 3.
Metabolite pathways in human.

2. Metabolite extraction technique

When it comes to extract the metabolites from the bio-fluid (blood, plasma, serum, saliva, or urine), mainly three methodologies are prevailing. These are as follows.

2.1 Protein precipitation

This technique is very useful for high throughput as it involves only centrifugation step when a precipitating agent is added to matrix (plasma, blood, or serum). The basic principle for this technique is the amount or volume of precipitating agent must be sufficient to precipitate the protein present in the matrix. Selection of precipitating agent also depends on the technique involved for analysis of the compound of interest. For example, trichloroacetic acid (TCA), perchloric acid (PCA), and zinc sulfate ($ZnSO_4$) are not suitable for mass spectrometer (MS) analysis, whereas these are suitable for high performance liquid chromatography (HPLC) analysis [12]. Organic solvents such as methanol or acetonitrile are suitable for both HPLC and LC–MS/MS analysis [13–15]. The amount of precipitating agent varies for precipitating the matrix. TCA or PCA can be used directly (20–50 μL) in 500 μL of matrix [16–19]. Organic solvent should be 2–3 times the volume of matrix. After centrifugation, either sample can be directly injected into the HPLC or LC–MS/MS or it can be concentrated in nitrogen evaporator followed by reconstitution. Centrifugation also plays an important role for this methodology [20]. Higher the revolution per minute (RPM) speed (~15,000 RPM) higher would be the sedimentation of particle and cleaner would be the sample. Flow of protein precipitation is shown in **Figure 4**.

The drawback of this technique is high matrix effect, low recovery if the compound of interest is associated with precipitated protein.

2.2 Liquid-Liquid extraction

The liquid-liquid extraction (LLE) technique [21–25] is one of the widely used methodologies to extract most of the metabolites from bio-matrix. The basic principle of this technique is portioning or separation of compound from one

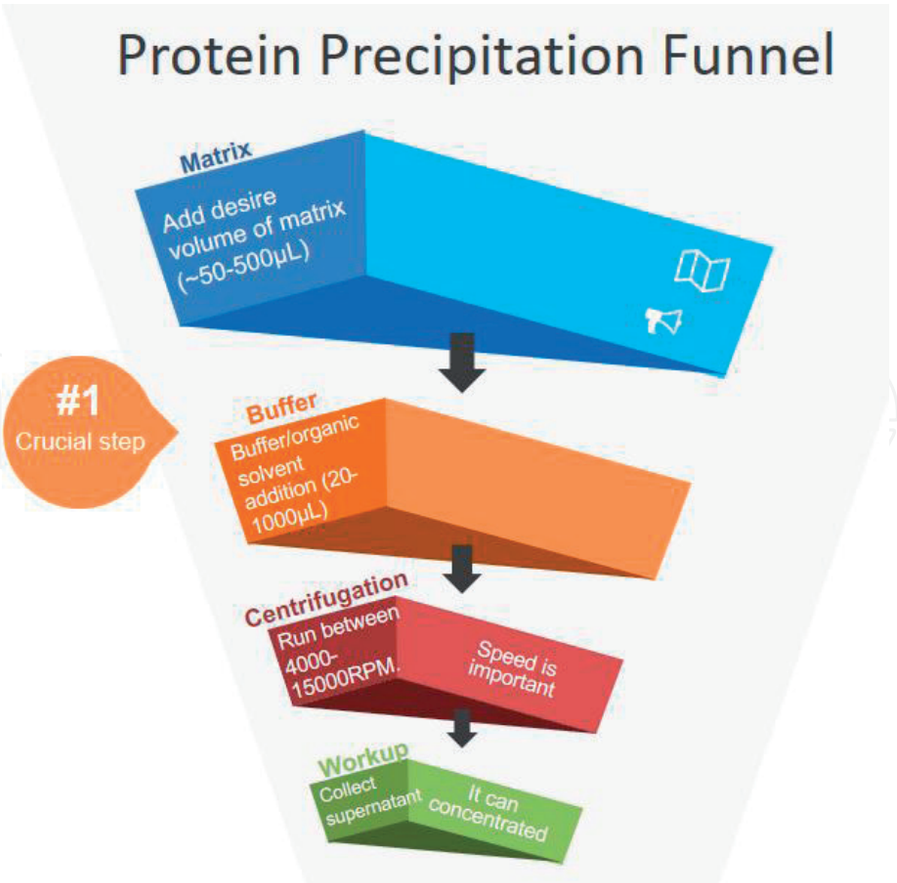

Figure 4.
Protein precipitation process.

liquid part to another one depending on its affinity. As we know, our body fluid (plasma … urine) is polar in nature; hence, less polar or nonpolar solvents such as ether/t-butyl methyl ether/ethyl acetate/dichloromethane are used to extract the compounds from the human matrix. The LLE technique can be used in many ways such as manual or automatic. Manual methodology would be cost effective when intelligent approach like freeze flash (using dry ice and methanol) is used along with vortexing the sample at high speed (2000–2500 RPM). Typical sample volume versus solvent volume is required in 1:3 ratio for proper extraction. Solvent needs to be evaporated in a nitrogen evaporator under constant temperature and pressure. The dried sample should be reconstituted in an appropriate solution for analysis on either HPLC or LC–MS/MS. Typical flow of the process is shown in **Figure 5**.

2.3 Solid-phase extraction

The cleanest process in extraction methodology is solid-phase extraction (SPE). This process involves the adsorption of compound on the solid surface (bed) of a polymeric membrane with a covalent and ionic interaction. Desorption of molecule takes place when a strong solvent/solution is passed through the surface of cartridge. Umpteen of SPE cartridge is available in the market staring from C18 to ionic (cation or anion) with weak and strong combination [26–28]. The cartridge can be of different platform like well plate of tube type. The polymeric membrane may vary from few milligram to few gram depending on the requirement of analysis. Matrix as low as 50 µL to as high as 3 mL can be employed for extraction using different format of SPE platform. Different kinds of protocols are used to

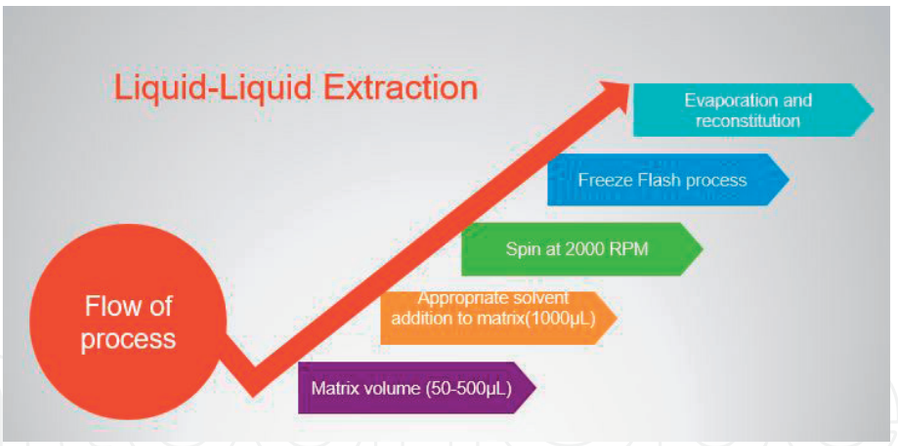

Figure 5.
Liquid liquid extraction methodology.

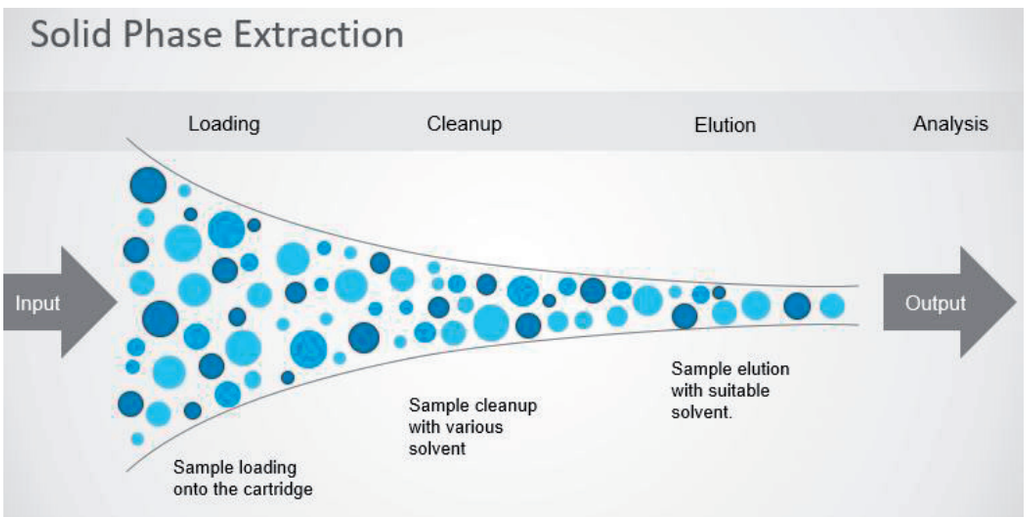

Figure 6.
Solid phase extraction technique.

clean up the sample from matrix, which depend on the various trials conducted during development stage. SPE unit can be operated in both negative pressure mode (using vacuum manifold) and positive pressure mode (using nitrogen gas). Clean sample can be either directly injected into the HPLC or LC–MSMS system or concentrated to get high recovery. Pictorial presentation on SPE process is shown in **Figure 6**.

3. Dry blood spot technique

When there is a challenge of low sample volume (e.g., neonatal), it is advisable to look for innovation in this area. Dry blood spot (DBS) is a very useful technique that can support the low volume sample analysis. Just a spot is required on the polymeric membrane that can be cut in a circular shape using specific tools. The cut part then can be dissolved in a suitable solvent and directly injected on the high sensitive instrument for analysis [29–31]. The major challenge for this method is sensitivity achievement on the analytical instrument. Mass spectrometers (latest models) are the only way out for such kind of analysis (**Figure 7**).

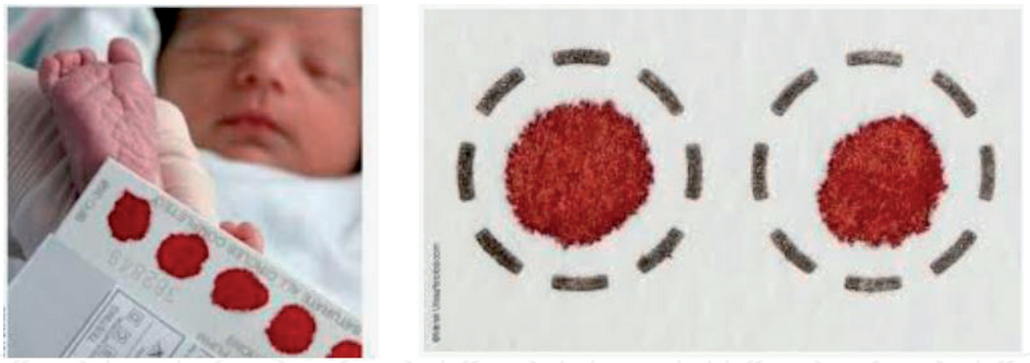

Figure 7.
Dry blood spot technique.

4. Metabolite analysis technique

There are a variety of analytical techniques that could be employed for metabolites identification and its analysis. Few of them are high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC–MSMS), nuclear magnetic resonance (NMR), ultraviolet visible spectrophotometer (UV), and infrared spectrophotometer (IR). Among all these, most reliable techniques are HPLC and LC–MSMS.

HPLC is a good technique for the analysis of formulation product in aqueous medium (nonhuman matrix) as there is no limitation of sample volume, concentration, and its detection. However, it would be a challenge when the analysis is to be done in human matrix due to obvious reason of low sample volume and hence its sensitivity on the instrument. Sensitivity on HPLC can be enhanced by replacing the UV detector by RF (fluorescence) detector provided the molecule if fluorescence is active. Derivatization methodology can also enhance the sensitivity of molecule by adding some auxochromes or chromophores to the main molecule moiety. Few examples are presented in **Table 1**, where parent molecules are not sensitive enough as such on HPLC analysis, however, using derivatizing reagent, sensitivity got enhanced.

Liquid chromatography when connected with mass spectrometer as detector, sensitivity of the molecule enhanced many folds by virtue of mass to charge (m/z) ratio detection. By enlarge, mass spectrometer is the only technique in the field of biomolecules and its analysis, by which one can quantitate the metabolites up to pg/ml level.

In the mass spectrometer, ion source plays a crucial role to ionize the molecule of interest. Basically, there are two kinds of ion sources predominantly used in

S. No	Molecule has low sensitivity (on HPLC)	Derivatizing agent	Complex has high sensitivity (on HPLC)
1	Ethinyl estradiol	Dansyl chloride	Ethinyl estradiol-Dansyl complex
2	Valproic acid	2-bromo-2′-acetonaphthone	Valproic-acid-2-bromo-2′-acetonaphthone complex
3	Alendronate	Diazomethane	Alendronate-Diazomethane complex
4	Mesalamine	Acetic anhydride	Mesalamine-Acetic anhydride complex

Table 1.
Molecule with derivatizing reagent.

entire pharma industry or academics. These are Electron spray ionization (ESI) and Atmospheric pressure chemical ionization (APCI).

4.1 Electro spray ionization (ESI)

The molecules of interest are first introduced into the ionization source of the mass spectrometer using an HPLC (or UPLC) system, where they are first ionized to acquire positive or negative charges. The liquid associated with molecules get evaporated due to high temperature. Thereafter, due to coulombic repulsion between the ion, charged particles are formed. The ions then travel through the mass analyzer and arrive at different parts of the detector according to their mass/charge (m/z) ratio. After the ions make contact with the detector, usable signals are generated and recorded by a computer system. The computer displays the signals graphically as a mass spectrum showing the relative abundance of the signals according to their m/z ratio. A typical pictorial diagram is shown in **Figure 8**.

4.2 Atmospheric pressure chemical ionization (APCI)

The APCI source is selected for molecules, which are thermally stable as the basic principle works on the fact of charge transfer from solvent molecule to the compound of interest in the ion source using corona discharge tube. The other operation in this ion source would remain same as ESI like transfer of ions from ion source to vacuum region and selective monitoring up to detector. APCI diagram is shown in **Figure 9**.

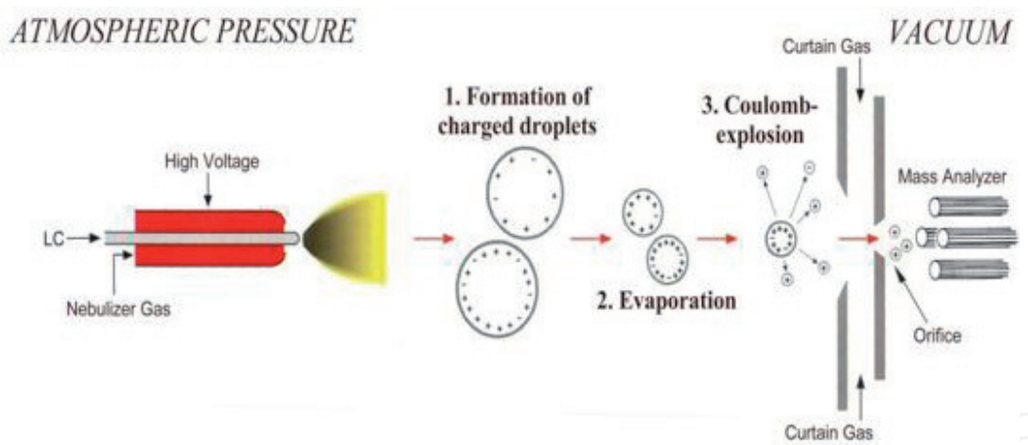

Figure 8.
Electro spray ionisation mechanism.

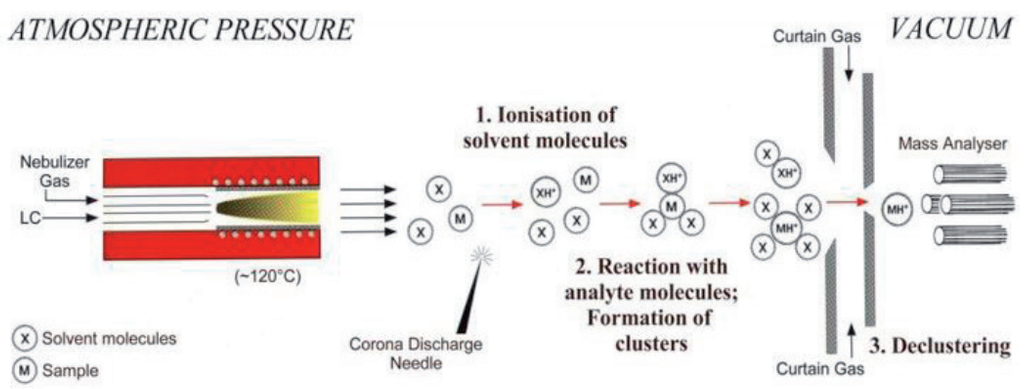

Figure 9.
Atmospheric pressure chemical ionisation mechanism.

S. No	Parameter	HPLC	LC–MS/MS
1	Run time	High (5–100 min)	Low (2–10 min)
2	Ratio of mobile phase	High aqueous (~70%) with low organic solvent (~30%)	Low aqueous (~30%) with high organic solvent (~70%)
3	Column	15–30 cm	5–15 cm
4	Efficiency	Low	High
5	Sensitivity	Low	High
6	Feasibility of analysis	Low	High
7	Injection volume	Low	High
8	Matrix volume	Low	High
9	Cost	Low	High

Table 2.
Comparison between HPLC and LC–MS/MS.

Both HPLC and LC-MS/MS techniques can be employed for identification and quantitation of metabolites in human matrix; however, both have some limitation and benefits. **Table 2** represents the basic difference between both the techniques:

5. Conclusions

Metabolites are the compounds generated by our body after the consumption of drug substance. Some of these metabolites are known, and most of them are unknown. They may be harmful, beneficial, or inactive in the body. Extraction and detection are thus very important to understand complete behavior of these metabolites. Sensitive analytical technique like LC–MS/MS is the most employed methodology by most of the pharmaceutical/diagnostic companies as one can detect parent and all possible metabolites in one single run within 10- to 50-min time in human matrix.

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Conflict of interest

There is no conflict of interest on publishing of this chapter.

Notes/Thanks/Other declarations

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