

Different Separation or Experimental Techniques for Clinical Chromatography: Small Review

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Abstract

Liquid chromatography is a widely popular technique in clinical laboratories and clinical applications. People evolved and developed various types of chromatography for decades after its initial idea published in 1960s. Mostly people use HPLC-MS, UHPLC techniques to purify antibody, drug isomers and enantiomers. Separating molecules of different sizes by modified HPLC and size exclusion chromatography saved solvent reduction by 90% in new instruments. Decades old thin layer chromatography modified into MALDI-TOF-MS for efficient identification of heredity related bulk amino acids secretion and the diseases associated with it. Single peptide branch is also efficiently isolated from a mixed protein sample with the help of MALDI-TOF-MS. Several Biotech companies e.g., Agilent, Thermo Fisher, GE, Sigma-Aldrich, Shimadzu, PerkinElmer, Dionex, Roche, BD Biosciences, and Illumina are the leading companies in chromatography instrumentation areas. They produced innovative liquid chromatography instruments by merging various modified parameters that reduced experimental time and toxic chemical usage. The target of clinical research or pharmaceutical industry is to reduce enantiomer or isomer separation, peak retention; quicker sample processing and flexibility to analyze most of the biological molecules. Column matrix, Injection volume, injection time, ionization and detectors (3D photo diode array chromatogram) are the key measuring parameters in these updated versions of liquid chromatography instruments. Liquid chromatography combined with mass spectrometry or micro fluidic device helps people to detect single nucleotide polymorphism in DNA or early stage of a disease like cancer.

Keywords: Chromatography; GC; LC; MS; HPLC; UHPLC; UPLC-MS; HPLC-TMS; MALDI-TOF-MS-ESI; Clinical Research

Abbreviations: AF/LC: Affinity chromatography/liquid chromatography; IAC: Ion affinity chromatography; HPLC: High performance liquid chromatography; MS: Mass spectrometry; UHPLC-MS: Ultra high performance liquid chromatography-mass spectrometry; TLC: Thin layer Chromatography; SFC: Supercritical fluid chromatography; GC: Gas Chromatography; LC: Liquid chromatography; GPC: Gel permeation chromatography; GFC: Gel filtration Chromatography; ESI: Electro spray ionization; MALDI-TOF-MS: Matrix assisted laser desorption/ionization time of flight mass spectrometry; PGC: Porous graphitic carbon chromatography; FRET: Fluorescence resonance energy transfer; QD: Quantum dot; TMS: Tandem mass spectrometry; CID: Collision induced dissociation

Introduction

Efficiency, accuracy and cost effectivity are the key parameters in clinical research or regular usage in clinical laboratories. Biological research revolutionized its technical field with the help of Physics and Chemistry. Continuous innovation and development in clinical and biological research makes instruments and techniques more efficient and easy to handle. Liquid chromatography is a highly precise and sophisticated technique. It has multidimensional applications. Not only disease diagnosis and disorders; but it is applicable to food industries QA/QC, Forensic and Environmental biology areas too [1-3]. Application of HPLC and mass spectrometry is priceless in pharmaceutical industry's research. Sample analysis in a shorter time frame is the target of liquid chromatography. Several research areas were explored by liquid chromatography such as oligosaccharides, oligonucleotides, proteins, sphingo lipids, allergy, allergen detection, rheumatoid arthritis, cancer, Alzheimer's disease etc. High throughput sphingo lipid separation from human serum was also possible by liquid chromatography [4]. These molecules were highly complex and diverse but crucial indicator of cell abnormalities.

In this review author put emphasis on different technical procedures and new combined experimental techniques that is widely used by multiple companies or research institutions, and explored how efficiently instruments extract or study biological molecules in cost effective ways. Several high throughput instruments- software's has developed to enhance clinical research and disease identification procedures.

Liquid chromatography (LC) is divided into four major groups and those are ion exchange, normal phase, reverse phase and size exclusion. Affinity chromatography is also a big part of liquid chromatography where antigen-antibody interaction or enzyme-inhibitor reaction takes place [5]. There are some limitations to identify or quantify clinical drugs or biological samples and that is often related to concentration of sample. People are now combining LC with mass spectrometry (MS) to address all the questions related to better analysis of samples [6-8]. Combination of GC and MS gives the highest level of sensitivity, specificity and reproducibility that is required to study the clinical trial drug and its stability [9,10]. All these technique helps to identify highly potent drugs as well as determine the potential of genotoxic impurities. Proteomics and Metabolomics research developed with HPLC and its advanced technical version of UHPLC, UPLC-MS, HPLC-TMS, and MALDI-TOF-MS-ESI etc. The theory behind HPLC

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is high atmospheric pressure, which helps to separate molecule into ionic forms. Technical efficiency comes from robotic platform that is used for auto sampling purposes. Small size column matrix or hybrid (metal) column matrix brought the dynamics to purify a broad range of biological molecules. Affinity based chiral separation is possible which shows the interaction of drug hormone with binding proteins.

Biological samples are often complicated and not easy to identify. Sometimes they exist in nature or in experimental platform at a very low concentration; for that reason concentration or mass measurement gets very difficult. The measurement of blood plasma glucose or fructose was difficult due to low concentration, but with the combined experimental approach like GC/MS it's possible now. Argininosuccinic aciduria (argininosuccinic lyase) test for new born is common in clinical practice. LC-MS/MS is a multiplex platform which lowers the cost of medical test. Halogenated hydrocarbon e.g., pesticides, PCB's, dioxins are preferred samples for GC method. HPLC is good technique to measure polycyclic aromatic hydrocarbon where fluorescent labeling is required.

HPLC-UHPLC

HPLC (high performance liquid chromatography) is a highly dynamic field and widely used in various research areas. Continuous innovation and development made HPLC application priceless in pharmaceutical industries QA/QC. HPLC is an important separation technique in biochemistry and intensely used to identify amino acids, nucleic acids, proteins, carbohydrates, hydrocarbons, antibiotics, steroids, and countless other inorganic substances [11-13]. HPLC grade solvents are always costly and sometimes not environment friendly; that's why research has drawn to that phase to reduce organic solvent usage. However it's always complicated to separate optical isomers. UHPLC (Ultra High Performance Liquid Chromatography) is recent and advanced version of HPLC that helps to analyze complex samples, and chiral molecules. UHPLC and supercritical fluid chromatography (SFC) is the recent approach to solve these problems. Supercritical fluid chromatography is a combination of SFC and GC (gas chromatography). UHPLC instrument's technique is based on Van Deemter equation $\{HETP=A+B/u+(Cs+Cm).u\}$, where linear velocity depends on pressure. The theory behind UHPLC is that heat energy helps to separate molecules. Small molecules produce frictional energy where reduction of external temperature (column wall) is required. Caution should be taken when thermo labile drugs are analyzed; due to frictional heat the structure of the drug can be changed or modified. UHPLC's column particle is a crucial element to consider for drug separation. Platinum showed the highest separation efficiency compare to nonporous, and silica based column.

pH sensitivity of column particles is also an important point to consider before starting a separation technique. New hybrid columns have the flexibility to separate acidic, basic and neutral drugs. Gradient elution can help to elute crude drugs by UHPLC in a shorter period of time compare to HPLC. Precision, reproducibility and linearity are the advantages of UHPLC compare to HPLC. Column particle size moved from 5 μm (HPLC) to 1.7 μm in UHPLC (Agilent or fisher's website). Attaching mass spectrometry with HPLC has given the dynamic to analyze unknown drugs or chemicals in shorter time frame. Hybrid column also keeps the option open to explore more hybrid column material in future. The sensitivity level of UHPLC has enhanced to the level where it can detect free polyamines in clinical samples or biological fluids. In UHPLC instruments flow rate, gradient, injection volume, UV detection, and liquid modifier are the critical parameters. In any column chromatography buffers and their accurate pH is very

important to get successful extraction of samples. Smaller internal diameter of the column reduces the use of analytes during experiments. Microbore column enhances mass sensitivity and reduces the sample volume up to 5 μL [12]. The internal size of the column could be 1-2 mm, and it's possible to pack with 3, 5, 10 micron particles. The mobile phase in super critical fluid chromatography (SFC) is gas e.g., carbon dioxide. This column is frequently used in clinical laboratories as well as in forensic toxicology. Combination of UHPLC, 2D-LC (2 dimension) and highly sensitive MS (Mass Spectrometry) revolutionized molecular biology or pharmaceutical research. UHPLC column material is superficially porous. It can operate at 8700 psi or 600 bar. In new UHPLC instruments 50,000 psi give better separation of molecules in longer columns. There are some limitation in protein/peptide identification either they are very small or very large. Caution should be taken when working with small peptides. Small peptides can be completely digested by enzymes. Large protein hydrolysis or digestion depends on arginine or lysine's distribution in the protein. The important application of HPLC-MS/MS is in therapeutic drug monitoring (TDM) field. This technique has high analytical specificity and potential to separate various ranges of molecules. It helped to diagnose and evaluate therapeutic drugs for carcinomas' and other diseases (Table 1) [14,15].

UPLC

UPLC is a modified version of HPLC. It's a trade mark of Waters Corporation. Simultaneous innovation of particle technology and instrumentation helped to launch this technique and instrument in 2004. The advantage of UPLC includes less band spreading and requires less time to run samples. In UPLC (Ultra Performance Liquid Chromatography) completely porous column material can be used. The size range of this column particle is 2 μm which is smaller than HPLC (5 μm). Higher amount of sample can be loaded (3 times higher loading capacity) in UPLC but it doesn't cause peak broadening. At the same time it can run at high pressure- 15000 psi or 1034 bar instrument. Higher pressure also helps to maximize flow rate in column. UPLC shows better peak separation of basic drugs and in a shorter period of time [16-18]. The key advantages of UPLC instruments are tolerance to high pressure, high temperature, and elevated mobile phase pH.

Mass Spectrometry (MS)

MS is an analytical chemistry technique that helps to identify type and amount of chemicals present in an unknown sample. By measuring mass to charge ratio and abundance of gas phase ions MS instrument detects unknown samples. This technique and instrument was launched in 1919s. MS technique involves very long capillary column where experimental sample gets ionized in a high vacuum area. The high electron impact causes disintegration of experimental sample and each fraction gets detected according to mass/charge ratio. It passes through a magnetic field which has specificity to mass/charge and gets detected in an aluminum block where secondary electron generates. To get better detection people are combining several techniques such as GC-MS (Gas Chromatography- Mass Spectrometry) LC-TMS (Liquid Chromatography-Tandem Mass Spectrometry) [19-37]. This new techniques has no limitation by molecular mass. In gas chromatography (GC) sample can be vaporized. Generally mobile phase is inert gas (helium or nitrogen). Stationary phase is microscopic layer of liquid or polymer. GC was introduced to world in 1947. GC-MS limited by molecular mass. GC-MS is suitable for studying plant terpenes and essential oils as well as clinical drugs. After fractionation fatty acids are easy to separate by GC but it's difficult with HPLC (www.Agilent.com/chem/metabolomics). These volatile substances do not ionize easily in LC-MS.

	UHPLC	GC-MS	LC-MS	MALDI-TOF	Immunoaffinity	Size Exclusion
Atmospheric Pressure/G	50,000 psi	He, N ₂ , H ₂	Gas-N ₂ , psi depends on instrument	Varies with instrument model	Varies with instrument model	Varies with instrument model
Laser/UV Application	UV	Laser	Laser, UV	N ₂ Laser beam- 337, 355, 266	UV (280 nm)	UV-280 nm, 230 nm, 210 nm, 410 etc., depends on samples
Time (total experiment)	45 minutes	16 minutes	1.18/30.00 min	Avg.1 hour, depends on plate size	Avg. 30 minutes	Avg. 30 minutes
Matrix	Core shell, hybrid particles, sephadex, 2, 3, 5, 10 micron particles	Inert material or sand	Phospholipid	Varies depends on biological samples	Sepharose (90 μm), metals, hybrid metals and Various IgG's	Superdex/Sephadex
Column (diameter and length)	diameter-1-2 mm Length-50-250 mm	Capillary glass, steel, copper column- 30-60 meter	0.1-1 mm or 150 mm	NA	1-5 ml	150 × 2.1 mm 4.6 × 50-150 mm 3.2 × 100 mm
Elution Solvent	Acetonitrile, Formic acid, Methanol	Acetonitrile, Formic Acid, Water	Ammonium Acetate, Formate, Hydrogen Carbonate, Formic/ Acetic Acid, Trifluoroacetic acid, Trialkylamine, TRIS, BIS-TRIS, propane	Acetonitrile, Water, Methanol, Chloroform, Propanol, Ethanol	Tris-HCL, NaCl	Acetonitrile in TFA

Table 1: A comparative table of experimental procedures and parameters.

Some of the new combined techniques are comparatively better than the other such as LC-TMS. It does not require molecular derivatives but GC-MS does. Standard mobile phase can be acetonitrile, formic acid, water containing ammonium acetate as proton donor. LC-TMS is a cost effective process and new instruments can process 1000 clinical sample at a time. LC-TMS is also useful for therapeutic drug monitoring purposes. It can detect active drug compound and metabolites. GC-MS or LC-MS is widely popular in toxicology study also. It can detect as many as 300 samples at a time. LC-MS is cost effective to analyze biological samples which are frequently used in clinical laboratories. LC-MS has more flexibility compare to GC; specially its good for research purposes where experimental sample contains various unknown metabolites. LC-MS is buffer and pH sensitive. Phosphate and sodium acetate did not show high efficiency in LC-MS. These volatile substances do not ionize easily in LC-MS. Before loading samples into MS instrument enzymatic digestion is required for proteins. Efficient digestion of small peptides makes analysis easier. Peptides are easy to ionize and act as positive ions. Trypsin is widely used enzyme to digest proteins. To improve analytical method people uses chymotrypsin for second digestion. These digestive enzyme generally targets C terminal side of the proteins; most of the time arginine and lysine. Generally mixed proteins are difficult to analyze in MS; it needs to be separated by 2D gel at first.

A detector is a crucial part in any experimental instrument. Optical detector such as UV has limitation because many analytes do not contain chromophore. Mass spectrometry data depends on additional ionization which gives better signal intensity, and nebulizer helps to enhance signal [19]. Evaporative light scattering detection, charged aerosol detections virtually covers any analytes (Thermo scientific/ Dionex, Corona charged aerosol particle). ESI and mass are the standard operating procedure in protein purification field. ESI and tandem mass are also equally popular purification and analysis method.

Tandem MS is very specific technique; here signal to noise ratio is very low which helps with clear and faster analysis. Combination of MS-ESI is a good technique which helps to study a broader range of biological samples. APCI is ionizing method where corona discharges on solvent spray and produces primary or mono ions. This technique reduces thermal decomposing. APCI and ESI together enhance MS efficiency. ESI actually works well with moderately polar molecule. APCI (Atmospheric pressure chemical ionization) and ESI (electro

spray ionization) strongly dependent on gas phase proton affinity. In this technique nebulizer gas flow helps to keep folded protein in its native three dimensional structures. Researchers argue that Electro sonic spray ionization (ESSI) is better than ESI. It describes that nebulizer modulates better air flow, better ionization technique, and voltage control; therefore it's better than ESI. In new ESSI technique protein doesn't lose its 3D structure even in acidic condition compare to ESI. Multiple charging is not possible with APCI; singly charged ions dominate here. ESI can be considered as soft ion source and causes small fragmentation whereas MS causes extensive fragmentation. ESI is not a recommended technique to use for lipid molecules.

Not only biomarker analysis or proteomics IA-MS method was used to extract antibody and verify reactivity during purification. Human genetics was first explored by IA-MS. This technique also helps to identify metabolites (up to molecular level) from frequently used biological samples [23]. MS signal can be reduced by ions present in biological samples e.g., plasma, urine, serum etc. are always complex bio-fluids [27,30,38]. Testosterone was widely analyzed with this technique. Immuno-precipitation helps to identify antibody from a protein mixture. A surrogate peptide with a protein of interest enhances the yield and sensitivity. SISCAPA (stable isotope standards and capture by antipeptide antibodies) is very efficient to analyze large protein mixture, and applied extensively to identify disease related biomarkers.

Protein identification procedure is divided into several parts; first stage is mass finger printing. Here mass peak and value is compared with molecular mass after hydrolysis, but people have found collision induced dissociation (CID) is more efficient technique than finger printing [26]. CID helps to identify single peptide where fast atom bombardment takes place for ionization. CID is more automated than 2 dimensional gel analysis. After HPLC separation the mass spectrometry takes place. Writing a robust algorithm for mass spectrometry or liquid chromatography is also a big challenge. The algorithm calculates backbone and side chains of peptides. Isotope tagging helps to differentiate peptides and its identification. Different isotope incorporates in proteins in different ways which helps to identify relative abundance of specific amino acid. Cells can grow with two different isotopes, and at the end of culture they can be mixed and prepare for analysis. The level of simplicity in new mass spectrometry instruments is unique. Samples can be loaded after extraction and centrifugation.

MALDI-TOF

MALDI-TOF (matrix assisted laser desorption/ionization time of flight mass spectroscopy) has been greatly used in bacterial taxonomy and proteomics. It can identify species up to sub species level. General formula for MALDI-TOF matrix is acetonitrile, water, trifluoroacetic acid (50:48:2). People used this technique widely to identify bacteria's and yeast's [20,24]. Bacterial and fungal cell smear or cell extract gets used for this experiment [31-37]. This technique measures peptides and other compounds in presence of salts. MALDI-TOF-MS was utilized in human genome project. It helps to detect SNPs or single nucleotide polymorphism in DNA helix. The frequency of SNPs can be 1 per 1000 nucleotides. SNPs are not very common in human genome especially in exon areas, and their patterns are not similar either. Recently scientist have discovered some disease specific SNPs and that can be used as markers. This technique is vastly used to detect proteins, peptides and nucleic acids [32]. Laser pulse in MALDI-TOF causes excitation of matrix molecules followed by energy transfer in gas phase matrix ion production. Bacterial sample volume should be optimal for MALDI-TOF. Too less or too high amount of sample can completely perturb the spectrum. Serial dilution of bacterial sample is good for MALDI-TOF analysis. MALDI-TOF-MS has good usage to find relative abundance of specific molecule in tissues or biological samples. It can also be used to study antibiotics and anti-micro biological samples. This technique is now frequently used in clinical microbiology as well as for high molecular weight viral protein identification purposes [34,39]. OCN (oscillating capillary nebulizer) drops small drops of aerosol on the surface of biological samples and prepares homogeneous surface i.e., reduces crystal size and controls solvent effects. MALDI-TOF is one of the pioneering techniques to analyze proteins but modern research prefers mass spectrometry with ESI. It gives better clarity to the result and analysis procedures. ESI is the standard ionization method for most of the liquid spectrometry. ESI generates ions that carry multiple charges whereas MALDI generates singly charged molecules. This step of technique is pulse based. People described that this technique was efficient to analyze post transcriptional changes in RNA.

Immunoaffinity Chromatography

Immunoaffinity chromatography (IAC) separates molecules from biochemical mixtures. It is a highly specific bonding between antigen and antibody, enzymes with substrates or ligands. DNA can also be extracted or purified by affinity chromatography. Four different types of non-covalent interaction take place during Immunoaffinity chromatography e.g., ionic interactions, hydrogen bonds, van der Waals interactions and hydrophobic interactions. Generating monoclonal antibody from a single immortal hybridoma cell line is the first step to purify through immune affinity chromatography. Recombinant antibodies are also equally important and can be fused with proteins and peptides (drugs and toxins) [38-40]. In affinity chromatography ligand gets attached with a solid matrix and target molecule remains in liquid phase. Macro porous uniform particle is good for IAC column during purification. Generally 300-500 Å pore size covers most of the antibody structures in immobile phase and helps to elute small to medium size targets (100-150 KDa). Magnetic particles are also available to use with affinity column ligands, such as protein A, protein G, streptavidin etc. (along with specific antibodies). Elution is difficult when several antibodies target the same antigen and its various epitope binding site. Targeting a single protein by multiple antibodies can denature antigen and damage the column. Polyclonal antibodies are possible to produce from multiple B cell clones. The sample run in affinity chromatography can be affected by pressure, ionic strength, temperature, dielectric constant and pH.

To enhance precision and better identification, two different experimental methods gets combined in new chromatography instruments such as IA and MS (immunoaffinity chromatography and mass spectrometry). Not only biomarker analysis or proteomics IA-MS method was helpful to extract antibody and verify reactivity during purification. Human genetics was first explored by IA-MS. This technique also helps to identify metabolites (up to molecular level) from frequently used biological samples. MS signal can be reduced by ions present in biological samples e.g., plasma, urine, serum etc. are always complex bio-fluids. Testosterone was widely analyzed with this technique. Immuno-precipitation helps to identify antibody from a protein mixture. A surrogate peptide with a protein of interest enhances the yield and sensitivity. In terms of sample volume IA-MS method has advantage over MS method. This technique involves a single antibody in bigger amount/volume. It has long incubation period which causes low sample throughput and can offset multi analytic detection. However elution gradient helps to get undamaged protein of interest and saves its activity.

Size Exclusion Chromatography

Size exclusion chromatography is based on molecular size of molecules [41]. It's also another version of affinity chromatography. Larger molecules run through the column faster than the small molecules; because they cannot enter in the porous matrix at stationary phase, whereas smaller molecules runs through the column matrix and takes longer time to elute. The stationary phase is made of chemically inert materials such as gel, porous inorganic solids, glass etc. This technique is also applicable to larger molecules e.g., proteins, polymers. Quality control purposes people can use this technique e.g., to identify the differences in properties of polymers. There are two basic types of size exclusion chromatography. One is gel permeation chromatography (GPC), and other one is hydrophobic which uses non aqueous mobile phase organic solvent. This technique helps to determine molecular weight distribution in synthetic polymers. Another exclusion technique is called GFC (gravity flow chromatography) where hydrophilic packing material separate, fractionate and measure molecular weight distribution of samples. Samples for GFC should be dissolved in water e.g., polysaccharides and proteins.

Lectin Affinity Chromatography

Lectin affinity chromatography helps to purify carbohydrate molecules [38,42]. Lectins are proteins and have different epitope binding sites for carbohydrates. Lectins are available in three different types such as merolectins, hololectins and chimero lectins. They are water soluble proteins and can be extracted with aqueous buffer (intechopen.com). Sepharose and Sephadex bind covalently with lectin. PGC is a version of size exclusion chromatography. PGC based approach can deliver information's about protein bound sugars. Several research group used PGC-LC-ESI-MS/MS for glycomics study in cancer patients (disease associated N glycosylation); because glycoprotein takes important role in cancer development as well as in pathogen invasion. ESI-MS and MALDI-MS help to study the complicated branched nature of carbohydrates/sugars. This technique has potential to separate structural isomers. Boronate was the earliest ligand used for clinical chromatography to determine glycohemoglobin in long term diabetes patients. Quantitative affinity chromatography such as LC is useful to measure drug hormone interactions. Lectin affinity chromatography is useful for Proteome analysis in stem cells (Agilent capillary 1100 system).

Paper Chromatography

Paper chromatography and thin layer chromatography (TLC) are frequently used methods in clinical practices. Generally on top of a glass plate silica gel, aluminum oxide or cellulose gets used as a thin layer of matrix. Non volatile sample is suitable for this experiment. Amino acids, carbohydrates, nucleotides, keto acids, hormones can be detected by paper chromatography or TLC from biological fluids (serum, urine, saliva, sweat) [43,44]. Several hereditary diseases can be detected by paper chromatography, where disease specific amino acid secrets in larger volume. TLC is good for qualitative and quantitative measurement of hormones from urine and plasma samples. This technique helps to identify the effects of phase 0 micro-dose clinical trial medicines. It also helps to identify drug of abuse from dried blood samples on paper.

Protein PEGylation

Protein PEGylation [41] is a technique to enlarge protein's size and eventual elution of proteins. It also increases proteins half-life (≤ 20 KDa) when circulates in the system as a drug or during its elution procedure. Protein gets attached with PEG by covalent bonds. The PEG-protein reaction is mainly controlled by temperature and pH. Time sensitivity is one of the drawbacks of this technique. Over exposure of PEG can cause tri PEGylation, and it doesn't show homogeneity. The new modified SERC technique is based on molecular size. It gives uniform product by controlling the time of PEGylation, moving reaction zone and subsequent removal of the product from column. PEG reaction is pulsed and can add up to 5000 Da molecular weight to targeted protein. PEGs and proteins act little differently in columns; however there is a linear relationship between PEG and protein (correlation coefficient $R^2 \geq 0.99$). PEGylation reaction utilizes UV light as detector.

Sandwich Immunoassay

Sandwich Immunoassay utilizes two antibodies to target a single analyte. The first antibody gets attached to the column matrix and second one helps to elute the analyte from the column. Aptamer based sandwich assay helps to detect hepatocellular carcinoma [45-47]. Aptamers are oligonucleotides with strong affinity towards proteins, nucleotides, and small cells. Powerless microfluidic device/chip helps to attach target DNA and microRNA at very minute level (e.g., 0.5 pM) [44]. This technique or sandwich assay will be efficient to detect early stage cancer within 20 minutes. MicroRNAs are related to lot of human diseases. Early stage cancer detection with very minute level of microRNA is always a challenge. Dr. Maeda's work showed promising future in early stage detection of cancer or point of care. Sandwich assay is powerful when combined with QD and CCD camera, and makes it easier to detect pathogen in very minute level. Urinary tract infection can also be measured by sandwich hybridization [45].

ELISA

This experiment is based on antigen antibody reaction. It started with radioactively labeled Ag or Ab in 1960s. For ELISA we use primary and secondary antibody for reaction. Monoclonal and polyclonal antibodies can grow following standard cloning techniques followed by affinity purification. Body fluid, environmental samples are frequently used for ELISA. This technique is still used in clinical laboratory as a gold standard for protein quantification. Sandwich ELISA is frequently used in immunology labs. This technique is time consuming no doubt; that's why a new technique called lab on a chip (LOC) or lab on a compact disc (LOCD) takes place. LOCD is an integrated microfluidic platform. The centrifugal force of the spinning disc helps fluid to

migrate from one chamber to another [48-50]. This new technique showed great efficiency where people can have their graphical result via Bluetooth or smart phone and shows 95% accuracy. This LOCD device is portable and will be a positive technique for remote detection.

FRET Assay

Quantum dot induced fluorescence resonance energy transfer is called FRET Assay. Here two adjacent molecules share excited state spectrum and energy passes through one molecule to other by transferring electrons. This technique was first described 50 years ago and it helps to study molecular interactions. Nucleic acid sandwich hybridization and FRET can be done together. The target sample concentration can be nM to μ M range. FRET test is possible to visualize by Zeiss confocal fluorescence microscope. The fluorophore or dye's excitation and emission spectrum should have overlap in the spectrum region [50]. The donor molecule does not need to be fluorescent.

Discussion

Phase zero clinical trial samples can be blood, urine, hormone, cell extract, tissue, water, enzyme digested proteins, DNA, RNA, and cerebrospinal fluid. Paper chromatography/strip analysis started with early diagnosis of hereditary diseases. GC chromatography is widely used in forensic biology as well as in clinical laboratories to test blood alcohol level.

Biological samples are widely analyzed by liquid chromatography and mass spectrometry. HPLC and mass spectrometry is also useful to study plant products; such as alkaloids, antimicrobial residue in milk, forensic samples, pesticides, and characterization of petroleum in molecular level. Time and pH sensitivity makes the biological samples more vulnerable to degrade or denature quickly. New HPLC instruments and ESI technique keeps protein in its native state especially immunoglobulin [51]. We need to study antibodies in its native 3-D state to find the docking site and bonding pattern of new sample or phase zero drugs. Various types of affinity columns are available now to purify antibodies for research or clinical trial purposes.

The required advancement in instrumentation areas enhanced the clarity of clinical trial phase zero drugs. With the help of advanced instruments and software's liquid chromatography showed the efficacy to identify novel bio molecules in a short period of time. It is cost effective and efficient for drug discovery projects [52,53]. For some samples LC is better than GC because of auto sampling facilities and produces plenty of derivatives. Stationary and mobile phase is capable of analyzing very polar molecules e.g., some amines, alcohols, carboxylic acids etc. It's easy to analyze biological samples in new LC instruments compare to GC; because derivatization is not required to analyze biological samples.

Sometimes it's difficult to identify soft bacteria's in MALDI-TOF-MS. ESI application takes place to smoothen cell surface for better detection. Matrix must have a chromophore to absorb energy from laser beam so that the protein of interest stays in its native state. Generally it produces singly charged molecule with a mass to charge (m/z) ratio which varies with sample. After ionization molecule enters into a free field where movement is coordinated according to their velocity. Bigger molecules move comparatively slower than the smaller molecules. Linear TOF method has capability to measure samples at femtomolar (10^{-15} mol/liter) and attomolar (10^{-18} mol/liter) level [23]. MALDI-TOF-MS technique has limitations to identify all bacterial samples. Not only sample preparation related complication, but a robust algorithm or software is essential to analyze data. In ESSI-MS

supersonic gas flow by nebulizer and electrosonic spray ionization (ESSI) helps to keep proteins in its three dimensional structure compare to ESI [54]. ESSI reduces sample flow rate. Sample flow rate still needs to be optimized to stop breakage of molecules and to identify velocity of different molecules.

MS-HPLC is better technique over MALDI-MS, and spectrum shows better separation of peaks. MALDI-TOF technique was used for SNP detections in human genome project but with larger DNA size signal intensity gets lost and spectrum loses its resolution. Recent MALDI-TOF-MS technique does not require PCR amplification step. SELDI-TOF-MS (surface enhanced laser desorption/ionization time-of-flight mass spectrometry) is another version of MALDI and can be considered as continuation of MALDI branch [55]. Ciphergen's Protein Chip system produces reproducible data. Sample preparation technique is quite different in SELDI. In SELDI sample deposits on a chip whereas in MALDI sample solution gets mixed with column matrix before use. In MALDI several fractionated micro technique is used to prepare sample which makes the procedure complicated, but SELDI technique is comparatively lot simpler. Protein interacts with micro chip according to their surface net charge in case of SELDI. Sample can be washed on spot; therefore co-crystallization of protein and matrix is possible which helps with detection step.

Through microfluidic device [22] people described how DNA and miRNA can be hybridized. It can detect miRNA up to 0.5 pM to 0.25 aM level. Early detection of miRNA level will save patients from cancer. They have merged microarray and microfluidic device together [44]. The advantage of this device is portable, power free; thus fulfills all the early detection parameters. Fluorescent labeling is not required in this instrument. This instrument holds the promise of significant marketing.

DNA and nickel affinity chromatography is very specific and helps to elute specific proteins. Here DNA incorporates into a cellulose matrix. Although DNA-protein bonding is very specific; sometimes it's hard to elute protein of interest. Harsher methodology is not advised when further analysis is required for protein of interest or immunoglobulin's. It damages protein's 3-D structure. ELISA is an unavoidable assay in immunology. ELISA technique also advanced from regular ELISA to sandwich ELISA for better detection purposes [48]. This technique is frequently used in clinical laboratories to detect various diseases.

Urine is frequently used biological sample in medical sector for diagnosis. Now Ion chromatography (AS4A anion chromatographic column) is useful to detect urine samples. It measure oxalate in samples, specifically heredity related diseases [52]. This analysis can be measured at pH 9.2 (Thermo fisher Scientific). This excretory product needs to be in its native state before analysis (mass spectrometry) and to identify exact secretion from human body. To overcome isotope related derivatization and time sensitivity people came up with new instruments and analysis procedures [2]. A research group suggested that three LC column and ESI spray helped to analyze urine sample in its native state bypassing the step of isotopes, and derivative formation (LC-MS-MS, instrument REMEDI HS, BioRad Diagnostics) [1,2]. The technique is called tandem mass spectrometry. MS/MS also used to identify heredity related metabolic disorder (dried blood sample) in new born and this process is call metabolite profiling [56,57]. Sample preparation is a rate limiting factor in biological field (Panny et al. - CDC report, 2001). Fragmentation occurs when working with small molecules. Significant background problem also appears in spectrum; more research needs to be done in this field. Efficient peak separation

is also possible by using less width column. Plasma mass spectroscopy (isotope labeled) is beneficiary for urine analysis because urine has tendency to form crystals rapidly. Modification of urine sample with ESI creates positive ions and helps with downstream analysis. In case of negative ion (organic acids) the sample can be loaded in MS without several dilutions. With the advancement in technology now people can analyze hundreds of samples at a time. New instruments showed time and cost affectivity by introducing auto sampler platform [5].

MS gives detail oriented analysis or data's of a protein however sample processing is complicated and need skilled hands. A single peptide string can distinguish a protein from a mixture of proteins. MALDI-MS is a good technical procedure however it's time consuming; and sometime a single spot (cut out from gel) represents more than one protein and appears to be a single peak in spectrum.

Amino acid profiling is possible by several techniques such as ion exchange chromatography, thin layer chromatography, paper electrophoresis, gas chromatography and MS. Abnormal metabolite detection is a routine practice in new born. All these techniques are time and labor sensitive. ESI-TMS is a faster procedure to profile amino acids.

Advancement in UHPLC application areas helped to reduce time and toxic chemical exposure. New UHPLC instruments process samples at very fast without losing resolution and sensitivity. New market available instruments are not limited by pH and high pressure. UHPLC instruments can run sample with impurities that's a major plus point for a phase zero drug or a mixed protein sample or to screen bulk bio-molecules for pharmaceutical purposes. Appearance of glycosylation was noticed in several diseases such as cancer or inflammation. Study of glycan structure is important because it helps with disease progression in cancer patients. PGC-LC-ESI-MS/MS is a valuable method to study glycoprotein and glycopeptides in depth. It also helps to separate isomers during drug synthesis [54]. Sphingo lipids are not only internal part of cell membranes but they keep cells in intact condition. Elevated level of sphingo lipids in human serum can indicate disruption of cell signaling in human body associated with particular diseases [56].

Protein purification by affinity chromatography in presence of DNA matrix is a highly specific method and helps to purify proteins associated with transcription, recombination, and replication related processes. Crude extract usage is an important characteristic of affinity chromatography. However too many proteins and DNA in the experimental sample reduces its efficiency. Chitin, lectins are widely used matrix for affinity chromatography. Before starting experiments people should study the coagulant factor of lectins with their compound of interest. The purification procedure could be vice versa e.g., if we want to purify antigen then antibody should be in the matrix. Steric interference can occur between support and substance in affinity chromatography by interfering with ligand-hydrocarbon spacer arms. Monoclonal antibody purification is always a complicated procedure. Traditional detection method like UV or fluorescence absorbance is not enough to detect broad range of biomolecules. Evaporative light scattering detection (ELSD) or charged aerosol detection brought dynamics in spectrometry (Paul et al. - Technical note 143, Thermofisher, Germany). Nebulization is the first step to produce small, aerosol particles in presence of high amount of solvents. Smaller charged molecules run with high velocity followed by enhanced signal for better detection. Pressure pump in HPLC is 40 MPa (400 atmosphere) but in UPLC it can be 100 MPa. The HPLC solvents flow through the column efficiently in presence of high atmospheric pressure.

Some high light of instruments produced by different companies

Agilent invented a sub 2 μm level column particle for 1290 infinity model. It has QbD aligned LC method that carries multidimensional operation mode. Some instruments of Agilent have method transfer facilities also e.g., from UHPLC to HPLC (ISET application) with robust S-matrix software's; along with broad range pH facilities. Gradient time period is 10-15 minutes and oven temperature should be set at 37°C. Sample volume for UHPLC is 1 μL and HPLC 5 μL . Result and spectrum (resolution, retention time, and critical peak pairs) is reproducible with very little deviations. Fusion QbD software platform helped to transfer UHPLC method to HPLC (Vinayak et al. - Agilent). This instrument is useful in pharmaceutical lab's QA/QC purposes. ACN and MeOH are organic modifier solvents required for this instrument. Chiral products are important to study in pharmaceutical industries. Agilent's Infinity 1200 series showed efficiency in this field. Six long chiral columns can be installed in 1200 series. Binary, ternary, quaternary isocratic solvent mixtures are used in this system. Biorad's REMEDEi's multicolumn instrument with UV identification facilities is capable to analyze 700 drugs. There is a drawback in this instrument though; it cannot separate same molecular weight compounds from those are practically different. ESI gives same spectra which is not the case with other instruments. Shimadzu Nexera X2 has features to analyze samples at superfast speed and with well separation mode (Shimadzu web site). UHPLC column requires 5 μL samples. Organic modifier is acetonitrile and required oven temperature is 40°C. Particle size varies from 1.6 micron to 5 micron. Smaller particle size helps to fulfill all these criteria's. UV light detects the sample. The columns have high pressure tolerance produced by this company (100 Mpa). Sample volume varies from model to model from 5 μL to 10 μL , depends on column particle. Normal phase and reverse HPLC efficiency depends on biological samples. Antioxidant showed better separation with normal phase HPLC at high concentration [8], whereas reverse phase HPLC-MS showed better separation of serum sphingo lipids at pM level [3].

Thermo fisher Scientific/Life Technologies Company's UHPLC column with integrated HPLC system provides very fast auto-sampling procedure within a short time cycle (Thermofisher web site). ACN and MeOH are the organic modifiers for these instruments. The required injection volume is 2 μL . Sample detection takes place by UV/Vis spectra. Column compartment temperature remains at 25°C (Karsten et al. - Dionex/Thermo fisher Scientific). POROS' resin beads column are also available in Thermofisher (10-20 micron particle size with protein A and G). It's good for purifying monoclonal antibody with HPLC. Same stainless steel column serves the purpose for affinity chromatography as well. They have high flow and high affinity with temperature flexibility from 2-40°C.

Chiral compounds exist in two enantiomeric (their molecular formula is same but structural arrangement is different) forms; and it's a major problem in pharmaceutical industry. One form can be a live saving drug at the same time the other form can be toxic to health. It's really a challenge to separate these isomers or enantiomers during drug synthesis. Enantiomer can be separated in several different techniques e.g., capillary electrophoresis, crystallization, membranes, chromatography, liquid-liquid extraction, enzyme kinetic resolution and supercritical fluid extraction. GC, CE, SFC, HPLC are the most widely used chromatography in pharmaceutical industries. Immobilized stationary phase generally helps to separate enantiomers or isomers. Supercritical technology uses carbon di oxide as environment friendly green solvent for separation. Urine samples are

frequently used for drug testing purposes and can be processed by GC-MS [2]. MS is an important platform for urine analysis because it's full of kidney and urinary tract proteins. Any abnormalities in those areas can be found by mass spectrometry; but data can vary from sample storage procedures to sample collection time. It's difficult to work with urine samples because of high salt concentration, and presence of lower amount of protein. pH also varies in wide range that makes urine sample difficult to deal with [1,5,27].

Troubleshoot with HPLC instrument and data

Pressure, peak shape retention is the problematic areas that appear frequently in liquid chromatography. Too high pressure can cause plugged frit, column contamination, plugged packing etc. Back flush of column is advised when these troubles arise. Precipitation from buffer can also happen. Low pressure causes leakage and low flow in column. Disrupted sample path can cause split peaks, peak tailing, broad peaks, double peaks if sample flows through different path in the column (Agilent Web site). It can happen when column packing material is loose. Mass spectrometry creates tailored peak when purification goes through an inefficient column matrix. Inaccurate buffer pH can dissolve silica. Peak tailing, broadening and loss of efficiency occur due to secondary interactions with molecules, column contamination, column aging, inefficient column loading etc. Fronting appears when column is overloaded. Peak broadening takes place due to void nature of column and large injection volume. Ghost peaks or broadened peaks shows up from late elution or from previous samples with high molecular weight e.g., protein or polymer. Stainless steel is good for sealing. Buffers are the critical parameters to keep proper peak retention and peak shape. Buffers can cause de-shaped peak when pH is completely outside of the range.

Noisy baseline can be a cause of several different issues. Those are mainly indicator of dirty flow in cell, failing detector lamp, pulses from pump if periodic, and temperature's effect on detector. Passing air bubble through detector or aging lamp can also raise noisy baseline. Chemical contamination in LC-MS may come from impure chemicals that are used for column packing purposes or as buffers. Some researchers mentioned that when they used the combination of electrochemical detector (NMR) with non aqueous eluent in ion exchange separation; they achieved better result in silica matrix than aqueous. New matrices' like polymeric, base deactivated silica's, pyrolysed carbon improved stability and high efficiency of certain class compounds during purification e.g., basic drugs. This research group mentioned that new column materials reduced solvent consumption but increased sensitivity. Micellar solvent in reverse phase liquid chromatography helped to determine drugs in biological fluids. When cells go through apoptosis due to cancer or bacterial or viral infections, it produces enormous amount of cell debris. With advancement in instrumentation area people can measure sub-cellular level of mRNA, DNA or proteins now. People get detail answers about what goes wrong when cells are under stress. HPLC and MS have given that opportunity to explore cells up to molecular level.

Conclusions

Instrumental advancement comes from trouble shooting and wishes to bring more detail oriented research data. Current MS, ESI, UPLC or UHPLC techniques helped to identify unknown clinical samples with the help of new hybrid column materials. Scientists are capable of collecting data with robust algorithms where people could not imagine or invade decades ago i.e., single amino acid change in biological samples (single amino acid, single DNA base, fat or oil

analysis from random samples). Now sample can be processed as low as in 1 minute with new HPLC/UHPLC instruments. People can process acidic, basic or neutral drugs with the help of ESI at very low concentrations. New instruments also solved isomer or enantiomer associated separation problems during drug synthesis or identification. New HPLC instruments have high pressure tolerance at the same time high amount of sample can be loaded in presence of fully porous column particle. Superficially porous column particle showed less purification efficiency than completely porous particle.

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