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Analytical Techniques in Pharmaceutical Analysis for Samples Separation, Characterization, Determination and its Handling

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ABSTRACT

Separations are key aspects of many modern analytical methods. Real world samples contain many analytes. Many analytical methods do not offer sufficient selectivity to be able to speciate all the analytes that might be present. Separation isolates analytes. Most separation methods involve separation of the analytes into distinct chemical species, followed by detection. Instrumental methods may be used to separate samples using chromatography, electrophoresis or field flow fractionation. The development of the pharmaceuticals brought a revolution in human health. These pharmaceutical would serve their intended only if they are free from impurities and administered in appropriate amount. To make drugs serve their purpose various chemical and instrumentation method were developed at regular intervals which are involved in the estimation of drugs. For this analytical instrumentation and methods plays an important role. This review highlights the role of the analytical instrumentation and analytical method in assessing the quality of the drug. The review highlights variety of analytical techniques such as chromatographic, spectroscopic, electrophoretic, titrimetric, microscopic and electrochemical and their corresponding methods that has been applied in the analysis of pharmaceuticals. Quality of pharmaceutical product largely depends upon the environment controls during its storage and handling. Each pharmaceutical product should be handled and stored under specified storage condition labelled on product information data sheet or product pack. Ion chromatography was based on the use of a low-capacity anion exchange separator column used with a basic eluent and a suppressor column. IC is a part of high-performance liquid chromatography used to separate and determine anions and cations.

Keywords: Analytical techniques, Sample separation, Chromatography, Spectroscopy, Electrochemical method, Microscopically method, Sample handling, Sample determination Electrophoresis.

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INTRODUCTION

With the great development of analytical instruments, interesting analytes can be directly detected [1]. However, it still remains a great challenge for the detection and quantification of those analytes with low-abundance due to the restriction of instrumental detection limit and interferences of complicated matrix [2]. Biological samples are particularly complicated samples with matrix interference. Therefore, prior to the analysis of trace biological targets, it is imperative for the isolation, separation and purification of raw samples [3, 4]. Although electrophoresis is low cost and simple to manipulate, it usually takes relatively long time and has poor repeatability [5]. Ultrafiltration has high separation efficiency, but it fails to obtain dry powder of target analytes and the membrane of ultrafiltration may exhibit adsorption toward biological macromolecules [6]. Among these techniques, solid-phase extraction (SPE) is one of the most important and powerful techniques because of its outstanding selectivity and recovery [7]. Although traditional SPE techniques where the adsorbents are packed into columns have been applied in many successful cases, but it is not suitable for coping with samples containing suspended solid or fouling components [8]. Batch separation technique, during which the adsorbents are incubated directly with the samples, can solve the above problems. Many new materials such as nano-materials and mesoporous materials have been employed in this mode [9, 10]. However, when using these materials as affinity adsorbents for the enrichment of biological target analytes, nonreversible adsorption and high-speed centrifugation are often unavoidable, which may result in sample loss and co-precipitation of unwanted interferents despite of their ability to remove salts and other contaminants [11]. Consequently, the application of these advanced materials as adsorbents is

restricted to a great extent. Therefore, a rapid, convenient, gentle and efficient sample preparation is urgently needed for biological analysis. In this case, with the use of magnetic materials, magnetic separation techniques have shown their usefulness. Magnetic separation technique is a batch-scale technique based on functionalized magnetic materials [8,12]. Magnetic materials are adsorbents particularly suitable for biological macromolecules due to their large surface area, good biocompatibility, easy functionalization and convenient manipulation. In a typical process of magnetic separation, magnetic materials, which exhibit affinity toward the isolated structure, are mixed with a sample containing target compounds. Within a period of incubation, target compounds bind to the magnetic particles. The whole magnetic complex is subsequently separated from the sample using an extra magnetic field. After washing out the contaminants, the isolated target compounds can be eluted and used for further work [12,13]. Magnetic separation techniques have several advantages in comparison with standard separation techniques used in various areas of biosciences [14]. The separation of enantiomers is a field of ever-growing importance in chemical industries, pharma, and food, and getting more important in different fields of bioanalysis (environmental, clinical). The capabilities of super- or subcritical fluid chromatography (SFC) regarding the separation of enantiomers are widely recognized. Due to the orthogonality of SFC compared to commonly applied chromatographic techniques like reversed phase liquid chromatography (RPLC) or hydrophilic interaction liquid chromatography (HILIC) [15,16] combined with a new generation of more robust instruments, SFC is becoming the method of choice for enantiopurity analysis [17]. Not only analytical but also preparative-scale separations of enantiomers are common in pharmaceutical industries but getting more important in different fields of bioanalysis. For method development general screening and optimization strategies for fast chiral separations in modern supercritical fluid chromatography are necessary [18,19] The determination of drugs and related substances in biological samples such as whole blood, plasma, serum, tissues and cells is referred to as bioanalysis. This term was coined in the 1970s in relation to various techniques designed for the study of pharmacokinetics of drugs [20,21]. To improve productivity, coupled with the increasing demands for green chemistry approaches in analytical determinations, the liquid-phase microextraction (LPME) method was introduced in the mid-to-late 1990s. This is a modified form of the LLE that utilizes microlitres of solvents for the extraction process. The LPME technique is able to overcome some of the problems that are often encountered in solid phase microextraction (SPME). These problems include, but are not restricted to, the bending of syringe, leaching of fibre coating material and the fragility of the fiber itself. The microextraction techniques received favorable responses and various modifications have been introduced, e.g., single-drop microextraction (SDME), continuous-flow microextraction (CFME) and hollow-fiber liquid-phase microextraction (HF-LPME). Details of these techniques are described elsewhere [22–25]. The drugs which are marketed may have different dosage forms. Formulation can be categorized according to the route of administration [26].

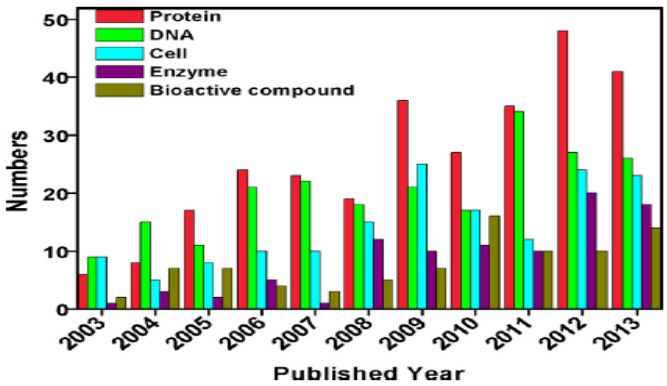

Fig1. Original papers on applications of magnetic separation techniques for enrichment of protein, nucleic acid, cell, bioactive compound and immobilization of enzyme from 2003 to 2013 (These papers were obtained from the Science Cita-tion Index Expanded (SCIE) Database of the Institute for Scientific Information (ISI)during January 2003–December 2013.).

2. Analytical Techniques for Sample Separation and its Determination

2.1 Titrimetric Techniques

Origin of the titrimetric method of analysis goes back to somewhere in the middle of the18th century. It was the year 1835 when Gay-Lussac invented the volumetric methods which subsequently leads to the origin of term titration. Although the assay method is very old yet there are signs of some modernization, i.e., spreading of non-aqueous titration method, expanding the field of application of titrimetric methods to (very) weak acids and bases as well as potentiometric end point detection improving the precision of the methods. With the development of functional group analysis procedures titrimetric methods have been shown to be beneficial in kinetic measurements which are in turn applied to establish reaction rates. These are many advantages associated with these methods which include saving time and labor, high precision and the fact that there is no need of using reference standards. In the past titrimetric methods have been used for the determination of captopril [27], albendozole [28] and gabapentin [29] in commercial dosage forms. Sparfloxacin [30] was determined by the nonaqueous titration method. In addition to its application in drug estimation titrimetry has been used in the past for the estimation of degradation products of the pharmaceuticals [31].

2.2 Chromatographic Techniques

2.2.1 Thin Layer Chromatography

Although an old technique yet it finds a lot of application in the field of pharmaceutical analysis. In thin layer chromatography, a solid phase, the adsorbent, is coated onto a solid support as a thin layer usually on a glass, plastic, or aluminium support. Several factors determine the efficiency of this type of chromatographic separation. First the adsorbent should show extreme selectivity toward the substances being separated so as to the dissimilarities in the rate of elution be large. For the separation of any given mixture, some adsorbents may be too strongly adsorbing or too weakly adsorbing. **Table 1** lists a number of adsorbents in the order of adsorptive power. Thin layer chromatography is a popular technique for the analysis of a wide variety of organic and inorganic materials, because of its distinctive advantages such as minimal sample clean-up, wide choice of mobile phases, flexibility in sample distinction, high sample loading capacity and low cost. TLC is a powerful tool for screening unknown materials in bulk drugs [32]. It provides a relatively high degree of assertion that all probable components of the drug are separated. The high specificity of TLC has been exploited to quantitative analytical purpose using spot elution followed by spectrophotometric measurement. TLC has been utilized for the determination of some steroids [33], pioglitazone [34], celecoxib[35] and noscapine [36]. TLC plays a crucial role in the early stage of drug development when information about the impurities and degradation products in drug substance and drug product is inadequate. Various impurities of pharmaceuticals have been identified and determined using TLC [37, 38].

Table 1 Chromatographic adsorbents: (approximate order is shown in the table, since it depends upon the substance being adsorbed, and the solvent used for elution).

Most strong adsorbent	Alumina	Al_2O_3
	Charcoal	C
	Florisil	MgO/SiO_2 (anhydrous)
Least strong adsorbent	Silica gel	SiO_2
Source:http://www.chem.wisc.edu/courses/342/Fall2004/TLC.pdf.		

2.2.2 High Performance Thin Layer Chromatography

With the advancement of the technique, high performance thin layer chromatography (HPTLC) emerged as an important instrument in drug analysis. HPTLC is a fast separation technique and flexible enough to analyze a wide variety of samples. This technique is advantageous in many means as it is simple to handle and requires a short analysis time to analyze the complex or the crude sample cleanup. HPTLC evaluates the entire chromatogram with a variety of parameters without time limits. Moreover, there is simultaneous but independent development of multiple samples and standards on each plate, leading to an increased reliability of results. HPTLC has been used to quantitate drugs as ethinyl estradiol and cyproterone[39], alfuzosin[40]and tramadol and pentazocine [41].

2.2.3 High-Performance Liquid Chromatography

HPLC is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems, in order to recognize better the role of individual molecules. It was in the year 1980, HPLC methods appeared for the first time for the assay of bulk drug materials [42]. As seen in **Table2,** this has become the principal method in USP XXVII [43] and to a lesser extent but one of the most widely used methods also in Ph. Eur. 4[44]. The refractive index detector is the detector of choice when one needs to detect analytes with restricted or no UV absorption such as alcohols, sugars, carbohydrates, fatty acids, and polymers. Decent trace detection performance is secured through a low noise. This detector is having the lowest sensitivity among all detectors but suitable at high analyte concentrations. Lakshmi and Rajesh utilized the refractive index detector to analyze the content of volgibose in pharmaceutical formulations [45]. The electrochemical detector responds to the substances that are either oxidizable or reducible and the electrical output results from an electron flow triggered by the chemical reaction that takes place at the surface of the electrode. This detector was applied recently to analyze the content of glutathione in human prostate cancer cells and lung adenocarcinoma cells [46].One of the most sensitive detectors among the LC detectors is fluorescence detector. Typically its sensitivity is 10–1000 times higher than that of the UV detector for strong UV absorbing materials used as an advantage in the measurement of specific fluorescent species in samples. One of the most important applications of fluorescence is the estimation of pharmaceuticals [47]. The application of various types of detector in HPLC is compared in **Fig 2.** It became the method-of-choice for analytical support in many stages of quality control and assurance within the pharmaceutical industry [48,49].

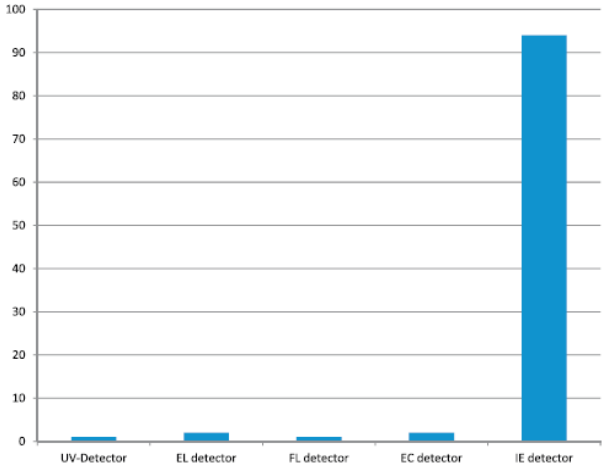

Fig 2 Usage of different detectors for HPLC analysis of drugs, Scale 0–100 represents use of the detector percentage. Source: R.N. Rao, V. Nagaraju. J. Pharm. Biomed. Anal. 2003, 33, 335–377.

Table 2 Proportion of various analytical methods prescribed for the assay of bulk Drug materials in ph.Eur. 4 and USP XXVIL

Method	Ph. Eur 4(%)	USP 27(%)
HPLC	15.5	44
GC	2	2.5
Titration	69.5	40.5
Acid- Base	57.5	29.5
Aqueous Mixture	21	5.5
Indicator	6.5	4.5
Potentiometric	14.5	1
Non aqueous	36.5	24
Indicator	9.5	14
Potentiometric	27	10
Redox(Iodometry, Nitritometry,etc)	6.5	5.5
Other (Complexometry,argentometry, etc)	5.5	5.5
UV-Vis spectrophotometry	9.5	8.5
Microbiological assay (antibiotics)	3	2.5
Other(IR, NMR,Polarimetry,fluorimetry,	0.5	2
Atomic absorption spectroscopy,polarography, gravimetry Etc)		

Source: S. Gorog/ Journal of pharmaceutical and Biomedical Analysis 36(2005)931-937.

2.2.4 Gas Chromatography

Moving ahead with another chromatographic technique, gas chromatography is a powerful separation technique for detection of volatile organic compounds. Combining separation and on-line detection allows accurate quantitative determination of complex mixtures, including traces of compounds down to parts per trillion in some specific cases. Gas liquid chromatography commands a substantial role in the analysis of pharmaceutical product [50]. The creation of high-molecular mass products such as polypeptides, or thermally unstable antibiotics confines the scope of this technique. Its main constraint rests in the comparative non-volatility of the drug substances therefore, derivatization is virtually compulsory. Recently, gas chromatography has been used for assay of drugs such as isotretinion [51], cocaine [52] and employed in the determination of residual solvents in betamethasone valerate [53].

2.2.5 Ion Exchange Chromatography

Ion exchange chromatography (IEC) is based on electrostatic interactions between charged patches on the surface of biomolecules and oppositely charged functional groups attached to a stationary phase via a spacer arm. The interactions are strongest when the ionic strength of the surrounding buffer is low, and binding can be modulated by changes in ionic strength and pH. Charges on molecules in solution and on the IEC resin are balanced by counter-ions, for example, salt and buffer ions, which are displaced when the target molecule binds to the charged functional group on the resin. The net binding charge of the protein will be the same as that of the counter-ions displaced from the resin, hence the term "ion exchange" [54]. Desorption of the adsorbed molecule is commonly executed by increasing the ionic strength of the buffer, and thereby eluting the protein by ionic competition. Alternatively, elution can be done by changing pH, which will change the net charge of the protein. For a more detailed description of the IEC technique, [55].IEC has been employed for several decades for the separation of small inorganic ions. However, it was not until hydrophilic materials of large pore size were introduced in the late 1950s that IEC of biological macromolecules became a useful separation tool [56]. IEC has since been successfully employed for protein purification and is today one of the most commonly used chromatographic separation modes for purification of pharmaceutical proteins and peptides. Most industrial purification processes comprise one or several IEC steps. The reason for its success is that it is considered a robust method and the principles are well characterized and well understood. The IEC resins typically have high binding capacities and offer good, and controllable, selectivity. Another advantage is that elution is done under mild conditions in which biomolecules maintain their native structures.

There are basically two different types of resins used for IEC. In cation exchange chromatography (CIEC), negatively charged ligands bind positively-charged molecules, whereas the opposite is true for anion exchange chromatography (AIEC). Overall, AIEC is the most frequently used type due to the fact that many recombinant proteins are acidic, and thus negatively charged at neutral pH. Furthermore, AIEC resins bind polynucleotides, DNA, and RNA (due to their negatively charged phosphate groups). AIEC is used for endotoxin and virus removal, and it is also a common method for virus and vaccine purification. IEC is very versatile for downstream processing of recombinant proteins, and can be used for capture as well as intermediate purification and polishing. In capture, the requirement for capacity andquantitative yield is predominant, whereas further downstream in intermediate purification and especially polishing, yield is often compromised by selecting conditions that deliver enhanced purity. Early in the purification process, the target molecule concentration is often low and IEC can be a very efficient concentration step, in addition to purifying the product. In most monoclonal antibody (mAb) purification platform processes, IEC contributes to at least one of two polishing steps following the Protein A affinity step [57,58]. For mAb polishing, CIEC is commonly used in bind-and-elute mode for the target molecule, which allows negatively charged impurities such as residual DNA, RNA, some host cell proteins (HCPs), leached Protein A, and endotoxin to be removed during loading or in the wash fraction. CIEC also has the power to separate antibody charge variants and aggregates during elution. Most mAbs are relatively basic molecules that enable the use of AIEC in flow-through (FT) mode for the product under conditions where viruses, DNA, leached Protein A, and acidic HCP bind. This can be done either using pure FT mode or under conditions where the target protein interacts weakly with the resin [59].

Examples

Polishing of Mabs Using CIEC [60]

This example describes a mAb polishing step using a high-capacity CIEC resin, Capto S Impact. The mAb, an IgG1 with a pI of 8.4, was produced by CHO cells and initially purified by

direct capture using Protein A chromatography. In this case, no intermediate purification step was required. The polishing by CIEC was optimized for capacity and separation of mAb monomer from aggregates by varying the pH and salt concentration under binding and elution conditions. Process optimization resulted in a QB10% of 109 mg mAb/mL of resin at pH 5.0 and 50 mM NaCl in the loading buffer. Elution was performed by applying a linear salt gradient from 50 to 400 mM NaCl for 20 column volumes (CV's). At a sample load of 76 g mAb/L resin, corresponding to 70% of QB10, the aggregate concentration was reduced from 2%–3% to 0.9%, the HCP concentration from >300 ppm to 170 ppm, and the concentration of leached Protein A from 3.6 to less than 1 ppm (i.e., below the limit of quantification), with a monomer yield of 93%. The chromatogram in Fig. 3 shows the good separation of mAb fragments, monomer, and aggregates. The fragments do not bind as strongly to the resin and consequently elute earlier in the gradient than the monomer. The aggregates, on the other hand, which have a higher net binding charge compared with the monomer, tend to interact more strongly with the resin and elute after the monomer in the gradient.

Sample: MAb in 50 mM sodium acetate, 50 mM NaCl, pH 5.0

Medium: Capto S ImpAct (B/E mode)

Column: Tricorn 5/100

Load: 76 mg MAb/mL medium (70% of QB10)

Residence time: 5.4 min

Binding buffer: 50 mM sodium acetate, 50 mM Nacl, pH 5.0

Wash: 5 CV of binding buffer

Elution buffer: 50 mM sodium acetate, 50 to 400 mM NaCl in 20 CV

System: ÄKTA system

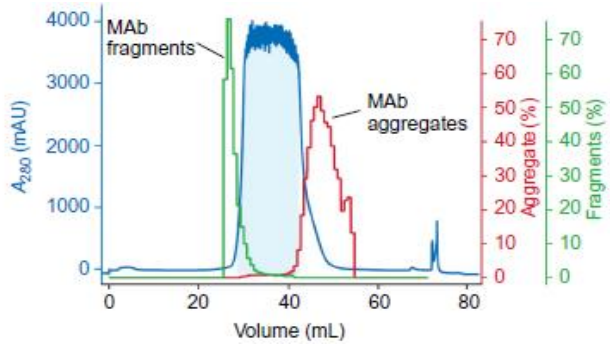

Fig. 3 Separation of mAb fragments and aggregates with Capto S Impact at high sample load. The green histogram shows the amount of fragments and the red histogram shows the amount of aggregates as the percentage of total protein amount in corresponding fraction. The light blue area under the UV trace corresponds to the pooled product fractions. Courtesy: GE Healthcare Bio-Sciences AB.

Purification of Type 5 Adenovirus Using AIEC [61]

Adenovirus was produced by HEK 293 cells in suspension. After cell harvest and lysis, the clarified supernatant containing virus was treated with Benzonase (Novagen, Madison, WI), and the pH was adjusted to 8.0, prior to capture on a high-capacity AIEC resin—Q Sepharose XL. The loading buffer for the capture step was 50 mM Tris, 2 mM MgCl2, 5% sucrose pH 8.0. After loading, a wash was performed with 300 mM NaCl in binding buffer, followed by elution using a linear gradient from 300 to 750 mM NaCl in three CVs. The collected viral peak from the Q Sepharose XL column (Fig. 4) was diluted to a conductivity of approximately 25 mS/cm and then further purified on a SOURCE 15Q column. In this second AIEC step, the column was washed with 300 mM NaCl in loading buffer followed by elution with a gradient from 300 to 600 mM NaCl for 3 CVs (Fig. 18.3B). The amount of free doublestranded DNA (dsDNA) was reduced from 13.0% to 1.6% over the SOURCE 15Q step. Finally, the virus was buffer exchanged using a size exclusion chromatography (SEC) column. Process evolution from gradient to step elution for the AIEC steps was also carried out. The step elution variant was scaled up to pilot production, and later used for second adenovirus project.

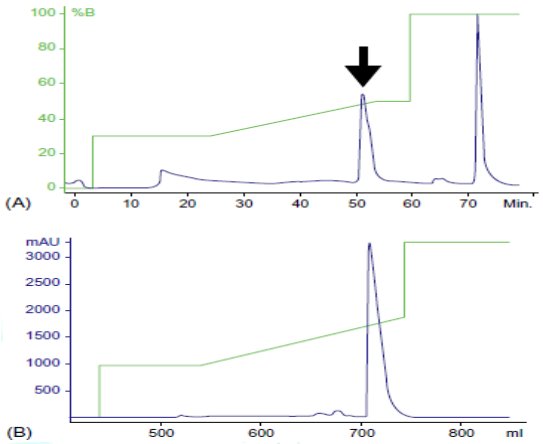

Fig. 4 After pH adjustment with 1 M TRIS, the virus in the lysis buffer was loaded directly onto the column at 113 cm/h. Immediately following the load, the column was washed with 30% buffer B for at least one column volume or until a stable baseline was achieved. The virus (arrow) was eluted from the column with the use of a linear gradient to 75% buffer B in three column volumes. (B) The diluted viral peak from the Q Sepharose XL column was then loaded onto a Source15Q column at a flow rate of 152 cm/h. Immediately following the load, the column was washed with 30% buffer B until a stable baseline was achieved. The virus was eluted from the column with the use of a linear gradient from 30% to 60% buffer B in three column volumes. Reproduced from Jendrek et al. Development of a production and purification method for type 5 adenovirus. BioProcess J. 5 (1) (2006) 37–42 with permission.

2.2.6 Supercritical Fluid Chromatography

In general, modern SFC separations are mainly performed on packed columns rather than on capillary columns. Consequently, none of the publications reviewed inhere reported the use of capillary columns for enantioseparation. As also known from achiral SFC, the main component of a chiral SFC method is the column. This parameter, obviously, determines if a method has the potential to succeed or not. Unless a chiral stationary phase (CSP) is used, it is dead capital. The broad enantiorecognition ability of polysaccharide based CSPs are reported and methodical screening strategies were developed to give the analyst evidence or at least empirical based guidance **[62,63].** Most of the chiral CSPs used in SFC are well known from chiral high performance liquid chromatography (HPLC). Noteworthy, chiral columns originally designed and marketed for chiral HPLC may easily be used in SFC as well **[64,65].** Application of columns whose utilization is recommended with high proportion of water as MP ensuring three-dimensional formation by hydration may be questionable for use in SFC. This might be the case for some

columns equipped with protein based chiral selectors (e.g. human serum albumin, α1-acid glycoprotein). Also, a lack of data and missing longtime experiences might keep column producers from recom-mending use in SFC officially. Thus, polysaccharide, cyclodextrin, and (glyco) protein based, as well as Pirkle-type CSPs are used in chiral SFC. General structure elements of these CSPs are exemplified in Fig 3. Although several types of CSPs and many brands are available on the market the dominating CSPs in chiral SFC are polysaccharide based. Former publications already pronounced the prominentrole of amylose and cellulose based CSPs **[66,67–71].**

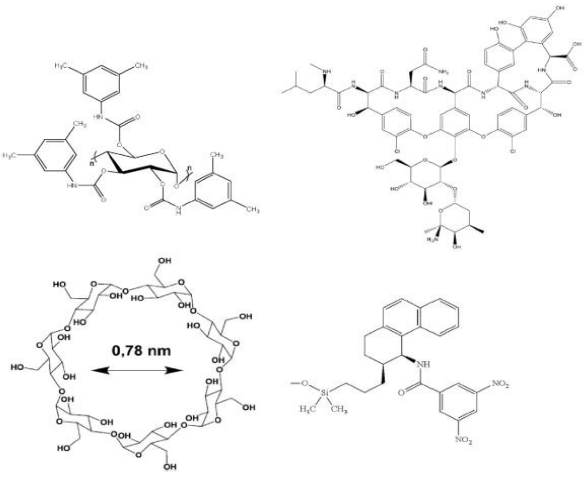

Fig. 5. Structure of chiral stationary phases (CSPs) as examples of polysaccharide, cyclodextrin, (glyco-)protein, and Pirkle-type based CSPs, displayed are amylose tris-(3,5-dimethylphenylcarbamate), i.e. polysaccharide based (upper left), vancomycin (protein, upper right), β_-cyclodextrin (lower left), and Whelk-O1 (Pirkle-type, lower right).

2.3 Electrophoresis

The capillary electrophoresis separation current is decoupled from the electrospray current through the sheath liquid reservoir [72,73,74,75], as seen in Fig. 6A. The design minimizes dilution associated with a sheath liquid by using a taper glass nanospray emitter.An on-line enzyme reactor successfully integrated with capillary electrophoresis and mass spectrometry utilized a solid support for enzyme [72,73] or protein [75] entrapment. The device is based on a monolithic microreactor positioned in-capillary that is made of acrylamide [72,73] or sulfonated-silica [75]. The polyacrylamide-based microreactor proved to be a compatible platform for enzyme immobilization while maintaining enzyme activity [72,73]. These microreactors were employed for two different enzymes. Immobilized alkaline phosphatase facilitated the determination of protein phosphorylation [73]. A trypsin monolith was developed for on-line protein digestion in a cell lysate. The integrated system was compatible with a 300 pg sample size. The in-line monolith microreactor decreased incubation time to minutes [72] as compare to off-line protein digestion, which required 24 h when performed in free solution [75]. This concept was expanded by using the in-capillary monolith to trap the targeted proteins rather than the trypsin [75]. The approach minimized the deactivation of the trypsin activity through immobilization to the capillary surface while preserving the benefits of on-line digestion. Additionally, the amount of digested proteins was not limited by the length of the immobilized enzymes zone in the capillary. This monolithic material contained sulfonate groups to facilitate electrostatic trapping and exhibited high surface area. Under acidic conditions, proteins were easily extracted from the crude sample and subject to multiple processing steps. The microreactor was used to reduce, alkylate, and digest entrapped proteins. Immobilizing the protein enables the use of larger concentrations of trypsin. Digestion of proteins derived from a cell lysate was completed in 10 min. The excellent efficiency of the capillary electrophoresis was well-suited to separate the complex peptide digest as shown in Fig. 6A. The median separation efficiency of the peaks in the electropherogram was 240,000theoretical plates. Using a 100 cm long capillary and a Q executive HF mass spectrometer, 975 proteins were identified from Xenopus laevis zygote homogenate.

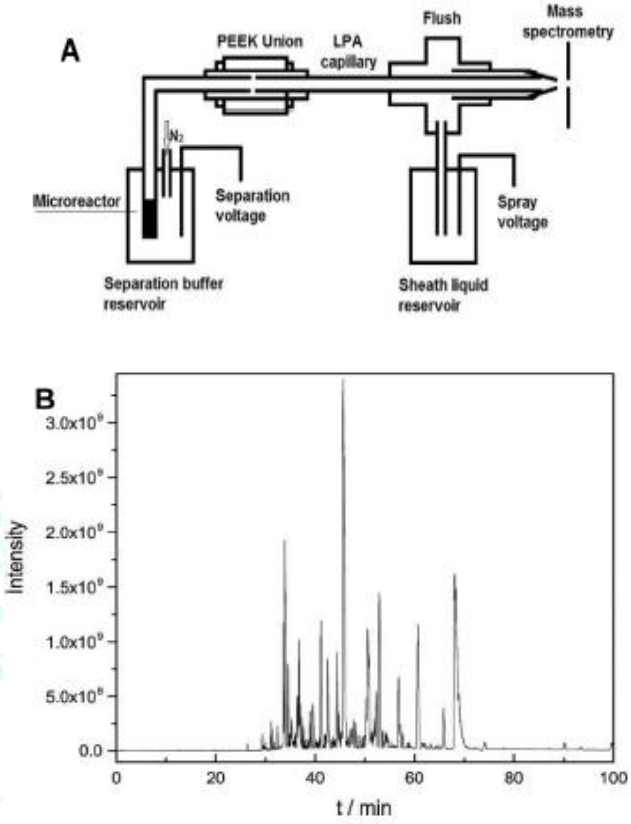

Fig. 6 Schematic of capillary zone electrophoresis coupled with mass spectrometry showing on-line protein digests. The schematic in A is of strong cation exchange based monolith based microreactor coupled with capillary zone electrophoresis mass spectrometry. The mass spectrum in B is of an on-line analysis of Xenopus laevis protein digests achieved by the set up used in A. Reprinted with permission from Z. Zhang, L. Sun, G. Zhu, O.F. Cox, P.W. Huber, N.J. Dovichi, Nearly 1000 Protein Identifications from 50 ng of Xenopus laevis Zygote Homogenate Using Online Sample Preparation on a Strong Cation Exchange Monolith Based Microreactor Coupled with Capillary Zone Electrophoresis, Analytical Chemistry 88(1) (2016)877–882. Copyright 2016 American Chemical Society.

2.4 Spectroscopic Techniques

2.4.1 UV-VIS Spectrophotometry

Another important group of methods which find an important place in pharmacopoeias are spectrophotometric methods based on natural UV absorption and chemical reactions [76]. Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. The advantages of these methods are low time and labor consumption. The precision of these methods is also excellent. The use of UV–

Vis spectrophotometry especially applied in the analysis of pharmaceutical dosage form has increased rapidly over the last few years [77-80]. The colorimetric methods are usually based on the following aspects: _

- Complex-formation reaction.
- Oxidation-reduction process.
- A catalytic effect.

It is important to mention that colorimetric methods are regularly used for the assay of bulk materials. For example, the blue tetrazolium assay is used for the determination of corticosteroid drug formulations [81,82]. The colorimetric method is also exploited for the determination of cardiac glycosides and is presented in European Pharmacopoeia. Several approaches using spectrophotometry for determination of active pharmaceutical ingredients in bulk drug and formulations have been reported and details of these methods are recorded in **Table 3.**

Table 3 Quantitative analysis of drugs in pharmaceutical formulations by UV–visible spectrophotometric procedures.

Reagent used	**Name of drug**	**ʎmax**	**Reference**
m-Cresol	Acetaminophen	640	Qureshi et al. (1992)
p-Chloranilic acid	Quetiapine fumarate Milrinone	520 519	Vinay and Revenasiddappa (2012)
2,3-Dichloro 5,6-dicyano1,4-benzoquinone	Duloxetine Amlodipine besylate	477 580	Toker and onal (2012) Rahman and Hoda (2003)
Chloranil	Dutasteride Lisinopril	525 520	Kumar et al. (2012) Rahman et al. (2007)
7, 7, 7, 8- Tetracyanoquinodimethane	Lisinopril Alendronate sodium	743 840	Rahman et al. (2005b) Raza and Haq (2011)
Iodine	Flunarizine dihydrochloride	380	El Walily et al. (1995) Helmy et al. (2012)
Potasium iodide and potassium iodate	Irbesartan	352	Rahman et al. (2005b)
Ninhydrine	Pregabalin	402.6	Bali and Gaur (2011)
Ascorbic acid	Lisinopril	530	Rahman et al. (2005c)
Folin ciocalteu phenol	Oxcarbazepine Ampicillin, amoxycillin, and carbenicillin	760 750, 770, 750	Gandhimathi and Ravi (2008) Ahmad et al. (2004)
Tris buffer	Diclofenac sodium	284, 305	Kramancheva et al. (1997)
Sodium metavanadate	Diltiazem HCl	750	Rahman and Azmi (2000)
Bromothymol blue	Rasagiline mesylate	414	Chennaiah et al. (2011)
Bromophenol blue	Rasagiline mesylate	414	Chennaiah et al. (2011)
Bromocresol green	Rasagiline mesylate	414	Chennaiah et al. (2011)
Potassium permanganate in alkaline medium	Isatin	60	ALOthman et al. (2013)
Brucine-sulfanilic acid in H2SO4 medium	Nicorandil	410	Rahman et al. (2004)
3-Methyl-2-benzothiazoline	Nicorandil	560	Rahman et al. (2004)
Cu (II) & eosin	Carbinoxamine	538	Ramadan and Mandil (2006)
Potassium ferricyanide and ammonium ferric sulfate	Pantoprazole sodium	725	Rahman et al. (2006b)
Chloramin T	Zidovudine Verapamil HCl	520 425	Basavaiah and Anil Kumar, 2007 Rahman and Hoda (2002)

2.4.2 Near Infrared Spectroscopy

Near infrared spectroscopy (NIRS) is a rapid and non-destructive procedure that provides multi component analysis of almost any matrix. In recent years, NIR spectroscopy has gained a wide appreciation within the pharmaceutical industry for raw material testing, product quality control and process monitoring. The growing pharmaceutical interest in NIR spectroscopy is probably a direct consequence of its major advantages over other analytical techniques, namely, an easy sample preparation without any pretreatments, the probability of separating the sample measurement position by use of fiber optic probes, and the expectation of chemical and physical sample parameters from one single spectrum. The major pharmacopoeias have generally adopted NIR techniques. The European Pharmacopoeia in chapter 2.2.40 [83] and United States pharmacopoeias (chapter 1119) [84] address the suitability of NIR instrumentation for application in pharmaceutical testing.

2.4.3 Nuclear Magnetic Resonance Spectroscopy

Since the first report appeared in 1996 [85] describing the use of NMR spectroscopy to screen for the drug molecules, the field of NMR based screening has proceeded promptly. Over the last few years, a variety of state-of-the art approaches have been presented and found a widespread application in both pharmaceutical and academic research. Recently NMR finds its application in quantitative analysis in order to determine the impurity of the drug [86], characterization of the composition of the drug products and in quantitation of drugs in pharmaceutical formulations and biological fluids [87,88], Many reviews on the application of NMR in pharmaceuticals have been published [89, 90].

2.4.4 Ion Mobility-Mass Spectrometery

Ion mobility-mass spectrometry (IM-MS) has gained the attention of numerous researchers because of its unique ability to separate ions based on size, shape, or charge in

addition to being coupled to a high mass accuracy time-of-flight mass analyzer. There are several different types of ion mobility that have been utilized for glycopeptide analysis. Travelling wave ion mobility-mass spectrometry (TWIMS-MS) [91], the most popular commercially available IM-MS instrument, has been used to thoroughly characterize an IgG1 mAb's glycosylation heterogeneity profile with glycopeptides of each glycoform being readily identified [92]. Li et al. found that TWIMS could separate peptides from glycopeptides into distinct trend lines that could be used for predicting glycosylation status of other peptides; they also noted that ion mobility reduced chemical noise to allow for the detection of lower abundant ions [93]. TWIMS-MS has also been used to distinguish epimeric glycopeptides derived from Muc 2 [94 Fig. 7A]. Two isomeric glycopeptides, same peptide backbone but differing only in the attachment of either α-GlcNAc or α-Gal-NAc, were partially separated with TWIMS-MS; interestingly, multiple overlapping conformers were identified for each glycopeptide. The authors confirmed the identity of each glycopeptide by using CID-IMS-MS [Fig. 7B]; the diagnostic oxonium ions were separated with TWIMS-MS, highlighting the utility and importance of characterizing product ions. High-field asymmetric wave ion mobility spectrometry (FAIMS) has also been used to rapidly separate coeluting isomeric O-linked glycopeptides, differing only in glycosylation site [Fig. 7C]. The coeluting peptides were separated by altering the correction voltage applied. Confirmation of the glycosylation sites was confirmed with electron transfer dissociation (ETD) [Fig. 7D and E]; the two glycosylation sites are readily identified by their diagnostic product ions [95]. The combination of FAIMS to separate isobaric peptides and ETD to confirm the glycosylati site has proven to be quite useful in analyzing complex glycopeptides mixtures.

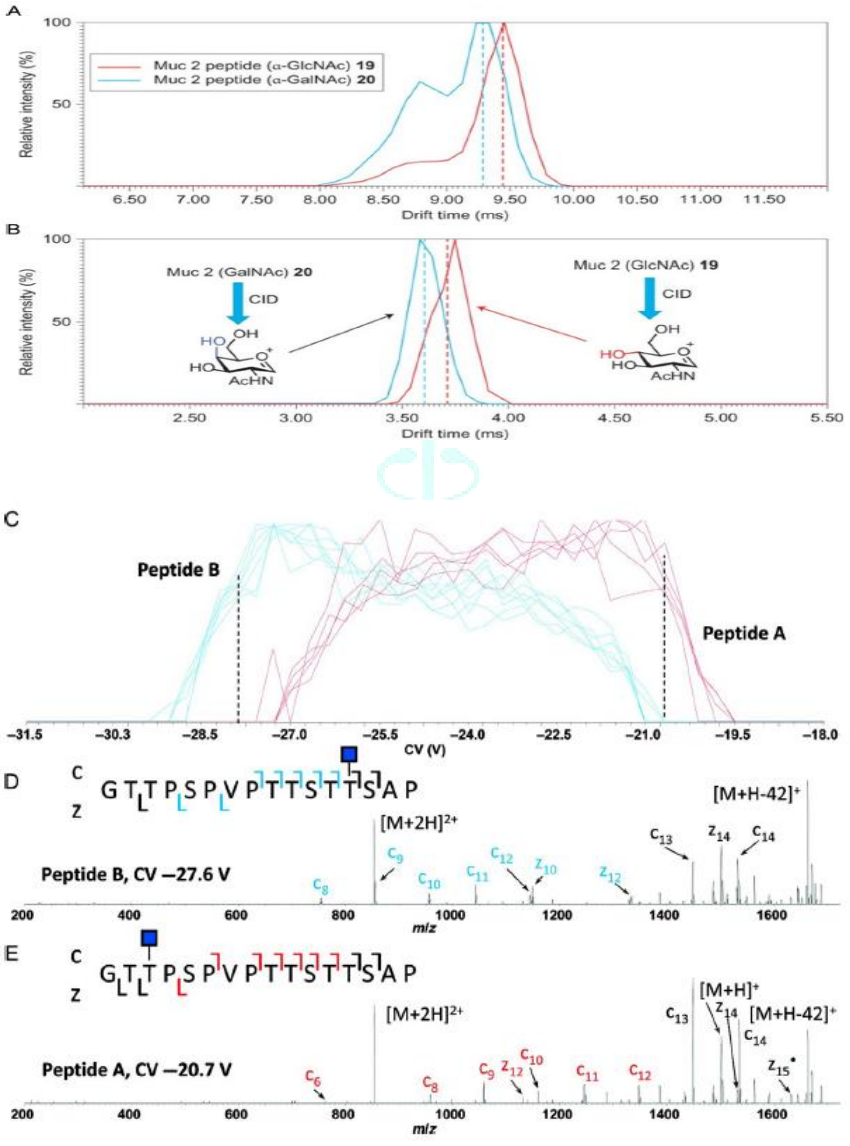

Fig. 7 Separation and analysis of glycopeptides by ion mobility-mass spectrometry.

[A]travelling wave ion mobility mass spectrometry (TWIMS) identified multiple conformers of the isobaric Muc2 glycopeptides (PTTTPITTTTTVTPTPTPTGTQT with GalNAc 19 and GlcNac 20). TWIMS was not able to differentiate between the two intact glycopeptides**; (B)** however, after CID; TWIMS-MS could distinguish the diagnostic oxonium ions from each Mucin glycopeptide**. (C)** High-field asymmetric wave ion mobility mass spectrometry (FAIMS-MS) separation of two isobaric O-linked glycopeptides, differing only in glycan site attachment. **(D, E)** Glycopeptide identity confirmed with ETD MS2; diagnostic c and z ions are indicated in blue and red.

2.5 Electrochemical Methods

The application of electrochemical techniques in the analysis of drugs and pharmaceuticals has increased greatly over the last few years. The renewed interest in electrochemical techniques can be attributed in part to more sophisticated instrumentation and to increase the understanding of the technique themselves. Here the application of various electrochemical modes in the analysis of drugs and pharmaceuticals is presented in Table 4. Moreover, a large number of electroanalytical methods are available for quantification of pharmaceuticals. An amberlite XAD-2 and titanium dioxide nanoparticles modified glassy carbon paste was developed for the determination of imipramine, trimipramine and desipramine. The electrochemical behaviour of these drugs was investigated using cyclic voltammetry, chronocoulometry, electrochemical impedence spectroscopy and adsorptive stripping differential pulse voltammetry [96]. The capsaicin modified carbon nanotube modified basal-plane pyrolitic graphite electrode or p-chloranil modified carbon paste electrodes have been developed for the determination of benzocaine and lidocaine. The electrochemically initiated formation of capsaicin-benzocaine adduct causes a linear decrease in the voltammetric signal corresponding to capsaicin which correlates to the added concentration of benzocaine [97]. A copper (II) complex and silver nanoparticles modified glassy carbon paste electrode was constructed and used for the determination of dopamine, levodopa, epinephrine and norepinephrine. The electrochemical behavior of these drugs was studied using cyclic voltammetry, electrochemical impedance spectroscopy, chronocoulometry and adsorptive stripping square-wave voltammetry techniques[98]. Adsorptive stripping differential pulse voltammetric method has been developed for the determination of venlafaxine and desvenlafaxine using Nafion-carbon nanotube composite glassy carbon electrode [99]. An electrochemical method based on potentiometric stripping analysis employing cryptand and carbon nanotube modified paste electrode has been proposed for the subnanomolar determination of bismuth [100].

Table 4: Determination of drug by various electrochemical techniques.

Techniques	Drugs determined	Remark	Reference
Voltammetry	B-blockers drugs	Nafion-coated glassy carbon electrode	Nigovic et al.(2011)
	Rosiglitazone	Square wave adsorptive stripping voltammetry	Al-Ghamdi and Hefnawy(2012)
	Leucovorin	Silver solid amalgam electrode	Selesovska et al.(2012)
	Secnidazole	Cathodic adsorptive stripping voltammetry	El-Sayed et al. (2010)
	Acetaminophen and tramadol	At glassy carbon paste electrode	Sanghavi and Srivastava(2011a)
	Dopamine	Differential pulse stripping voltammetry	Abdoljavadi and Masrournia (2011)
	Atenolol	Using nanoglod modified indium tin oxide electrode	Goyal et al.(2005)
Polrography	Nifedipine		Jeyaseelan et al. (2011)
	Anti-cancer drug, vitamin k3		TasA et al. (2011)
	Ciclopirox olamine		Ibrahim and El-Enany(2003)
Amperometry	Diclofenac		Gimenes et al. (2011)
	Verapamil		Ortuno et al.(2005)
Potentiometry	N-acetyl-L-Cysteine		Prkic et al.(2011)
	Pentoxifylline		Alarfaj and EL-Tohamy (2011)

2.6 Microscopic Techniques

2.6.1 Scanning Photoelectron Microscopy

Scanning photoelectron microscopy (SPEM) is a microspectroscopic and spectromicroscopic technique measuring XPS at the µm scale and providing spectromicroscopic imaging data based on the XPS spectroscopic features(e.g. S2_t2_ around 162.3eV [101,102]). It is one of the most useful techniques for investigation of surface electronic characteristics e.g. chemical bonding states and chemical potential shifts [103], and imaging with high spatial resolution [104]. SPEM provides high quality information from the top few nm of the sample surface with both high spatial and energy resolutions [104]. This is in contrast to scanning electron microscopy (SEM) and transmission electron microscopy (TEM) which provide very high spatial resolution, due to the shorter wavelengths of electron beams as compared to those of the X-rays used for SPEM and PEEM, but have poor spectroscopic capabilities in terms of energy resolution and/or surface sensitivity due to limited energy-filtered electron and photonemission. The primary strength of SPEM is its separate imaging and micro spectroscopy capabilities, which allow independent optimisation of energy resolution, lateral resolution and acquisition time for each mode [105]. Moreover, additional analysers for detecting e.g. transmitted, reflected or fluorescence signals can be attached to SPEM, further extending its analytical capabilities [106]. Fig. 5 shows a schematic setup of the ESCA microscopy beamline at the Elettra synchrotron facility in Trieste, Italy. The role of the Fresnel zone plate optics (FZP) is to demagnify a beam spot to a diameter of 150 nm (or less). Because FZPs are a diffractive optic, an order sorting aperture (OSA) is put between the sample and FZP to cut off undesired diffraction orders [107]. Emitted photoelectrons are collected by a SPECS-PHOIBOS 100 hemispherical analyser and detected bya48-channel electron detector where each channel measures electrons with a specific (narrow) kinetic energy range defined by the selected energy window [104,108].

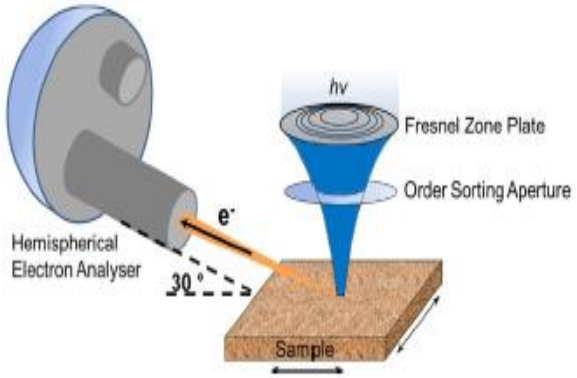

Fig. 8. SPEM setup at Elettra, Trieste, Italy

2.6.2 Photoemission Electron Microscopy

Photoemission electron microscopy(PEEM),generally offering similar capabilities to SPEM, is a non-destructive, synchrotron-/laboratory-based spectromicroscopy technique and is also a unique surface sensitive instrument providing images with high spatial resolution and full field [109,110]. PEEM is easily moved and installed to fit different beam lines or even normal laboratory environments using laser or Hg lamp UV light sources. It is actually the only soft X-ray imaging technique that has been used more frequently in normal laboratories than at synchrotron beamlines [111]. The presence of the energy filter reduces the chromatic aberrations in the electron optics and enables the selection of optimum lens setting as well as the position and size of contrast and field aperture for high signal intensity, thereby improving the lateral resolution [112]. PEEM is regarded as the most promising approach to photoemission-based imaging with high lateral resolution [113]. Further advancement in resolution will be highly dependent upon improvements in instrumental electrical and mechanical stability, as well as the development of better detectors [114], to achieve a resolution below 1 nm [115].

2.7 Electrophoretic Methods

Another important instrument essential for the analysis of pharmaceuticals is capillary electrophoresis (CE). CE is a relatively new analytical technique based on the separation of charged analytes through a small capillary under the impact of an electric field. In this technique solutes are perceived as peak as they pass through the detector and the area of individual peak is proportional to their concentration, which allows quantitative estimations. In addition to pharmaceutical studies it finds an application in the analysis of biopolymer analysis and inorganic ions. CE analysis is generally more effective, can be performed on a quicker time scale, requires only a small amount, lesser up to Nano liter injection volumes and in most cases, takes place under aqueous conditions. These four characteristics of CE have proven to be beneficial to many pharmaceutical applications. Several reports have appeared on the application of this technique in the routine drug analysis [116-118]. Capillary electrophoresis (CE) has long been employed for the rapid and reproducible separation and analysis of fluorescently labeled glycans and intact glycoproteins. CE has been employed for the rapid separation of glycopeptides before analysis by mass spectrometry. [119] developed a method utilizing CE coupled to ESI-orthogonal accelerating time-of-flight mass spectrometry (CE-ESI-oaTOF-MS) to rapidly analyze N- and O-glycopeptides derived from erythropoietin (EPO), a commonly used glycoprotein hormone abused in athletic competitions. Their method also discovered a novel sulfated sialoform glycopeptide. Lew et al. developed a rapid method for therapeutic antibody quality control utilizing CE-MS to analyze the tryptic digest of Trastuzumab; the relative abundance of 14-glycoforms on one glycopeptide were readily attained [120 Fig. 9]. Barroso et al. coupled CE-TOF-MS and μLC-TOF-MS for a clinical research application; their method was able to detect the relative glycoform abundance of human Transferrin Tf glycopeptide to predict the alcohol dependence of individuals utilizing a small serum sample [121].

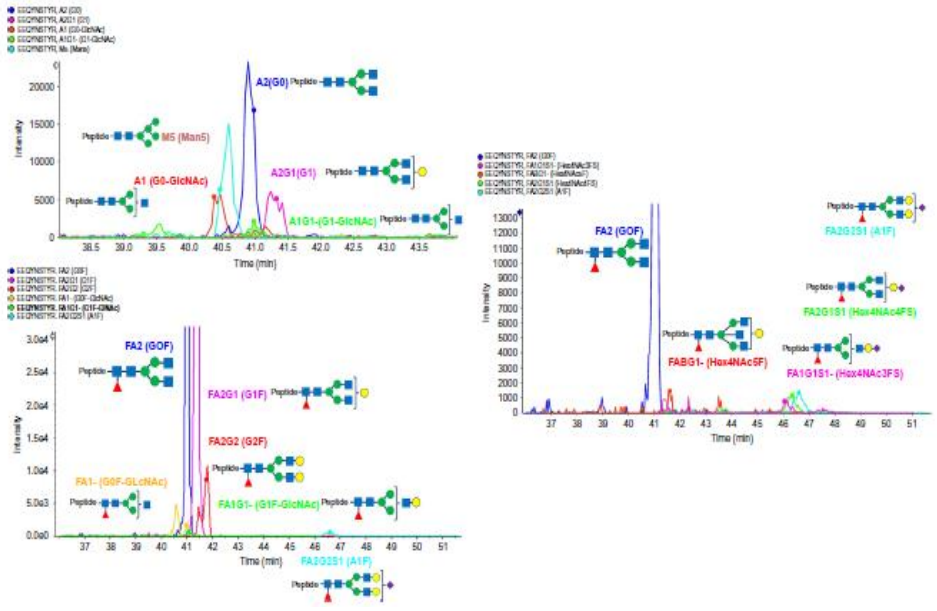

Fig. 9 Capillary electrophoresis mass spectrometry (CE-MS) of therapeutic antibody. CE-MS quantified 14-glycoforms on one glycopeptides from Trastuzumab. Reproduced with permission from Lew, C., Gallegos-Perez, J. L., Fonslow, B., Lies, M., & Guttman, A. (2015). Rapid level-3 characterization of therapeutic antibodies by capillary electrophoresis electrospray ionization mass spectrometry. Journal of Chromatographic Science, 53(3), 443–449. doi:10.1093/chromsci/bmu229.

2.8 Sample Handling

2.8.1 Biological Specimens

Once a drug is introduced into a biological system, it is subjected to the processes of absorption, distribution, metabolism, and excretion, which are subjects of pharmacokinetics. Some basic principles governing the interactions between drugs and the human biological system, including drug introduction, absorption, distribution, and excretion will be addressed. Such knowledge facilitates the proper selection of specimens for analysis, sample pretreatment, and analytical data interpretation. Various biological specimens, including plasma, serum, blood, urine, saliva, milk, sweat, breath, hair, nails, skin, tissues, feces and stomach contents, are available for drug analysis (**Table 5**). Blood and urine are the specimens most commonly used in clinical and forensic analyses. Drugs and their metabolites can generally be detected for a few hours to a few days in blood and urine (**Fig. 10**). Alternative specimens such as hair sweat and oral fluid (saliva) can provide unique and sometimes additional information. The advantages of these specimens include ease of sample collection and sample stability. In sampling from these specimens, factors that must be considered include collection method, collection time, and the characteristics of the individual (e.g., age, sex, circadian rhythm, before or after a meal or exercise, and season of the year). Furthermore, in storing biological specimens, it is necessary to consider the effects of temperature, pH, ionic strength, light, oxygen, humidity and enzymes. Factors and measures used to avoid contamination and alterations during sampling, storage and sample preparation are shown in Table 5. This section describes the characteristics, collection and handling of these specimens.

Serum, Plasma and whole Blood

Blood is perhaps the most useful type of biological sample for the identification and quantification of drugs and for the interpretation of toxicologically significant data. Blood is a complex fluid containing solubilized proteins, dissolved fats and salts, and suspended cells. The major constituents, the red blood cells (erythrocytes), can be separated from the clear fluid (plasma)

Table 5: Biological specimens available for drug analysis

1.Liquids and gases	Blood	Wholeblood,plasma,serum
	Urine	
	Secretion and effusion	Saliva,tears,sweat,milk,cerebrospinal fluid, gastric juice, bile, semen, liquor amnii
	Breath	
2.Solids and semisolids	Feces	
	Keratinaceous tissues	Hair, skin, nails.
	Organs and other tissues	Brain,liver,lung,kidney,muscle,fat,bone,stomach contents

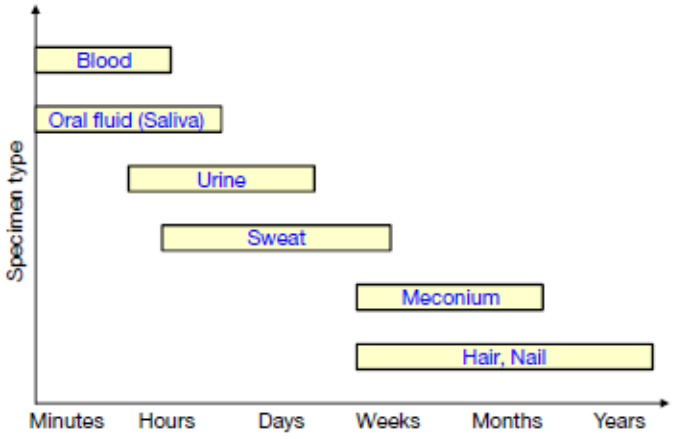

Fig. 10 Approximate periods for detecting compounds in biologic specimens.

Table-6 Factors and measures for contaminations and alterations during sampling, storage and sample preparation in pharmaceutical and biomedical analysis by centrifugation.

Contamination and adsorption	Factors	Measures
	1.Contamination	
	Experimental apparatus	Washing of apparatus
	Reagents(impurities, additives, decomposition compounds)	Conservation in alright container Environmental clarification
	Enviroment(volatile materials, dust, microbial products, experimenter)	
	2.Adsorption to experimental apparatus	Choice of appropriate material
		Correction by addition of analogue
	3.Drying and moisture absorption	Operation in a closed system
Chemical alteration	1.oxygen	Addition of antioxidants, chelating agents
	2.Temperature	Storage in liquid nitrogen, freezer or refrigerator
	3.Moisture	Dry disposal of sample, addition to desiccant to container
	4.Light	Shading, use of brown vials
Biological alteration	1.Vital reactions, metabolism	Addition of metabolic inhibitor, storage at low temperature
	2.Enzymatic reactions	Addition of enzyme inhibitor, storage at low temperature
	3.Growth of microbes	Addition of fungicides, storage at low temperature

If blood is allowed to stand without the addition of anticoagulating agents, the red cells will eventually clot and the resulting fluid, serum, can be decanted. In plastic tubes, clotting may be delayed for more than 1 hour and some hemolysis can take place. Plasma is obtained from blood collected with heparin or another anticlotting agent. Because heparin is a mucopolysaccharide and may interact with some analytes, ethylenediamine tetraacetic acid (EDTA) or other anticoagulant may be preferable.EDTA should not be used when analyzing metallic and organometallic compounds. As matrices for drug analysis, the significant difference between plasma and serum is that serum does not contain fibrinogen and several clotting factors (2.5%–5% of proteins).In general, plasma or serum is routinely used for drug analysis. A method developed for plasma can normally be applied without modification to serum. In contrast, whole blood may be the sample of choice when trying to detect the presence of a drug, or when quantitative information is needed during assays of deteriorated blood samples, where complete separation of plasma or serum from red cells is not possible. Dried blood spots are frequently analyzed. That is, small drops of whole blood are collected and airdried on filter paper prior to analysis. This technique can result in the cost-effective shipment and storage of samples at room temperature, with only a small volume of solvent (5 mL) needed to extract the analytes. A frequent drawback in blood collection is sample hemolysis, diluting analytes and interfering with serum assays. Because serum and plasma contain high concentrations of proteins, drugs may bind to these proteins to varying degrees, depending on the individual physicochemical properties of these drugs. In general, acidic and neutral drugs bind primarily to albumin, whereas basic drugs bind primarily to acid glycoprotein. Only free drug is available for extravascular distribution and elimination, as well as being able to cross cellular membranes and interact with drug receptors. Direct analysis of drugs in serum or plasma from which protein has been removed may therefore not reflect total drug concentration. Therefore, protein is often denatured prior to extraction with an organic solvent. Alternatively, an appropriate adjustment of pH may dissociate drugs strongly bound to proteins.[122-125].

Urine

Urine is one of the most commonly used biological matrices for drug analysis, particularly because of its relative ease of collection. Usually, urine is sampled over a 24-h period into precleaned bottles. Preservatives may be required to stabilize the analyte, particularly if it is susceptible to bacterial degradation. To guarantee the stability of the urine, the time between sampling and analysis should be kept as short as possible. After through shaking, the urine is divided into several samples and the aliquots arefrozen immediately. As a matrix, urine has moderate complexity and usually contains both organic and inorganic constituents, as well as a relatively high salt content. Urine contents are highly variable, because of the dilution resulting from the body's attempts to maintain water balance. Therefore, results obtained from urine samples are typically normalized by expressing drug concentration per gram creatinine. Compared with blood, plasma, or serum, urine is relatively free of protein, making possible the direct extraction of an analyte with an organic solvent. However, the interpretation of results obtained from urine samples is complicated by several factors, such as the amount of urine excreted, variations in pH and ionic strength, and the time lapse after intake of the drug. Variations in urine pH and ionic strength can affect the elimination of many drugs, as well as their extraction efficiency when effects on the drug's acid/base balance are significant. The other components of the sample may compete with the analyte for adsorption to the extractant, further reducing the amount extracted. These problems may be controlled for by using an appropriate internal standard, or by adding a standard compound to normalize quantification. The internal standard should be structurally related to the other compounds. If urine samples must be significantly diluted prior to extraction, because of very high drug concentrations, matrix variability is less significant.[122-125]

Saliva

Saliva is a colorless fluid excreted into the oral cavity from three principle glands. The parotid gland, exiting at the top of the mouth, secretes saliva derived mainly from blood plasma (serous fluid). The sublingual glands, exiting at the

sides of the mouth, excrete both serous fluid and mucin. The submandibular glands, exiting at the base of the tongue, also excrete both serous fluid and mucin. Saliva is approximately 99% water, 0.3% protein (mostly enzymes) and 0.3% mucin with the balance being salts. Mucin is responsible for the stickiness of saliva. The low protein concentration in saliva minimizes drug binding compared with plasma. Therefore, saliva can be used to estimate the actual, protein-unbound, circulating concentration of some drugs and their metabolites at the time of collection. The pH of unstimulated saliva ranges from 5.6–7.0, increasing with stimulation to a maximum of 8.0. Therefore, drug concentrations in saliva partially depend on the pH of the saliva and the degree of stimulation. Saliva must be collected under certain stimulation conditions, because saliva flow and composition vary over a 24-h period. Compared with urine collection, saliva collection is less invasive and causes fewer concerns about violation of privacy and adulteration of samples. Compared with blood, saliva samples can be directly analyzed without a prior extraction step. These advantages have promoted numerous investigations and several review articles on the suitability of saliva in TDM and in forensic applications. Saliva volume may be increased by stimulating saliva flow, for example by chewing gum; sucking on candy; placing citric acid on the tongue; adsorption on cotton rolls or administration of pilocarpine. Material for stimulation of saliva must be carefully selected because lipophilic drugs may be adsorbed onto the material. For identification purposes, adsorption could be employed to directly extract the drug from the saliva while inside the mouth. OraSure® both stimulates saliva flow and collects the saliva on an absorbent pad. Salisoft® tubes containing a polypropylene–polyethylene swab are also used for collection of saliva by chewing. Other devices have been developed to collect saliva from selected glands by placing the end of the device over the gland and applying suction. Although selected gland secretions can reduce saliva/plasma ratios and minimize oral contamination, most studies utilize mixed saliva specimens because their collection is less invasive. Incubating collected saliva at room temperature may release CO2, increasing the pH and generating sediment. In addition, growth of bacteria in saliva may alter its composition. Therefore, if not analyzed immediately after collection, saliva must be stored in a freezer.[122-125]

Hair

Hair is frequently utilized for drug analysis as its collection is relatively noninvasive, and it provides a historical record of exposure, due to the relatively long lifetime of drugs in hair (Fig. 7). However, drug concentration may vary by hair type, due to variations in drug affinity. The primary advantage of hair as a matrix for analysis is that it is fairly nonpolar, thereby tending to adsorb tends to absorb parent drug molecules, as they are usually less polar than their metabolites. This makes hair an ideal matrix for the nonpolar extraction of analytes, particularly when the parent drug is extensively metabolized and often non detectable in other tissues. Hair patterns, colors, textures, diameter and growth rates vary considerably by gender, age and race/ethnicity, as well as at different areas of the body. The average rate of hair growth in the vertex region of the scalp is 0.44 mm/day (range, 0.38–0.48 mm) for men and0.45 mm/day (range, 0.4–0.55 mm/day) for women. Although the mechanisms of drug incorporation into hair have not been sufficiently clarified, drugs are thought to be distributed into hair by two processes: direct incorporation from blood into growing hair shafts and/or adsorption from other media such as sweat and smoke or powders from the environment. The most important considerations in hair sampling are collection from an anatomical location in which hairs are relatively uniform, collection at a uniform distance from the scalp, collection of a sufficient sample for the number of tests to be performed, prevention of contamination and accurate identification of the samples. Hair is mainly collected from an area at the back of the head, an area in which hair growth rate varies less than in other areas and the hair is less subject to factors associated with age and sex. At least 200–250 mg should be collected because of the relatively large interindividual variability. When head hair is not available or too short, pubic or axillae hairs could be collected. The problem of external contamination is very important, as people may be exposed to a smoky or dusty environment. Another important concern is the change in drug concentration induced by cosmetic hair treatments. Various pretreatment procedures for release of drugs from hair have been proposed including methanol sonication and incubation with 0.1–1.0 M sodium hydroxide, 0.1 M hydrochloric acid, water, buffers, 0.1% sodium dodecyl sulfate, acetone, pronase, methanol-5 M hydrochloric acid, proteinase K, and b-glucuronidase-arylsulfatase. With the exception of methanol sonication, the resulting mixture from the pretreatment step must be further purified prior to analysis. [126-128].

2.8.2 Medicinal Plants

Plant-derived medicines such as crude drugs and traditional Chinese medicines consist of complex matrices, with their size, color and shape varying among individual plants. These formulations may also contain molds and insects, due to contamination and inadequate storage. In analyzing plant-derived medicines, it is first necessary to confirm the origin of the plants, the presence or absence of contaminants and their identity, and the state of dryness. Matrices of medicinal plants may be solid, particulate or a mixture of organic compounds in an aqueous solution, and may consist of many components, such as macromolecules and small molecules. Solid medicinal plants require only grinding before extraction, but may be freeze-dried to generate a homogeneous powder. Liquid samples are most often freeze-dried, but may also be treated as solid samples. Sonication during solvent extraction may also improve the recovery of active components from medicinal plants. Soxhlet, shaking, and stirring are the conventional techniques most commonly used for the extraction of active components from medicinal plants. Solid samples are frequently extracted with pure or aqueous methanol, ethanol, acetonitrile or acetone, with or without the addition of small amounts of acids, using simple soaking, mixing, shaking or Soxhlet extraction. [129-132]

CONCLUSION

The main aim of sample separation is to decrease the complexity of material mixture. Various techniques are used to carry out the separation of the material mixture. The review also highlights the advancement of the techniques beginning from the older titrimetric method and to advanced electrophoretic method. Sample determination is carry out by the above techniques mention such as titrimetric techniques, chromatographic techniques, and spectroscopic technique. The review highlights more about sample determination in electrochemical method, on which various compounds are been determined. Sample handling were explain by means of biological specimens on how the serum, plasma , urine , saliva and hair can be stored, analysed and able to known their concentration based on their ph ranges. Pharmaceutical drugs are to serve the human to make them free from potential illness or prevention of the disease. For the medicine to serve its

intended purpose they should be free from impurity or other interference which harm humans. The review is aimed at focusing on separation, determination and handling of samples with various analytical instruments in the pharmaceutical analysis.

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