



Review Article

Comprehensive Review on Manufacturing Process and Characterization of Biosimilar Drugs

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Abstract

According to the World Health Organization, the prevalence of deadly neoplasms and endocrine, blood, and immune disorders will observe an incredible rise in coming decades followed by increased demand of affordable treatments. Keeping this incident in mind, the development of similar drugs is increasing globally. The sanction of such drugs depends on the conformity with international regulations, which starts with physical presentation of similarities that lie in their physicochemical and functional properties in contrast to the relating product. Consecutive clinical studies are done to physically present similar pharmacological behaviour and to curtail the distrust in relation to their safety points and effectiveness. Here we are to present a physical and biological testing of biosimilar and its reference product, by making use of different procedures and approaches, to judge the physicochemical and biological peculiarities with every possible impact it can cause on its pharmacokinetics, pharmacodynamics, and immunogenicity.

Keywords: Biosimilar; pharmacodynamics; efficacy; extensively.

Introduction

Biological or biopharmaceuticals are drugs that are manufactured from living cells with the help of various biological processes and simulate certain biological products and substances like hormones (Dranitsaris *et al.*, 2011). Biosimilar drugs are simply the mimics of the original biological drugs that have been authorized, hence they are similar but not exacts (Schellekens and Casadevall, 2004). The effective substance of Biosimilars medicine is very analogous to one of the biological reference medicine and is used in same quantity and treats the same disease (Roger, 2006). Biosimilars are entity based, regulatory based and market based (Duerden, 2007).

Biosimilars are a new species of drugs that target at providing the same safety and providing effective results similar to that reference biological drug. The active protein structure of biological makes them more exposed to cause an acute and chronic immune response (Schellekens and Casadevall, 2004). The overall risk is moderate with Biosimilars, but administrative steps are required because of structural complexity, manufacturing process and risk for immunogenicity (Haselbeck, 2003). 100 biopharmaceutical companies are actively involved in research and development, manufacturing and marketing of bio similar curative products in only India. There were 14 therapeutic drug that were easily available in 50 brands in 2005; but the number has elevated to 20 therapeutic drugs in 250 brands

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in 2011. Therapeutic products include insulin, erythropoietin, chorionic gonadotropin, streptokinase, interferon and heparin. The accelerated growth of Biosimilars market offers enormous possibility for manufacturing, research and development companies (Kumar and Singh, 2014). EMEA-CHMP has published guidelines of product specificity relating to similarities in safety, effectiveness and quality.(Guideline, 2005).

Biologics is way too complex than other fields. The complex 3D structure of biologic products is resolved by the production process. However, even minor changes in these processes can cause great changes in macromolecular structure and capability of biologic drugs .Even minor variations in these processes can cause tremendous affect. For example, impurity levels or the extent of material gathering and post-translational alterations which can lead to serious safety applications such as loss of effectiveness, abominable immunogenic responses (immunogenicity) and other adverse events. The European Medicines Agency as well as the FDA has recognized that bio similar products don't act identical to the original medicine version. They act differently (Schellekens, 2009), and have also published huge collection of regulatory archives (Cai *et al.*, 2013).

Scientific advancements in recombinant genetic engineering techniques and refinements in manufacturing processes has made its path to success at a very great pace in the field where biological proteins are used in disease treatment (Cai *et al.*, 2011; Tsiftoglou *et al.*, 2013; Lee *et al.*, 2012; Nowatzke *et al.*, 2011). Biological medicines play pivotal role in treatment for harmful and fatal diseases, such as cancer, hepatitis, autoimmune disorders, neurodegenerative diseases, and orphan diseases. But the treatment cost with a biological medicine is costly as compared to a "classical" chemical or synthetic medicine (Chu and Pugatch, 2009). Biosimilars medicines are being launched in the market where the copyrights or patents of certain original biological drugs have terminated. This is a best way to release cheap alternatives in the market and bloom the competition. Like this these medicines are easily available and accessible for the patient easiness. There will be an economic benefit to health sector because of these alternatives.(Camacho *et al.*, 2014).

Manufacturing Procedure of Bio Similar

- A biosimilar is medicine that is very similar to a sanctioned original product with respect to their structural and functional features and possess a very low degree of differences as compared to the original biological drug in terms of safety, purity, and capability.
- Small molecule generics are produced through chemical synthesis using a manufacturing process that is done under controlled environment and one can generally predict the results coming (Oldfield, 2011), the production of biosimilar is complicated

and the curative effects of the biologic molecule rely greatly on each individual step of the development process (Kuhlmann and Covic, 2006; Lee *et al.*, 2012).

- Biosimilars are developed from living cells through genetic engineering and their production relies on developed cell-expression systems and well-controlled manufacturing processes (Fig. 1).
- Many of these steps require full development by the new manufacturer because the knowledge gained through the production of reference product is not publically available (Lee *et al.*, 2012).
- Production rooted from living cells results in a dissimilar mixture of parent drug, abridged chunks and structural isoforms,(Lee *et al.*, 2012) and the products have an in-rooted sensitivity to post-translational variations of the parent drug or fragments.(Lee *et al.*, 2012; Tsiftoglou *et al.*, 2013; Oldfield, 2011; Kuhlmann and Covic, 2006; Cai *et al.*, 2013; Wiecek and Mikhail, 2006).
- The initial protein sequence of a biosimilar is exact to the reference product; there can be a little modification in 3-dimensional structure as a result of changes in the manufacturing process that will occur between products,

Analysis of Biosimilars

For the registration of Biosimilars drugs, "bio similarity" needs to be authentic between the physiochemical and biological frameworks of bio similar and originator clusters. In order to test the safety results and effectiveness of Biosimilars as compared with reference many medical, preclinical and, clinical studies must be done as per regulatory guidelines developed by EMA and the FDA. The development and validation of bio analytical methods is very essential (Phinney, 2014). ICH guidelines, ICH Q5E ,and Q6B (Baber, 1994) provide well-explained guidelines on physiochemical and structural features that could be considered well fitting in the appraisal of comparability as listed below:

- Sequence of Amino acid;
- Composition of Amino acid;
- Peptide map;
- Structure of Carbohydrate;
- Sequence of Terminal amino acid;
- Sulfhydryl groups and disulphide bridges;
- Molecular weight;
- Extinction coefficient;
- Electrophoretic pattern;
- Liquid chromatographic patterns;

• Spectroscopic patterns

• Pattern of Isoform;

The advanced analytical technique such as mass spectrometers employed in the assessment of physiochemical parameters extremely sensitive to concentration and sample matrix. Isolation or purification of an active constituent from the reference formulation is carried out by extraction method; therefore, there could be possibility to a change in physiochemical or structural properties of active constituent and ultimately offers influence on comparability. Along with to physiochemical and structural elucidation, quantification of product and process related impurities should also include in the evaluation of Biosimilar (Cai *et al.*, 2013).

Manufacturing Process

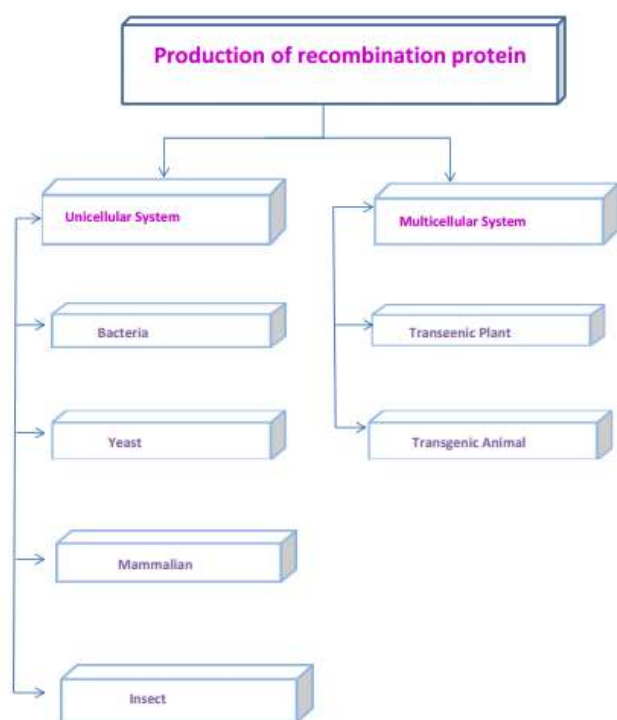


Fig. 1: Manufacturing process of biosimilar

Pharmacokinetic (PK) Assays

The consensus within the industry that produce PK assays for biosimilar sequences is that one assay, when used, should be able to measure both biosimilar and originator components in biological matrices (Cai *et al.*, 2011; Cai *et al.*, 2013). These assays are obligated to quantify the biosimilar and reference products such that they have similar precision and accuracy in order to qualify for non-clinical and clinical studies. In order to fulfil these requirements, we should utilize a single analytical standard to develop a single PK assay that measures the biosimilar and reference products in serum matrix. (Colbert *et al.*, 2014).

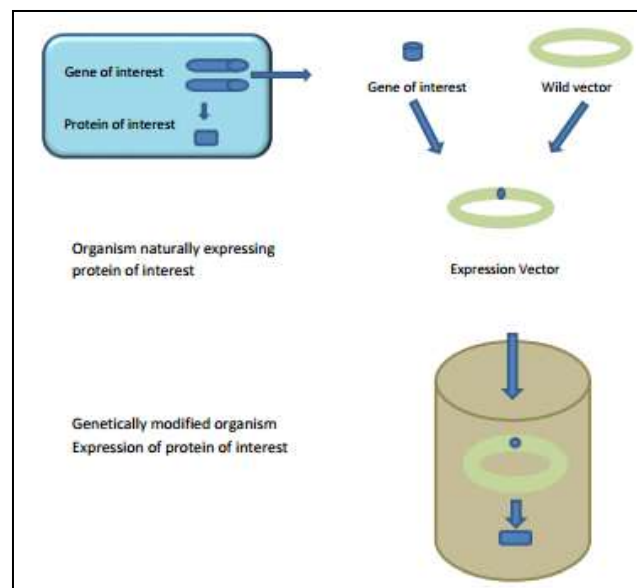


Fig. 2: Insert gene of interest into vector

Immunogenicity Assay

Most programs that deal with the production of therapeutic protein utilize this assay that consists of three step assays; screening, confirmatory and bioassay that decide neutralizing antibodies (Swanson, 2007). Screening assay is highly sensitive, has moderate selectivity and high throughput (Koren *et al.*, 2008). The confirmatory assay, on the other hand, is also highly sensitive but eliminates false-positive results due to greater degree of selectivity. After passing through these two assays, the samples are then examined with a bioassay to separate out the samples that can neutralize the biological effect of the drug. If equal or less immunogenicity than the originator arm is shown, it means that the biosimilar drug is equal, or less, immunogenic than the originator. The biggest advantage of using the ‘one-assay’ approach is that it does not have the ‘between assay’ variability introduced by the ‘two-assay’ approach (Cai *et al.*, 2012). ELISA and electrochemiluminescent assay (ECL) used to assess immunogenicity in comparison trials versus its reference medicinal products.

Basic ELISA

ELISA (enzyme-linked immunosorbent assay) or enzyme immunoassay (EIA), is a plate-based assay technique which detects and measures substances such as hormones, proteins, peptides and antibodies.

Basic ELISA is distinguished from other antibody-based assays in terms of its separation technique and measurement results. Specific and non-specific interactions are detached via serial binding to a solid surface which is usually a polystyrene multi wellplate. Its measurement results can also be attained (Fig. 3).

First of all, the antigen in a sample is immobilized to the wall of the wells of a microtiter plate by either using a “capture antibody” or direct adsorption to the plate’s surface. The capture antibody, mainly used in a specific ELISA type called “sandwich ELISA”, has to be specific to the target antigen. After this step, a detection antibody is added to form an antigen-antibody complex by binding to the adsorbed antigen. The detection antibody either provides a binding site for a labelled secondary antibody or is either directly conjugated to an enzyme, such as horseradish peroxidase (HRP). In general, the four main categories into which ELISAs can be grouped are: direct, indirect, sandwich, and competitive ELISAs.

Electrochemical-luminescence (ECL)

Due to high demands on purity, sensitive analytical techniques are essential in the biopharmaceutical industry. Electrochemical-luminescence (ECL), a new technology from Meso Scale Discovery is more sensitive and can analyse multiple impurities simultaneously and hence can possibly replace ELISA. The three constituents that are required for the detection reaction are; a molecule which has the ability to accept and donate electrons, an electrode and a reporter molecule which has the luminescent capability. The electron carrier in the system is tripropylamine (TPA) and the reporter molecule is a ruthenium (II) tris-bipyridine-(4-methyl-sulfone) NHS ester, known as a

SulfoTAG™ according to MSD. TPA donates an electron to the electrode while in vicinity to the electrode. Then TPA turns into a radical by losing a proton (H+) to the aqueous phase. Side by side, the SulfoTAG loses an electron to the electrode and then electron is transferred to the radical, which excites the SulfoTAG. A photon, with wavelength ~ 620 nm is emitted when it returns to the original energy state. (Yang *et al.*, 1994, Lu *et al.*, 2006).

Ion Exchange Chromatography

Ion chromatography (or ion-exchange chromatography) is a separation technique that uses the affinity to ion exchanger to separate ions and polar molecules. It is applicable on almost any kind of charged molecule like large proteins, amino acids and small nucleotides. There are two types of ion chromatography; anion-exchange and cation-exchange.

Huge amount of studies during the last decade describe charge profiles of proteins and ion exchange chromatography (IEX) is the standard procedure to characterize the protein charge variants (Lau *et al.*, 2010). Molecules are separated based on their respective charged groups in this technique. Analyte molecules are retained on the column following coulombic (ionic) interactions. Molecules interact electrostatically with opposite charges on the stationary phase matrix which consists of an immobile matrix of charged ionisable functional groups or ligands (Fig. 4).

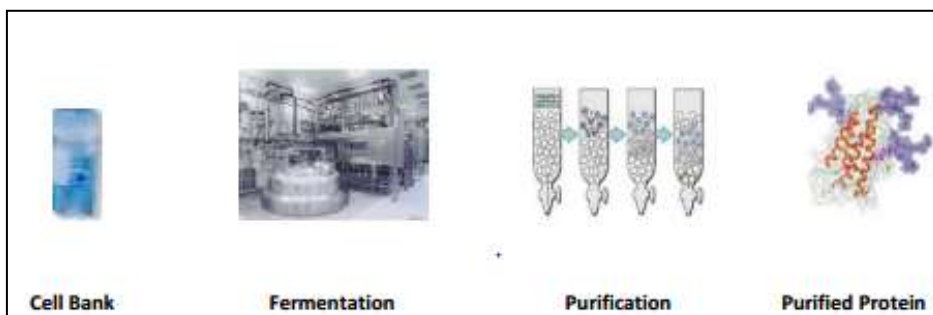


Fig. 3: Steps for biological products manufacturing

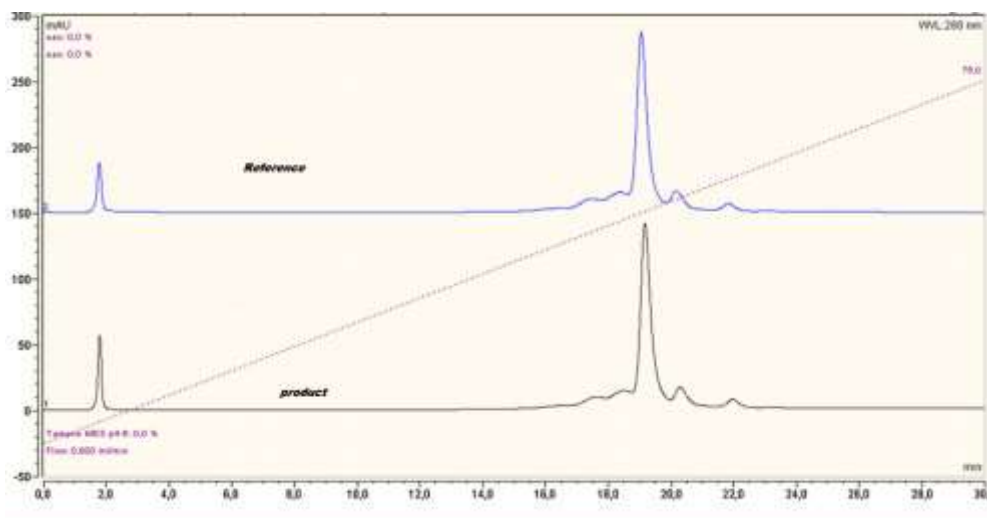


Fig. 4: Typical ion-exchange chromatography profiles of the two biosimilar products (reference and biosimilar product), no difference was observed.

RP-HPLC Peptide Mapping Analysis

It is the chemical or enzymatic handling of a protein to produce peptide fragments and then separate and identify these fragments in a productive way. For bio-therapeutic proteins and peptides, peptide mapping is detailed examination for the identification of minor and even isobaric differences in protein primary structure which include errors in the transcription of complementary DNA, point mutations. Peptide mapping by RP-HPLC is often used for quality control of therapeutic proteins. Since tryptic digestion generates a complex mixture of peptides (typically 25-100, depending on the size of the protein). Separation optimization is often carried out intuitively by altering both column length and gradient slope. A protein is selectively sliced by chemical digestion or enzymes (proteases) into smaller fragments in peptide mapping. The peptide fragments are typically analysed by gradient reversed-phase high performance liquid chromatography (HPLC) using an acetonitrile (ACN)/water gradient with added trifluoroacetic acid (TFA) after the digestion of protein. Critical traits of effective peptide mapping analyses are retention time precision and peak area precision.

Amino Acid Analysis

Amino acid was analysed by performing reaction with the hydrolysis peptide bonds through 6 M HCL, pre column derivatization followed by the o-phthalaldehyde and 9-fluoronyl methylchloroformate (FMOC-Cl) separated fluorescence exposure by RP-HPLC. Firstly samples were de-salted by PD-10 column (GE Healthcare) then aliquot containing internal standards hydrolysis tubes. The internal standard mixture sample is hydrolysed by 6M HCL at 110°C for 24 hour under concentrated pressure. Now dried hydrolysates under concentrated pressure, and transferred to HPLC vials by reconstituted in 50ml of 0.1M HCL. The HPLC ampoules were then full on the illustration plate of an Agilent 1200 HPLC system for automated derivatization of the unconventional amino acids. All accepted proline primary amino acid contained amino acid as derivatized OPA in presence of 3-mercaptopropionic acid. Amino acid was derivatization norcaline by using internal standard. By the conduction of RP-HPLC amino acid is analysed with the fluorescence detection. Using of the standard mixture amino acid retention times and fluorescence response and calibrated for the calculation of sample of the amino acid concentration we used the units of pmol/ml a curve plot show for the response ratio vs from the respective calibration. The amount ratio and the related molar ratio to leucine was calculated (Jung *et al.*, 2014).

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is a technique used to depict the stability of a protein or other biomolecule directly in its natural form. When heated at a constant rate, the heat change associated with the molecule's thermal

denaturation is measured. Macromolecules and macromolecular assemblies (>5000 Daltons), such as proteins and nucleic acids can form well-defined structures that experience thermally-induced conformational changes. During these rearrangements, the heat is absorbed due to redistribution of non-covalent bonds. This heat is measured by differential scanning calorimeters. A biomolecule in solution is in equilibrium between its native (folded) and denatured (unfolded) conformations. Molecule becomes more stable with the higher thermal transition midpoint (T_m). DSC is used to measure the enthalpy (ΔH) of unfolding resulted from heat-induced denaturation.

Size-Exclusion Chromatography (SEC-HPLC)

Size-exclusion chromatography (SEC), also known as molecular sieve chromatography, is a chromatographic method which involves separation of molecules in solution based on their size and molecular weight. Its speed and reproducibility gives it an edge over others for routine and validated analyses (Brange *et al.*, 1992; Yu *et al.*, 2008). The column used is filled with a porous material. Due to permeation deep into pores, smaller dissolved molecules flow more slowly through the column whereas large dissolved molecules flow quickly through the column because they do not enter the pores. As a result, larger molecules elute from the column earlier than smaller molecules and hence, the molecules are separated by size. This is the separation principle of size exclusion chromatography.

Agarose gel electrophoresis (AGE)

Agarose is a natural linear polymer. It is mined from seaweed and when heated in a buffer and allowed to cool, forms a gel matrix by hydrogen-bonding. For most applications, only a single-component agarose is needed without any polymerization catalysts. Therefore, agarose gels are simple and fast to prepare.

Agarose gel concentration

Size of fragments to be resolved determines the percentage of agarose used. The concentration of agarose is referred to as a percentage of agarose to volume of buffer (w/v), and agarose gels are normally in the range of 0.2% to 3% (Smith, 1993). The faster the migration of the DNA fragments, the lower the concentration of agarose.

Electrophoretic buffer systems

Buffers are an integral part of any electrophoresis technique because the effective maintenance of pH within the matrix determines the effective separation of nucleic acids. Moreover, the electrophoresis buffer's composition and ionic strength (salt content) affects the electrophoretic mobility of DNA (Somma and Querci, 2006).

Preparation of agarose solution for casting the gel

Dissolve the Agarose by placing the flasks in boiling water and allow it to cool to lukewarm. Cover the sides of a tray using cello tape and place the comb about 1 cm from the top

of the tray. Pour the Agarose carefully without making any bubbles, cool it for 20 mins and take off the combs and uncover the cello tape.

Loading Buffer

This buffer is added to the DNA fragment that has to be electrophoresed. To rise the density of the DNA solutions glycerol or sucrose is added, a part from that the samples goes into the tank i.e. running buffer and not descend into the gel patch. Xylene cyanol or bromophenol blue are the dyes accommodated in the gel loading buffer to enable the examination of the sample during loading of the gel and process of electrophoresis.

Voltage/current applied

Higher the voltage or current more rapidly the DNA drift, but too much high voltage or too much low voltage has a disadvantage. In the former, thin lines of different colors on the band appears particularly for the DNA whose size fall between $12 \geq 15$ kb. In addition to that the temperature as well as the current of the buffer extends. This will ultimately deliquesce the gel. For standard size gel it is advisable not to exceed 5-8 V/cm and 75 mA or 100 mA for mini gels. In later case the movability of small (≤ 1 kb) DNA is decreased and band expansion occur due to separation and dispersal.

Visualizing the DNA

The DNA can be localized inside the agarose gel directly by using colorant with small concentration of interpolate strikingly bright Ethidium Bromide dye under ultraviolet light.

Procedure

The DNA sample is combined with the loading dye and filled into the well with caution by using capillary tube or pipetman. Now as the sample loaded in respective wells the negative terminal i.e. cathode is connected at the top end of the gel and that the positive terminal i.e. anode is connected towards the bottom of respective gel. The utmost volume loaded on to well-formed from 1.5 mm thickness tooth of the comb is 30 μ l. by switching on the D.C. power pack the process of electrophoresis starts and gel starts running at 5v/cm. as soon as the bromophenol blue dye reached 1 cm above the bottom end the power supply is disconnected and both the gel along with platform is stained in plastic tray containing 0.5 μ g/ml ethidium bromide in germ free distilled water. After 30-45 minutes the platform and gel is washed out with distilled water and the gel is gently inserted into the UV trans-illuminator by keeping the platform in oblique direction once the UV light is switched on DNA bands are seen and photographed at f 5.6 for almost 10 seconds using orange filter.

Surface Plasmon Resonance

The first biosensor developed almost a decade ago based on surface Plasmon resonance (SPR), the need of this technique has increased undeviatingly (Jönsson *et al.*, 1991).

To study the molecular interactions surface Plasmon resonance (SPR) binding analysis methodology is used (Schuck, 1997; Ramakrishnan *et al.*, 2006). To reveal the interactions of two different molecules (one is mobile and one is fixed on thin gold film) SPR which is an optical techniques is used. As described in the work, an amine coupling reaction is used to immobilize affinity purified fusion polypeptides on a sensor chip that is inserted into the flow chamber of a Biacore 3000 instrument. As the second polypeptide is added the flow through analyse to chamber cause adhesion to the immobilized polypeptide ligand, producing a slight change in refractive index at gold surface (Kretschmann and Raether, 1968), which can be measured with accuracy. Through the ratio of rate constants binding affinities can be achieved tends to give uncomplicated characterization of protein-protein interaction. Without the use of radioactive or fluorescent labeling of polypeptides SPR can detect mass concentration (Stenberg *et al.*, 1991) before measurement, presenting a huge favor in reducing time and complication of the studies (Fig. 5).

In the fields of biochemistry, biology and medical sciences surface Plasmon resonance (SPR) become a significant optical bio sensing technology reason being real time, label free and noninvasive nature (Karlsson, 2004). SPR device is based on the Kretschmann configuration (Kretschmann, 1972) and composed of the following components: light source, prism, gold film, and detector. A testing cell adheres to the gold film and may be filled with different solutions to be studied. When a light beam spread in the prism and experience the interaction of the gold film and the solution, TIR takes place and the fading wave forms as long as the incident angle is greater than the critical angle. Normally, the intensity of the reflected light does not change with the incident angle under the condition of TIR. However, at a specific angle larger than the critical angle, the fading wave exhilarates the delocalized electrons or Plasmon's of the gold film, causing SPR; the intensity of the reflected light becomes smaller intensely at this point. SPR angle is defined as the incident angle at which the minimum reflectivity is observed (Tang *et al.*, 2010).

Protein Quantification by Mass Spectrometry

The very first stage to characterize the proteins structurally is the determination of molecular weight. There are two types of light Ionization Techniques, MALDI (Matrix-Assisted Laser Desorption Ionization and ES/ESI (Electrospray Ionization). As compared to ESI-MS, the MALDI-MS technique analyses intact proteins or peptides which contain greater amount of impurities (e.g., salts). MALDI causes fragmentation of the protein during ionization that can be used to get more information about the fragments. This technique requires sample to be placed in matrix that absorbs appropriate wavelength light. Matrix generates heat and forms ions of analyte and what is around it. The mass measurement precision and accuracy can be

further improved by using all the observed multiply charged ions (typically better than 0.01% for masses up to 100 kDa) (Smith *et al.*, 1990). Two different approaches are applied to determine the sequence of a protein (Fig. 6): bottom-up (Henzel *et al.*, 1993) and top-down (Ge *et al.*, 2002).

Amino Acid Analysis

Hydrolysis of peptide bonds with 6 M HCl was performed which was trailed by pre-column derivatization using o-phthalaldehyde (OPA) and 9-fluorenylmethylchloroformate (FMOC-Cl), separation by RP-HPLC, and fluorescence detection in order to carry out this analysis. Columns were first used to de-salt the samples and then aliquoted into hydrolysis tubes containing internal standards. Hydrolysis of the sample/internal standard mixtures was then carried out under reduced pressure using 6 M HCl for 24 h at 110°C. The hydrolysates were dried under reduced pressure, re-formed in 50ml of 0.1 M HCl and transferred to HPLC vials. For deriving liberated amino acids automatically, the HPLC vials were then laden onto the sample tray of HPLC system. Derivatization of amino acids containing a primary amino group (all except proline) was achieved with o-phthalaldehyde (OPA) in the presence of 3-mercaptopropionic acid. Proline was derivatized by reacting it with FMOC-Cl. Norvaline was utilized as the internal standard used for amino acid derivatizations except for proline derivatization in which

sarcosine was employed. RP-HPLC with fluorescence detection was utilized to analyse the amino acids. A standard mixture of amino acids helped in calibrating retention times and fluorescence responses were calibrated. Using plots of the response ratio vs. the amount ratios shown from respective calibration curves, we calculated the amino acid concentrations of samples in units of pmol/ml. Then we calculated the molar ratio with respect to leucine.

Sterility Testing

To carry out this testing, aseptic conditions were frequently observed by suitable selection of the working area and appropriated controls as stated in on GMP documents (24, 1995).

Test article under sterile testing should be monitored for the traces of elements that will affect the microorganisms' development within the growth media under analysis. This testing is usually known as the Bacteriostasis/Fungistasis (B/F) test or sterility test validation, qualification or verification [10]. Given sample type is passed through the method suitability test only once if no additional changes occurred to the product or any formulation or manufacturing process did not take place. However, it could be executed frequently to confirm no significant in the product or process affecting the sterility assay results (24, 1995).

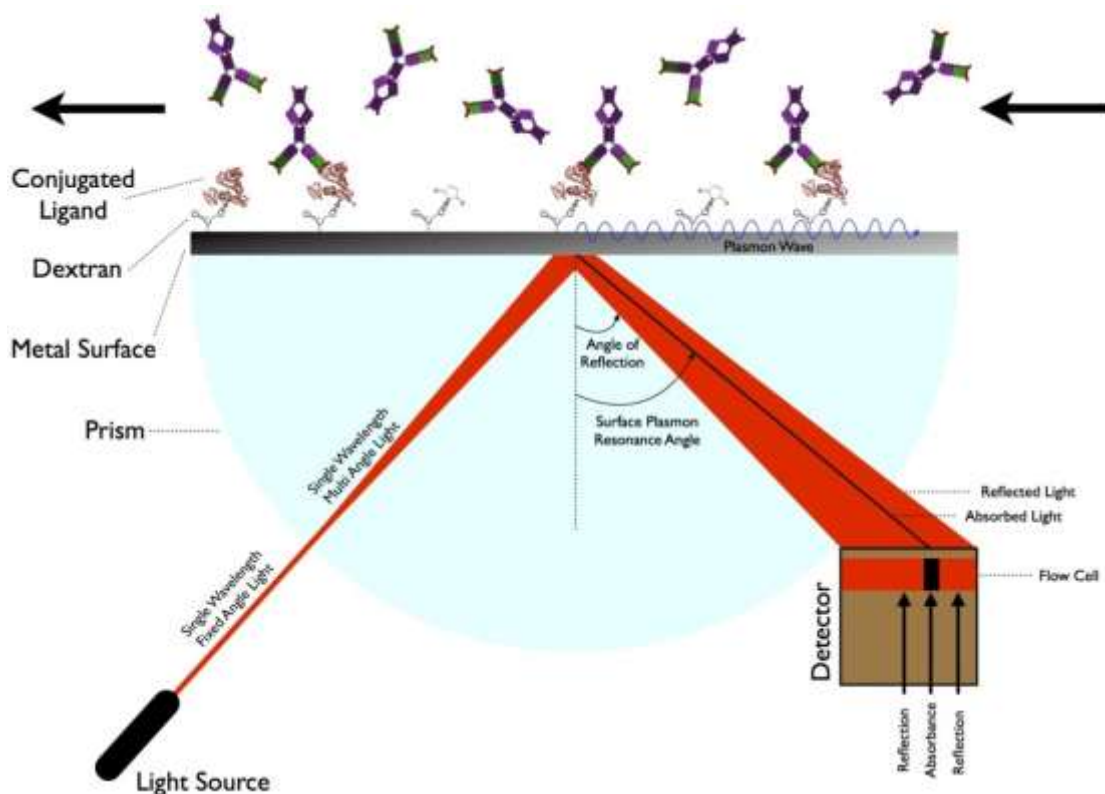


Fig. 5: Surface Plasmon Resonance principle

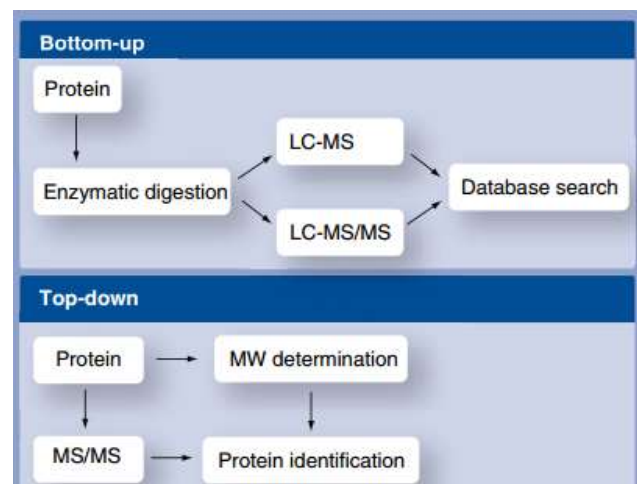


Fig. 6: Bottom up and top down technique (Chen and Pramanik, 2008)

Safety Pharmacology

Drugs or biomaterials can affect the functioning of vital organ systems, which includes the cardiovascular and central nervous systems. Evaluation of these effects before human exposure is necessary either studying them separately or as additions to toxicity studies. *In vitro* tests to determine the biological and pharmacological activity at the cellular level are often followed by safety tests in animal systems. Generally, there is no need of standard safety pharmacology studies (as commonly conducted for pharmaceuticals) for biotechnology-derived products. Expected and unexpected pharmacological effects of the test materials are outlined in these studies based specifically on factors related to required clinical activity.

Duration

Adjustable - dependent on the test system

Test System/Animal System

In Vivo: selected pharmacologically relevant species; if there is no previous information or there is difficulty in deciding the species relevance, more than one species is specified. *In Vitro*: relevant animal species are used to extract cell lines; may forecast the optimal species most suitable for *in vivo* studies. Dose Administration Variable, dependent on the likely effective dose range and test system.

Parameters

In Vivo:

Safety studies may be mandatory for the following systems and specific target organs:

1. Respiratory (lungs and bronchi)
2. Gastrointestinal and hepatic (esophagus, stomach, intestines, liver)
3. Renal (kidney)
4. Blood
5. Cardiovascular (heart and blood vessels)

6. Nervous/neurobehavioral (CNS and behavior)
7. Endocrine (thyroid and other endocrine glands)

In Vitro:

1. Biological activity at cellular stage can be easily evaluated on following criterion
2. Receptor occupancy
3. Receptor affinity
4. Binding and transport kinetics
5. Production and or secretion of specific proteins in response to test material (for example, antibody production in response to test antigen)
6. Other pharmacological effects on cellular function.

Inactivation Test

Every individual purified bulk material is to be tested on mice for effective inactivation of the virus before preservatives and other substances are added and induced. The test should be performed with undiluted purified bulk material injected into the cerebrum into at least 20 mice, but each should be weighing between 15 and 20 g. These mice shall be kept under observation for about 14 days. Any symptoms caused by the virus shall be observed by Immuno-florescence evaluation procedures. At the end of the observation period, no cytopathetic effects should be observed.

Considerations for phase III clinical comparability trials:

The purpose of Phase III clinical comparability trial is to eliminate defects that prevail regarding the efficiency and safety of the biosimilar products in relation to the reference product following completion of physicochemical, biologic, and preclinical investigations, as well as PK, PD and immunogenicity investigations in humans (Food and Administration, 2012). It also depends on the available history of safety measures with the reference product may lead to extensive phase III clinical investigation of the biosimilar, whereas along and relatively safe marketing history may warrant as elective and targeted approach (Food and Administration, 2012).

In order to carry on biosimilar developments, phase III clinical equivalency trials should physically show that whether there is an increased or decreased activity in relation to reference product. (Food and Administration, 2012). The goal is to demonstrate that any level of variation in efficiency or safety between the biosimilar and reference product is less as compared to the standard “clinical equivalency” margin (Dranitsaris *et al.*, 2013). Such an equivalence margin is the foundational crux of the trial and is based on the historical difference noted between the treatment effect or the reference product versus placebo (Kay and Smolen, 2013). Trial designs for biosimilar development are not unlike designs for any other biologic

product with similar considerations of patient population, sample size, endpoints, and study duration. Clinical development of a biosimilar also provides a constricted as compared to the reference product.

Conclusion

Extensive structural and functional comparison of biosimilars and reference products is the foundation of biosimilar development. The comparison exercise must be scientifically tailored using state-of-the-art analytical tools and sensitive tests to detect small product-related differences between the biosimilar and the reference product. Structural differences determine the amount of biological, non-clinical and clinical studies that are needed for development of biosimilar proteins. The primary amino-acid sequence must be the same for the biosimilar and the reference product in Europe and in the USA. As review edhere, a number of biosimilar techniques are mentioned. As highlighted here, the correct technique selection results in saving significant time and money.

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