Drug-Discovery and Development: *In Vitro* studies

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By

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TO WHOMSOEVER IT MAY CONCERN

This is to certify that **Vishal Patel**, M.Sc. (Microbiology) student of Nirma University, Ahmedabad, has successfully completed his internship from 6th February 2019 to 24th April 2019 in our organization, Oxygen Healthcare Research Pvt. Ltd.

We found him sincere, hardworking, technically sound and result oriented. He worked well as part of a team during his internship. We take this opportunity to thank him and wish him all the best for his future.

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1) Abbreviations

ADME	Absorption, Distribution, Metabolism and Excretion
ATC	Automatic Temperature compensation
APPI	Atmospheric Pressure Photoionization
CRL	Complete Response Letter
DMPK	Drug Metabolism and Pharmacokinetic
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
DAD	Diode Array Detector
ESI	Electrospray ionization
FMO	Flavin-contain Monooxygenase
FDA	Food and Drug Administration
HLM	Human Liver Microsomes
HTS	High Throughput Screening
HPLC	High Performance Liquid Chromatography
IND	Investigational New Drug
NDA	New Drug Application
NPLC	Normal Phase Liquid Chromatography
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PUDFA	Prescription Drug User Fee Act
PBS	Phosphate Buffer Saline
PHA	Polycyclic Aromatic Hydrocarbon
PK	Pharmacokinetic
UV	Ultraviolet
UGT	UDP-glucosyltransferase

2) Introduction

Drug is a chemical substance when administered to the organism cause biological effect. The drug can be beneficial or harmful. The process of designing or discovering new drug is known as Drug discovery. The main aim of drug discovery is to de3sign new molecule which selectively bind to target site [(1)]. Due to the development in combinatorial chemistry there is an increase in discovering of new diverse compounds. By the combination of combinatorial chemistry and high throughput screening, vast number compounds are examining for their in-vitro potency [(2)].

The approach is helpful to discover new structure with superb in-vitro potency. Drug discovery involves the identification of candidates, synthesis, characterization, screening of compound with

therapeutic efficiency. Drug discovery is a lengthy, expensive, difficult as well as disadvantage of low success rate [3]. Various Steps of drug discovery process are:

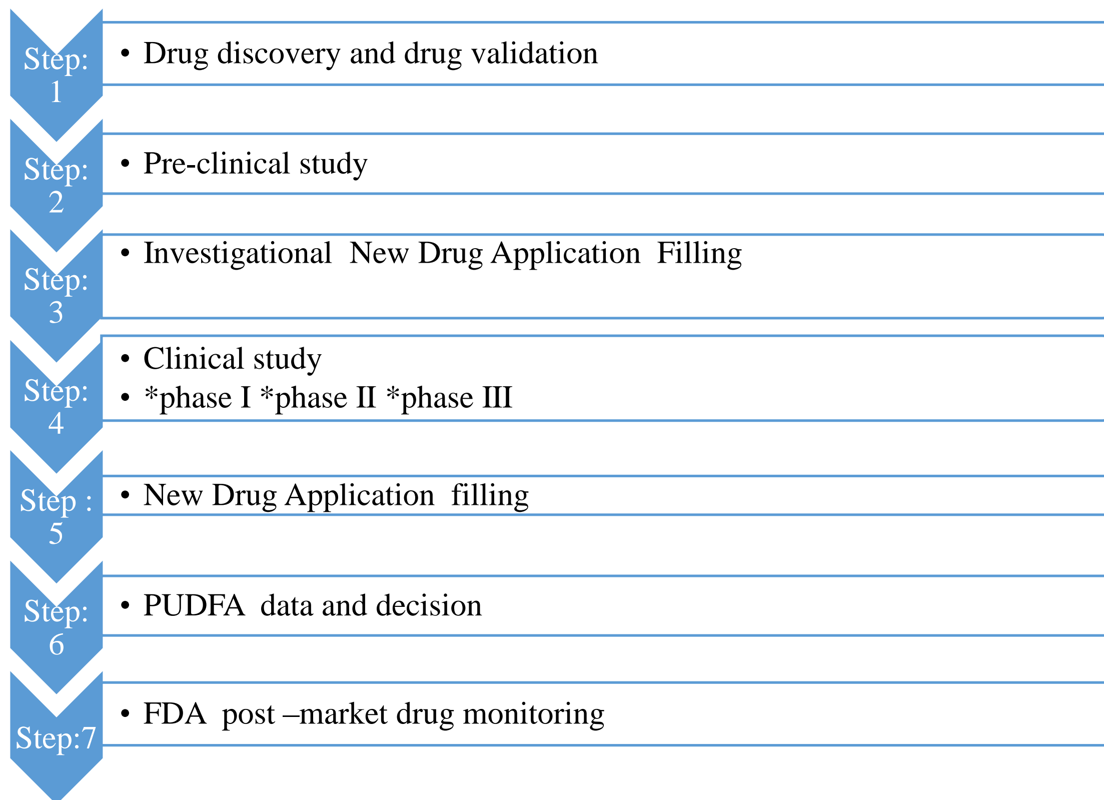

Figure 1: Drug discovery process

Drug Discovery and Development [4]

The gene or protein involved in infection are identified and number of test compounds are tested. The promising compound which show positive effect on the target are validated and further enter into the next step.

Preclinical Studies

This stage is divided into two parts: ***In vitro*** and ***in vivo*** studies.

- Examination of drug molecules interaction in laboratory environment [test-tubes] is known as *In Vitro* studies.

- Examination of drug molecules in animal models and other living cell culture. Safety remains the main priority.

At this stage the number of test compounds come down from 1000 to 1 or 5 compound.

Investigational New Drug (IND) Applications

The filling of investigational new drug application to FDA prior to the clinical trial. The result of preclinical testing, including side effects and other safety features of an experimental drug and manufacturing process will be verified by FDA.

Clinical studies

The compounds which pass successfully the pre-clinical stage enters the clinical phase:

1. **Phase: I:** A relatively small group of healthy people such as dozen too few dozens. The behavior of drug such as absorption, elimination as well as side effects and desired activity of drug is verified.
2. **Phase: II**: The high number of volunteer [patient]. In this phase, optimum drug is selected.
3. **Phase: III:** The high number of patient, up to few hundred to thousands. This phase is longer and costlier as compared to other phase involve in drug discovery process. Safety and efficacy play a big role in this phase.

New Drug Applications (NDA)

There is need for filling new drug application to FDA. This contains all information regarding the research and safety data examined during each of the prior steps. If the NDA is accepted by PDUFA, data is study for next 10 months for a standard application.

PDUFA Data and Decision

After the release of data by PDUFA, FDA makes the decision.
FDA has three choices:
It may approve a drug,
It may deny a drug or
It will request for additional information though CRL.

Post-market study

After the approval of drug by FDA, the developer has to submit long-term safety report consist detail report which contain information regarding any adverse effect cause by drug to the FDA.

3) Review of Literature

The success rate of drug discovery and development in last two decades is increased due to Drug Metabolism and pharmacokinetic.
This success mainly is due to the following three reasons:

1. Application of DMPK in the early stage of drug discovery.
2. DMPK scientist have better understanding regarding the drug metabolic enzymes, transporter difference amongst species and individual.

3. Better understanding regarding the optimization of DMPK property – absorption, distribution, metabolism and excretion (ADME) are essential parameter in drug discovery process.

ADME data of test compound obtained from the *in vitro* and *in vivo* studies help in predicting behavior of drug in patients and also help in making decision regarding holding or terminating a drug candidate. Incomplete ADME data leads to the failure of the drug-discovery process. ADME studies is carried out in three ways: in vitro, in vivo and in silico model. *In vitro* ADME parameter includes permeability, metabolic stability, protein binding, blood to plasma partition, inhibition or induction by cytochrome P450 enzymes, cell proliferation, toxicity etc., *in vivo* studies provides information about drug oral bioavailability, exposures, distribution, clearance, duration of exposure for a drug and in metabolism. In silico model helps in predicting drug behaviors on the basis of physiochemical properties of drug candidates in combination with crystal structure of protein and data obtained from ADME parameters in laboratory [(5)]. Various assays involved in in vitro study are as follows:

3.1) Lipophilicity assay

Lipophilicity plays important role in studying pharmacokinetic behavior of drugs as well as influences a varying property related to potency, pharmacokinetic and toxicology profile such as absorption, binding property and distribution into tissue [(6)]. It is also important factor for determining the solubility of compound [(7)]. Lipophilicity is the ability of chemical compound to dissolve in fats, oils, lipids etc. It is important for the biological activity as the affinity for a lipophilic environment facilitates the transport of chemical through membranes in biological system and formation of complexes between compounds and receptor binding sites. High lipophilic compound is preferred target for metabolism and has high clearance rate as well as increase in the permeability, protein binding, volume of distribution, and decrease in the solubility of compound [(8)]. Lipophilicity positively correlating with the high plasma protein binding [(7)]. The assay which measures the lipophilic characteristics are logP and logD. LogP is used when all solutes are in neutral state and logD is used when all solutes are in ionic state [(8)]. The distribution between an aqueous phase and a hydrophobic phase is key parameter in identifying the lipophilic characteristic of compound [(7)]. Reverse phase HPLC and *in silico* method is widely used to analyse lipophilic characteristic of the compound [(8)]. The role of

Lipophilicity assay in drug discovery is to determine whether the compound is lipophilic or hydrophilic or whether the drug compound is able to pass the lipid bilayer in the cell membrane [(9)].

3.2) Aqueous solubility

Most drug discovery experiment assume the complete dissolution of the compound as it is important for the pharmacological processes *in vitro and in-vivo*. Compound's precipitation decreases the concentration in solution which reduce the pharmacological process. Compound's partial dissolution or super saturation causes unreliable result in pharmacokinetic study, in structure-activity relationship study and in in-vivo potency [(10)].

Solubility of compound is key parameter in formulation of drug for in vivo dosing to achieve optimum exposure (10) and also it reflects the bioavailability of compound (11). Dissolution in the gastro-intestinal tract or in lungs fluid mainly depend on many factors including physiochemical, physiological and formulation parameter (12). Lower solubility of compound cause limitation in formulation and routes of administration, especially oral dosing and also in absorption of compound from the gastrointestinal tract (11).

Aqueous solubility of compound is measured by two ways: kinetic and thermodynamic solubility. Typically, in early stage of drug discovery, kinetic aqueous solubility method is used because it is fast and well suited for HTS [(13)]. Kinetic aqueous solubility is solubility of the compound at specific time interval within aqueous medium in non-equilibrium state where as in thermodynamic aqueous solubility is when the system is in equilibrium position.

Major difference between kinetic and thermodynamic solubility is at initial steps. In kinetic solubility, compound is added into aqueous medium after dissolving the compound in organic solvents e.g. DMSO. In thermodynamic solubility, solid form of compound is added into the aqueous medium. There is also difference in incubation time period [(12)].

3.3 Microsomal stability assay

In Vitro drug metabolism study is widely used to estimate hepatic drug clearance which play important role in drug discovery process. Most orally administrated drug is cleared by liver and also important for accurate measurement of hepatic metabolism as well as for assessment of

clearance effect. These processes help in evaluation and optimization of drug and also in estimation of early human doses and exposures in clinical [14].

In liver, microsome plays important role in drug metabolism. Microsomes are important part of liver which contain membrane-bound drug metabolizing enzymes such as phase I (CYPs, Flavin-containing monooxygenases (FMO), esterase, amidases, and epoxide hydrolases) and also the phase II(UGT) enzymes. Liver microsomes are widely used in *in vitro* study as it is good indicator for the CYP and UDP-glucosyltransferases (UGT) involved in the metabolism of a drug and also used for drug-interaction studies [15].

Mostly 60% of marketed drugs are cleared metabolically by cytochromes 450 enzymes and majority of activity are done by this family enzymes [14]. Recombinant P450s and HLMs, are important in *vitro* tools for determination the amount and route of the metabolism of new chemical compound and also in screening the inhibitory effect of drug-metabolizing enzymes [14].

Different species liver microsomes are used for metabolic study such as rat, mouse, dog, human etc. Metabolic stability of compound in *in vitro* is mainly determined by intrinsic clearance rate [CL_{int}] and in *in vivo* study by determining f_{inc} (in-vitro incubation) [14]. For studying the microsomal stability of phase I NADPH is used as co-factor and for phase II UGT is used as co-factor.

3.4 Plasma stability assay

After the administration of drug through different routes (oral, intravenous, intramuscular), mostly

drugs are distributed to target tissue via circulatory system (blood). During the residual time in blood stream, some of the compound undergoes decomposition by the enzymatic activity. This cause poor effect on pharmacokinetic study, difficulty in bio analysis for in vivo pharmacokinetic studies and inability to develop clinical candidates [16]. Plasma stability results could be useful in drug discovery programs for the identification of lead molecules and the subsequent optimization of chemical structure [17].

Stability of drug is essential for maintaining acceptable drug concentration and half-life in order to achieve desirable pharmacological effects [16]. Unstable compounds are tending to have rapid clearance, short half-life, and poor in vivo performance because compounds are continuously degrading even after the blood sample taken out from animals. Circulatory hydrolytic

metabolites complicate and slows down the drug development process. Therefore, pharmaceutical company cannot advance compounds which are highly prone to degrade in plasma except prodrug, ante drug
etc. [(16)]

Blood contain various type of enzymes such as cholinesterase, aldolase, lipase, dehydropeptidase, alkaline phosphatase and acid phosphatase. Different species has different concentration of enzymes depend on gender, species, disease state, age and race. If the compound has affinity of binding with this enzyme, has a hydrolysable group (amide, ester, lactac, lactone etc.) in right position and is reactive, it can be decomposed in plasma [(16)].

Screening of plasma stability provides the useful information to prioritize compounds for the in vivo study and provide useful information for making the modification in compound to improvement stability in plasma. Plasma stability is very useful for screening of prodrug and ante
drug, where rapid conversion in plasma is desirable [(16)].

3.5 Plasma-protein binding assay

Plasma protein binding is a reversible reaction between the drug and proteins in plasma due to hydrophobic and electrostatic interaction such as van der waal and hydrogen bonding. Pharmacokinetic properties such as volume of distribution, clearance and elimination is mainly affected by this type of interaction [(18)]. Only free fraction of drug can pass to the cell membrane. High protein binding has less fraction of free drug for the therapeutic effect as compared to lower binding capability [(18)]. High protein binding is associated with high Lipophilicity which has low free-drug concentration in the systemic circulation [(19)].

The amount of protein binding may impact the efficacy and toxicology of a drug as well as believed that only free drug concentration has therapeutic effect. Determination of plasma protein binding in animal and human before phase I clinical trial as it is helpful in evaluation of drug-drug interaction. In a drug discovery process, the free fraction and interensic clearance of drug from *in vivo* plasma protein binding experiment helpful in structural designing as well as prioritize compound for further *in vitro* studies [(19)]. Disease such as hepatic or renal impairment, pregnancy etc., alters the plasma protein binding effect which effect on therapeutic efficiency of the drug.

Blood contain around 55% of plasma. It is an aqueous solution which contains around 92% water, 7% protein, and other solute such as organic ions. Plasma protein contains albumin, globulin, clotting factor and regulatory protein. Albumin and α1-acid glycoprotein are the most important protein in plasma protein binding with drug [18]. Equilibrium dialysis is a method to identify the plasma protein binding.

3.6 CYP450 Inhibition assay

Drug- Drug interact with therapeutic agent which may inhibition or induction of drug metabolizing enzymes or drug transporter. Inhibition of these enzymes and transporter increase half-life as well as increase toxicity effect by therapeutic agent or induction can cause visa-versa effect [20]. Drug-drug interaction may result into several side effect, earlier termination of compound as well as restriction or withdrawal of compound from market [21]. Drug-drug interaction plays important role in clinical trial as well as in discovery and development of new drug [22].

There are two polycyclic aromatic hydrocarbons (PAH) - inducible form of cytochromeP450 in most of mammalian species. Various isoforms of the p450IA gene sub-family are as c and d in rat, form P_1 and P_3 in the mouse and form 6 and 4 in the rabbit respectively [23]. Cytochrome P450 is important drug metabolizing enzymes present in human liver microsomes plays prominent role in drug metabolism. There are mainly five major isozymes which contribute to the metabolism of the vast majority of the drugs as compared with other enzymes. These are CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 which plays an important role in the metabolism of drug. Other important isozymes which recently gained importance are CYP3A5, CYP2B6[22].

3.7 Permeability assay

For better effect of orally administrated drug, it should be bioavailable to the target site. Intestinal epithelial cells provide barrier against passage of various molecule [24] and dysfunction leads to the development of the several diseases [25]. Cell-cell interaction, cell-extracellular matrix contacts, and inflammatory factors play an important role in endothelial cell barrier [25].

Intestinal epithelial mucosa provides physical as well as biochemical barrier which limits the drug absorption and also provide defense mechanism against harmful substance. Active uptake, efflux systems as well as intestinal metabolic enzymes plays a significant effect on the

Absorption of the administrated drug mainly mediated by active uptake, efflux system as well as intestinal metabolizing enzymes [26]. Caco-2 cell monolayer is used to mimic the permeability process in *in vitro*.

Caco-2 cells are intestinal cell line derived from colorectal carcinoma which shows similarity in morphology as well as functional character with *in vivo* intestinal epithelial cell barrier. This assay provides information regarding transport of drug across a cell barrier and also helpful in predicting mechanism of drug transport as well as in improving formulation strategies [24]. This assay is mainly used to predict the amount of drug absorbed across the intestinal epithelial cell membranes.

3.8 Cell-based assays

Cell-based functional assays plays important role in early phase of the drug-discovery process. Cell based assays are in higher demand because of its advantage in predictability, possibility of automation, multiplexing and miniaturization. Cell-based assay involves assay which measure cell proliferation, toxicity, production of markers, motility, activation of specific signaling pathways and change in morphology [27].

Cell-based assays for HTS include mainly three types: second messenger assays, reporter gene assays and cell proliferation assays. Secondary messenger assay mainly monitors signal transduction which is cause by activation of cell-surface receptors, reporter gene assays mainly monitor cellular response and cell proliferation/toxicity assay mainly monitor cell growth or death in response on the basis of external stimuli or stress [28].

Cell based assay mainly depend on reporter gene technology. Reporter gene assays are very sensitive because of ability to amplify the signal produced by cell signaling [27]. Cell based assay has ability to distinguish between the agonists and antagonists information of compounds such as permeability, stability and cytotoxicity associated with the compounds [28].

Cell-based assays are performed in biologically relevant microenvironment and provide comparison between the whole organism and *in vitro* system [28]. These assays help in predicting tissues specific response as well as in target identification, validation, lead identification, lead optimization and toxicology screening in early stage of drug discovery. The main component of cell-based assay includes cells, device for culturing the cells and detection system for quantification of cells or cellular activities [28].

There are different types of cell-based assay such as:

1. Cell viability assay: mainly measure the ratio of live and dead cell by determining the integrity of cell membrane, redox potential of cell population or by the activity of cellular enzymes. This assay mainly determines the effect of drug and is used to optimise the cell culture condition.
2. Cell proliferation assay: mainly determines the growth rate of cell population. This assay plays an important role in regular tissue and cellular homeostasis for proper growth, development and maintenance of organism.
3. Cytotoxicity assay: mainly assess the number of live and dead cells after the administration of drug or pharmacological agents.
4. Cell Senescence assay: mainly detect senescence markers associated with β-galactosidase activity which reflects cell membrane integrity.
5. Cell death assay: mainly measure cell death by measuring three parameter apoptosis, autophagy and necrosis. Apoptosis is defined as controlled or programmed cell death in response to extrinsic or intrinsic signals. Exposure of phosphatidylserine on the extracellular face of the plasma membrane, activation of caspases, disruption of mitochondrial membrane potential, cell shrinkage, DNA fragmentation and DNA condensation are the hallmark of apoptosis cell death. Autophagy is selective degradation of intracellular target such as misfolded protein and damaged organelles. Necrosis is the swelling of cell and destruction of plasma membrane as well as organelles. Necrosis involves both programmed as well as accidental cell death [(29)].

4) Material and method

4.1 Aqueous Solubility

4.1.1 Kinetic Aqueous Solubility

- Test plate: 96-wells UV plates (Costar, CAT # 3635 (Sigma))
- Incubation Time: 4 hours
- No of Replicates: Triplicate
- Buffer System: 10 mM, pH 7.4 Phosphate Buffered Saline (PBS, CAT# P5368, (Sigma)).
- DMSO Concentration: Up to maximum 2.0 % DMSO in Aqueous solubility
- Deliverables: Determined λmax, Aqueous Solubility, Solubility Classification.
- Instrument: UV/Visible Spectrophotometer by EPOCH, Biotek Instruments Inc., (Gen 5 2.06 software)

Procedure

a) λmax determination and Linearity Protocol: Determine λmax of the test compound by scanning from 200 nm to 400 nm, step 1 nm in UV/Visible spectrophotometer.

b) Linearity Plot: Prepare a 12-points linearity plot by two fold dilution in triplicates from 1000 µM work stock in DMSO in a UV-absorbance 96-wells plate. Record the absorbance of each linearity plot at the respective determined Lambda max. using the UV/Visible spectrophotometer.

c) Kinetic Aqueous Solubility

   - Addition of compounds in buffer:
   - Step-1: Spike 5 µL of the test compound from 50 mM 100% DMSO stock in to 1 mL of PBS (pH 7.4) buffer in a volumetric test tube to give 0.5% DMSO final concentration.
   - Step-2: Observe for any evidence of turbidity/precipitation visually.
   - Step-3: If the compound doesn't gets precipitate, then repeat step-1 above until there is observation of any precipitate "but" spiking will be done up to a final maximum concentration of 2.0 % DMSO in the buffer.

- Step-4: If the compound gets precipitated then stop the spiking process at that respective final DMSO concentration and record the physical changes/colour/precipitation/turbidity observed.
- Incubation: Wrap the volumetric test-tube with Para film and incubate on a rotator at 20 rpm, 25°C for 4 hours.
- Sample preparation: Post-incubation of 4 hours, samples of aqueous solubility in the test tubes are filtered using 0.45 µm filters (Millipore, CAT# SLHNX13NL) and the filtrate is aliquoted on to a UV absorbance 96-wells plate for further spectroscopy analysis.
- Analysis: Record the UV absorbance of the test samples using the UV/Visible spectrophotometer at the respective determined λmax.
- Calculations: Calculate the kinetic aqueous solubility (µM) of the test compound using the linearity plot.

4.1.2 **Thermodynamic Aqueous solubility**

- Test plates: 96-wells UV plates (Costar, CAT # 3635 (Sigma))
- Incubation Time: 24 hours
- No of Replicates: Triplicate
- Buffer System: 10 mM, pH 7.4 Phosphate Buffered Saline (PBS, CAT# P5368, (Sigma)).
- Deliverables: Determined λmax, Aqueous Solubility, Solubility Classification
- Half Life, Intrinsic clearance and Clearance category
- Instrument: UV/Visible Spectrophotometer by EPOCH, Biotech Instruments Inc.,
- (Gen 5 2.06 software)

Procedure

a) λmax determination: Determine λmax of the test compound by scanning from 200 nm to 700 nm, step 1 nm in UV/Visible spectrophotometer.
b) Linearity Plot: Prepare a 12-points linearity plot by two-fold dilution in triplicates from 4000 µM or 1000 µM work stock in DMSO in a UV-absorbance 96-wells plate. Record the absorbance of each linearity plot at the respective determined Lambda max. Using the UV/Visible spectrophotometer.
c) Aqueous Solubility (Thermodynamic)

- Addition of compounds in Buffer:
- Step-1: add ~ 1.0 mg of compounds in to 1 mL of 10 mM, pH 7.4 Phosphate Buffered Saline in eppendrof tube.
- Step-2: Observe for any evidence of turbidity/precipitation visually at 0 hr.
- Incubation: Wrap the eppendrof tube with Para film and incubate on a rotator at 20 rpm, 25°C for 24 hrs.
- Sample preparation: Post-incubation of 24 hrs., observe for any evidence of turbidity/precipitation visually. Samples of aqueous solubility in the test tubes are filtered using 0.45 µm filters (Sartorius, CAT# 1776C) and the filtrate is aliquoted on to a UV absorbance 96-wells plate for further spectroscopy analysis.
- Analysis: Record the UV absorbance of the test samples using the UV/Visible spectrophotometer at the respective determined λmax.
- Calculations: Calculate the thermodynamic aqueous solubility (µM) of the test compound using the linearity plot.

4.1.3 Formulation solubility

Recommended conditions – For 1.0 mg test items.

- 100 µL DMSO (CAS# 67-68-5-Finar)
- 400 µL PEG 400 (CAT# 26475/01-Qualigens fine chemicals)
- 5 x 100 µL Water

Procedure

Step-1: Add DMSO 101.8 µL in compound, vortex it.

Step-2: Add PEG 400 400 µL in earlier soluble compound, vortex it.

Step-3: Add Water 100 µL in vial, vortex it.

Step-4: Add Water 100 µL in vial, vortex it.

Step-5: Add Water 100 µL in vial, vortex it.

Step-6: Add Water 100 µL in vial, vortex it.

Step-7: Add Water 100 µL in vial, vortex it.

4.2 Microsomal stability study

Requirements

- Test System: Human Liver Microsomes (HLM)(CAT# 452116- Corning)
- Test compound concentration: 1 µM
- Time point: 0, 15, 30, 60, 90 and 120 minutes
- No of replicates: Two
- Final Protein concentration: 0.5 mg/mL
- Final NADPH concentration: 1.0 mM
- Phosphate buffer saline (pH 7.4): 10 mM
- Final DMSO concentration: 1%
- Deliverables: Half Life, Intrinsic Clearance and Clearance category
- Bioanalysis: LC-MS/MS (Agilent 6410QQQ)

Procedure

- Pre-incubation mixture with cofactor: 1 µL test Cpd. + 50 µL of cofactor + 436 µL of PBS (pre-incubate for 10 min at 37˚C)
- 120 min w/o cofactor: 1 µL of test cpd. + 482 µL of PBS (Incubate for 120 min at 37˚C)
- 0 min sample: 487 µL of pre-incubation mixture + 1000 µL of acetonitrile containing internal standard + 13.0 µL of microsomes
- Incubation mixture (different time-points):13 µL of microsomes + 487 µL of pre-incubated mixtures (incubated for other time points 15, 30, 60, 90 and 120 min 37°C)
- Sample preparation: 150 µL incubation mixture + 300 µL of 70% acetonitrile 30%water with 0.1% Formic Acid containing 1µM internal standard + vortex 5 min at 1200 rpm+ Centrifuge 10 min @ 13000 rpm. Supernatant injected in to LC-MS/MS for quantification

4.3 Plasma stability assay

Requirements

- Test compound concentration: 10 µM
- Time points: 0, 15, 30, 60, 120 and 240 minutes(time points can be change as require by client)

- Incubation temperature: 37°C
- No of replicates: Two
- Test system: Human Plasma
- Final DMSO concentration: 2%
- Deliverables: % parent compound remaining at each time point
- Bioanalysis: LC-MS/MS (Agilent 6410QQQ)

Procedure

- Pre-incubation mixture: Human plasma incubated at 37° C for 15 min.
- Preparation of Master-mixture: 14 µL test cmpd. (0.5mM, 100% DMSO) + 636 µL of pre-incubated Human Plasma
- 0 min sample: 100 µL of pre-incubation mixture + 400 µL of acetonitrile with IS (Incubated for other time points 15, 30, 60, 120 and 240 min at 37°C) and proceed the same for every time points.
- Sample preparation: Vortex 5 min @ 1200 rpm and Centrifuge 10 min at 10000 rpm. Supernatant injected in to LC-MS/MS for quantification

4.4 Plasma protein binding assay

Requirement

- Test System: Plasma (Human), using RED device (PI-89809-Thermo Scientific)
- Test compound concentration: 1 µM
- Incubation time: 4 hr.
- No of replicates: Two (2)
- Phosphate Buffer Saline, pH 7.4 : 10 mM
- Final DMSO concentration: 0.01%
- Deliverables: % Binding and % Recovery
- Bio analysis: LC-MS/MS (Agilent 6410QQQ)

Procedure

- Pre-incubation: 450 µl Plasma pre-incubate for 15 min at 37˚ C
- Reaction mixture preparation: 7 µL of test cpd. (from 0.5 mM stock) + 343 µl plasma (pre-incubated)

- For 0 min sample preparation:100 µL of PBS + 100 µL reaction mix., add 300 µL of acetonitrile containing internal standard immediately, Vortexed and keep it on ice for 30 min. and then centrifuged at 10000 rpm for 10 min at 4°C.
- For 4 hr. sample preparation: In RED chamber add 200 µL of reaction mix. at the same time add 350 µL PBS buffer In WHITE chamber, seal the plate and put on an orbital shaker at 300 rpm for 4 hr. After incubation take 100 µL from RED device as well as from WHITE device and add 100 µL buffer (for RED) and 100 µL plain plasma (for WHITE) internal standard + 500 µL chilled ACN immediately to it. Vortexed it, keep it on ice for 30 min. and then centrifuged at 10000 rpm for 10 min. Supernatant injected in to LC-MS/MS for quantification.

5) Result and discussion

5.1 Aqueous Solubility

5.1.1 Kinetic Aqueous Solubility

Aim of this experiment to determine the solubility (kinetic) of the test compound (ketoconazole and quinidine).

a) Linearity plot

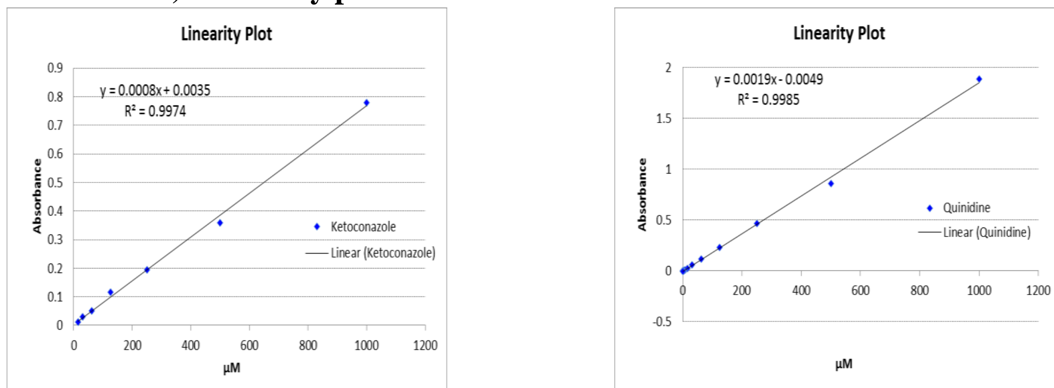

Figure 2: Linearity plot of test compound (ketoconazole and Quinidine)

b) Aqueous solubility (Quinidine)

Curve Name	Curve Formula	A	B	R2
Quinidine	Y=A*X+B	0.0019	-0.0049	0.9985

Table 1(a): formula for the measuring aqueous solubility

Test Sample		
Sample	**Value**	**Mean Result**
Quinidine	1.694	1.710
	1.72	
	1.717	
Absorbance of Quinidine at λ max 332 nm =	**1.710**	
Aqueous Solubility (µM) =	**903**	

Table: 1(b): Values of mean, λmax. and aqueous solubility

c) **Aqueous solubility:(Ketoconazole)**

Curve Name	Curve Formula	A	B	R2
Ketoconazole	Y=A*X+B	0.0008	0.0035	0.9974

Table 2 (a): formula for the measuring the aqueous solubility

Test Sample		
Sample	**Value**	**Mean Result**
KETOCONAZOLE	0.011	0.0107
	0.011	
	0.01	

Absorbance of KETOCONAZOLE at λ max 303 nm =	**0.0107**
Aqueous Solubility (μM) =	**9**

Table:2(b): Values of mean, λmax. Value and aqueous solubility

*5.1.*2 Thermodynamic Aqueous Solubility

Aim of this experiment to determine the solubility (thermodynamic) of the test compounds (Quinidine and Ketoconazole).

a) Linearity plot:

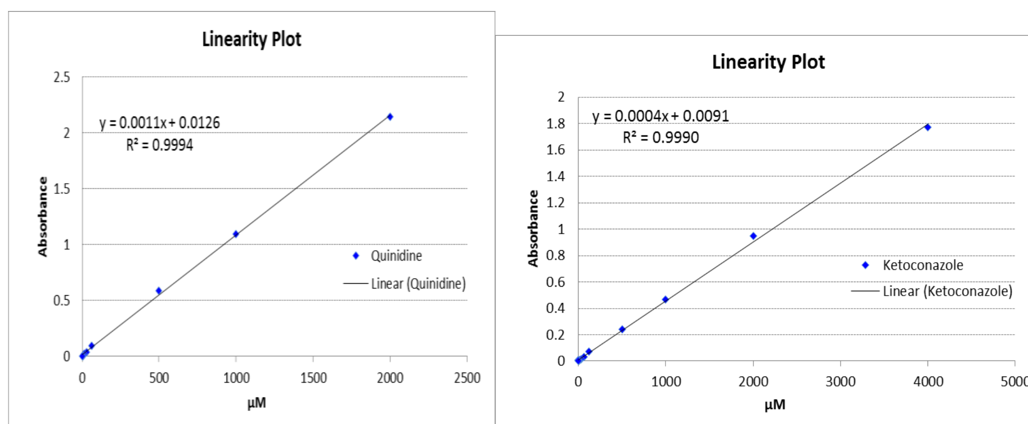

Figure 3: Linearity plot of test compound (Quinidine and ketoconazole)

a) Aqueous solubility (Quinidine)

Curve Name	Curve Formula	A	B	R2
Quinidine	Y=A*X+B	0.0004	0.0091	0.9990

Table 3(a): formula for the measuring the aqueous solubility

Test Sample			
Sample	**Value**	**Mean Result**	
Quinidine	2.288	2.307	
	2.318		
	2.316		
Absorbance of Quinidine at λ max 332 nm =			**2.307**
Aqueous Solubility (µM) =			**2086**

Table: 3(b): Values of absorbance mean, λmax. and aqueous solubility

b) Aqueous solubility (Ketoconazole)

Curve Name	Curve Formula	A	B	R2
Ketoconazole	Y=A*X+B	0.0004	0.0091	0.9990

Table 4(a): Formula for the measuring the aqueous solubility

Test Sample			
Sample	**Value**	**Mean Result**	
Ketoconazole	0.202	0.201	
	0.2		
	0.2		
Absorbance of Ketoconazole at λ max 303 nm =			**0.201**
Aqueous Solubility (µM) =			**479**

Table 4(b): Values of absorbance mean, λmax. And aqueous solubility

Figure 1 and 2 show the linearity plot of test compound (ketoconazole and quinidine) kinetic as well as thermodynamics solubility. Linearity plot help in the calculating the aqueous solubility of the compound. Linearity plot is performed with two-fold dilution, which show interception with most of the point.

Table 1(a) ,2(a), 3(a) and 4(a) shows the formula use for the calculation aqueous solubility of compound(Y=A*X+B), where A is slope is intercept and Y is mean of absorbance. Table 2(b) and 3(b) shows the result of the aqueous solubility of the quinidine and ketoconazole obtain with the help of formula (Y=A*X+B). From the above result it shows that kinetic aqueous solubility of the quinidine is 903µM, ketoconazole is 9µM as well as thermodynamic aqueous solubility of quinidine is 2086µM and for ketoconazole is 479 µM Which shows that the quinidine has the high solubility and the ketoconazole has lower solubility.

Drug can be taken in various forms such powder, solid or liquid and different route of administration like intravenous, intramuscular as well as by inhalation. Solubility is an important parameter in the early stage drug discovery. Kinetic aqueous solubility is the solubility of the compound at particular time within aqueous medium when the system is not in equilibrium state where as thermodynamic solubility is performed when system is in equilibrium state. Solubility of compound in gastrointestinal or lung fluid depend the various parameter such as physiochemical, physiological and formulation. On the basis of the concentration (1000µM kinetic) and (4000µM thermodynamics) used for linearity, Ketoconazole has lower solubility (9µM in kinetic), (479 µM in thermodynamic) which cause effect on further *in vitro* process as well as required high amount of the compound for reaching to the therapeutic target. Poor solubility cause reduction in the dissolution of compound in intestinal or lung fluid as well as increase concentration of the compound in the body fluid which increase the toxicity. Similarly, on the basis of the concentration (1000µM kinetic) and (4000µM thermodynamic) used for linearity, Quinidine has higher solubility (903µM in kinetic), (2086µM in thermodynamic) which shows that compound is highly soluble in the body fluid. Quinidine shows positive effect on solubility characteristic which helps in enhancing the further *in vitro* studies in drug discovery process as well as help in the lead optimization process. Higher solubility increases the success rate for further *in vitro* studies.

5.1.3 Formulation solubility

Name of compound: xxxx

Weighed weight of compound: 1.018 mgs

Steps	solvent	Amount(μl)	Observation
Step:1	DMSO	101.8	Soluble
Step:2	PEG400	400	Clear solution
Step:3	water	100	Clear solution
Step:4	water	100	Clear solution
Step:5	water	100	Clear solution
Step:6	water	100	Clear solution

Table: 5 Result of dissolution of compound in different solvents.

Above result table states that compound xxxx shows the solubility in different solvent and it state that above mention solvent can be used as vehicle agent's formulation *in vivo* studies. Formulation solubility is performed before the *in vivo* studies. Solubility and dissolution of drugs are the major parameter in the development of pre-formulation before selection of *in vivo* dosage. Formulation of drug is performed on various solvents such as DMSO, PEG400, water, solutol etc. Poor solubility of compound also effects in the formulation development; hence solubility play an important role in the drug discovery process.

5.2 Microsomal stability assay

Aim of this experiment is to determine the metabolic stability of the test compound (Verapamil and Diphenhydramine)

	Average Parameters (Human Liver Microsomes)						
Compound's Name	**Half-life (min)**	**% Rem @ 30 min**	**% Rem @ 60 min**	**% Rem @ 120 min**	**% Rem @ 120 min W/O Cofactor**	**CL int protein (μL/min/mg protein)**	**Classification**
Verapamil	16.96	18.72	9.15	-	99.13	81.76	High
Diphenhydramine	>120	82.39	77.57	72.84	91.19	4.45	Low

Table 5: Metabolic stability of test compounds (Verapamil and Diphenhydramine)

Above result table shows that half-life of verapamil is 16.96 min. and Diphenhydramine is >120 (min), hence verapamil is highly cleared and unstable as well as diphenhydramine has lower clearance and higher stability.

Liver is the place where almost 60% of the drug metabolism takes place. In liver, microsomes play

an important role as it contains enzymes required for drug metabolism. Metabolism and stability of the drug is important parameter for efficient effect on the target sites, more the compound is stable has more half-life and get more time for metabolism in the liver as well as lower stability causes fast excretion of the compound without reaching to the target sites. From the above result table, verapamil has a lower stability and high clearance values which excreted faster this cause lower concentration of drug to be acting on the target site with minimal effect on the target sites. Diphenhydramine shows high stability and higher half-life. For this it can be concluded that sufficient amount of diphenhydramine is present for metabolism as well as to act on the target site. But if compound is highly stable, it may be concentrated and not metabolized which may cause the toxicity therefore, moderately stable compound is preferred for any drug development.

5.3 Plasma stability assay

Aim of this experiment is to determine the stability of test compound in

	Average Parameters (Human Plasma Stability)						
Compound's Name	**Half-life (min)**	**% Rem @ 0 min**	**% Rem @ 120**	**% Rem @ 240**	**% Rem**	**% Rem**	**% Rem**

			min	**min**	**@ 360 min**	**@ 720 min**	**@ 1440 min**
Propantheline bromide	64.68	100.00	31.74	7.64	3.27	0.22	0.05

Plasma (Propantheline bromide)

Table 6: Plasma stability of test compound (Propantheline bromide)

From the above result table, it can be concluded that Propantheline bromide has half-life of 64.68 min., which shows that Propantheline bromide is moderately stable in plasma. After the administration of drug through different route, the drug is distributed to the target tissue though the circulatory system (blood). Blood has plasma which contain various types of enzymes (cholinesterase, aldolase, lipase etc.) which degrade some functional group present in drug. Functional group such as amide, ester, lactac, lactone etc. having drugs are highly prone to be degraded in the plasma. Unstable compounds are tending to have rapid clearance, short half-life, and poor *in vivo* performance which cause effect on the pharmacokinetic study as well as highly stable compound cause's concentration in plasma which may cause the toxicity effect. Above result shows that Propantheline bromide is moderately stable having half-life 64.68 min., which state that sufficient concentration is present for acting on the target site. Compound which shows neither high nor low plasma stability are preferred for further in vitro studies.

5.4 Plasma-protein binding assay

Aim of this experiment is to determine the binding affinity of protein in the plasma with test compound in plasma (Ketoconazole and Metoprolol).

Low	<90% bound
Medium	90% - 98% bound
High	98% - 99.9% bound
Very High	>99.9% bound

Table 7: classification scheme for plasma protein binding

Human Plasma Protein Binding Assay					
Compound ID	**Species / Plasma**	**% Unbound in Plasma Mean Value (n = 2) ***	**% Bound in Plasma**	**% Recovery (n = 2)**	**Classification**
Ketoconazole	Human	0.66	99.34	96.55	High
Metoprolol	Human	84.28	15.72	85.75	Low

Note:*% *Unbound is the percent of free fraction of test compound in total plasma following 4 hrs dialysis.*

Table 8: result summary of the test compound

From the above result table, it can be concluded that ketoconazole has high plasma-protein binding affinity around 99.34% and Metoprolol has low plasma-protein binding affinity 15.72. Plasma protein binding assay helps in drug-drug interaction studies. Plasma contain various types of protein such as albumin, globulin, clotting factor and regulatory protein etc. out of which albumin and α1-acid glycoprotein has high affinity for binding with drug. Drug with high protein binding efficiency has low free fraction of drug left for therapeutic purpose as well as it may cause the toxicity effect and low protein binding efficiency has vice-versa effect. Ketoconazole has high protein binding efficiency which shows that most concentration of the compound is bounded to the plasma protein as well as has a lower free fraction of drug for acting on the target sites and which may cause toxicity effect. Metoprolol has lower protein binding efficiency which shows that lower concentration of drug is bounded to plasma protein and also has higher free fraction of compound for acting on the target site.

6) Instruments used in in vitro studies

6.1 UV-Visible spectrophotometer

6.1.1 Introduction

Every chemical compound absorbs, transmits, or reflects light (electromagnetic radiation) over a certain range of wavelength. Spectrophotometry is a measurement of how much a chemical substance absorbs or transmits. It is a tool that hinges on the quantitative analysis of molecules depending on how much light is absorbed by colored compounds. Spectrophotometry uses photometers known as spectrophotometers that can measure a light beam's intensity as a function of its color (wavelength). Important features of spectrophotometers are spectral bandwidth (the range of colors it can transmit through the test sample), the percentage of sample-transmission, the logarithmic range of sample-absorption and sometimes a percentage of reflectance measurement.

A spectrophotometer is commonly used for the measurement of transmittance or reflectance of solutions, transparent or opaque solids, such as polished glass or gases. Although many biochemical are colored, as in, they absorb visible light and therefore can be measured by colorimetric procedures, even colorless biochemical can often be converted to colored compounds suitable for chromogenic color-forming reactions to yield compounds suitable for colorimetric analysis [30-37].

6.1.2 Principle

Spectroscopy is based on the Beer-Lambert's Law. This law states that whenever beam of monochromatic light is passed through a solution with an absorbing substance, the decreasing rate
of the light intensity along with the thickness of the absorbing solution is actually proportional to the concentration of the solution and the incident light.

This law is expressed through this equation:

$A = \log (I0/I) = ECI$

(A: absorbance, I0: intensity of light upon a sample cell, l: the intensity of light, C: concentration, L: length of the sample cell and E: molar absorptivity)

On the basis of the Beer-Lambert law, it has been stated that the size of molecule is directly proportional to absorbsonce of light.

6.1.3 Instrumentation

The essential components of a spectrophotometer instrumentation include:

I. A stable and cheap radiant energy source
II. A Monochromators is used to break the polychromatic radiation into component wavelength (or) bands of wavelengths.
III. Transport vessels (cuvettes) or 96-wellplate, for holding the sample
IV. A photosensitive detector
V. Readout system

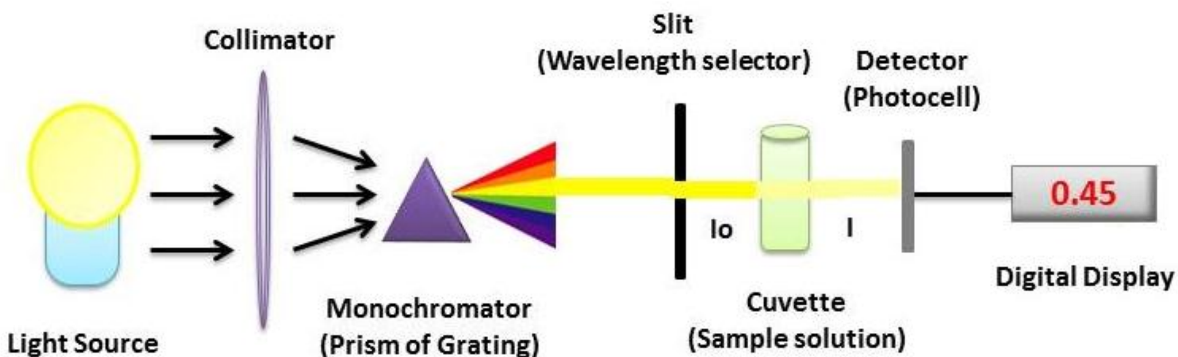

Figure 4: Basic instrumentation of a spectrophotometer source
https://www.biochemden.com/spectrophotometer-instrumentation-principle/

I. Radiation energy source

Materials which can be excited to high energy states by a high voltage electric discharge(or) by electrical heating serve as excellent radiant energy sources. Sources of Ultraviolet radiation: Most commonly used sources of UV radiation are the hydrogen lamp and the

deuterium lamp. Xenon lamp may also be used for UV radiation, but the radiation produced is not as stable as the hydrogen lamp. Sources of visible radiation: “Tungsten filament” lamp is the most commonly used source for visible radiation. It is inexpensive and emails continuous radiation in the range between 350 and 2500nm. “Carbon arc” which provides more intense visible radiation is used in a small number of commercially available instruments.

II. Wave length selectors

Filters: “Gelatin” filters are made of a layer of gelatin, colored with organic dyes and sealed between glass plates.

Monochromators: A Monochromators resolves polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands.

III. Sample Container

Sample containers are also one of the parts of Spectrophotometer instrumentation. Samples to be studied in the ultraviolet (or) visible region are usually glasses (or) solutions and are put in cells known as “CUVETTES”. Cuvettes meant for the visible region are made up of either ordinary glass (or) sometimes quartz. Most of the spectrophotometric studies are made in solutions, the solvents assume prime importance.

The most important factor in choosing the solvent is that the solvent should not absorb (optically transparent) in the same region as the solute.

IV. Detection devices

Most detectors depend on the photoelectric effect. The current is then proportional to the light intensity and therefore a measure of it. Important requirements for a detector include:

- High sensitivity to allow the detection of low levels of radiant energy
- Short response time
- Long term stability
- An electric signal which easily amplified for typical readout apparatus.

V. Amplification and Readout

Radiation detectors generate electronic signals which are proportional to the transmitter light. These signals need to be translated into a form that is easy to interpret. This is accomplished by using amplifiers, ammeters, potentiometers and potentiometric recorders.

6.1.4 Types of Spectrophotometer

- Colorimeters: In a colorimeter, a sample is exposed to a single wavelength of light, or is scanned with many different wavelengths of light. The light is in the visible band of the electromagnetic spectrum.
- IR Spectrometers: Chemists use infrared (IR) spectrometers to measure the response of a sample to infrared light. The device sends a range of IR wavelengths through the sample to record the absorbance. IR spectroscopy is also called vibrational or rotational spectroscopy because the vibrational and rotational frequencies of atoms bonded to each other, are the same as the frequencies of IR radiation.
- Atomic Spectrometers: Atomic spectrometers are used to find the elemental composition of samples and to determine the concentrations of each element.
- Mass Spectrometers: Mass spectrometers are used to analyze and identify the chemical structure of molecules, especially large and complex ones.
- UV Spectrometers: Ultraviolet (UV) spectroscopy works on a principle similar to that of colorimetry, except it uses ultraviolet light. UV spectroscopy is also called electronic spectroscopy, because the results depend on the electrons in the chemical bonds of the sample compound. Researchers use UV spectrometers to study chemical bonding and to determine the concentrations of substances (nucleic acids for example) that do not interact with visible light.
-

6.1.5 Application of Spectrophotometer

- Concentration Measurement
- Detection of Impurities
- Structure elucidation of organic compounds
- Chemical Kinetics
- Detection of functional groups
- Molecular weight determination

-

6.1.6 SOP

- Instrument i.e. Biotek EPOCH multi-plate reader was kept switch-on for auto calibration before use.
- Protocol separated in three parts

a) Lambda max search

- Range for lambda max was selected on the basis of compound appearance whether it lies between UV or Visible range.
- Lambda max was determined by graph at which optical density was maximum[OD].

b) Linearity

- 12-point serial dilution of sample was carried out from 1000µM with 2 fold serial dilution.
- Based on the lambda max value, linearity was performed.

c) **Aqueous solubity**

- After incubation of compounds aqueous solubility protocol was prepared with their individual's lambda max.
- According to protocol samples were placed into their respective wells with blank.
- Reading were taken and with the help of linearity and reading of aqueous solubility, compounds solubility was observed

6.2 LC-MS/MS

6.2.1 Introduction

Liquid chromatography-mass spectrophotometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities. It separate and measure ions based on their mass-to-charge(m/z) ratio. It is generally used for the bioanalysis purpose in *in vitro* lab, impurity profiling, detection of trace substances in a given sample [38-46].

6.2.2 Principle

LC-MS involves use of HPLC, in which individual component in a mixture are first separated followed by ionization and separation of the ions on the basis of their mass/charge ratio. The separated ion are analyzed by photo or electron multiplier tube detector which identified and quantifies each ions. The major advantage of this technology include sensitivity, specificity and precision at molecular level.

There are different types of columns to separate molecules based on various criteria:

a) Molecular exclusion columns- that separate molecules by weight, these columns use tiny beads with pores in them, this causes small molecules to take a longer route and therefore take longer to pass through
b) Normal phase liquid chromatography columns (NPLC)- that separate molecules depending on their polarity, these columns are made out of silica with polar functional groups covalently attached, polar molecules take longer to pass through these columns because they are attracted to it through dipole-dipole interactions
c) Reveres phase chromatography columns (RPLC)- they're made of silica with non-polar groups equivalently attached, here, non-polar molecules stay behind interacting with the stationary phase through Van Der Waals forces and the hydrophobic effect and take longer to pass through
d) Ionic exchange columns- that separate molecules by charge, in these columns molecules with formal charges take longer to pass through by electrostatic interactions.

Mass spectrometer contains four main components:

1. Ion source- ionization of sample take place by various ionizer such as ESI, APPI etc.
2. Mass analyzer- mass analysed based on mass/charge ratio.
3. Detector: the abundance of the separated ions is measured as an electric signal such as photo diode etc.
4. Recording device: detector signal convert into suitable form for further study.

6.2.3 Instrumentation

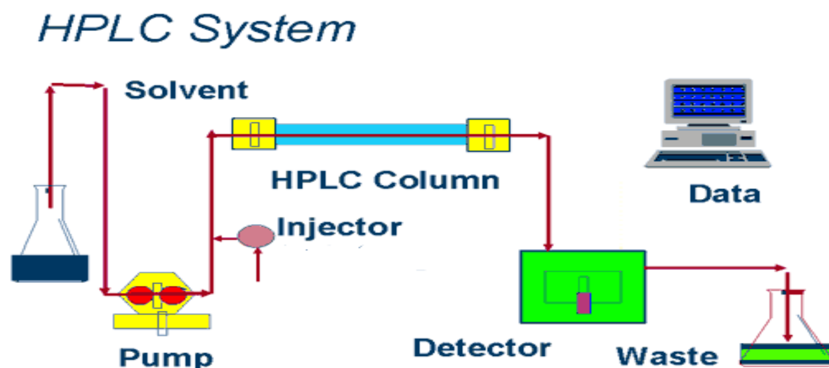

Figure 5: schematic diagram of HPLC system

http://fareastzareul1994.blogspot.com/2018/04/experiment-2-high-performance-liquid.html

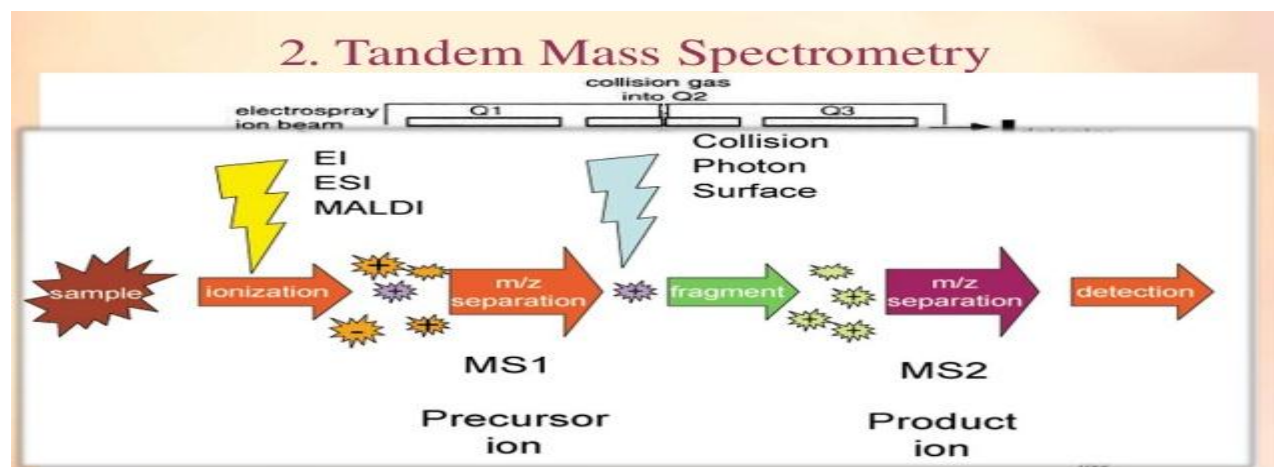

Figure 6: schematic diagram of mass spectrometry process

https://image.slidesharecdn.com/ms-150414021727-conversion-gate01/95/mass-spectrometry-in-pharmacognosy-17-638.jpg?cb=1428978594

- The parts of LC-MS/MS are as follows,
    a. Degasser: A degasser is a device used in drilling to remove gasses from drilling fluid (mobile phase) which could otherwise form bubbles.
    b. Binary/Quaternary Pump: This is the function of the pump (also called the "solvent delivery system"): to maintain a constant flow of mobile phase through the HPLC regardless of the pressure (back pressure) caused by the flow resistance of the packed column. There are two types as binary and quaternary pumps.

c. Auto-liquid Sampler: HPLC auto samplers are automated tools designed to quickly and accurately load samples to be run through a high performance liquid chromatograph so they can be analyzed by having their components separated, identified, and quantified.

d. Thermostat Controlled Column Compartment: Column is present here and used for separation of the analytes or sample and compartment can be thermostatically controlled. The reversed-phase HPLC column is the most versatile and commonly used column type and can be used for a wide range of different types of analytes. Normal-phase HPLC columns have polar packing. The mobile phase is nonpolar and therefore usually an organic solvent such as hexane or methylene chloride.

e. DAD (Diode Array Detector): Diode Array HPLC Detectors are most commonly used to record the ultraviolet and visible (UV-Vis) absorption spectra of samples that are passing through a high-pressure liquid chromatograph. An advantage to diode array HPLC detectors is the ability to select the best wavelength for analysis.

f. Tandem Mass Detector: Tandem mass spectrometry, also known as MS/MS or MS2, involves multiple steps of mass spectrometry selection, with some form of fragmentation occurring in between the stages. In a tandem mass spectrometer, ions are formed in the ion source and separated by mass-to-charge ratio in the first stage of mass spectrometry (MS1). Ions of a particular mass-to-charge ratio (precursor ions) are selected and fragment ions (product ions) are created by collision-induced dissociation, ion-molecule reaction, photo dissociation, or other process. The resulting ions are then separated and detected in a second stage of mass spectrometry (MS2).

6.2.4 SOP

- Separation of sample by using an HPLC column where the analytes are differentially partitioned between the mobile phase and the stationary phase.
- Samples are processed by using organic phase as per the optimized gradient process.
- The separated sample species are the sprayed into electron spray ionizer where it is converted to ions in the gas phase, the majority of eluent is pumped into the waste.

- Mass analyzer sorts ions according to their mass to charge ratio.
- The detector is used to count ions emerging from the mass analyzer and amplify the signal generated from the ions.
- All the mass analysis and detection is carried out under high vacuum-established using the combination of roughing and turbo molecular pumps.

6.3 Sonicator

6.3.1 SOP (47-49)

- The sonicating water bath is typically used for cleaning glassware, breaking up clumps of cells, or removing cells from surfaces. It is not usually used for cell lysis – use the probe (above) for this purpose.
- Ensure the sonicating water bath contains water to at least 5 cm depth. The water level should ideally come up to the same level as your sample
- Place samples (e.g. a falcon tube containing a cell or soil suspension) inside a glass beaker or similar vessel and fill the beaker with water to the same level as the water bath.
- Decontaminate earmuffs (supplied in the sonicator room) by wiping with 80% v/v ethanol, then put these on so they completely cover your ears.
- Place the beaker in the water bath and switch on the power (you may also need to switch the timer to 'hold'.
- Sonicate for a maximum of 5 minutes at a time. Allow the machine to rest for 5 minutes between each 5-minute burst if you require longer times.
- Switch off machine, take off earmuffs and again decontaminate these.

6.4 pH Meter

6.4.1 SOP (50-53)

pH Calibration

- Turn the meter's power on by pressing the "power" button.
- Attach the automatic temperature compensation (ATC) probe if it is available
- and/or is not with the electrode.

- Check that the measurement mode is pH. If not, press the "MODE" button until
- "pH” appears on the LCD display.
- Consult the quick reference guide at the bottom of the meter or nearby for help if needed.
- Always use fresh, unused, unexpired pH buffers for calibration. Buffers should be at the same temperature as the testing solutions.
- Rinse the pH electrode with distilled water and then with the buffer being used for calibration
- Dip the pH electrode into a neutral pH buffer (i.e., pH 7.00). Stir the buffer with a magnetic bar (at a moderate rate for ~30 s) for best results.
- Press the "CAL/MEAS" (calibration [or Standardization]/Measurement) button to select the 'calibration (standardization)' function. Set the buffer pH value on the meter display to 7.00.
- When the "reading" is stable, press the "ENTER" button to accept. The primary reading will flash briefly before the secondary display begins scrolling through the remaining available buffers.
- Rinse the pH electrode with distilled water and then with the buffer to be used for next calibration (i.e., pH 4.01).
- Dip the pH electrode into the next buffer of pH 4.01. The meter display should be locked on the buffer value.
- When the "reading" is stable, press "ENTER" to accept. The primary reading will flash briefly, then display the percent efficiency (slope) before the secondary display begins scrolling through the remaining the available buffers.
- Repeat steps previous to calibrate the pH 10.01 buffer.
- The meter will automatically return to measurement mode upon the successful completion of the 3-point calibration.
- Notes:
- The standard buffers of pH 4.01 and 10.01 may be replaced with other appropriate buffers per the testing samples' pH range.

1. For a single (neutral)-, or 2-point calibration, press the "CAL/MEAS" button to return to the measurements after completion of the calibration.
2. Calibration with more than 3 points may be used for more precise measurements.
3. It is recommended to perform the calibration at the beginning of each day. For very precise work the pH meter should be calibrated before each measurement.
4. Manually adjust the pH values of the buffers if the temperature differs from the standard room temperature and no ATC probe is attached.

***pH* Measurements**

- Confirm that the meter is on the pH measurement mode.
- Thoroughly rinse the pH electrode between measurements with distilled water to prevent carryover contamination of the tested solutions. Gently blot the electrode on
  a laboratory cleaning tissue to remove the excess rinse water. Do not rub the bulb since this can cause a static charge buildup. Alternatively, rinse the electrode with the testing solution.
- Dip the pH electrode into a testing solution or suspension. Stir the solution with a magnetic bar (~30 s) with the same stirring rate as for calibration for best results.
- The pH is completed when the pH reading is stable.
- If needed and available, press the "HOLD" button to freeze the measured reading. Press again to resume live reading.
- Record the pH value (and temperature if needed) by writing down or pressing the "MEMORY" button (if applicable) to store the value into memory.
- Repeat steps above for multiple measurements.
- Thoroughly rinse and store the electrode in storage solution once all measurements are compound.
- Notes:
  1. The pH probe response time in each buffer should be no longer than 60 s, but may be longer for some testing solutions/slurries.

2. The electrode probe should be cleaned using pH-electrode cleaning solution once a month, or whenever it is dirty. A 0.1 M HCl solution can be used for general cleaning. Diluted liquid detergent and household laundry bleach may be used for cleaning grease and bacterial contaminations. However, to avoid unexpected problems, the best practice is to always refer to the electrode manufacturer recommendations.
3. The pH electrode bulb should be moist at all times. Keep it in the electrode storage solution that comes with the electrode. Use pH 4 buffer solution if no storage solution is available. Use pH 7 buffer solution for a short time if neither are available.

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