Section (i): Introduction to drugs history

A pharmaceutical drug also referred to as a medicine or medication, officially called medicinal product, can be loosely defined as any chemical substance formulated or compounded as single active ingredient or in combination of other pharmacologically active substance. It may be in a separate but packed in a single unit or packed as combination product intended for internal or external use in the medical diagnosis, cure, treatment or prevention of disease[1-3]. The term "pharmaceutical" is derived from the Greek word pharmakeutikos (from pharmakeutēs 'druggist', from pharmakon 'drug') [4].

Drug discovery

In the fields of medicine, biotechnology and pharmacology, drug discovery is the process by which new candidate medications are discovered. Historically, drugs were discovered through identifying the active ingredient from traditional remedies or by serendipitous discovery. Later, chemical libraries of synthetic small molecules, natural products or extracts were screened in intact cells or whole organisms to identify substances that have a desirable therapeutic effect in a process known as classical pharmacology. Since sequencing of the human genome which allowed rapid cloning and synthesis of large quantities of purified proteins, it has become common practice to use high throughput screening of large compounds against isolated biological targets which are hypothesized to be disease modifying in a process known as reverse pharmacology. Hits from these screens are then tested in cells and then in animals for efficacy. Even more recently, scientists have been able to understand the shape of biological molecules at the atomic level, and to use that knowledge to design the drugs.
Modern drug discovery involves the identification of screening hits, medicinal chemistry and optimization of those hits to increase the affinity, selectivity (to reduce the potential side effects), efficacy/potency, metabolic stability (to increase the half-life), and oral bioavailability. Once a compound that fulfils all these requirements has been identified, the process of drug development will be started prior to clinical trials. One or more of these steps may, but not necessarily, involve computer-aided drug design.

Despite advances in technology and understanding of biological systems, drug discovery is still a lengthy, expensive, difficult, and inefficient process with low rate of new therapeutic discovery [5]. In 2010, the research and development cost of each new molecular entity was approximately US$1.8 billions [6]. Drug discovery is done by pharmaceutical companies, with research assistance from universities. The "final product" of drug discovery is a patent on the potential drug. The drug requires very expensive Phase I, II and III clinical trials, and most of them fail. Small companies have a critical role, often selling the rights to larger companies that have the resources to run the clinical trials.

**Drug development**

Drug development is a blanket term used to define the process of bringing a new drug to the market once a lead compound has been identified through the process of drug discovery. It includes pre-clinical research (microorganisms/animals) and clinical trials (on humans) and may include the step of obtaining regulatory approval to market the drug.

**Regulation**
The regulation of drugs varies by jurisdiction. In some countries, such as the United States of America, they are regulated at the national level by a single agency. In other jurisdictions they are regulated at state level or at both state and national levels by various bodies as in Australia. The therapeutic goods regulation is designed mainly to protect the health and safety of the population. Regulation is aimed at ensuring the safety, quality, and efficacy of the therapeutic goods which are covered under the scope of the regulation. In most jurisdictions, therapeutic goods must be registered before they are allowed to be marketed. There is usually some degree of restriction for the availability of certain therapeutic goods depending on their risk to consumers.

Depending upon the jurisdiction, medications may be divided into over-the-counter drugs (OTC) which may be available without special restrictions, and prescription only medicines (POM), which must be prescribed by a licensed medical practitioner. The precise distinction between OTC and POM depends on the legal jurisdiction. A third category, behind-the-counter medications (BTMs), is implemented in some jurisdictions. BTMs do not require a prescription, but must be kept in the dispensary, not visible to the public, and only be sold by a pharmacist or pharmacy technician. Doctors may also prescribe prescription drugs for off-label use purposes for which the drugs were not originally approved by the regulatory agency. The classification of pharmaco-therapeutic referrals helps to guide the referral process between pharmacists and doctors.

The International Narcotics Control Board of the United Nations imposes a world law of prohibition of certain medicines. They publish a lengthy list of chemicals and plants whose trade and consumption (where applicable) is forbidden. OTC medicines are sold without restriction as
they are considered safe enough that most people will not hurt themselves accidentally by taking it as instructed. Many countries, such as the United Kingdom have a third category of pharmacy medicines which can only be sold in registered pharmacies or under the supervision of a pharmacist.

**Classification of drugs**

**Classification based their origin**

Pharmaceuticals or drugs are classified on the basis of their origin.

1. Drugs from natural origin: Herbal or plant or mineral origin. Some drug substances are of marine origin.
2. Drugs from chemical as well as natural origin: Derived from partial herbal and partial chemical synthesis examples: steroidal drugs
3. Drugs derived from chemical synthesis.
4. Drugs derived from animal origin: hormones and enzymes.
5. Drugs derived from microbial origin: Antibiotics.
6. Drugs derived by biotechnology genetic-engineering and hybridoma techniques.
7. Drugs derived from radioactive substances.

**Classification based their pharmacological properties**

Drugs are classified into various groups on the basis of their pharmacological properties like mode of action and their pharmacological action or activity such as chemical properties, mode or route of administration, biological system affected or their therapeutic effects. An
elaborate and widely used classification system is the Anatomical Therapeutic Chemical Classification System (ATC system). The World Health Organization keeps a list of essential medicines.

1. Antipyretics: reducing fever (pyrexia/pyresis)
2. Analgesics: reducing pain (painkillers)
3. Antimalarial drugs: treating malaria
4. Antibiotics: inhibiting germ growth
5. Antiseptics: prevention of germ growth near burns, cuts and wounds
6. Mood stabilizers: lithium and valpromide
7. Hormone replacements: Premarin
8. Oral contraceptives: Enovid, "biphasic" pill, and "triphasic" pill
9. Stimulants: methylphenidate (Ritalin)
10. Tranquilizers: meprobamate, chlorpromazine, reserpine, chlordiazepoxide, diazepam, and alprazolam
11. Statins: lovastatin, pravastatin, and simvastatin
Section (ii): Chromatographic Instrumentation

Chromatography is a method used to purify individual chemical compounds from mixtures of compounds. It is often used for preparative applications on scales from micrograms up to kilograms. The main advantage of column chromatography is the relatively low cost and disposability of the stationary phase used in the process. The latter prevents cross-contamination and stationary phase degradation due to recycling.

The history of chromatography spans from the mid-19th century to the 21st century. Chromatography, literally "color writing" was used and named in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll (which is green) and carotenoids (which are orange and yellow). New forms of chromatography developed in 1930’s and 1940’s, made the technique useful for a wide range of separation processes and chemical analysis tasks, especially in biochemistry.

The first printed description was in 1903, in the proceedings of the Warsaw Society of Naturalists, a section of biology. The term chromatography was first used in print in 1906 by Mikhail Tsvet a Russian Scientist in his two papers about chlorophyll in the German botanical journal, Berichte der Deutschen Botanischen Gesellschaft. In 1907 he demonstrated his chromatograph for the German Botanical Society. Interestingly, Mikhail's surname "Цвет" (Tsvet) means "color" in Russian. So there is the possibility that his naming the procedure chromatography (literally "color writing") was a way that he could make sure that he, a commoner in Tsarist Russia, could be immortalized.

In a 1903 lecture (published in 1905) using filter paper, Tsvet has described the properties of living plant fibbers in his experiments on plant pigments, a precursor to paper
chromatography. He found that he could extract some pigments, such as orange carotenes and yellow xanthophylls from leaves with non-polar solvents, but others such as chlorophyll required polar solvents. He reasoned that chlorophyll was held to the plant tissue by adsorption, and that stronger solvents were necessary to overcome the adsorption. To test this, he applied dissolved pigments to filter paper, allowed the solvent to evaporate and then applied different solvents to see which could extract the pigments from the filter paper. He found the same pattern as from leaf extractions: carotene could be extracted from filter paper using non-polar solvents, but chlorophyll required polar solvents.

*Normal–phase chromatography*

Normal–phase chromatography was one of the first kind of high performance liquid chromatography (HPLC) that chemists developed. Also known as normal-phase HPLC (NP-HPLC), this method separates analytes based on their affinity for a polar stationary surface such as silica. Hence it is based on the analyte ability to engage in polar interactions (such as hydrogen-bonding or dipole-dipole type of interactions) with the sorbent surface. NP-HPLC uses a non-polar, non-aqueous mobile phase (e.g. chloroform), and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity. The interaction strength depends not only on the functional groups present in the structure of the analyte molecule, but also on steric factors. The effect of steric hindrance on interaction strength allows this method to resolve (separate) structural isomers.

The use of more polar solvents in the mobile phase will decrease the retention time of analytes, whereas more hydrophobic solvents tend to induce slower elution (increased retention
times). Very polar solvents such as traces of water in the mobile phase tend to adsorb to the solid surface of the stationary phase forming a stationary bound (water) layer which is considered to play an active role in retention. This behaviour is somewhat peculiar to normal phase chromatography because it is governed almost exclusively by an adsorptive mechanism (i.e. analytes interact with a solid surface rather than with the solvated layer of a ligand attached to the sorbent surface). Adsorption chromatography is still widely used for structural isomer separations in both column and thin-layer chromatography formats on activated (dried) silica or alumina supports [7-9].

Partition and NP-HPLC methods fall out of favour in 1970s with the development of reversed-phase HPLC because of their poor reproducibility of retention times due to the presence of water or protic organic solvent layer on the surface of the silica or alumina. This layer changes with any change in the composition of the mobile phase (e.g. moisture level) causing drifting retention times. Recently, partition chromatography has become popular again with the development of HILIC bonded phases which demonstrate improved reproducibility, and due to a better understanding of the range of usefulness of the technique.

**Displacement chromatography**

The basic principle of displacement chromatography is, a molecule with a high affinity for the chromatographic matrix (the displacer) will compete effectively for binding sites, and thus displaces all molecules with lesser affinities. There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in the form of narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels
down the column in elution mode depends on many factors. But for two substances to travel at
different speeds, and thereby be resolved, there must be substantial difference in some
interaction between the bio molecules and the chromatographic matrix. Operating parameters are
adjusted to maximize the effect of this difference. In many cases, baseline separation of the
peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks
to elution mode chromatography, especially at the preparative scale are operational complexity
due to gradient solvent pumping and low throughput due to low column loadings. Displacement
chromatography has advantages attributed to Arne Tiselius [10] who in 1943 first classified the
modes of chromatography as frontal, elution, and displacement. Displacement chromatography
found a variety of applications including isolation of transuranic elements [11] and biochemical
entities [12]. The technique was redeveloped by Csaba Horvath [13] who employed modern
high-pressure columns and equipment. It has since found many applications, particularly in the
realm of biological macromolecule purification.

**Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)**

Reversed phase high performance liquid chromatography (RP-HPLC) has a non-polar
stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase
is silica which is surface modified with RMe₂SiCl, where R is a straight chain alkyl group such
as C₁₈H₃₇ or C₈H₁₇. With such stationary phases, retention time is longer for molecules which are
less polar, while polar molecules elute more readily (early in the analysis). An investigator can
increase retention times by adding more water to the mobile phase, thereby making the affinity
of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now
more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding
more organic solvent to the eluent. RP-HPLC is so commonly used that it is often incorrectly
referred to as "HPLC" without further specification. The pharmaceutical industry regularly employs RP-HPLC to qualify drugs before their release.

RP-HPLC operates on the principle of hydrophobic interactions, which originate from the high symmetry in the dipolar water structure and plays the most important role in all processes in life science. RP-HPLC allows the measurement of these interactive forces. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand on the stationary phase. This solvophobic effect is dominated by the force of water for "cavity-reduction" around the analyte and the C$_{18}$-chain versus the complex of both. The energy released in this process is proportional to the surface tension of the eluent (water: $7.3\times10^{-6}$ Jcm$^{-2}$, methanol: $2.2\times10^{-6}$ Jcm$^{-2}$) and to the hydrophobic surface of the analyte and the ligand respectively. The retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically reducing the polarity and the surface tension of the aqueous mobile phase during the course of analysis.

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C, and generally non-polar atomic bonds, such as S-S and others) is retained longer because it is non-interacting with the water structure. On the other hand, analytes with higher polar surface area (conferred by the presence of polar groups, such as -OH, -NH$_2$, and COO$^-$ or -NH$_3^+$ in their structure) are less retained as they are better integrated into water. Such interactions are subject to steric effects that very large molecule and may have only restricted access to the pores of the stationary phase, where the interactions with surface ligands (alkyl chains) take place. Such surface hindrance typically results in less retention. Retention time increases with hydrophobic
(non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly, organic compounds with single C-C-bonds elute later than those with a C=C or C≡C bonds, as the double or triple bond is shorter than a single C-C bond.

**Ultra performance liquid chromatography instrumentation (UPLC)**

UPLC can be regarded as a new invention for liquid chromatography. UPLC refers to Ultra Performance Liquid Chromatography. UPLC brings dramatic improvements in sensitivity, resolution, and speed of analysis. It has instrumentation that operates at higher pressure than that used in HPLC, and this system uses fine particles (less than 2.5µm) and mobile phases at high linear velocities decreases the length of the column, reduces solvent consumption and saves time. This review introduces the theory of UPLC, and it summarizes some of the most recent works in the field. The schematic diagram of UPLC is shown in the Figure 2.1.

![Fig 2.1: Schematic diagram of UPLC](image-url)
According to the Van Demeter equation, as the particle size decreases to less than 2.5 µm, there is a significant gain in efficiency, while the efficiency does not diminish at increased flow rates or linear velocities [14-15]. Therefore by using smaller particles, speed and peak capacity (number of peaks resolved per unit time in gradient separations) can be extended to new limits, termed UPLC. The technology takes full advantage of chromatographic principles to run separations using columns packed with smaller particles(less than 2.5 µm) and/or higher flow rates for increased speed. This gives superior resolution and sensitivity [16-17]. Now a days in industrial area UPLC refers for some of the most recent work field.

**Principle of UPLC:**

The UPLC is based on the principal of use of stationary phase consisting of particles less than 2.5 µm (while HPLC columns are typically filled with particles of 3 to 5 µm). The underlying principles of this evolution are governed by the Van Deemter equation [18], which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency).

\[
H = A + \frac{B}{v} + Cv
\]

Where;

- \( A, B \) and \( C \) are constants
- \( v \) is the linear velocity, the carrier gas flow rate.

- The \( A \) term is independent of velocity and represents "eddy" mixing. It is smallest when the packed column particles are small and uniform.
- The \( B \) term represents axial diffusion or the natural diffusion tendency of molecules. This effect is diminished at high flow rates and so this term is divided by \( v \).
The $C$ term is due to kinetic resistance to equilibrium in the separation process. The kinetic resistance is the time lag involved in moving from the gas phase to the packing stationary phase and back again. The greater the flow of gas, the more a molecule on the packing tends to lag behind molecules in the mobile phase. Thus term is proportional to $v$.

**Instrumentation:**

**Sample injection:**

In UPLC, sample introduction is critical. Conventional injection valves, either automated or manual, are not designed and hardened to work at extreme pressure. To protect the column from extreme pressure fluctuations, the injection process must be relatively pulse-free and the swept volume of the device also needs to be minimal to reduce potential band spreading. A fast injection cycle time is needed to fully capitalize on the speed afforded by UPLC, which in turn requires a high sample capacity. Low volume injections with minimal carryover are also required to increase sensitivity. There are also direct injection approaches for biological samples. The schematic diagram of binary solvent manager and sample manager are shown in Figures 2.2 and 2.3.
Injection technique

Three injection methods are available:
1. **Full Loop Injection** Method/Mode as the injection technique
   - Inject 100% of nominal loop volume

2. **Partial Loop Injection** Method/Mode using “**Needle Overfill**” as the injection technique
   - Can inject from 10% to 80% of total loop volume

3. **Partial Loop Injection** Method/Mode using “**Pressure Assist**” as the injection technique
   - Can inject from 10% to 50% of total loop volume

1. **Full loop injection Mode**

   ![Typical two position 6 port valve making a Full loop Injection]

   **Fig 2.4: Full loop injection mode**

   The full loop mode (Figure 2.4) is the preferred technique when performing fast, qualitative analysis and whenever accuracy and precision are the primary concerns. We can use all sample loop sizes. Smaller loops require larger overfill factors than larger loops. A different injection volume requires a different loop mentioned in Table 2.1.

   **Table 2.1: Default overfill factors for ACQUITY UPLC sample loops**
The sample and air gap are aspirated with the sample loop in the load position. The metering syringe actuates drawing into the tip of the sample needle an air gap and a sample volume equal to the specified injection volume times the overfill factor (Example: For a 20 µL full loop injection using a 4 X overfill factor, a total of 80 µL is drawn). The system pressurizes to 150 psi and compresses the air gaps before and after the sample. The sample volume flows into and through the sample loop (Example: For a 20 µL full loop injection using a 4 x overfill factor, 20 µL of sample is centered in the loop, with 5 µL before the sample loop and 55 µL after the sample loop). The valve actuates switching the sample loop to the injection position. Mobile phase from the solvent manager carries the sample to the column. The wash cycle follows injection.
2. **Partial loop mode using “Needle Overfill”**

![Diagram of partial loop using needle overfill](image)

The Pre-Sample cushion, the sample, and the Post Sample cushion are aspirated from the sample vial, while the leading air gap is aspirated from the home position.

This mode (Figure 2.5) is the best general purpose mode for partial loop injection. It provides the best partial loop accuracy, precision and linearity for samples including strong and weak acids and bases, hydrophilic compounds and hydrophobic compounds. This mode is the first choice, except where clear indications for the other modes exist or sample volume is limited. In this mode only mobile phase and sample are injected into the column. Air gaps or weak wash solvent are not injected into the column. Cycle time depends on aspiration rate. Smaller loops require more time. It performs optically when injection volumes are maintained within a range 10 to 75 % of nominal loop volume. This mode requires more sample than the partial loop mode.

With the sample loop in the injection position, the metering syringe actuates drawing into the tip of the sample needle an air gap and a sample volume equal to the specified injection volume plus the needle overfill flush volume. The metering syringe continues to pull the sample...
aliquot through the sample needle and through the injection valve until the pre-sample and sample injector volume passes through the injection valve. The valve actuates switching the sample loop to the load position. Then the sample volume is pushed into the sample loop. The valve actuates bringing the sample loop into the injection position. The sample is loaded into the column. No weak wash injected into column (mobile phase and sample injected) and sample does not come into contact with weak wash. This is recommended for partial loop injections, especially from small loops because accuracy is improved compared to pressure assist partial loop injections.

3. **Partial loop injection mode with “Pressure Assist”**

![Diagram](image)

Fig 2.6: **Partial Loop using Pressure Assist**

The partial loop with pressure assist injection mode (Figure 2.6) is used for peptide analysis and for those situations where the sample volume is very limited, where analysis time takes precedence over any other concern, or where the injection volume is very large. This mode
has a shorter cycle time than partial loop with needle overfill mode. The injection range is lower for each loop size and the performance depends on how well the weak wash solvent matches the mobile phase and the sample diluent. The injection volume is maintained in the range 10 – 50% of the loop volume. The sample must be able to fully dissolve in the weak wash solvent. When the analysis time is the primary goal, the preferable mode is the injection volume should be large and sample volume is to be limited. For large scale loop injections, 20 and 50 µL loops are recommended.

With the sample loop in the load position, the metering syringe actuates drawing into the tip of the sample needle an air gap and a sample volume equal to the specified injection volume. The system pressurizes to 150 psi and compresses the air gaps before and after the sample. The sample volume is pushed into the sample loop and the valve actuates bringing the sample loop into the injection position. The sample is loaded into the column and the wash cycle follows injection. In this mode accuracy is generally lower when compared to partial loop with needle overfill.

**Washing the sample needle**

Washing the needle is an optional procedure, helpful when suspect a carryover problem. It flushes strong and/or weak wash solvent through the needle and injection port, removing contaminants from inside and outside the sample needle, the external piercing needle, and the injection port.

**UPLC Columns**

By using 1.7 µm particle packed columns (Figure 2.7), the resolution of chromatographic separation can be increased which is the primary principle of the UPLC method. Separation of
the components of a sample requires a bonded phase that provides both retention and selectivity. Four bonded phases are available for UPLC separations: ACQUITY UPLC™ BEH C$_{18}$ and C$_{8}$ (straight chain alkyl columns), ACQUITY UPLC BEH Shield RP$_{18}$ and ACQUITY UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C$_{6}$ alkyl). Each column chemistry provides a different combination of hydrophobicity, silanol activity, hydrolytic stability and chemical interaction with analytes.

**Fig 2.7: UPLC columns**

ACQUITY UPLC BEH C$_{18}$ and C$_{8}$ columns are considered as the universal columns of choice for most UPLC separations by providing the widest pH range. They incorporate trifunctional ligand bonding chemistries which produce superior low pH stability. This low pH stability is combined with the high pH stability of the 1.7 µm BEH particles to deliver the widest usable pH operating range. ACQUITY UPLC BEH Shield RP$_{18}$ columns are designed to provide selectivity that complements the ACQUITY UPLC BEH C$_{18}$ and C$_{8}$ phases. ACQUITY UPLC BEH Phenyl columns utilize a tri functional C$_{6}$ alkyl tether between the phenyl ring and the silyl functionality. This ligand, combined with the same proprietary end capping processes as the ACQUITY UPLC BEH C$_{18}$ and C$_{8}$ columns, provides long column lifetimes and excellent peak shape. This unique combination of ligand and end capping on the 1.7 µm BEH particle creates a new dimension in selectivity allowing a quick match to the existing HPLC column.
An internal dimension (ID) of 2.1 mm column is used. For maximum resolution, a 100 mm length is chosen and for faster analysis and higher sample throughput, 50 mm column is preferable. Half-height peak widths of less than one second are obtained with 1.7µm particles, which gives significant challenges for the detector. In order to integrate an analyte peak accurately and reproducibly, the detector sampling rate must be high enough to capture enough data points across the peak. The detector cell must have minimal dispersion (volume) to preserve separation efficiency. Conceptually, the sensitivity increase for UPLC detection should be 2-3 times higher than HPLC separations, depending on the detection technique. Mass spectral detection is significantly enhanced by UPLC; increased peak concentrations with reduced chromatographic dispersion at lower flow rates promote increased source ionization efficiencies.

The ACQUITY UPLC system consists of a binary solvent manager, sample manager including the column heater, detector, and optional sample organizer. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient. There are built-in solvent select valves to choose from up to four solvents. There is a 15,000 psi pressure limit (about 1000 bar) to take full advantage of the sub-2µm particles. The sample manager also incorporates several technology advancements. Using pressure assisted sample introduction, low dispersion is maintained through the injection process, and a series of pressure transducers facilitate self-monitoring and diagnostics. It uses needle-in-needle sampling for improved ruggedness and needle calibration sensor increases accuracy. Injection cycle time is 25 seconds without a wash and 60 sec with a dual wash used to further decrease carry over. A variety of micro titer plate formats (deep well, mid height, or vials) can also be accommodated in a thermostatically controlled environment. Using the optional sample organizer, the sample
manager can inject up to 22 micro titer plates. The sample manager also controls the column heater. Column temperatures up to 65°C can be attained. To minimize sample dispersion, a “pivot out” design allows the column outlet to be placed in closer proximity to the source inlet of an MS detector.

**Detectors**

For UPLC detection, the tunable UV/Visible detector is used which includes new electronics and firmware to support Ethernet communications at the high data rates. Conventional absorbance-based optical detectors are concentration sensitive detectors, and for UPLC use, the flow cell volume would have to be reduced in standard UV/Visible detectors to maintain concentration and signal. According to Beer’s Law, smaller volume conventional flow cells would also reduce the path length upon which the signal strength depends. A reduction in cross-section means the light path is reduced, and transmission drops with increasing noise. Therefore, if a conventional HPLC flow cell were used, UPLC sensitivity would be compromised. The ACQUITY tunable UV/Visible detector cell consists of a light guided flow cell equivalent to an optical fiber. Light is efficiently transferred down the flow cell in an internal reflectance mode that still maintains a 10 mm flow cell path length. Tubing and connections in the system are efficiently routed to maintain low dispersion and to take advantage of leak detectors that interact with the software to alert the user to potential problems [19-26]. The schematic diagram of PDA detector is shown in Figure 2.8.
Comparison between HPLC and UPLC

The characteristics of HPLC and UPLC and advantages of UPLC over HPLC are summarized in Table 2.2.

Table 2.2: Differences between HPLC and UPLC

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HPLC</th>
<th>UPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size</td>
<td>&lt;4 µm</td>
<td>1.7 µm</td>
</tr>
<tr>
<td>Maximum backpressure</td>
<td>35-40 MPa</td>
<td>103.5 MPa</td>
</tr>
<tr>
<td>Analytical column</td>
<td>Alltima C18</td>
<td>Acquity UPLC BEH C18</td>
</tr>
<tr>
<td>Column dimensions</td>
<td>150 X 3.2 mm</td>
<td>150 X 2.1 mm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20µL</td>
<td>3-5µL</td>
</tr>
<tr>
<td>Pressure limit</td>
<td>Up to 4000 psi</td>
<td>15000 psi</td>
</tr>
<tr>
<td>Total run time</td>
<td>10 min</td>
<td>1.5 min</td>
</tr>
</tbody>
</table>
**Types of UPLC instruments by Waters**

*The ACQUITY UPLC I*

This instrument provides the most powerful solution to the most critical need in separation science today – successfully analyzing compounds that are limited in amount or availability amid a complex matrix, more rapidly than ever before. The instrument is developed to produce the most accurate and reproducible separations, getting the most information possible and accelerate laboratory results. Complex separation challenges require LC systems designed to maximize the benefits of sub-2-µm particle columns integrated in a system designed to optimize performance.

*The ACQUITY UPLC H*

It is a streamlined system that brings together the flexibility and simplicity of quaternary solvent blending and a flow-through-needle injector to deliver the advanced performance expected of UPLC type separations – high resolution, sensitivity and improved throughput while maintaining the robustness and reliability that ACQUITY systems are known for. Choosing the ACQUITY UPLC H enables to continue running existing HPLC methods on a forward looking LC platform that allows to confidently and seamlessly transition to UPLC separations, using integrated system tools and reliable column kits for method transfer and method development that simplify migration.

*Nano ACQUITY Ultra Performance LC (UPLC) System*

It is designed for nano-scale, capillary, and narrow-bore separations to attain the highest chromatographic resolution, sensitivity, and reproducibility. Direct nano-flow offers significant improvements over conventional nano-flow separations and technologies. It improves
peak capacity and peak shape, and increases the number of components that can be detected per separation. The system’s 10,000 psi operating pressure capability allows for superior high-peak capacity separations by operating longer columns packed with sub-2 micron particles. It is optimized for high-resolution identification and 2D-LC separations at precise nano flow rates. The nano ACQUITY UPLC System provides solutions for biomarker discovery and proteomics applications, for protein identification and characterization.

The PATROL™ UPLC® Process Analyzer

This is a real-time Process Analytical Technology (PAT) system that detects and quantifies complex multiple component manufacturing samples and final product directly on the production floor. Designed with the same enabling technology that drives the ACQUITY UPLC® System, PATROL UPLC moves existing liquid chromatography (LC) analysis from off-line Quality Control (QC) laboratories directly to the manufacturing process, resulting in significant improvements in production efficiency. It also reduces process cycle times, so that more product can be produced with existing resources which enables manufactures to produce more material that consistently meet or exceed the product specifications, potentially eliminating variability, failed batches or the need to re-work material assuring the end-product quality, including final release testing.

The PATROL UPLC process analyzer is an ideal solution for pharmaceutical, biopharmaceutical, petrochemical, and food manufacturers that are under increased internal and external pressure to evaluate PAT programs and techniques. Global regulatory initiatives, such as the U.S. Food and Drug Administration and European Medicines Agency Critical Path and PAT Initiatives and manufacturing quality-by-design programs, such as Six Sigma, are driving corporations to assess and implement novel PAT solutions such as the PATROL UPLC System.
Advantages of UPLC

Various advantages of UPLC are as follows:

• Decreases run time and increases sensitivity.
• Provides the selectivity, sensitivity, and dynamic range of LC analysis.
• Maintains resolution performance.
• Expands scope of Multi residue Methods.
• UPLC’s fast resolving power quickly quantifies related and unrelated compounds.
• Faster analysis through the use of a novel separation material of very fine particle size.
• Operation cost is reduced.
• Less solvent consumption.
• Reduces process cycle times, so that more product can be produced with existing resources.
• Delivers real-time analysis in step with manufacturing processes.
• Increases sample throughput and enables manufacturers to produce more material that consistently meet or exceeds the product specifications, potentially eliminating variability, failed batches, or the need to re-work material.
• Assures end-product quality, including final release testing [26].

Disadvantages of UPLC

Due to increased pressure required, the maintenance cost is more and reduces the life of the columns of these types. So far performances similar or even higher have been demonstrated by using stationary phases of size around 2 µm without the adverse effects of high pressure. In addition, the phases of less than 2 µm are generally non-re-generable and thus have limited use [26].
To improve the separation results of HPLC, various modifications have been brought in UPLC. It improved in three areas: chromatographic resolution, speed and sensitivity of analysis. It uses fine particles and saves time and reduces solvent consumption. The specific advantages of UPLC over HPLC are given in Table 2.3.

Table 2.3: *Comparison between HPLC and UPLC*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Description</th>
<th>HPLC</th>
<th>UPLC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Pump</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Flow Rate</td>
<td>0.05 - 5 mL min^-1</td>
<td>0.001-2 mL min^-1</td>
<td>Design to work at very high precision at lower flow rates</td>
</tr>
<tr>
<td>2</td>
<td>Backpressure</td>
<td>5000 psi</td>
<td>Upto15000 psi</td>
<td>Can take sub2 micron particle size very effectively. Lower the particle size more the surface area and better separation</td>
</tr>
<tr>
<td>3</td>
<td>Delay volume</td>
<td>650 uL</td>
<td>120 uL</td>
<td>Lower the delay volume more sharper the peak and help to increase resolution</td>
</tr>
<tr>
<td>4</td>
<td>Pump design</td>
<td>Quaternary</td>
<td>Binary with four solvent options</td>
<td>Automatic switch over valve helps to play with four solvent effectively</td>
</tr>
<tr>
<td></td>
<td><strong>Injection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Auto sampler</td>
<td>120, 2 mL vials</td>
<td>384 microtiter plates or 96, 2mL vials max 8,448 samples with optional sample organizer</td>
<td>Design to give very high throughput</td>
</tr>
<tr>
<td>2</td>
<td>Injection volume range</td>
<td>0.1 - 100 uL</td>
<td>0.1 - 50 uL in 0.1uL increment</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Carry over</td>
<td>less than 0.1 %</td>
<td>less than 0.005 %</td>
<td>Very high precision at lower injection volume and very less carry over at there are two wash mode strong and weak</td>
</tr>
<tr>
<td>S.No.</td>
<td>Description</td>
<td>HPLC</td>
<td>UPLC</td>
<td>Remarks</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td></td>
<td><strong>Detector</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Wavelength range</td>
<td>190-700 nm</td>
<td>190-500 nm</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Optical resolution</td>
<td>1.2 nm</td>
<td>1.2 nm</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Baseline noise</td>
<td>3.0*10^{-5} AU</td>
<td>10*10^{-6} AU</td>
<td>Very low noise and better signal to noise ratio</td>
</tr>
<tr>
<td>4</td>
<td>Cell volume</td>
<td>8uL</td>
<td>500 nL</td>
<td>Light guided flow cell gives detector better signal to noise ratio and hence better sensitivity</td>
</tr>
<tr>
<td>5</td>
<td>Acquisition speed</td>
<td>20-80 hz</td>
<td>upto 80hz</td>
<td>Faster actuation allows the detector to detect the peaks hence increases speed</td>
</tr>
<tr>
<td></td>
<td><strong>Column chemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Oven</td>
<td>10-60 °C</td>
<td>10-90 °C</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Size</td>
<td>20 to 250 mm length</td>
<td>20-150 mm length</td>
<td>Can be used as normal HPLC application also</td>
</tr>
<tr>
<td>3</td>
<td>E card facility</td>
<td>Not available</td>
<td>Available</td>
<td>Tracks the history of the column and all QC documents are stored in the chip</td>
</tr>
<tr>
<td>4</td>
<td>Particle size</td>
<td>2.1 µm to 10 µm or more</td>
<td>1.7µm to 10 µm or more</td>
<td>Lower particle size gives the UPLC options</td>
</tr>
</tbody>
</table>
References

1. Definition and classification of drug or pharmaceutical regulatory aspects of drug approval, 2013.


5. Anson, Blake D, Ma Junyi, He Jia-Qiang, Identifying cardiotoxic compounds, Genetic Engineering & Biotechnology News, 2009; 34.


10. A. Tiselius, Displacement development in adsorption analysis, 1943.


