

THERAPEUTIC DRUG MONITORING AND HPLC: A REVIEW

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Introduction

Modern therapeutics involves the use of drugs for the treatment of the disease states. The goal of therapeutics is to achieve a desired beneficial effect with minimal adverse effects.¹ The fundamental tenet of clinical pharmacokinetics is that a relationship exists between the pharmacological effects of a drug and an accessible concentration of the drug (e.g., in blood or plasma). This relationship has been documented for many drugs and is of benefit in the therapeutic management of patients. In most cases, the concentration of drug at its sites of action will be related to the concentration of drug in the systemic circulation. The pharmacological effect that results may be the clinical effect desired, a toxic effect, or in some cases an effect unrelated to the known therapeutic efficacy or toxicity. Clinical pharmacokinetics attempts to provide both a quantitative relationship between dose and effect and a framework within which to interpret measurements of concentrations of drugs in biological fluids and their adjustment through changes in dosing for the benefit of the patient.² Each drug exhibits its own therapeutic range below the lower limit of which the drug doesn't show any response and above which shows the signs of toxicity.

What is TDM?

Therapeutic drug monitoring (TDM) is a measurement made in laboratory of a parameter which, with appropriate interpretation, will directly influence prescribing procedures. Commonly the measurement in a biological matrix (plasma, serum, blood, saliva, urine) is of a prescribed xenobiotic, but it may also be of an endogenous compound prescribed as a replacement therapy in an individual who is physiologically or pathologically deficient in that compound.³

From a clinical standpoint, the goals of TDM are to assist the clinical team managing the patient in optimising pharmacotherapy to maximise efficacy while minimising drug related toxicity. The basic assumption of TDM is that the circulating concentration of a drug correlates better with pharmacological effect than does the dosage of the drug given to the patient. Like any diagnostic test, the measurement of plasma level is justified only when the information provided is of potential therapeutic benefit. The clinical value of plasma level monitoring depends on how precisely the treatment outcome can be defined. When a therapeutic outcome can be objectively and replicably quantified, such as during antithrombotic therapy with coumarin derivatives, little additional information is gained by plasma levels. On the other hand when a precise therapeutic end point is difficult to define monitoring of drug levels may be of considerable therapeutic assistance.

What are the causes for variations in drug response?

There are two major sources of variability between individual patients in drug response

- 1) **Pharmacokinetic variability**⁴ (dose and plasma concentration variation)
- 2) **Pharmacodynamic variability**⁴ (variation in drug concentration at the receptor and the response). Although it causes variation in drug response, it does not alter the concentration of drug in plasma.

Factors affecting the concentration of drug in the plasma (mainly pharmacokinetic factors):

- 1) **Patient compliance**⁴: this is a major issue in long term therapy. Due to lack of information and understanding about the disease, unpleasant side-effects, fear of addiction, conflicting medical advice and in many instances patients stop taking medications on their own when they have decreased seizure frequency/ or become fit free(in case of seizures) and then falsely believe that they are cured.

2) **Age:** neonates eliminate drug slowly, children eliminate drug twice as fast as adults, and elderly eliminate drugs at a slower rate than adults. This produces definite variation in plasma levels of the drug.

3) **Physiology:** females have smaller body size and weight and need lower doses, they also have greater body fat and hence occasionally drugs which are redistributed into fat will show delayed release and toxicity, pregnancy also leads to altered pharmacokinetics.

4) **Pharmacokinetics:** Pharmacokinetics includes a sequence of processes occurring in succession from the point of drug dosing till excretion.

Absorption: Absorption of the drug is an important variable that depends on the dosage of the drug, dosage form of the drug, the time of the day when the drug is taken (chronopharmacology), the release rate from the drug formulation (slow release & extended release) and solubility of the drug (lipid soluble drugs are better absorbed than water soluble drugs).

Distribution: Once the drug is absorbed it is distributed in the serum transported in blood bound to plasma proteins. Only the free form of the drug will be distributed and reach the target site. Any alteration with the amount of plasma proteins affects the concentration of the drug in the plasma.

Metabolism: Significant factors that affect serum drug levels include the liver metabolism and renal excretion of the drug and its metabolites. Smoking, alcohol and presence of other drugs (steroid hormones) also affect the concentration of drug by means of metabolism due to induction of enzyme systems in liver leading to decrease in plasma levels. Also few drugs (ketoconazole) inhibit enzyme systems to cause increase in plasma levels.

Clearance⁴: Most drugs are excreted by either through biliary route or urinary route, so any disease affecting liver and biliary tract and kidneys and urinary tract alter the excretion kinetics, leading to increase in serum levels of the same.

5) **Disease:** hepatic, renal and cardiovascular and gastrointestinal disorders affect drug levels by hampering absorption or elimination of the drugs.

6) **Drug interactions:** in a developing country like ours it is essential to ascertain whether patient is taking concomitant medications in the form of ayurvedic, homeopathic, unani which contains preparations which alter the pharmacokinetic profiles of the drug in question or interact to change the blood levels.

A well known example is that of '*shankapusphi*' a memory enhancer when taken in a patient on phenytoin lead to spontaneous loss of seizure control due to induction of CYP450 enzyme systems metabolising phenytoin at faster rates.

7) **Interpopulation(ethnic variations):** the standard therapeutic ranges for interpretation of TDM data are derived from population studies done in developed world. Normograms used for dose adjustment is based on pharmacokinetic data obtained in developed countries. Studies have found that effective anticonvulsant dosage may be lower by 10-15% in Indians than Europeans.

8) **System of medicine and quality of medications:** it has been found that ayurvedic drugs contain phenytoin and phenobarbitone. Hence it is essential to elicit from the patient any past history regarding use of alternative medications. Also use of substandard generic drugs may affect the bioavailability of drug.

9) **Genetic polymorphisms:** phenotyping methods have identified that there are fast metabolisers and slow metabolisers e.g: phenytoin(CYP2C9,CYP2C19), isoniazide, nortriptyline, imipramine.

When is Therapeutic drug monitoring useful?

TDM is useful when the following criteria are met namely,

- 1) the drug in question has narrow therapeutic range.
- 2) a direct relationship exists between the drug or drug metabolite levels in plasma and the pharmacological or toxic effects,
- 3) the therapeutic effect cannot be readily assessed by the clinical observation,
- 4) large individual variability in steady state plasma concentration exists at any given dose
- 5) appropriate analytic techniques are available to determine the drug and metabolite levels.

TDM is unnecessary when,

- 1) clinical outcome is unrelated either to dose or to plasma concentration
- 2) dosage need not be individualized
- 3) the pharmacological effects can be clinically quantified
- 4) when concentration effect relationship remains unestablished,
- 5) drugs with wide therapeutic range such as beta blockers and calcium channel blockers.

What are the major indications for which TDM is done? ^{5,6}

While there may be specific individual circumstances for TDM, most indications can be summarized as follows:

1. Low therapeutic index
2. Poorly defined clinical end point
3. Non compliance
4. Therapeutic failure
5. Drugs with saturable metabolism
6. Wide variation in the metabolism of drugs
7. Major organ failure
8. Prevention of adverse drug effects.

For which all drugs has TDM found to have an established value?

TDM has been found to have a significant value when treating various disorders encountering the following drugs,

1. Cardiovascular drugs⁷: Amiodarone, Digoxin, Digitoxin, Disopyramide, Lignocaine, Procainamide, Propranolol and Quinidine
2. Antibiotics⁸: Gentamycin, Amikacin and Tobramycin
3. Antidepressants: Lithium and Tricyclic antidepressants
4. Antiepileptic drugs⁹: Phenytoin, phenobarbitone benzodiazepines, carbamazepine, Valproic acid and Ethosuximide
5. Bronchodilators: Theophylline
6. Anti-Cancer drugs: Methotrexate
7. Immunosuppressives: Cyclosporine

How do we do TDM?

TDM is measurement of the plasma concentration of the drug after a dose is given by any route and subsequent sample collection(usually plasma) and its estimation by any of the methods narrated below,

1) Chemical assays:

- 1) Spectrophotometry
- 2) Chromatography

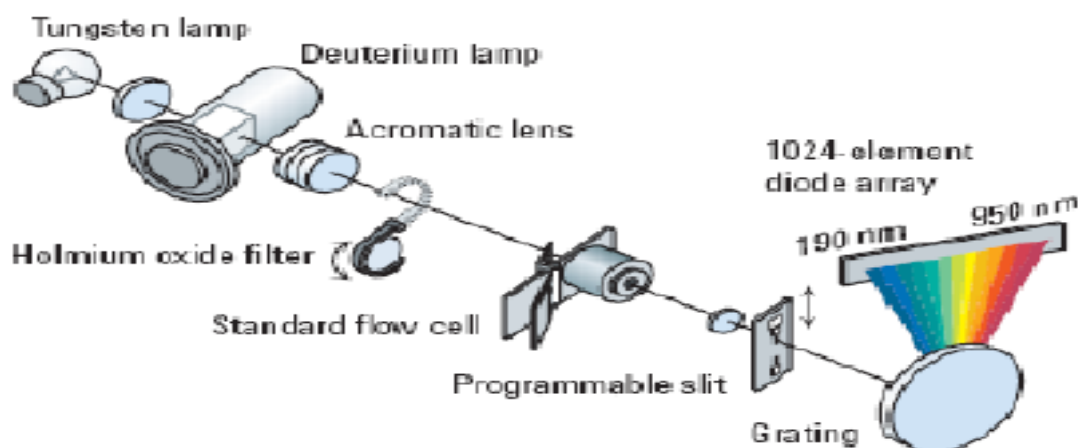
2) Immunological assays:

- 1) Radio immuno assay (RIA)
- 2) Enzyme immuno assay (EIA)
- 3) Fluorescence polarisation immunoassay (FPIA)

Spectrophotometry : the principle of spectrophotometry is scattering of the incoming rays of light into various components by the analyte (plasma containing the drug in question) and measuring the amount of scattering of the constituent wavelengths at the receiver end of the photometer.

Based on the type of light source we have,

1. Visible light spectrophotometry
2. Ultra violet spectrophotometry
3. Infra red spectrophotometry
4. Fluorescence spectrophotometry
5. Flame photometry



Prior to advent of GLC and HPLC, drug samples were analyzed by spectrophotometric methods. Solvent extraction schemes coupled with a spectrophotometric finish can still provide a much derived simplicity in assay procedure when the level of sensitivity required is not too low. i.e. in the $\mu\text{g}/\text{ml}$ range. However the drawbacks are large volume of samples, complex extraction procedures and interference by other compounds.

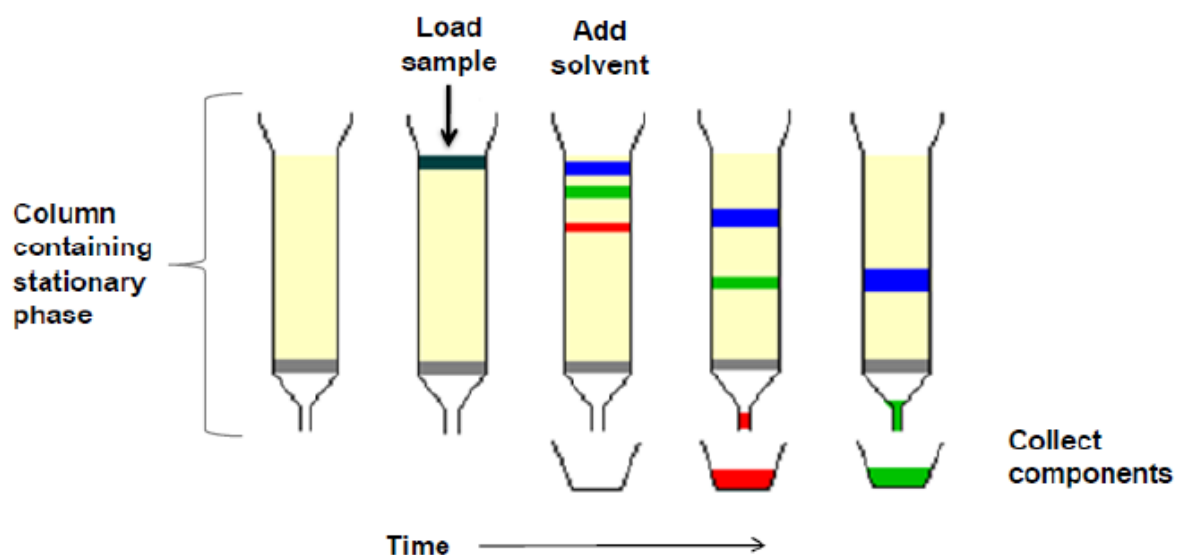
Chromatography: is based on different retention times of the analytes in a suitably packed column, when flushed with a suitable carrier or eluant (solvent).

- Types: **1) Gas-Liquid Chromatography (GLC)**
2) High Performance Liquid Chromatography (HPLC)
3) Liquid Solid Chromatography (LSC)
4) Partition Chromatography (PC)

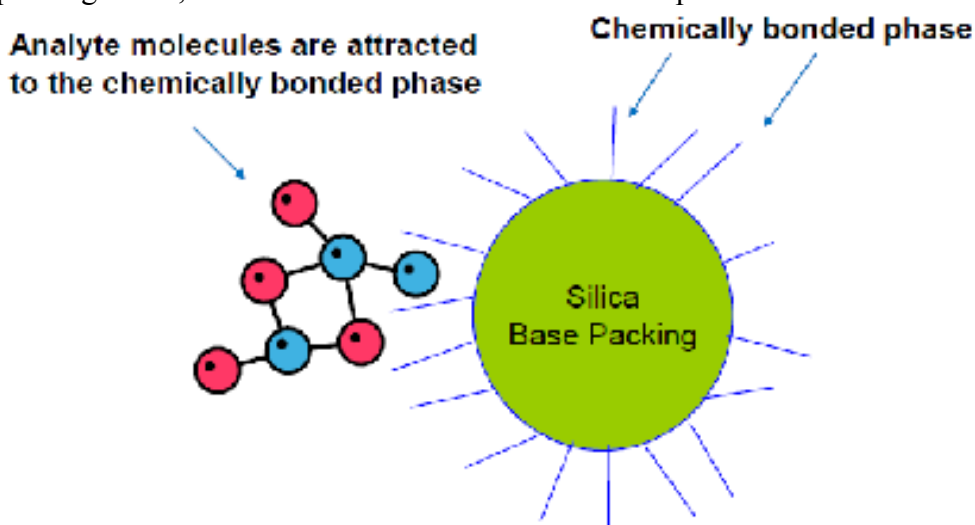
Among the above types, HPLC is the most frequently used method to determine drug concentrations for various reasons, hence HPLC will be discussed in detail in subsequent pages.

High Performance Liquid Chromatography (HPLC)^{5,6}:**1. Introduction HPLC.**

High Performance Liquid Chromatography (HPLC) is one mode of chromatography, one of the most used analytical techniques. Chromatographic process can be defined as separation technique involving mass-transfer between stationary and mobile phase. HPLC utilises a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

**2. Theory**

HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing beads, tend to interact with the surface adsorption sites.



Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process:

Hydrophobic (non-specific) interactions are the main ones in **reversed-phase (RP)**.

Dipole-dipole (polar) interactions are dominant in **normal phase (NP)** mode.

Ionic interactions are responsible for the retention in **ion-exchange** chromatography.

All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface. The weaker the eluent interaction, the longer the analyte will be retained on the surface.

SEC (size-exclusion chromatography) is another case. It is the separation of the mixture by the molecular size of its components. The basic principle of SEC separation is that the bigger the molecule, the less possibility there is for it to penetrate into the adsorbent pore space. So, the bigger the molecule the less it will be retained.

3. Types of HPLC

There are many ways to classify liquid column chromatography. If this classification is based on the nature of the stationary phase and the separation process, three modes can be specified.

a. Adsorption chromatography: the stationary phase is an adsorbent (like silica gel or any other silica based packing) and the separation is based on repeated adsorption-desorption steps.

b. Ion-exchange chromatography: the stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

c. Size exclusion chromatography: the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size.

Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later. This technique is also called gel filtration or gel permeation chromatography.

Concerning the first type, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography.

1) In **normal phase chromatography**, the stationary bed is strongly polar in nature (e.g. silica gel), and the mobile phase is nonpolar (such as n-hexane). Polar samples are thus retained on the polar surface of the column packing for longer than less polar materials.

2) **Reversed-phase chromatography** is the inverse of this. The stationary bed is (nonpolar) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained.

Reverse phase chromatography is used for almost 90% of all chromatographic applications.

Eluent polarity plays the major role in all types of HPLC. There are two elution types: isocratic and gradient. In the first type, constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run.

HPLC as compared with the classical LC technique is characterised by:

- _ high resolution
- _ small diameter (4.6 mm), stainless steel, glass or titanium columns;
- _ column packing with very small (3, 5 and 10 μm) particles;
- _ relatively high inlet pressures and controlled flow of the mobile phase;
- _ continuous flow detectors capable of handling small flow rates and detecting very small

amounts;

_ rapid analysis;

Initially, pressure was selected as the principal criterion of modern liquid chromatography and thus the name was "high pressure liquid chromatography" or HPLC. This was, however, an unfortunate term because it seems to indicate that the improved performance is primarily due to the high pressure. This is, however, not true. In fact, high performance is the result of many factors: very small particles of narrow distribution range and uniform pore size and distribution, high pressure column slurry packing techniques, accurate low volume sample injectors, and sensitive low volume detectors and, of course, good pumping systems. Naturally, pressure is needed to permit a given flow rate of the mobile phase.

4. Stationary Phases (Adsorbents)

HPLC separations are based on the surface interactions, and depend on the types of the adsorption sites. Modern HPLC adsorbents are the small rigid porous particles with high surface area.

Main adsorbent parameters are:

_ Particle size: 3 to 10 μm

_ Particle size distribution: as narrow as possible, usually within 10% of the mean;

The last parameter in the list represents an adsorbent surface chemistry.

Depending on the type of the ligand attached to the surface, the adsorbent could be normal phase (-OH, -NH₂), or reversed-phase (C₅, C₈, C₁₈ CN, NH₂), and even anion(CH₂NR₃+OH⁻), or cation (R-SO₃-H⁺) exchangers.

5. Mobile phases

In HPLC, the type and composition of the eluent is one of the variables influencing the separation.

Despite the large variety of solvents used in HPLC, there are several common properties:

-Purity

-Detector compatibility

-Solubility of the sample

-Low viscosity

-Chemical inertness

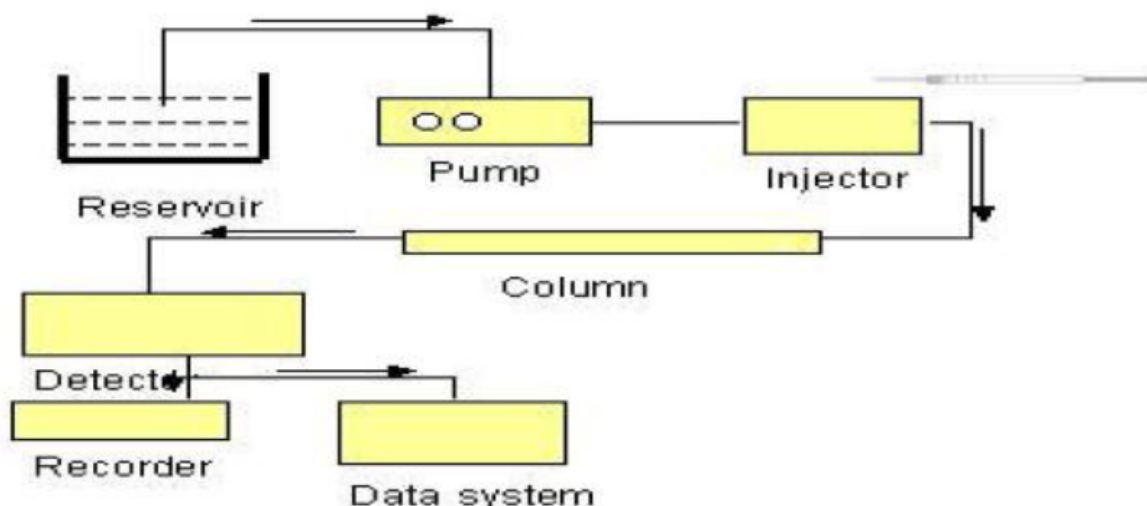
For normal phase mode, solvents are mainly nonpolar; for reversed-phase, eluents are usually a mixture of water with some polar organic solvent such as acetonitrile or methanol.

Size-exclusion HPLC has special requirements. SEC eluents have to dissolve polymers, but the most important is that SEC eluent has to suppress possible interactions of the sample molecule with the surface of the packing material.

6. Instrumentation of HPLC system

HPLC instrumentation includes a pump, injector, column, detector and data system. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometre size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or peak) on the recorder.

Detection of the eluting components is important, and this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyse the chromatographic data, computer, integrator, and other data processing equipment are frequently used.



7. Functional description of the instrument

Mobile phase reservoir

Pump

Injector

Column

Detector

Data system

7.1 Mobile phase reservoir, filtering

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Helium purging and storage of the solvent under helium is not sufficient for degassing aqueous solvents. It is useful to apply a vacuum for 5-10 min. and then keep the solvent under a helium atmosphere.

7.2 Pump

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations.

The most important advantages are: higher resolution, faster analyses, and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts. Many separation problems can be resolved with larger particle packings that require less pressure.

Flow rate stability is another important pump feature that distinguishes pumps.

Very stable flow rates are usually not essential for analytical chromatography.

However, if the user plans to use a system in size exclusion mode, then there must be a pump which provides an extremely stable flow rate.

An additional feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods. The degree of flow control also varies with pump expense. More expensive pumps include such state-of-the-art technology as electronic feedback and multiheaded configurations.

Modern pumps have the following parameters:

Flow rate range: 0.01 to 5 mL/min

Flow rate stability: not more than 1%

For SEC flow rate stability should be less than 0.2%

Maximum pressure: up to 300 hPa.

It is desirable to have an integrated degassing system, either helium purging, or membrane filtering.

7.3 Injector

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of autosamplers and microprocessors.

In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, loss in efficiency or all of these.

It is always best to remove particles from the sample by filtering over a 5 µm filter, or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns.

Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance. Typical sample mass with 4.6 mm ID columns range from the nanogram level up to about 2 mg diluted in 20 ml of solvent. In general, it will be noted that much less sample preparation is required in LC than in GC since unwanted or interfering compounds, or both, may often be extracted, or eliminated, by selective detection.

7.4 Column

Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 µm) particles. The internal diameter of the columns is usually 4.6 mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), then larger diameter columns may be needed. Packing the column tubing with small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase prepacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment.

In general, LC columns are fairly durable and one can expect a long service life unless they are used in some manner which is intrinsically destructive, as for example, with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples. It is wise to inject some test mixture (under fixed conditions) into a column when new, and to retain the chromatogram.

If questionable results are obtained later, the test mixture can be injected again under specified conditions. The two chromatograms may be compared to establish whether or not the column is still useful.

7.5 Detector

Today, optical detectors are used most frequently in liquid chromatographic systems. These detectors pass a beam of light through the flowing column effluent as it passes through a low volume (~ 10 µl) flow cell. The variations in light intensity caused by UV absorption, fluorescence emission or change in refractive index, from the sample components passing through the cell, are monitored as changes in the output voltage. These voltage changes are

recorded on a strip chart recorder and frequently are fed into a computer to provide retention time and peak area data.

The most commonly used detector in LC is the ultraviolet absorption detector (fig.8). A variable wavelength detector of this type, capable of monitoring from 190 to 400 nm, will be found suitable for the detection of the majority samples.

Other detectors in common use include: Photo Diode Array UV detector (PAD), refractive index (RI), fluorescence (FLU), electrochemical (EC). The RI detector is universal but also the less sensitive one. FLU and EC detectors are quite sensitive (up to 10-15 pmole) but also quite selective.

7.6 Data system

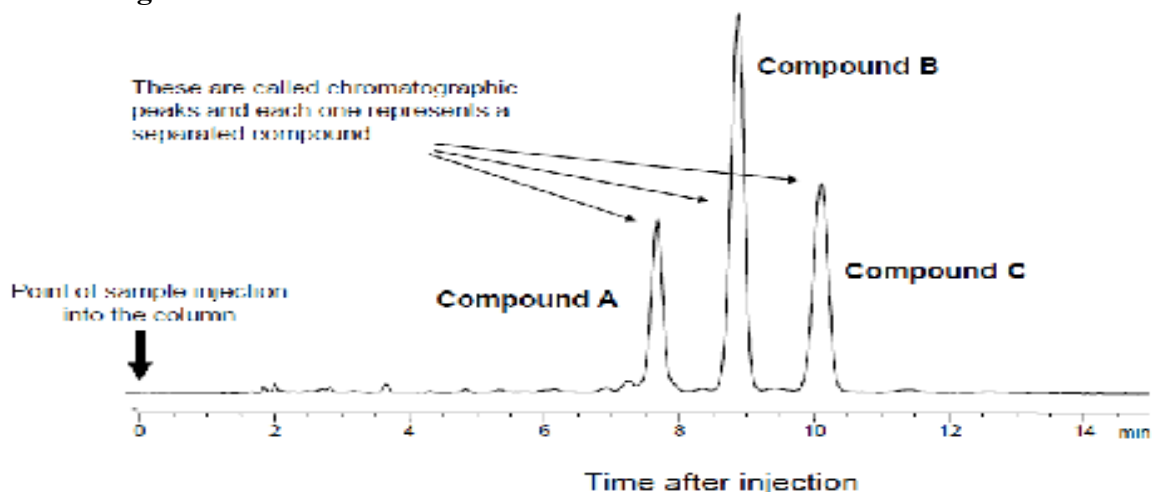
Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time.

The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention. There are several types of data systems, each differing in terms of available features. In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient. If higher control levels are desired, a more intelligent device is necessary, such as a data station or minicomputer. The advantages of intelligent processors in chromatographs are found in several areas. First, additional automation options become easier to implement. Secondly, complex data analysis becomes more feasible. These analysis options include such features as run parameter optimisation and deconvolution (i.e. resolution) of overlapping peaks. Finally, software safeguards can be designed to reduce accidental misuse of the system.

How does a chromatogram look like?

The components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.

These separated components are detected at the exit of this tube (column) by a flow-through device (detector) that measures their amount. An output from this detector is called a “**liquid chromatogram**”.



What is HPLC used for?

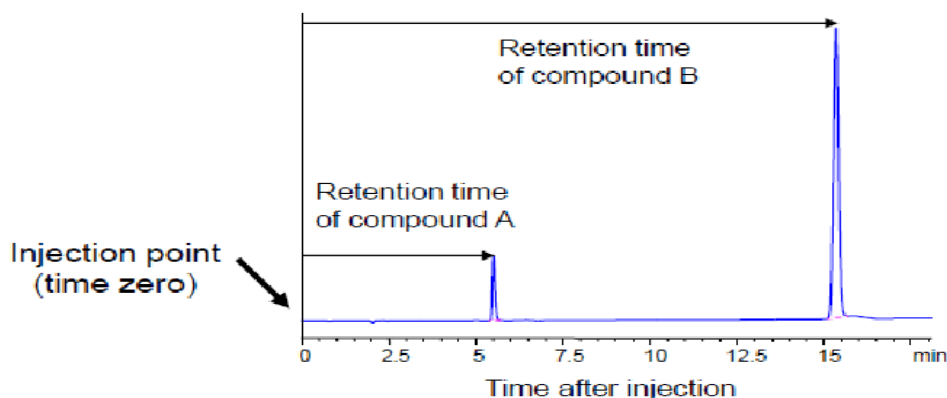
Separation and analysis of **non-volatile** or **thermally-unstable compounds** HPLC is optimum for the separation of chemical and biological compounds that are non-volatile.

Typical non-volatile compounds are:

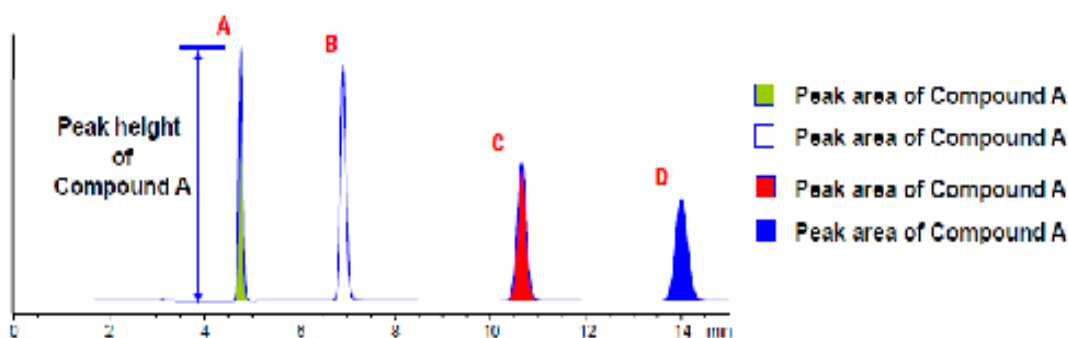
- 1) Pharmaceuticals like aspirin, ibuprofen, or acetaminophen (Tylenol)
- 2) Salts like sodium chloride and potassium phosphate
- 3) Proteins like egg white or blood protein
- 4) Organic chemicals like polymers (e.g. polystyrene, polyethylene)
- 5) Heavy hydrocarbons like asphalt or motor oil
- 6) Natural products like ginseng, herbal medicines, plant extracts
- 7) Thermally unstable compounds like trinitrotoluene (TNT), enzymes

HPLC is useful for

1. **Qualitative analysis:** Identification (ID) of individual compounds in the sample. The most common parameter for compound ID is its retention time (the time it takes for that specific compound to elute from the column after injection).



2. **Quantitative analysis:** The measurement of the amount of a compound in a sample. There are two main ways to interpret a chromatogram to perform quantification namely,
 - 1) determination of the peak height of chromatographic peak as measured from the baseline;
 - 2) determination of the peak area.



3. Preparation of pure compounds:

By collecting the chromatographic peaks at the exit of the detector, and by concentrating the compound by removing/ evaporating the solvent, a pure substance can be prepared. This methodology is called Preparative chromatography.

4. Trace analysis: A trace compound is a compound that is of interest but its concentration very low, usually less than 1% by weight, often parts per million (ppm) or lower.

The determination of trace compounds is very important in pharmaceutical, biological, toxicology, and environmental studies since even a trace substance can be harmful or poisonous.

Immunological assays:

are made up of 3 components: i) the antigen ii) the antibody iii) the antigen-antibody complex.

1) Enzyme Immuno assay: the drug to be assayed is contained in a solution, such as serum or plasma. Antibodies specific to the drug are added to the solution along with an enzyme substrate and NAD. Enzyme labelled drug is then added to the mixture. Competition for the antibody sites occurs between the drug in the patient sample, and enzyme labelled drug. When enzyme labelled drug binds to the antibody, enzymatic activity is significantly reduced due to steric exclusion of the substrate by the antibody. Only the free enzyme- labelled drug can act on the substrate, converting NAD to NADH in the process. Enzyme activity, which is directly proportional to concentration of drug in the sample, is measured spectrophotometrically at 340nm.

These techniques offer some advantages over RIA in that no radioactive tracer is required; there is no need to separate the bound from the unbound fractions. However the potential for cross reactivity still exists. Burgess *et al* compared serum phenytoin concentration in patients with normal renal functions and in patients with end stage renal disease using EMIT and GLC and found that in patients with renal insufficiency and EMIT values were 90% higher than GLC values, Digoxin RIA remains as one of the most precise and sensitive methods for quantitation of digoxin in patients serum.

2) Radio immuno assay (RIA): It is sensitive, reasonably precise but requires the use of radionucleides. Upon reaction with the drug there is dislodgement of the radio-isotope which can be detected to know the corresponding amount of the drug present in the sample.

Cross reactivity with other closely reacted drugs is a potential problem with this technique. Besides it is not possible to find out the optically active isomer. The hazards of using 24 radioactive material is a considerable limitation of this method.

3) Fluorescence polarization Immunoassay(FPIA) : is considered as one of the most accurate and sensitive methods. It uses a competitive binding immunoassay methodology to allow tracer labelled antigen and patient antigen to compete for the binding sites on the antibody molecules. When the amount of analyte in the unknown specimen is high, more analyte will bind to the antibody leaving the antigen-tracer free in solution, i.e. small molecule rotating rapidly. When the amount of the antigen in the specimen is low, less will bind to the antibody, and more antigen-tracer will be bound, i.e. large molecule rotating slowly.

High concentration = more free tracer = decreased polarised vertical light

Low concentration = less free tracer = increased polarised vertical light.

This assay procedure combines competitive protein binding with fluorescence polarization to give direct measurement without the need for a separation procedure. The advantages of this method are accuracy, precision and short turn around time. Apple *et al* compared three methods viz., FPIA, EMIT and HPLC for measurement of total and free phenytoin levels in

uremic patients and found interferences in EMIT assays were minimal and that FPIA and HPLC determinations are in agreement.

So when do we sample for blood required for TDM?

Timing of the sampling is very critical in determining the serum levels because different drugs exhibit varied half-lives and hence timing also varies for different drugs.

Drug concentration determinations must always be interpreted in the context of the clinical data. Therapeutic ranges are available but should be used only as a guide. Many factors alter the effect of a drug concentration at the site of action, e.g., serum concentration of Digoxin that is therapeutic for most patients may be excessive for a patient with hypokalemia. Furthermore range of serum drug concentration require adjustment when other drugs with synergistic or antagonistic actions are administered concomitantly.

1) **Patient demographics:** a) patient's age

b) patient's weight and height

c) acute/ chronic disease

2) **Dosing history:** Dose/frequency of drug administration

3) **Time of sampling:** C_{ssmax} (peak), C_{ssmin} (trough) or C_{ss} (mean)

C_{ssmax} : 1-2 hr after oral dose, 4-6 hr after sustained release or 1hr after injected dose.

C_{ssmin} : 10-20 min after oral dose, just before injected dose for injected drug.

C_{ss} : Midway between 2 consecutive doses.

An important part of therapeutic drug monitoring is the timing of the blood collection. The importance of proper timing of a sample is not given sufficient attention while ordering measurement of a plasma concentration. When a drug is administered, the blood concentration increases until it reaches a peak and then the concentration begins to fall. The lowest concentration (trough) is usually just before the next dose. The time required for the serum concentration of a drug to decrease by 50% is called the half-life of the drug. When a drug is administered in intervals approximately equal to its half-life, a steady state concentration will be achieved after 4-5 half-lives. For drugs with a long half-life, there is little difference between the steady state peak and trough concentrations. For drugs with a short half-life, the differences between the peak and trough concentrations can be significant and both are usually measured (i.e. Aminoglycosides). Drugs that are given intravenously require time to redistribute into the different body compartments. In general, intravenous medications can be sampled 30-60 minutes post administration.

For drugs with a long half life such a Phenytoin atleast 4 to 5 half lives must elapse before a sample is taken. A knowledge of usual half life ranges will thus be useful.

4) **Patient's other drug intake:** frequency, doses and actual time when drug taken.

Major causes of unexpected serum concentration in patients:

The most important causes of unexpected serum concentrations are non compliance, inappropriate dosage, malabsorption, poor bioavailability, drug interactions, hepatic or renal disease altered protein binding and genetic factors.

How do we correct a faulty dosing by a treating physician?

If these factors cannot be eliminated, a dosage adjustment is required. For drugs with linear kinetics the following formula may be used:

$$\text{New dose} = \text{Old dose} \times \left[\frac{C_{ss(\text{predicted})}}{C_{ss(\text{measured})}} \right]^{1/2}$$

Sample timing for some important drugs:

- a) **Phenytoin:** Since phenytoin has a long half life a single daily dose may be employed and so the timing of concentration monitoring is not critical.
- b) **Carbamazepine:** Its half life may be as long as 48 h following a single dose. A trough concentration taken just after a dose together with a peak level three hours later is ideal.
- c) **Digoxin:** The measurement must be made atleast six hours after a dose to avoid inappropriate high levels.
- d) **Theophylline:** This drug has a narrow therapeutic index and timing of sampling is not critical if the patient is receiving one of the slow release formulations.
- e) **Lithium:** A 12 hr sample gives the most precise guide to dosage adjustment.
- f) **Phenobarbitone:** Any time sample is sufficient
- g) **Gentamicin:** Pre dose peak; 0.5 hr after i.v. and 1 hr after i.m.

Can only free drug concentration be taken as a measure of serum values during TDM?

Development of new filtration devices (equilibrium dialysis, ultrafiltration, ultracentrifugation) has made it possible to measure free unbound drug levels in serum. The advantages are that the free concentration is independent of changes in plasma binding and is the pharmacologically active concentration. The disadvantages are that it is time consuming, expensive and therapeutic ranges do not yet exist for many drugs.

Can saliva be used as sample for TDM?

The concentration of a drug in saliva is proportional to the concentration of the unbound rather than to the total of bound and unbound drugs in plasma. The practice of measuring drugs in saliva is appealing because it is non invasive.

However it has its limitations viz., some substances such as lithium are actively secreted into the saliva rather than by passive process. Drug binding to salivary proteins may produce discrepancies in plasma/salivary ratios, e.g. Phenytoin. Drugs may also bind to oral cell debris, e.g. Propranolol, Salivary flow may be reduced in patients taking anti cholinergic drugs. Preparations used to stimulate salivary flow might interfere with drug estimation e.g. lemon flavored sweets interfere with Amitriptyline estimations.

What happens at the extremes of age?

Variability in response to drugs occurs at extremes of age. Elderly patients are more sensitive to the CNS depressant effect of drugs but are less sensitive to cardiovascular effects of Propranolol. On the other hand young children are more sensitive to CNS depression effects of morphine. However more data are needed on the effects of age on pharmacokinetic and pharmacodynamics of drugs to allow optional individualization of dosage.

What happens during pregnancy?

Little has been published on the monitoring of plasma drug levels during pregnancy. Plasma drug levels of Phenytoin and Phenobarbitone tend to reduce during pregnancy.

So these are various short comings of TDM that is it cannot be applied to persons with ages in the extremities and pregnant women as their pharmacokinetic variations account for varied plasma distribution. Hence the values may vary from that of general population, leading to lessened significance to normal values and failure of TDM.

Cost effectiveness:

The measurement of drug levels in body fluids must be cost effective. The cost of performing an individual test is determined by the summing equipment, personnel, supply and overhead expenditure for a given period of time and dividing that amount but the number of assays performed in the same time interval. The fee charges are then determined by the test's cost plus desired profit. The foregoing calculations produce an unreasonably expensive fee although high fee for unique tests requiring special methods may not be unreasonable. Cost-benefit analysis of Gentamicin dosage regimens of burn patients with gram negative septicaemia showed that a cost benefit ratio of 8.7 to 1 10 with decreased mortality and increased economic productivity. Mungall et al showed that use of clinical pharmacokinetics by therapeutic drug monitoring service offered substantial benefits like fewer adverse reactions, shorter intensive care unit stay and shorter overall hospital stay.

Clinical usefulness of TDM:

TDM data provides the clinician with greater insight into the factors determining the patient's response to drug therapy. For example when a patient fails to respond to a usual therapeutic dose, measurement of plasma level can help to distinguish a noncompliant patient and a patient who is a true non-responder. TDM also provides useful information regarding individual variations in drug utilization patterns and alteration in drug utilization as a consequence of altered physiological state or disease process.

TDM is a useful adjunct in treating many patients provided the potential pit falls and problems are considered. TDM cannot compensate for errors in diagnosis, poor choice of drug, errors in dispensing and dosages, errors in sample timing, non-compliance, etc. However, when used in combination with good clinical observation, it can lead to optimal drug therapy.

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