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Student Objectives for this Chapter

- Describe the need and techniques for separation of a drug from the experimental matrix
- Describe methods use to quantitate drug concentrations
- Understand the Advantages and Disadvantages of some these methods

---

Topics to be covered in this chapter

- Sample Preparation
- Spectroscopic Analysis
- Chromatographic Separation
- High Performance (Pressure) Liquid Chromatography (HPLC)
- Gas Liquid Chromatography (GLC)
- Radioimmunoassay (RIA)
- Enzyme Multiplied Immunoassay (EMIT)
- Fluorescence Polarization Immunoassay (FPIA)
- Comparison of Clinical Analytical Assay Methods

Pharmaceutical Analysis generally involves two steps; a) separation of the compound of interest and b) quantitation of the compound. Often, the better the separation the easier the quantitation.

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Sample Preparation

Separation (from Dosage Form or Biological Sample)

High concentrations of a drug in a sample means milligrams of drug in a liter or kilogram of sample matrix. The needle (1 mg) in a haystack (1 kg) means looking for and accurately measuring 1 part in 1,000,000. Low drug concentration make this ratio 10 to 1000 times larger. Separating the drug from most of the background material can be very useful in the determination of drug concentrations. The presence of similar compounds such as precursors, degradation products or metabolites makes the separation even more of a challenge.

Some useful techniques include:

- Centrifugation - removing excipients and/or macromolecules

  A significant separation can be achieved at times by simple centrifugation. Centrifugation of whole blood after clot formation produces serum with no red blood cells and fewer plasma proteins. Centrifugation of whole blood pretreated with anticoagulants such as heparin, EDTA or citrate produces plasma. Protein can be removed from plasma by centrifugation after the addition of trichloroacetic acid (TCA) or acetonitrile.

- Extraction - using pH/pKa partitioning or lipid/aqueous solubility differences

  Extraction can be used to remove unwanted interfering compounds or to concentrate the compound(s) of interest. The use of various organic solvents of differing polarity and/or aqueous buffers can provide excellent resolution based on the solubility of the free compounds of interest and/or their salt forms. Extraction may be used to remove lipophilic interfering compounds or to remove the desirable compounds into a cleaner environment.

- Ultrafiltration

  One method of separating free drug from plasma samples is ultrafiltration. The sample can be forced (by centrifugation) through a membrane filter. The filtrate is protein free solution of the compound.

- Chromatography - Adsorption, partitioning, size, or charge

  Chromatography can be used in a clean-up mode or in a sensitive analytical mode. In the clean-up mode the compound of interest may be adsorbed tightly onto a column while the interfering compounds are washed through it and subsequently flushed from the column with a 'stronger' eluting solvent. In the analytical mode the challenge is to choose the right column (stationary phase) and mobile phase so that the compound is eluted within a reasonable time and well resolved from other components in the sample.

- Immunoassay

  The very tight, strong binding between a compound and an antibody can be very useful as a method drug analysis. Using controlled, limited amounts of antibody and labelled drug it is possible analyse the drug sample.

- Other methods used in drug analysis might include dialysis and electrophoris

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Drug Quantitation

The other part of the process is recognizing (the right needle) and counting/measuring the number of drug molecules. Each drug has physical and chemical properties that can be exploited. In some cases a radiolabelled drug may be required.

Useful quantitation techniques include:

- Spectrophotometric
  - Absorbance

    Most compounds include chromophores within their molecular structure. Thus these compounds absorb electromagnetic energy in the visible (350 - 700 nm) and/or ultraviolet range (200 - 350 nm). Within specific concentration ranges the amount of energy absorbed is proportional to the concentration of the compound in the sample.

  - Fluorescence

    A number of compounds which absorb light energy are also able to re-emit some of that energy as light at a higher wavelength (lower energy). The emitted energy can be measured and correlated with the concentration of the compound.
- Radioactivity

  Radioactive atoms can be chemically incorporated into a compound of interest and subsequently used to quantitate the compound. The quality of the method depends on how well the radiolabel remains with the compound of interest.
- Electrochemical

  Various compounds will undergo oxidation or reduction under the influence of an electrical potential. These electrochemical reactions result in an electrical charge which can be detected and used as a measure of the drug concentration.
- Other techniques include optical activity, conductivity, refractive index, mass spectrometry

---

Spectroscopic Analysis

Spectroscopic Analysis includes Absorption Spectroscopy and Fluorescence Spectroscopy

Absorption Spectroscopy

- Theory
  - Beer-Lambert's Law
- Instrumentation
  - Single Beam
  - Double Beam
- Application

Theory

Absorption by molecules in solution produces changes in electronic transitions as well as vibrational and rotational changes. For example the carbonyl group bonds contain sigma and pi electrons. These electrons may transition from bonding to antibonding levels.

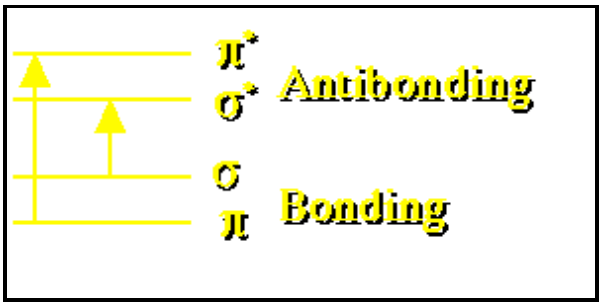

Figure 24.3.1 Sigma and Pi Bond Levels

Each of these transitions would result in a single peak in the absorbance / wavelength spectrum except for the broadening effect of the rotational and vibrational transitions.

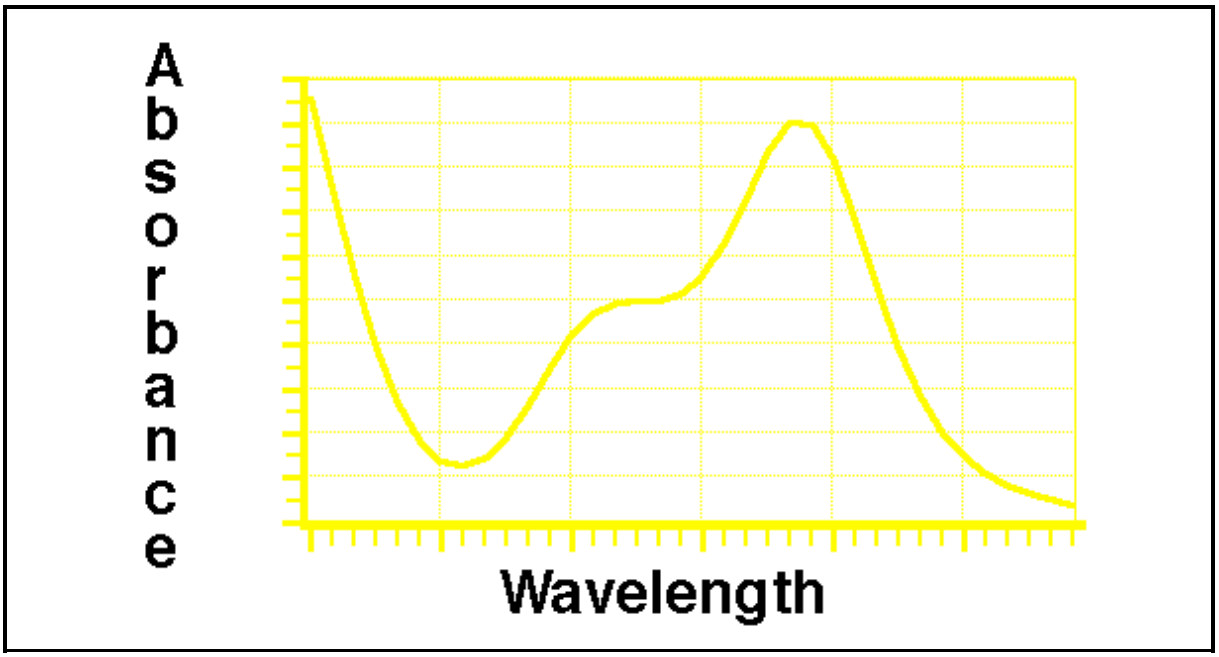

Figure 24.3.2 Plot of Absorbance versus Wavelength

As light passes through a compound in solution the intensity is reduced.

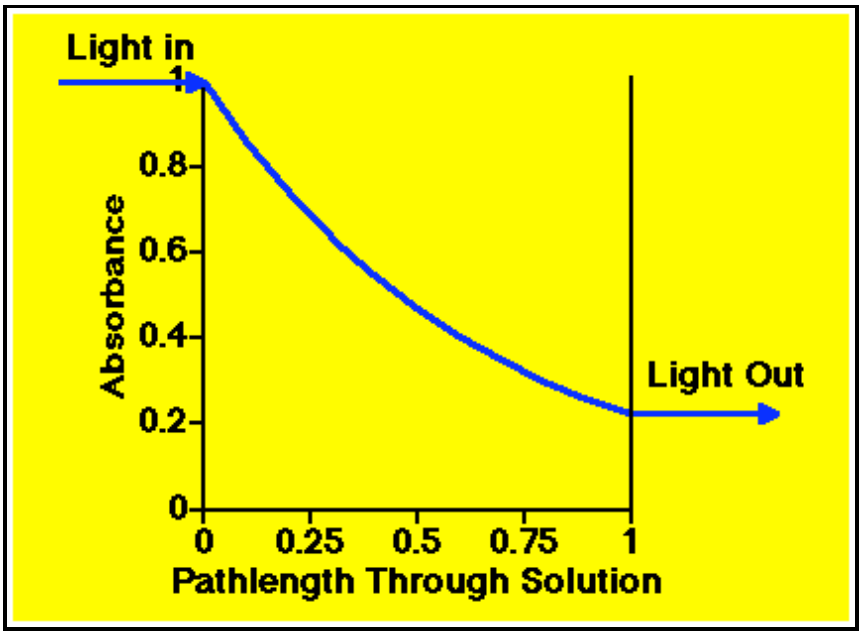

Figure 24.3.3 Light Absorbed through a Solution

The longer the pathlength the more light is absorbed. Also, the higher the concentration of compound in solution the more light is absorbed. Absorbance is proportional to pathlength and the concentration (Beer-Lambert's law)

$$A = a \bullet b \bullet c$$

Equation 24.3.1 Beer-Lambert Law for Light Absorption

where

a = absorptivity (ε, epsilon - molar absorptivity includes pathlength and wavelength)

b = pathlength (commonly 1 cm)

c = concentration (molar if molar absorptivity)

If b is 1 cm and c is in g/100ml the absorptivity is given as $A^{1\%}_{1\ cm}$ at wavelength (lambda).

Absorptivity may also be called the extinction coefficient or absorption coefficient

Instrumentation

Single Beam Spectrophotometer

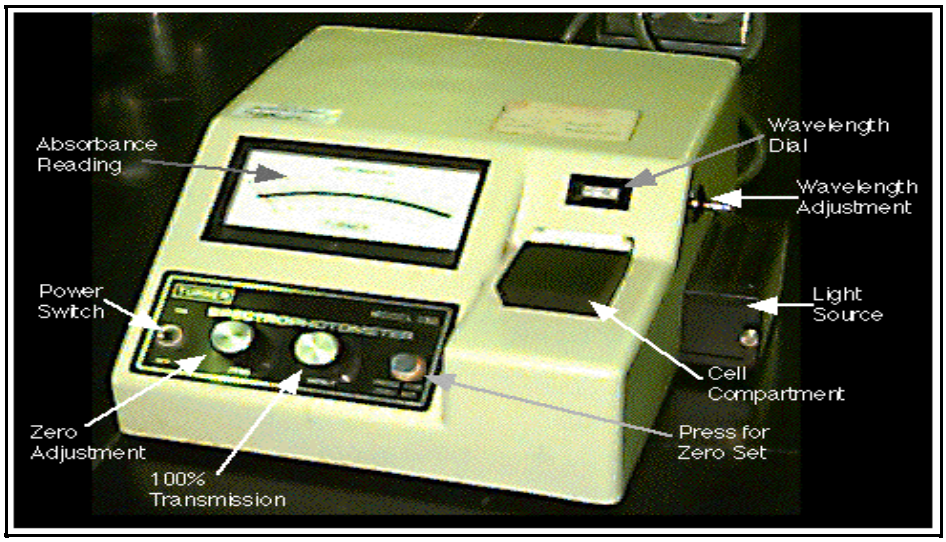

Figure 24.3.4 Turner Model 330 Spectrophotometer

The Turner model 330 single beam spectrophotometer has a cell holder for the sample and dials for zero adjustment, 100% transmission and wavelength. Absorbance is read from the upper scale on the meter.

Calibration of the Spectrophotometer

The spectrophotometer should be turned on at least 30 minutes before calibration. Adjust the wavelength to the required setting. With the 'Press for Zero Set' button depressed adjust the 'Zero Adjustment' dial until the meter needle is aligned with 0 % transmission (lower scale). Then place an appropriate blank in a cell in the cell holder and adjust the '100 % Transmission' dial until the meter needle is aligned with 100 % transmission (lower scale). The blank can be replaced with the sample(s) of interest and the absorbance read from the upper scale. This procedure should be made for each wavelength of interest. The spectrophotometer should remain on until all the required readings are made.

Double Beam Spectrophotometer

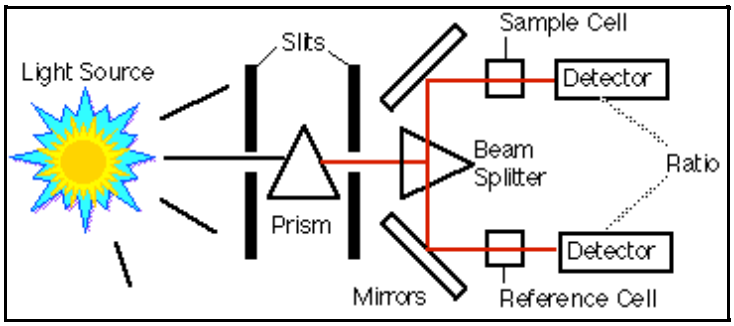

Figure 24.3.5 Schematic of a Double Beam Spectrophotometer
Redrawn from: Bauer, H.H., Christian, G.D., and O'Reilly, J.E. 1978 Instrumental Analysis, Figure 7.14, page 187

Application

Laboratory Exercises

Analysis of Drugs by Visible Spectroscopy

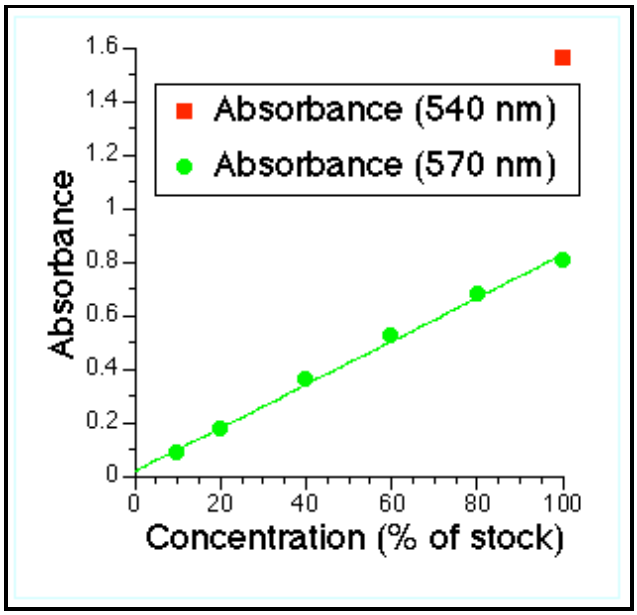

Figure 24.3.6 Absorbance versus Concentration

Extraction of Salicylic Acid

One Compartment - IV Bolus

One Compartment - IV Infusion

One Compartment - Oral

One Compartment Model - IV Bolus - Multiple Dose

Fluorescence Spectroscopy

- Molecule absorbs energy and immediately (10^{-6} to 10^{-8} sec) emits energy at a higher wavelength (lower energy) Phosphorescence is similar but involves a slower emission step (> 10^{-4} sec).
- Excitation and emission wavelengths specific to the compound
- Emission measured at 90° to the excitation light path
- Emission proportional to drug concentration

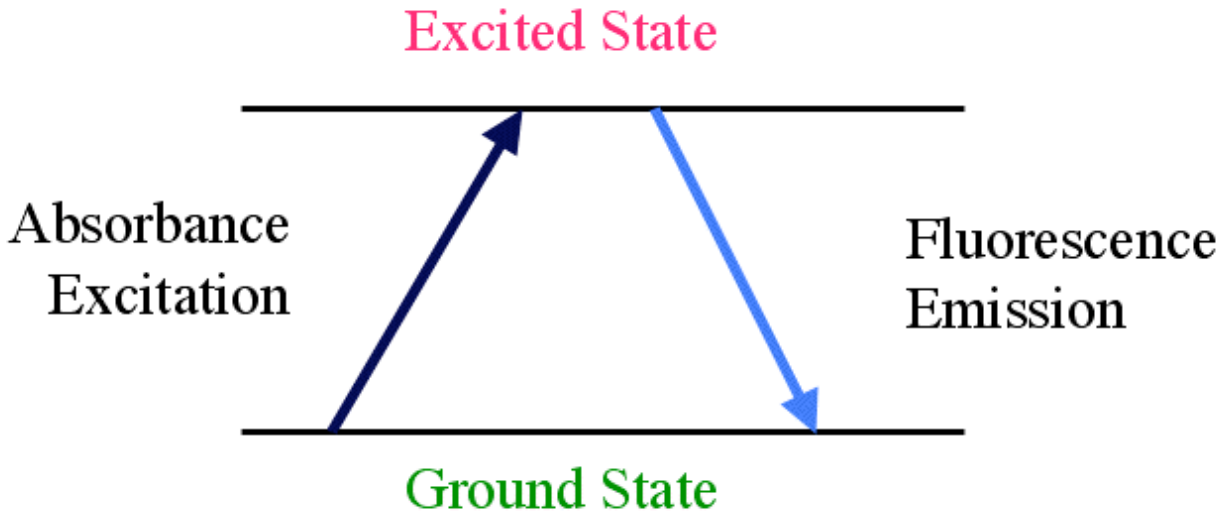

Fig 24.3.7 Absorption to an Excited Energy State followed Fluorescence Emission

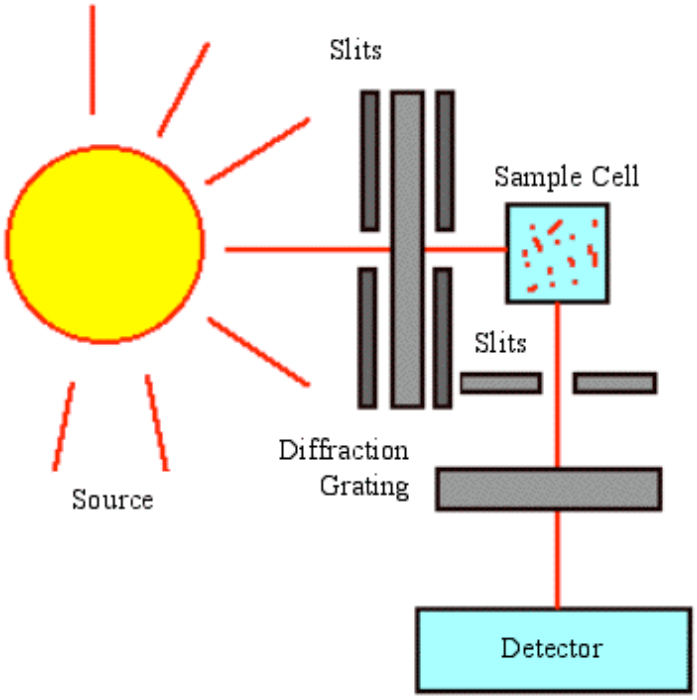

Fig 24.3.8 Schematic of a Fluorescence Detector
Redrawn from: Instrumental Analysis by Bauer, Christian and O'Reilly, 1978, page 235

References

- Beer's Law at Sheffield Hallam University
- UV-Visible Absorption Spectroscopy at Sheffield Hallam University
- Search for Spectroscopic Analysis at Goggle

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Chromatographic Separation

Chromatography is based on the separation of substances of interest by their differing affinity between a mobile phase and stationary phase. The mobile phase is usually a liquid or a gas while the stationary phase is usually a solid but may be an immobilized liquid.

Relative affinity may be based on relative solubility, adsorption, size or charge. Differences in solubility are expressed by partitioning between the mobile and stationary phases. Adsorption differences cause the separation of molecules in a non aqueous environment. Permeation (gel permeation) chromatography is based on smaller molecules being retained by inclusion without smaller pores of the gel. Separation by ion-exchange chromatography is based on the exchange of ions in the mobile phase with ions on the stationary phase. As such it is better suited to purification than separation between similar materials.

Commonly used chromatography methods are high performance/pressure liquid chromatography (HPLC), gas liquid chromatography (GLC) and thin layer chromatography (TLC).

click the arrow
to start the chromatography

Figure 24.4.1 Column chromatography with a multi-component sample

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References

- Chromatography
- Search for Chromatographic Separation at Goggle

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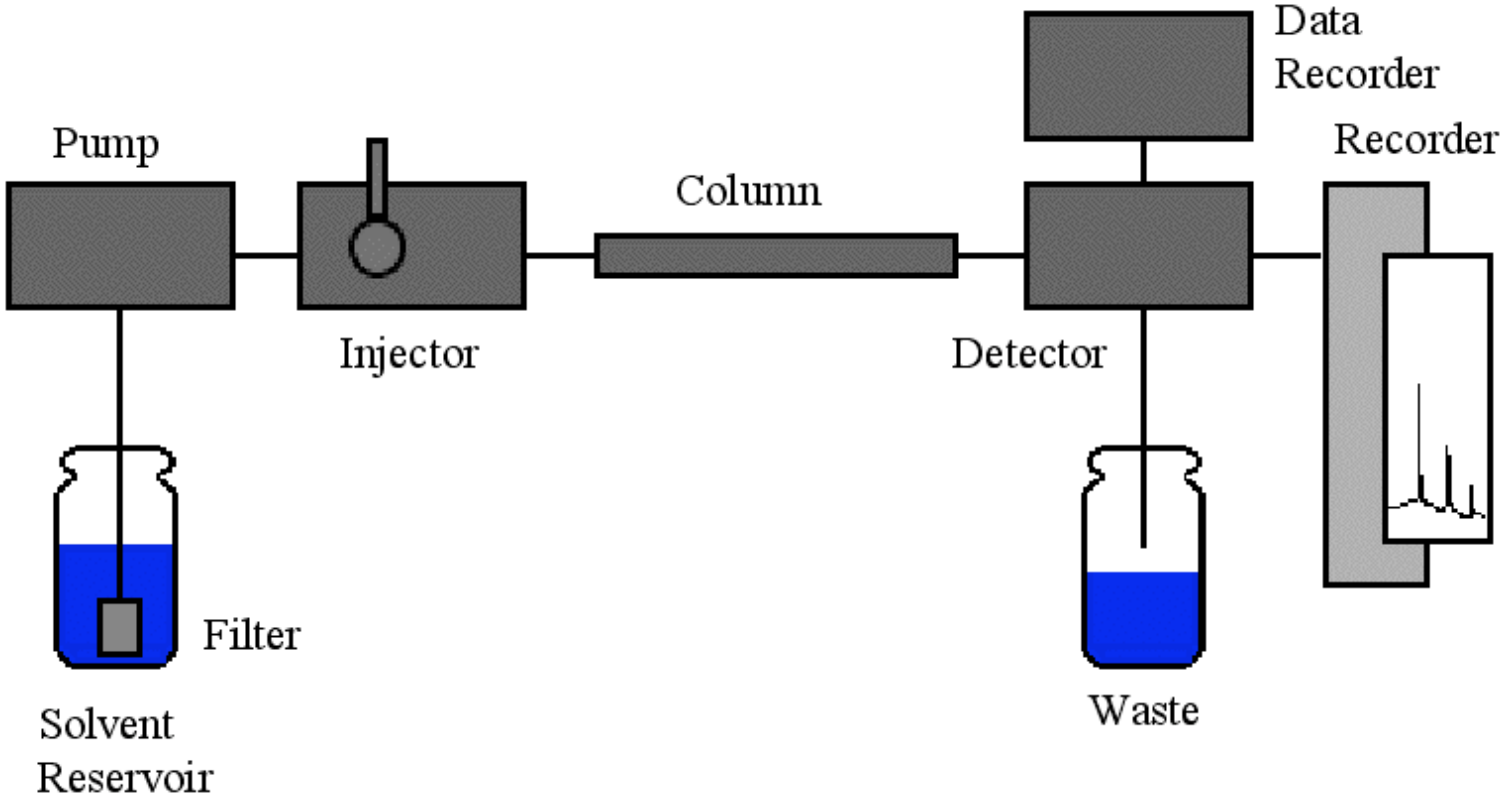
High Performance (Pressure) Liquid Chromatography (HPLC)

The General Approach

- Collect blood sample
- Separate plasma (partial clean-up)
- Extraction into organic solvent (polarity/pH)
- Injection onto column
- Separation on column
- Quantitation with detector
- Record and analyse results

HPLC Instrumentation

- Solvent Reservoir
- Pump
    - High pressure - 1000 to 5000 psi
- Injector
    - Low pressure - stop flow
    - High pressure value
- Column
    - Normal Phase - organic (water-free) mobile phase
        - Silica gel - non-aqueous
        - Adsorption
    - Reverse phase (C8, C18) - aqueous mobile phase
        - Partitioning
    - Ion-exchange - aqueous mobile phase
    - Molecular sieve - aqueous mobile phase
        - Size
- Detector
    - Specific
        - Absorbance
        - Fluorescence
        - Electrochemical
    - Non-specific
        - Refractive index
        - Radioactivity
        - Conductivity
- Recorder



Click on Figure 24.5.1 to
download a demonstration HyperCard stack
written by Keith Brain at the Welsh School of Pharmacy
Please let Keith know if you are using this in your teaching.
Requires HyperCard or HyperCard Player from Apple

Fig 24.5.1 Diagram illustrating a Typical HPLC System
Redrawn from: Pieper and Rutledge, Laboratory Techniques for Pharmacists, Upjohn 1989, page 27, figure 6

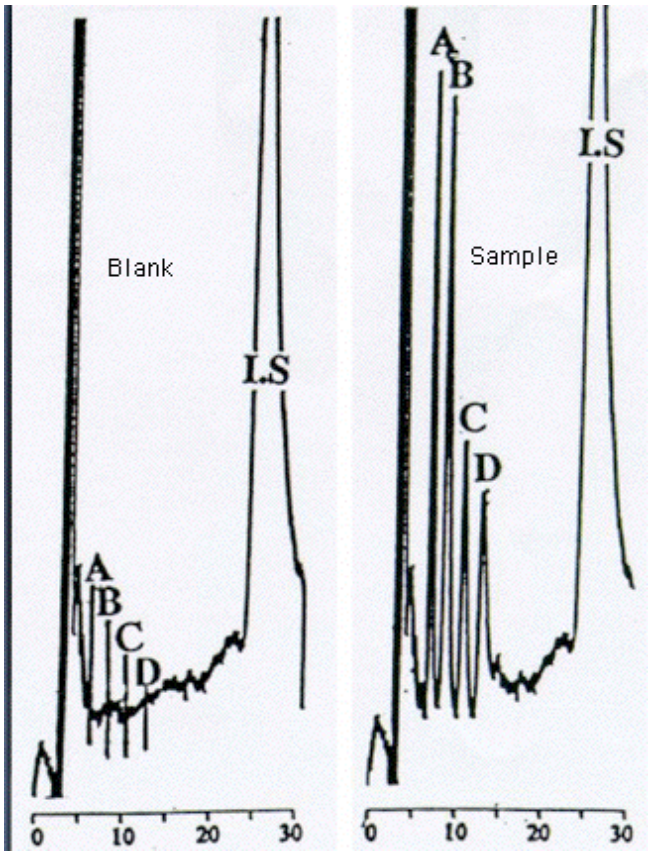


Figure 3.5.2 Example Chromatogram

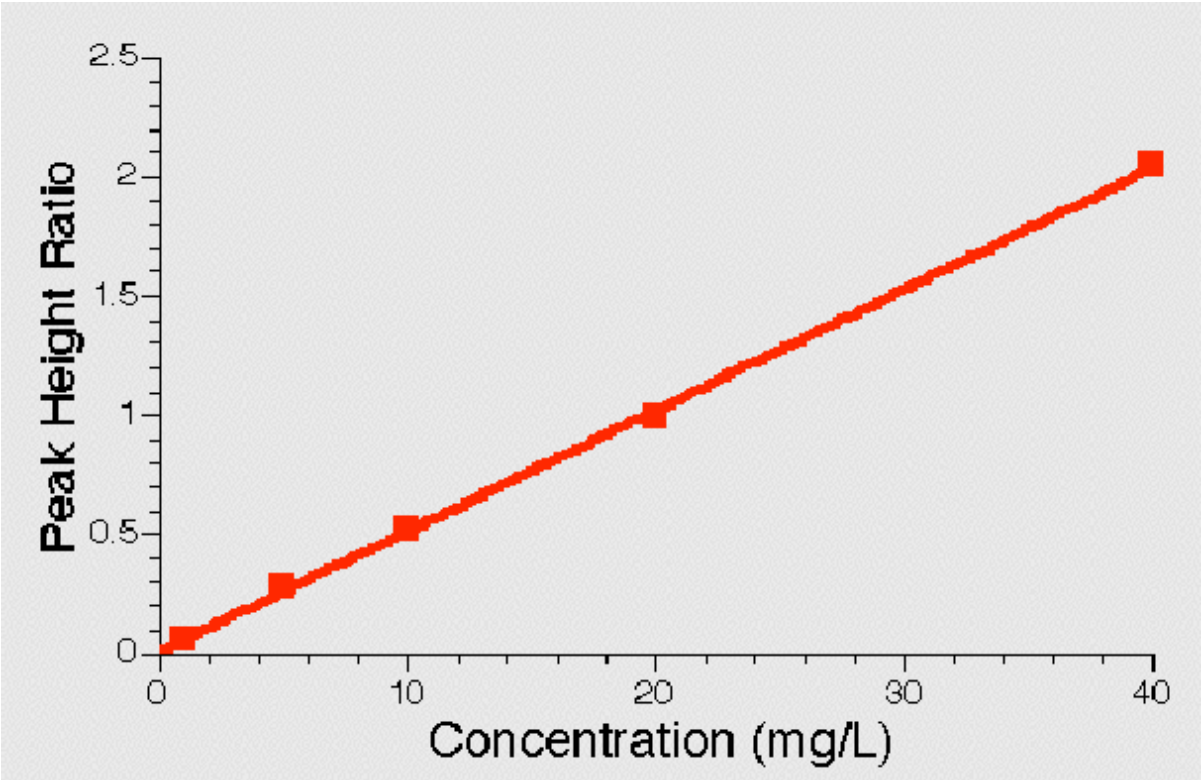


Figure 3.5.3 Example Standard Curve - Peak Height Ratio versus Concentration

References

- High Performance Liquid Chromatography (HPLC): A Users Guide
- High Performance Liquid Chromatography of Mebudipine: Application to Pharmacokinetic Study

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Gas Liquid Chromatography (GLC)

GLC Instrumentation

- Gas cylinder and regulator
  - Nitrogen
  - Helium
  - Argon
  - Hydrogen
- Injection port
- Column
  - Gas-solid - adsorption
  - Gas-liquid - High boiling point stationary phase
  - Glass, steel, capillary glass
- Column oven
- Detector
  - Flame ionization (FID) general purpose - modest sensitivity
  - Nitrogen phosphorus FID specific for N and P
  - Electron capture (EC)
  - Mass spectrometry (MS)
- Recorder

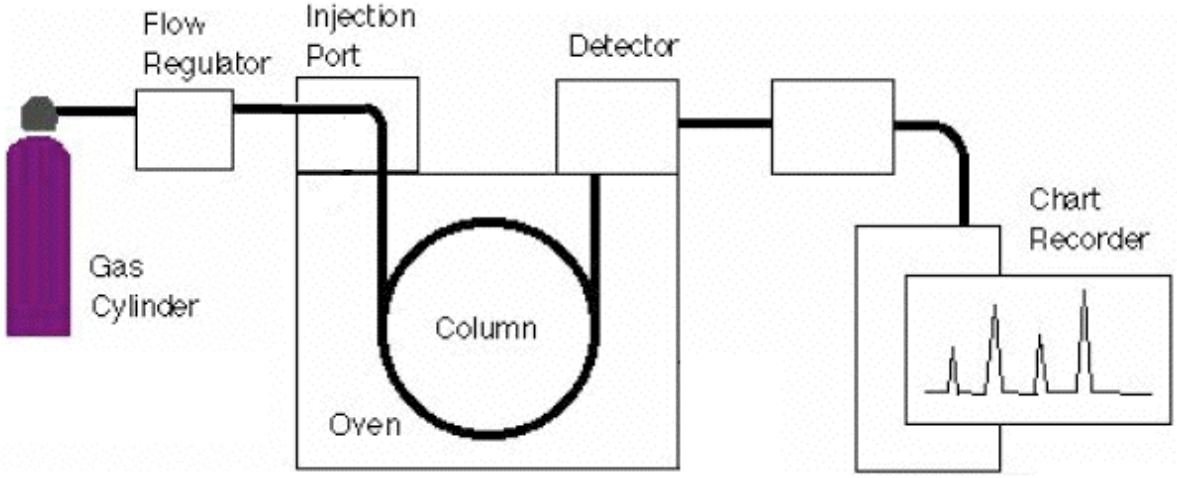

Fig 24.6.1 Diagram illustrating a Typical GLC System
Redrawn from: Pieper and Rutledge, Laboratory Techniques for Pharmacists, Upjohn 1989, page 20, figure 2

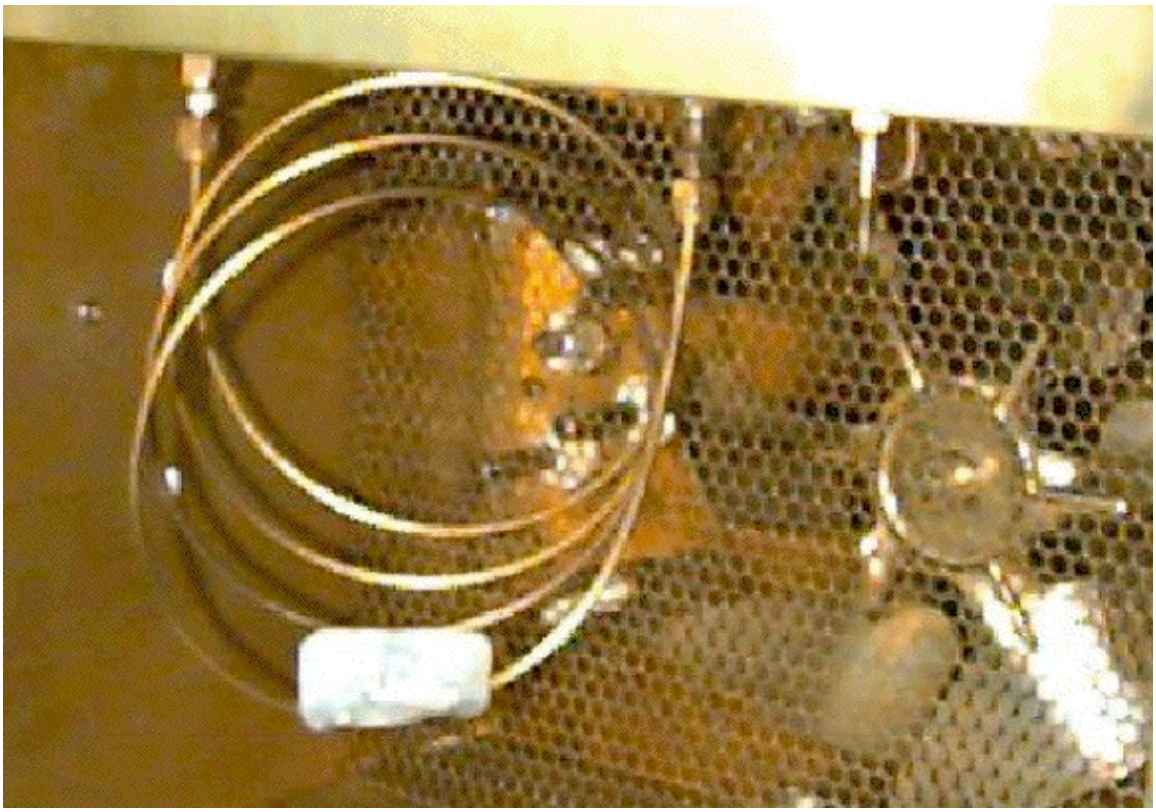

Fig 24.6.2 Steel column installed in Oven

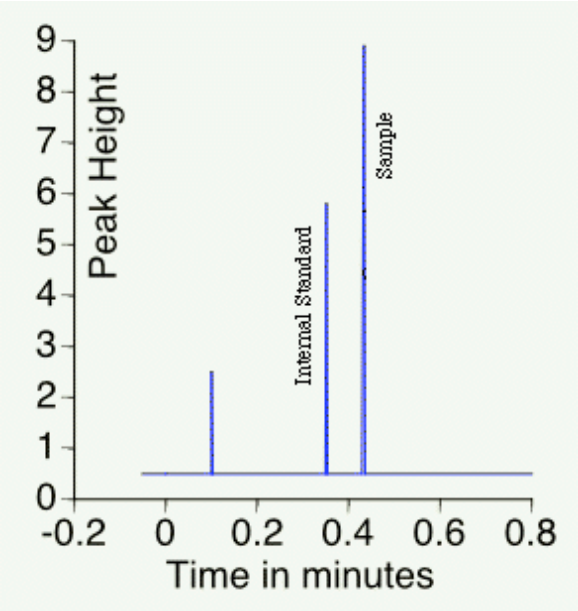

Fig 24.6.3 Typical chromatogram

References

- Gas Chromatography

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Radioimmunoassay (RIA)

RIA involves the separation of the drug using the specificity of antibody - antigen binding and quantitation using radioactivity.

Components of RIA Assay Kit

- Drug
- Antibody
- Labelled Drug

General Procedure for Performing a RIA Analysis

- Mix sample containing drug with fixed quantity of labelled drug and antibody
- Allow to equilibrate - incubate
- Separate drug bound to antibody from unbound drug
    - Charcoal adsorption of antibody (and bound drug)
    - Antibody - antibody binding precipitates bound drug
    - Antibody bonded to container
- Measure radioactivity associated with bound labelled drug
    - low drug concentration means more bound radioactivity and higher measurement
    - high drug concentration means less bound radioactivity and lower measurement
- Determine standard curve
    - Non-linear plot of radioactivity versus concentration
    - Logit-log concentration plot is linear

A Blank and Three Standard Samples

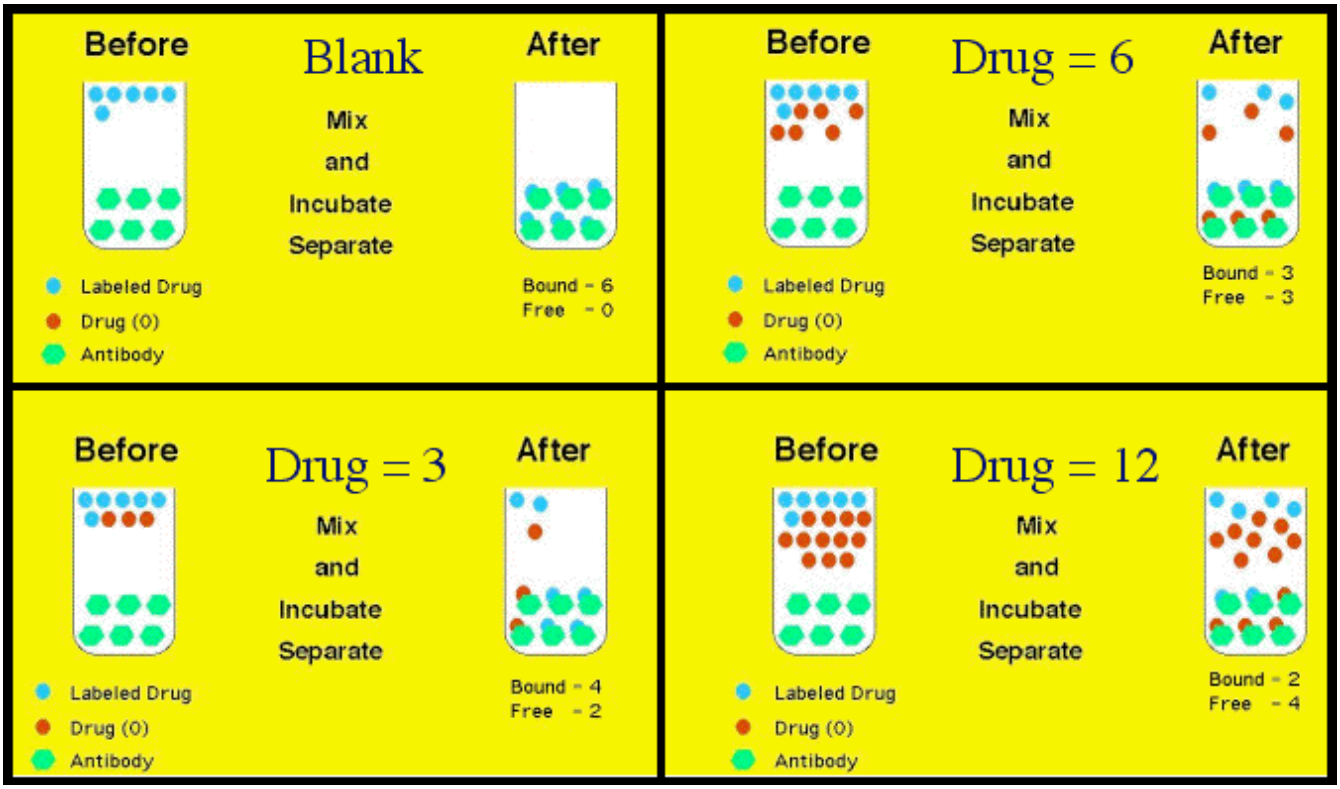

Fig 3.7.1 RIA before and after Incubation - Blank and Three Standard Samples

Table 24.7.1 Bound and Free Drug Concentrations

Total [Drug]	Bound [Drug]	Free [Drug]
0	6	0
3	4	2
6	3	3
12	2	4

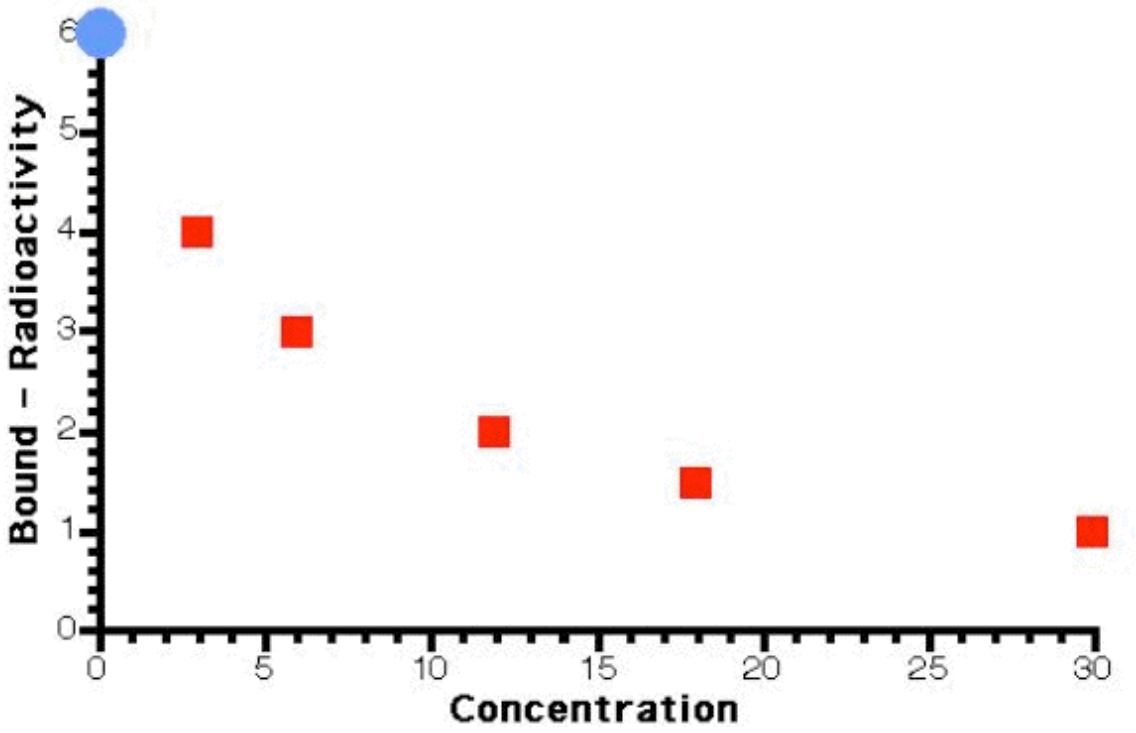

Fig 24.7.2 Plot of Bound versus Total Drug Concentration

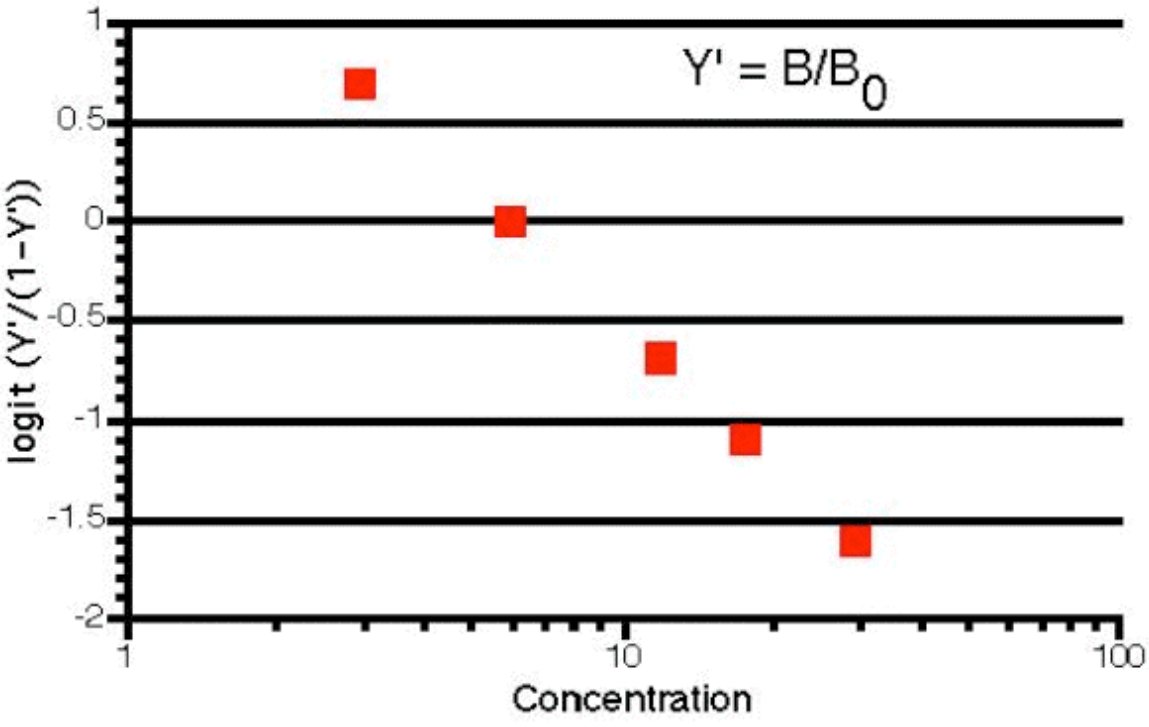

Fig 24.7.3 Logit versus Log Total C Plot

References

- Search for Radioimmunoassay at Goggle

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Enzyme Multiplied Immunoassay (EMIT)

Separation using the specificity of antibody - antigen binding and quantitation using enzyme reaction

Components of the EMIT Assay Method

- Drug
- Antibody
- Substrate
- Enzyme bound to drug

General Procedure for EMIT Assay

- Mix sample containing drug with fixed quantity of enzyme bound drug, and antibody
- Add substrate
- Measure absorbance at 15 and 45 seconds after substrate addition
- Quantitate by measuring enzyme-substrate reaction (by UV - visible spectroscopy)
- Δ Absorbance from Reaction rate from Drug concentration
- Non linear relationship between Δ Absorbance and Concentration
- Determine standard curve

Fig 24.8.1 EMIT Assay Components in Action
Redrawn from: Pieper and Rutledge, Laboratory Techniques for Pharmacists, Upjohn 1989, page 40, figure 12

References

- Search for Enzyme Multiplied Immunoassay at Goggle

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Fluorescence Polarization Immunoassay (FPIA)

FPIA Procedure

- Fluorescein-labelled drug competes with unlabelled drug for antibody
- Sample excited with plane polarized light (490 nm)
- Fluorescein emits plane polarized light (520 nm)
- Small, free drug-fluorescein, rotates faster leading to less emission
- Larger, antibody-drug-fluorescein, rotates slower and produces more emission
- Drug in sample competes for antibody with fluorescein bound drug
- More drug in the sample; less fluorescein labelled drug bound to antibody; lower emission of plane polarized light
- Higher drug concentration results in lower light emission values

- Available for a variety of drugs
- Advantages
  - Rapid turnaround times, sensitivity, ease of operation
- Disadvantages
  - Background interference in serum sample (requires blank measurement)

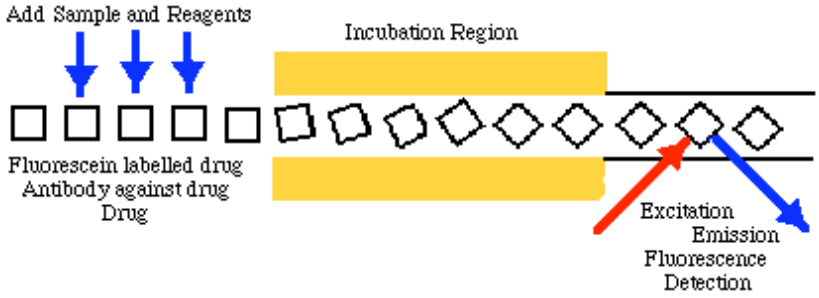

Fig 24.9.1 FPIA Equipment Set-up
See Pieper and Rutledge, Laboratory Techniques for Pharmacists, Upjohn 1989, page 43, figure 14 for a more detailed image

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References

- Determining Digoxin Pharmacokinetics by Fluorescence Polarization Immunoassay
- Search for Fluorescence Polarization Immunoassay at Goggle

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Comparison of Clinical Assay Methods

Basis for Comparison of Methods

- Sample (Type and size)
- Analysis Time
- Sensitivity, Specificity
- Accuracy, Precision
- Ease of Use, Versatility
- Cost of Equipment
- Cost of Supplies

Table 24.10.1 Comparison of Assay Methods

	HPLC	GLC	RIA	EMIT	FPIA
Separation Method	Chromatography	Chromatography	Immunoassay	Immunoassay	Immunoassay
Quantitation Method	Various including absorption, fluorescence, electrochemical, radioactivity, refractive index	Various including flame ionization, thermal conductivity, electron capture, mass spectrometry	Radioactivity	Kinetic via Absorption	Fluorescence
Sample Size (μl)	100 - 500	100 - 500	100	50	50
Sample Type*	P, S, U, Sa	P, S, U, Sa	P, S, U, Sa	P, S, U	P, S, U
Time (hr)	0.5	0.5	0.25	0.1 - 0.3	0.1 - 0.2
Sensitivity**	1	1	2	3	2
Specificity**	1	1	4	4	3
Accuracy**	2	2	2	2	2
Precision**	3	3	2	1	1
Training**	5	5	3	3	2
Equipment Cost**	4	4	5	3	4
Reagent Cost**	3	2	2	3	2
Clinical Application**	4	5	4	2	1
Research Application**	1	2	3	3	3

* **P**lasma, **S**erum, **U**rine, **Sa**liva

** 1 excellent - 5 poor

Redrawn from: Pieper and Rutledge, Laboratory Techniques for Pharmacists, Upjohn 1989, page 47, table 3

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