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Method Development and Validation of Efavirenz by UV-Spectrophotometry

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Abstract

The method developed for the determination of Efavirenz in tablet dosage form was found to be simple, sensitivity, precise, selective, rapid and economical. Efavirenz exhibited maximum absorption at 291nm and obeyed Beer's law in the concentration range of 10-50µg/ml. The proposed method for determination of Efavirenz showed linear regression y=0.181x+1.071 with correlation coefficient (R2) of 0.999. Interday and Intraday studies showed high degree of repeatability of an analytical method under normal operating conditions. The %RSD for precision, which was less than 2%.It indicates that the method is precise. Recoveries obtained do not differ significantly from 100% showed that there was no interference from the common excipients used in tablet formulation indicating accuracy and reliability of the method. The proposed method can be used for drug analysis in routine quality control & method proves to be more economical than the published standard methods.

1. Introduction

Analytical chemistry basically concerned with the determination of chemical composition of matter however, identification of substance, the elucidation of its structure and quantitative analysis of its composition are the aspects covered by modern analytical techniques.

Analytical chemistry may be derived as the science and art of determining the composition of material terms of the elements of compounds contained. By analytical techniques both qualitative analysis and quantitative analysis can be done.

The qualitative and quantitative analysis can be done by various analytical methods, techniques can be revised and some of them give accurate results, example spectrometric methods. Analytical instrumentation provides the lower limit of detection required to assure the safety. The instrumentation plays an important role in the production and evaluation of new production.

Analytical method is a specific application of a technique to solve an analytical problem. Analytical chemistry involves the application of range of techniques and methodologies to obtain and access quantitative and structural information on the nature of matter.

The quantitative estimation is the method to determine how much of each constituent is in the sample. Estimation of given drug or medicinal in it. The quantitative analysis was gravimetric, made possible by the invention of a precise balance. It was soon found that carefully calibrated glasses were

made considerable saving of time through the volumetric measurement of gravimetrically standardized solutions.

The instrumental analysis, a physical property of a drug is utilised to determine its chemical composition. A study of physical properties of drug molecules is the prerequisite for product formulation and often leads to a better understanding of the inner-relationship between molecular structure and drug action.

A. Quantitative Analysis :

This is to refer the identity of the product i.e., it yields useful clues from which the molecular or automatic species the structural features, or the functional groups in the sample can be classified.

B. Quantitative Analysis :

This is refer the purity of the product i.e., the results are in the form of numerical data corresponding to the concentration of analysis, the required information is obtain by measuring a physical property that is characteristically related to the component of interest the analyte. The most important aspect of analysis is quantitative chemical analysis.

Methods of analysis

Generally analytical methods are classified into:

- Chemically analysis
- Instrumental method

❖ Chemical analysis
1.Volumetric methods
2. Gravimetric methods

Chemical Analysis

Table.1: Classification of Chemical Analysis

Technique	**Property used**
Gravimetry	Weight of the analyte
Tritrimetry	Volume of standard reagent solution reacting with analyte.
Thermal Analysis	Physical changes in the analyte when heated or cooled
Electro chemical Analysis	Electrical properties of the analyte in the solution
Radio chemical Analysis	Characterizing the ionizing nuclear radiation emitted by the analyte.

Analytical Techniques

1. Titrimetric methods

A. Acid-Base Titrations

1. Direction Titrations

a. Titration of an acid by a base.

- Titration of liberated acid.
- Non- Aqueous

b. Titration of base by an acid.

- Titration metal salts
- Non-aqueous

2. Residual Titrations

a. Precipatation Titrations

b. Redox Titrations

2. Gravimetric methods

- Weigh drug after extraction.
- Weigh a derivative after separation
- Weighing residue after ignition.

3. Spectrophotometric Methods

- Dye complex method
- Colorimetric method
- Ultra Violet method
- Flourimetric method
- Flame photometry
- Automatic absorption spectroscopy
- Infrared spectrophotometry
- Raman spectroscopy
- X-ray spectroscopy
- Mass spectroscopy

4. Electro analytical methods

- Photometry
- Voltametry
- Colorimetry

- Electrogravimetry
- Conductance Techniques

5. Chromatographic methods

- Thin layer chromatography
- Paper chromatography
- Column chromatography
- Gas chromatography
- High performance liquid chromatography(HPLC)

6. Miscellaneous Methods

- Thermal analysis
- Kinetic Techniques
- Enzyme assay

7. Hyphenated technique

- **GC-MS** -(Gas Chromatography-Mass Spectrometry)
- **LC-MS-**(Liquid Chromatography-Mass Spectrometry
- **GC-IS-**(Gas Chromatography-Infrared Spectroscopy)
- **ICP-MS-**(Inductively Coupled Plasma Mass-Spectroscopy)

SPECTROSCOPY

1. Automatic absorption and emission spectroscopy (**AAS/AES):** To analyze alkali and alkaline earth metals in the dilute solutions, natural liquids and extracts at trace levels.

2. Ultra violet visible spectroscopy **(UV/Vis):** To analyze molecular (organic) and ionic species capable of absorbing at **UV** or visible wavelengths in dilute solutions.

3. Fourier transform Infrared spectroscopy **(FT-IR):** To analyze only molecular compounds (organic compounds, Natural products, Polymers. etc.).

4. Fourier transform Raman spectroscopy **(FT-Raman):** To analyze only molecular (organic) compounds which are not responding well in the IR region and hence, it is an alternate to IR.

5. Nuclear magnetic resonance spectroscopy **(NMR):** To identify and characterize the organic and inorganic compounds.

6. Microwave spectroscopy: To analyze simple gaseous molecules in far IR region to study their stereochemistry.

7. Electron spin resonance spectroscopy **(ESR):** To study formation and life time of the free radicals formed in organic reactions and also finds application in biological works.

8. Molecular fluorescence spectroscopy**:** To study molecular and ionic compounds in dilute solutions capable of giving fluorescence finds application in vitamin analysis.

Introduction to spectroscopy:

Spectroscopy and spectrography are terms used to refer to the measurement of radiation intensity as a function of wavelength and are often used to describe experimental spectroscopic methods. Spectral measurement devices are referred to as spectrometers, spectrophotometers, spectrographs or spectral analyzers.

Daily observations of colour can related to spectroscopy. Neon lighting is a direct application of automatic spectroscopy. Neon and other noble gases have characteristic emission these frequencies (colour). Neon lamps use collision of electrons with the gas to excite these emissions. Inks, dyes and paints include chemical compounds selected for their spectral characteristics in order to generate specific colours and hues. A commonly encountered molecular spectrum is that of nitrogen dioxide. Gaseous nitrogen dioxide has a characteristic red absorption feature, and this gives air polluted with nitrogen dioxide a reddish brown colour. Rayleigh scattering is a spectroscopic scattering phenomenon that accounts for the colour of the sky.

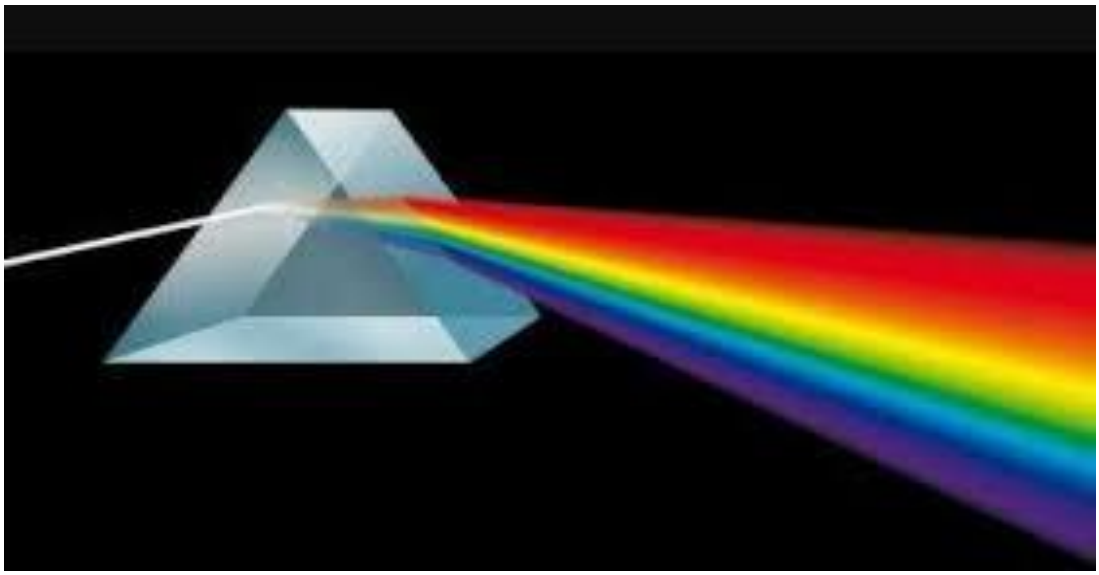
Spectroscopic studies were central to the development of quantum mechanics and include Max Plank's explanation of blackbody radiation Albert Einstein's explanation of the photoelectric effect and Niles Bohr's explanation of automatic structure and spectra. Spectroscopy is used in physical and analytical chemistry because atoms and molecules have unique spectra. As a result these spectra can be used to direct, identify and quantity information about the atoms and molecules. Spectroscopy is also used in astronomy and remote sensing on earth. Most research telescopes have spectrographs. The measured spectra are used to determine the chemical composition and properties of astronomical objects (such as their temperature and velocity).

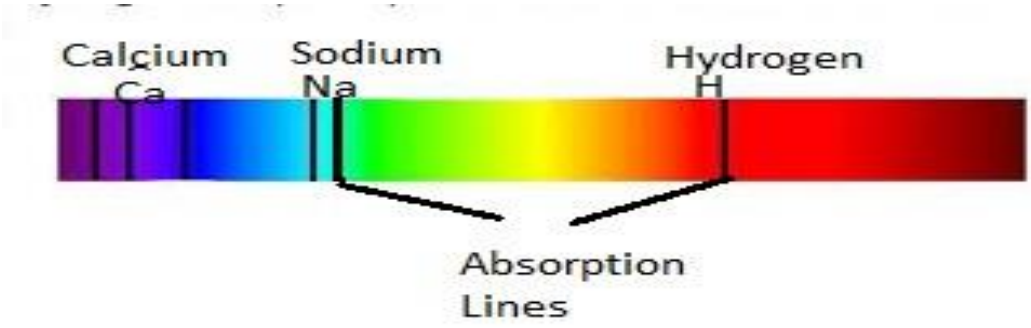
One of the central concepts in spectroscopy is a resonance and its corresponding resonant frequency. Resonances were first characterized in mechanical systems such as pendulums. Mechanical systems that vibrate or oscillate will experience large amplitude oscillations when they are driven at their resonant frequency. A plot of amplitude vs. Excitation frequency will have a peak centred at the resonance frequency. This plot is one type of spectrum, with the peak often referred to as a spectral line, and most spectral lines have a similar appearance.

In quantum mechanical systems, the analogous resonance is a coupling of two quantum mechanical stationary states of one system, such as an atom , via an oscillatory source of energy such as a photon. The coupling of two states is strongest when the energy source matches the energy different between the two states. The energy **(*E*)** of a photon is related to its frequency **(*v*) by *E*=hν** where *h* is plank's constant, and so a spectrum of the system response vs Photon frequency will peak at the resonant frequency or energy. Particles such as electrons and neutrons have a comparable relationship, the de Broglie relations, between their kinetic energy and their wavelength and frequency and therefore can also excite resonant interactions.

Spectra of atoms and molecules often consist of spectral lines, each one representing a resonance between two different quantum states. The explanation of these series, and the spectral pattern associated with them, were one of the experimental enigmas that drove the development and acceptance of quantum mechanics. The hydrogen spectral series in particular was first successfully explained by the Rutherford-Bohr quantum model of the hydrogen atom. In some cases spectral lines are well separated and distinguishable, but spectral lines can also overlap and appear to be a single transition if the density of energy states is high enough. Named series of lines include the principal, sharp, diffuse and fundamental series.

Spectroscopy is a sufficiently broad field that many sub-disciplines exist, each with numerous implementations of specific spectroscopic techniques. The various implementations and techniques can be classified in several ways.





ABSORPTION SPECTRUM

Types of radioactive energy

Types of spectroscopy are distinguished by the type of radioactive energy involved in the interaction. In many applications, the spectrum is determined by measuring changes in the intensity or frequency of this energy. The types of radioactive energy studied include:

- Electromagnetic radiation was first source of energy used for spectroscopic studies. Techniques that employ electromagnetic radiation are typically classified by the wavelength region of the spectrum and include microwave, terahertz, infrared, near infrared, visible and ultraviolet, x-ray and gamma spectroscopy.
- Particles, due to their de Broglie wavelength, can also be a source of radioactive energy and both electrons and neutrons are commonly used. For a particle, its energy determines its wavelength.
- Acoustic spectroscopy involves radiated pressure waves.
- Mechanical methods can be employed to impart radiating energy, similar to acoustic waves, to solid materials.

Nature of the interaction

Types of spectroscopy can also be distinguished by the nature of the interaction between the energy and the material. These interactions include**:** [1]

- Absorption occurs when energy from the radioactive source is absorbed by material. Absorption is often determined by measuring the fraction of energy transmitted through the material; absorption will decrease the transmitted portion.
- Emission indicates that radioactive energy is related by the material. A material's blackbody spectrum is a spontaneous emission spectrum determined by its temperature; this feature can be measured in the infrared by instruments such as the Atmospheric Emitted Radiance Interferometer **(**AERI**).**[4] Emission can also be induced by outer sources of energy such as flames or sparks or electromagnetic radiation in the case of fluorescence.
- Elastic scattering and reflection spectroscopy determine how incident radiation is reflected or scattered by a material. Crystallography employees the scattering of high energy radiation, such as x-rays and electrons, to examine the arrangement of atoms in proteins and solid crystals.
- Impedance spectroscopy studies the ability of a medium to impede or slow the transmittance of energy. For optical applications, this is characterized by the index of refraction.
- Inelastic scattering phenomenon involves an exchange of energy between the radiation and the matter that shifts the wavelength of the scattered radiation. These include Raman and Compton scattering.
- Coherent or resonance spectroscopies are techniques where the radioactive energy couples two quantum states of the material in a coherent interaction that is sustained by the radiating field. The coherent can be disrupted by other interactions, such as particle collisions and energy transfer, and so often require high intensity radiation to be sustained. Nuclear magnetic resonance **(NMR)** spectroscopy is a widely used resonance method and ultrafast laser methods are also now possible in the infrared and visible spectral regions.

Types of material

Spectroscopic studies are designed so that the radiant energy interacts with specific types of matter.

Atoms

Atomic spectroscopy was the first application of spectroscopy developed. Atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES) involve visible and ultraviolet light. These absorptions and emissions, often referred to as atomic spectral lines, are due to electronic transitions of outer shell electrons as they rise and fall from one electron orbit to another. Atoms also have distinct x-ray spectra that are attributable to the excitation of inner shell electrons to excited states.

Atoms of different elements have distinct spectra and therefore atomic spectroscopy allows for the identification and quantitation of a sample's elemental composition. Robert Bunsen and Gustav Kirchhoff discovered new elements by observing their emission spectra. Atomic absorption lines are observed in the solar spectrum and referred to as Fraunhofer lines after their discovered. A comprehensive explanation of the hydrogen spectrum was an early success of quantum mechanics and explained the Lamb shift observed in the hydrogen spectrum led to the development of quantum electrodynamics.

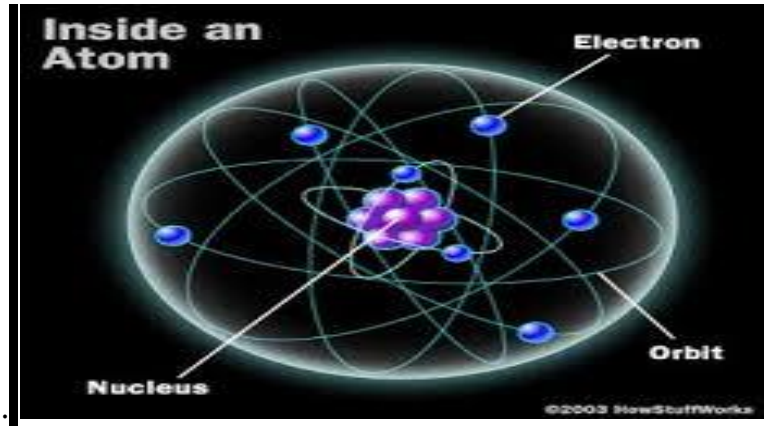


Fig.1: Structure of Atoms Modern implementations of atomic spectroscopy for studying visible and ultraviolet transitions include flame emission spectroscopy, inductively coupled plasma atomic emission spectroscopy, glow discharge spectroscopy, microwave induced plasma spectroscopy, and spark or emission spectroscopy. Techniques for studying x-ray spectra include X-ray spectroscopy and X-ray fluorescence (**XEF).**

Molecules

The combination of atoms into molecules leads to the creation of unique types of energetic states and therefore unique spectra of the transitions between these states. Molecular spectra can be obtained due to electron spin states (electron paramagnetic resonance), molecular rotations, molecular vibration and electronic states. Rotations are collective motions of the atomic nuclei and typically lead to spectra in the microwave and millimetre-wave spectral regions; rotational spectroscopy and microwave spectroscopy and microwave spectroscopy are synonymous. Vibrations are relative motions of the atomic nuclei and studied by both infrared and Raman spectroscopy as well as fluorescence spectroscopy.

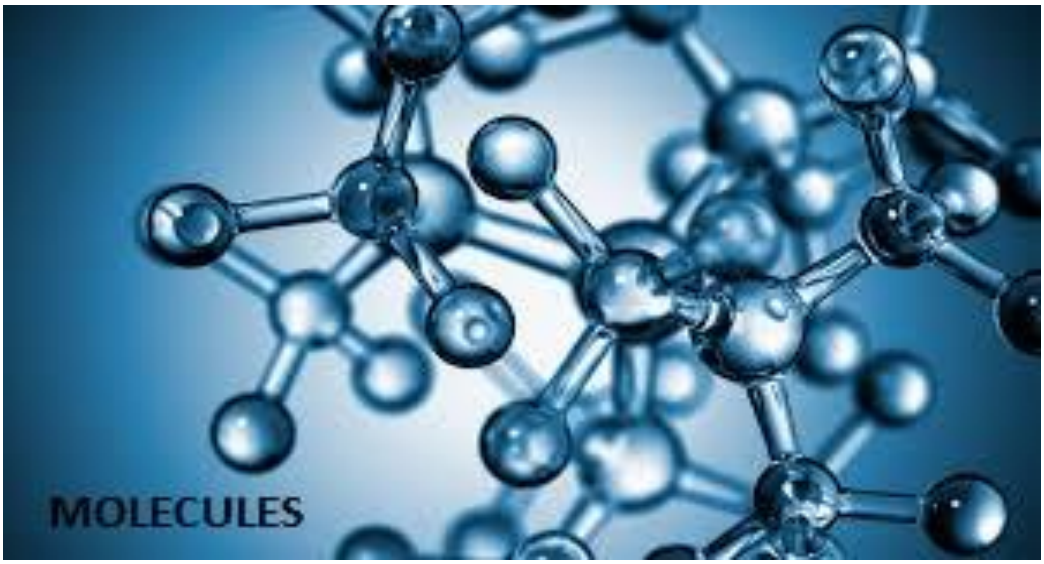


Fig 2: Structure Of Molecules

Studies in molecular spectroscopy led to the development of the first maser and contributed to the subsequent development of the laser.

Crystals and extended materials

The combination of atoms or molecules into crystals or other extended forms leads to the creation of additional energetic states. These states are the numerous and therefore have a high density of states. This high density often makes the spectra weaker and less distinct, i.e., broader. For instance , blackbody radiation is due to the thermal motions of atoms and molecules within a material. Acoustic and mechanical responses are due to collective motions as well. Pure crystals, though, can have distinct spectral transitions, and the crystal arrangement also has an effect on the observed molecular spectra, The regular lattice structure of crystals also scatters x-rays, electrons or neutrons allowing for crystallographic studies.

Nuclei

Nuclei also have distinct energy states that are widely separated and lead to gamma ray spectra. Distinct nuclear spin states can have their energy separated by a magnetic field, and this allows for NMR spectroscopy.

UV SPECTROSCOPY

Ultraviolet and visible spectrometers have been in general use for the last 35 years and over this period have become the most important analytical instrument in the modern day laboratory. In many applications other techniques could be employed but none rival UV-Visible spectrometry for its simplicity, versatility, speed, accuracy and cost-effectiveness. This description outlines the basic principles for those new to UV-Visible spectrometry. It is intended purely as a brief introduction to the technique and it is Thermo Spectronic's policy to continually add to this range of documentation for further details, as they become available.

Radiation is a Form of energy and we are constantly reminded of its presence via our sense of sight and ability to feel radiant heat. It may be considered in terms of a wave motion where the wavelength, λ, is the distance between two successive peaks.

The frequency, ν, is the number of peaks passing a given point per second. These terms are related so that:

C= νλ

Where **c** is the velocity of light in a vacuum

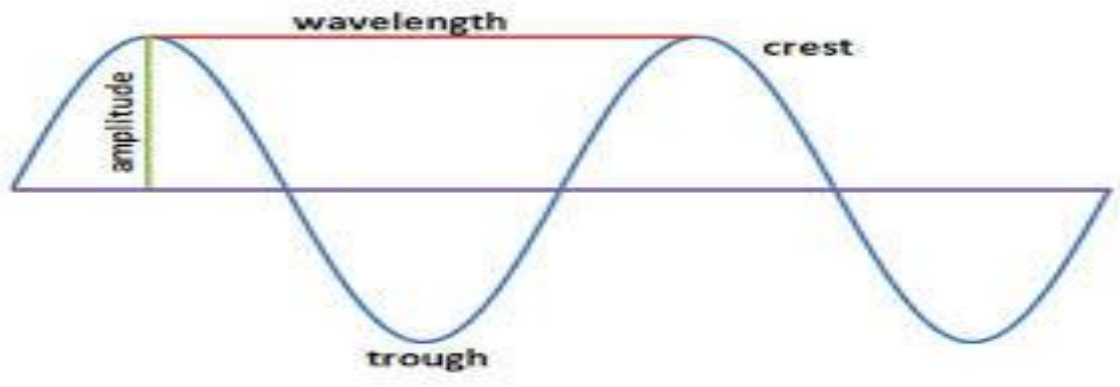


Fig.3: Wave length

The full electromagnetic radiation spectrum is continuous and each region merges slowly into the next. For spectroscopy purposes, we choose to characterize light in the ultraviolet and visible regions in terms of wavelength expressed in nanometres. Other units which may be encountered, but whose use is now, discouraged are the Angstrom(Å) and the millimicron (mμ).

1nm = 1mu = 10A =10^{-9} meters.

For convenience of reference, definitions of the various spectral regions have been set by the Joint Committee On Nomenclature in Applied Spectroscopy.

Table.2: Various wavelengths and regions

Region	Wavelength (nm)
Far ultraviolet	10-200
Near ultraviolet	200-300
Visible	380-780
Near infrared	780-3000
Middle infrared	3000-30,000
Far infrared	30,000-300,000
Microwave	300,000-1,000,000,000

The human eye is only sensitive to a tiny proportion of the total electromagnetic spectrum between approximately 380 and 780 nm and within this area we perceive the colours of the rainbow from violet through to red. If the full electromagnetic spectrum shown in Figure was redrawn on a linear scale and the visible region was represented by the length of one centimetre, then the boundary between radio and microwaves would have to be drawn approximately 25 kilometers away.

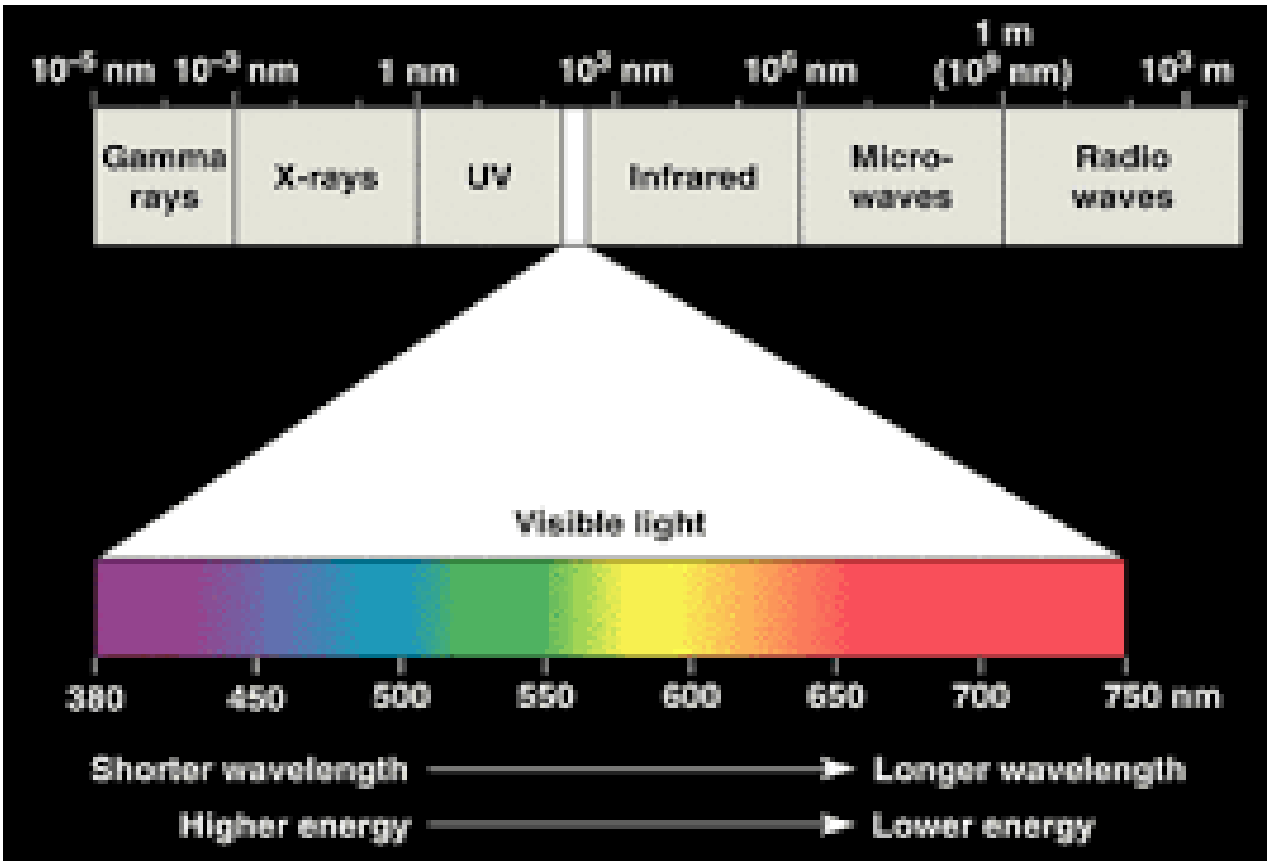


Fig.4: Visible radiation

Besides the sun, the most conveniently available source of visible radiation with which we are familiar is the tungsten lamp. If the current in the circuit supplying such a lamp is gradually increased from zero, the lamp filament at first can be felt to be emitting warmth, then glows dull red and the gradually brightens until it is emitting an intense white light and a considerable amount of heat.

The radiation from normal hot slides is made up of many wavelengths and the energy emitted at any particular wavelength depends largely on the temperature of the solid and is predictable from probability theory. The curves in the below Figure show the energy distribution for a tungsten filament at three different temperatures. Such radiation is known as ‘blackbody nation’. Note how the emitted energy increases with temperature and how the wavelength of maximum energy shifts to shorter wavelengths. More recently it has become common practice to use a variant of this the tungsten – halogen lamp. The quartz envelope transmits radiation well into the UV region. For the region itself the most common source is the deuterium lamp and a UV Visible spectrometer will usually have both lamp types to cover the entire wavelength range.

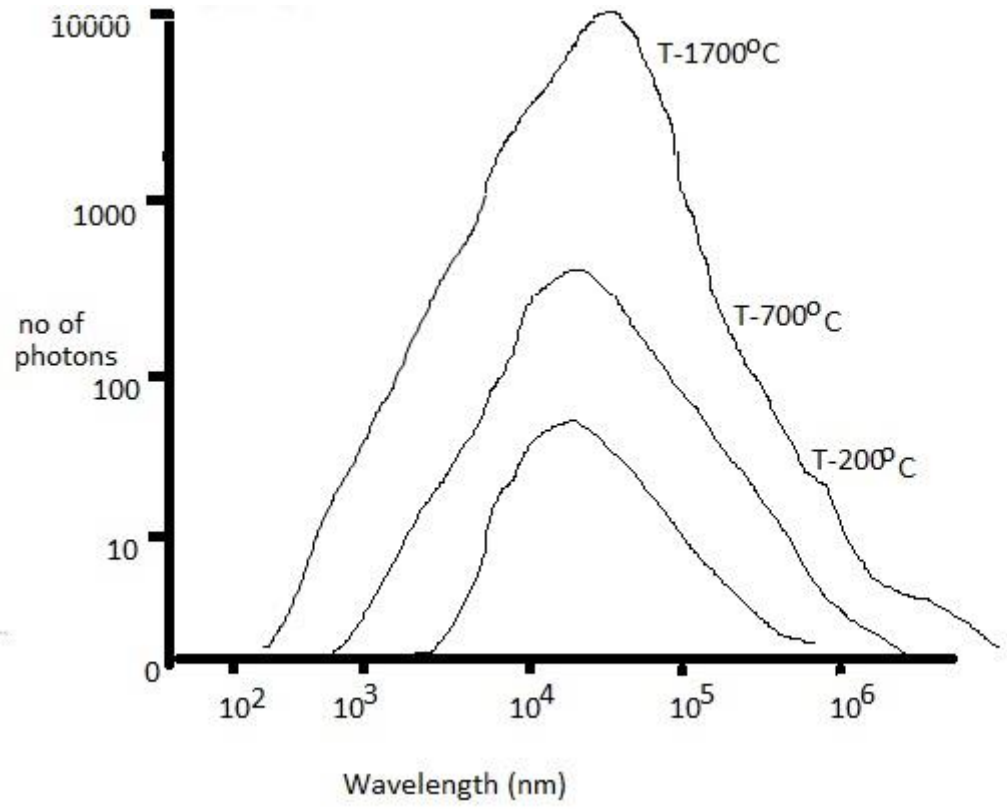


Fig.5: Wavelength Range

Quantum Theory

To gain an understanding of the origins of practical absorption spectrometry, a short diversion into quantum theory is necessary. For this purpose, it is best to think of radiation as a stream of particles known as photons instead of the waves considered earlier. Atoms and molecules exist in a number of defined energy states or levels and a change of level requires the absorption or emission of an integral number of a unit of energy called a quantum, or in our context, a photon.

The energy of a photon absorbed or emitted during a transition from one molecular energy level to another is given by equation.

$$e=h\nu$$

Where h is known as Plank's constant and v is the frequency of the photon. We have already seen that

$$c=\nu\lambda,$$

Therefore,

$$E=hc/\lambda$$

Thus, the shorter the wavelength, the greater the energy of the photon and vice versa. A molecule of any substance has internal energy which can be considered as the sum of the energy of its electrons, the energy of vibration between its constituent atoms and the energy associated with rotation of the molecule. The electronic energy levels of simple molecules are widely separated and usually only the absorption of a high energy photon, that is one of very short wavelength, can excite a molecule from one level to another.

In complex molecules the energy levels are more closely spaced and photons of near ultraviolet and visible light can effect the transition. These substances, therefore, will absorb light in some areas of the near ultraviolet and visible regions. The vibrational energy states of the various parts of a molecule are much closer together than the electronic energy levels and photons of lower energy (longer wavelength) are sufficient to bring about vibrational changes. Light absorption due to only to vibrational changes occurs in the infrared region. The rotational energy states of molecules are so closely spaced that light in the far infrared and microwave regions of the electromagnetic spectrum has enough to cause these small changes.

For ultraviolet and visible wavelength, one should expect from this discussion that the absorption spectrum of a molecule (i.e., a plot of its degree of absorption against the wavelength of the incident radiation) should show a few very sharp lines. Each line should occur at a wavelength where the energy of an incident photon exactly matches the energy required to excite an electronic transition.

In practice it is found that the ultraviolet and visible spectrum of most molecules consists of a few humps rather than sharp lines. These humps show than the molecule is absorbing radiation over a band of wavelengths. One reason for this band, rather than line absorption is that an electronic level transition is usually accompanied by a simultaneous change between the more numerous vibrational levels. Thus, a photon with a little too much or too little energy to be accepted by the molecule for a 'pure' electronic transition can be utilized for a transition between one of the vibrational levels associated with the lower electronic state to one of the vibrational levels of a higher electronic state.

If the difference in electronic energy is 'E' and the difference in vibrational energy is 'e', then photons with energies of E, E+e, E+2e, E-e, E-2e, etc., will be absorbed.

Furthermore, each of the many vibrational levels associated with the electronic states also has a large number of rotational levels associated with it. Thus a transition can consist of a large electronic component, a smaller vibrational element and an even smaller rotational change. The rotational contribution to the transition has the effect of filling in the gaps in the vibrational fine structure.

In addition, when molecules are closely packed together as they normally are in solutions, they exert influences on each other which slightly disturb the already numerous, and almost infinite energy levels and blur the sharp spectral lines into bands. These effects can be seen in the spectra of benzene as a vapour and in solutions. In the vapour, the transitions between the vibration level are visible as bands superimposed on the main electronic transition bands.

In solution they merge together and at high temperature or pressure even the electronic bands can blur to produce single wide band such as that enclosed by the dotted line in the figure.

General Chemical Origins

When white light falls upon a sample, the light may be totally reflected, in which case the substance appears white or the light may be totally absorbed, in which case the substance will appear black. If, however, only a portion of the light is absorbed and the balance is reflected, the sample appears yellow-green and if yellow is absorbed, the sample appears blue. The colours are described as complementary.

However, many substances which appear colourless do have absorption spectra. In this instance, the absorption will take place in the infra-red or ultraviolet and not in the visible region.

Table.3: Wavelength and Colour absorption

Colour Absorbed	Colour Observed	Absorbed Radiation(nm)
Violet	Yellow-Green	400-435
Blue	Yellow	435-480
Green-Blue	Orange	480-490
Blue-Green	Red	490-500
Green	Purple	500-560
Yellow-Green	Violet	560-580
Yellow	Blue	580-595
Orange	Green-Blue	595-605
Red	Blue-Green	605-750

A close relationship exists between the colour of a substance and its electronic structure. A molecule or ion will exhibit absorption in the visible or ultraviolet region when radiation cause an electronic transition within its structure. Thus, the absorption of light by a sample in the ultraviolet or visible region is accompanied by a change in the electronic state of the molecules in the molecule.

The energy supplied by the light will promote electrons from their ground state orbital's to higher energy, excited state orbital's or antibonding orbital's. Potentially, three types of ground state orbital's may be involved:

σ (bonding) molecular as in

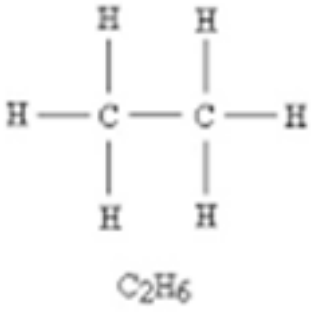


Fig.6: Structure of Ethane

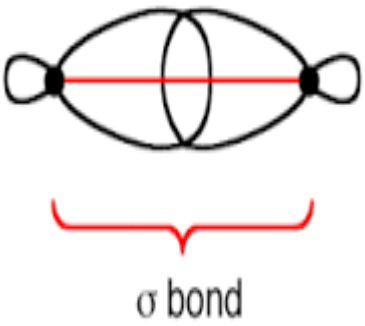


Fig.7: Structure of Sigma bond

π (bonding) molecular orbital as in

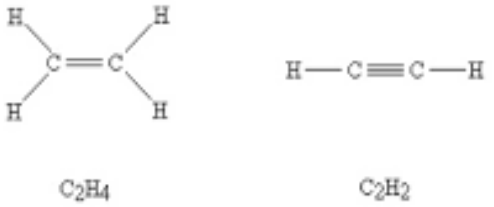


Fig.8: **Structure of Ethene**

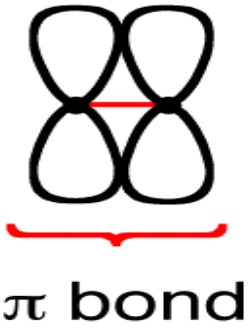


Fig.9: **Structure of Pi bond**

n (non-bonding) atomic orbital as in

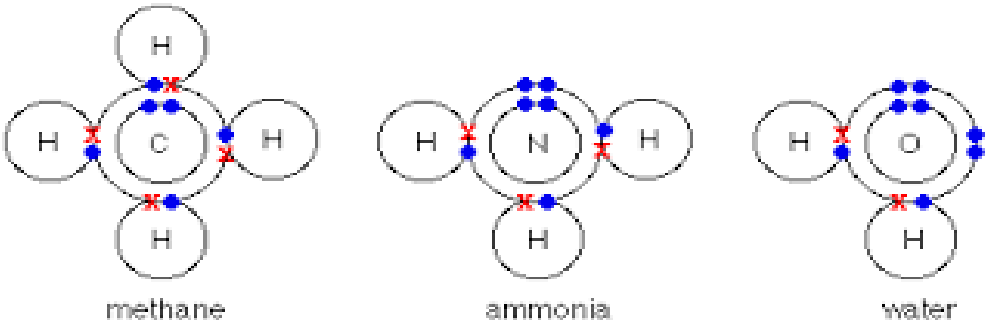


Fig.10. Non bonding atomic orbital's

In addition, two types of anti-bonding orbital's may be involved in the transition:

i. σ* (sigma star) orbital
ii. **π*** (pi star) orbital

(There is no such thing an **n*** antibonding orbital as the n electrons do not form bonds).A transition in which a bonding electron is excited to an antibonding σ orbital is referred to as σ to σ* transition. In the same way π to π* represents the transition of one electron of a lone pair (non-bonding electron pair) to an antibonding π orbital. Thus the following electronic transitions can occur by the absorption of ultraviolet and visible light.

σ to σ*
n to σ*
n to π*
π to π*.

Figure illustrates the general pattern of energy levels and the fact that the transitions are brought about by the absorption of different amounts of energy.

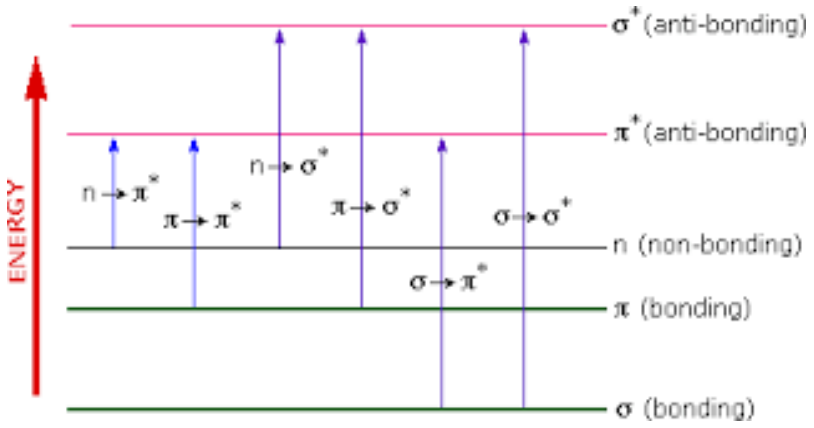


Fig.11: Electron transitions

Both s to σ* and n to σ* transition require a great deal of energy and therefore occur in the far ultraviolet region or weakly in the far ultraviolet region or weakly in the region 180-240nm.Consequentiy, saturated groups do not exhibit strong absorption in the ordinary ultraviolet region. Transition of the n to π* and π to π* type occur in molecules with unsaturated centres; they require less energy and occur at longer wavelengths than transitions to σ* anti bonding orbital. Below Table illustrates the type of transition and the resulting maximum wavelength.

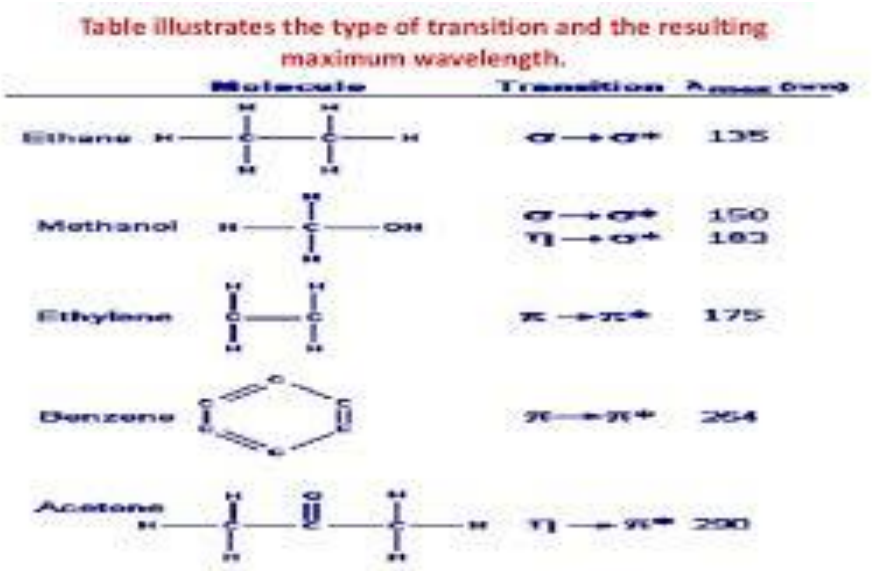


Fig.12: Type of transition and maximum wavelength

It will be seen presently that the wavelength of maximum absorption and the intensity of absorption are determined by molecular structure. Transitions to π* anti bonding orbital which occur in the ultraviolet region for a particular molecule may well take place in the visible region if the molecular structure is modified. Many inorganic compounds in solution also show absorption in the visible region. These include salts of elements with incomplete inner electron shells (mainly transition metals) whose

ions are complexed by hydration e.g. $[Cu(H_2O4)]_2^+$. Such absorptions arise from a charge transfer process, where

Electrons are moved from one part of the system to another by the energy provided by the visible light.

Correlation of Molecular structure and spectra Conjugation

π to π* transitions, when occurring in isolated groups in a molecule, give rise to absorptions of fairy low intensity. However, conjugation of unsaturated groups in a molecule produces a remarkable effect upon the absorption spectrum. The wavelength of maximum absorption moves to a longer wavelength and the absorption intensity may often increase.

The same effect occurs when groups containing n electrons are conjugated with a π electron group. Aromatic systems, which contain p electrons, absorb strongly in the ultraviolet. In general, the greater the length of a conjugated system in a molecule, the nearer the λmax comes to the visible region. Thus, the characteristic energy of a transition and hence the wavelength of absorption is a property of a group of atoms rather than the electrons themselves. When such absorption occurs, two types of groups can influence the resulting absorption spectrum of the molecule: Chromophores and Auxochromes.

Chromophores

A chromophore (literally colour-bearing) group is a functional group, not conjugated with another group, which exhibits a characteristic absorption spectrum in the ultraviolet or visible region. Some of the more important chromophoric groups are:

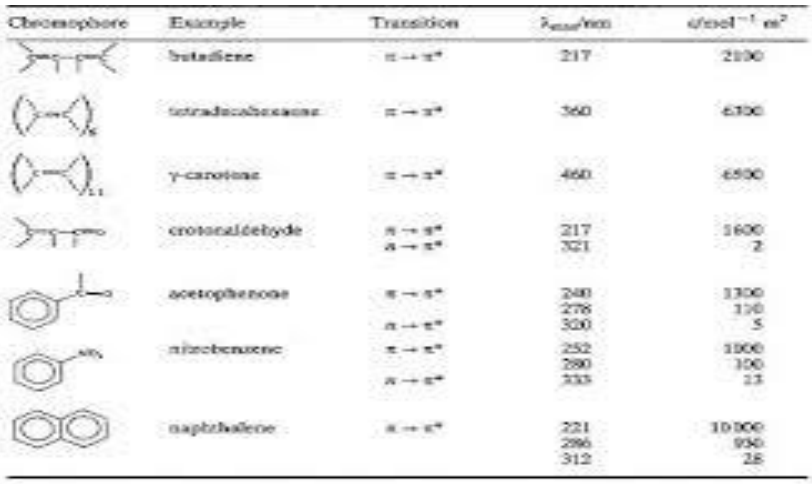


Fig.13: Chromophores

If any of the simple Chromophores is conjugated with another (of the same type or different type) a multiple chromophore is formed having a new absorption band which is more intense and at a longer wavelength that the strong bands of the simple Chromophores.

This displacement of an absorption maximum towards a longer wavelength (i.e. from blue to red) is termed a bathochromic shift. The displacement of an absorption maximum from the red to ultraviolet is hypsochromic shift.

Auxochromes:

The colour of a molecule may be intensified by groups called 800nm region, but will affect the spectrum of the chromophore to which it is attached. The most important auxochromic groups are OH,NH_2,CH_3 and NO_2 and their properties are acidic (phenolic) or basic. The actual effect of an auxochrome on a chromophore depends on the polarity of the auxochrome, e.g. groups like CH_3-,CH_3CH_2- and CL-have very little effect, usually a small red shift of 5-10nm. Other groups such as –NH_2 and –NO_2 are very popular and completely alter the spectra of Chromophores such as:

Fig.14: Auxochromes

In general it should be possible to predict the effect of non-polar or weakly polar auxochrome, but the effect off strongly polar auxochrome is difficult to predict. In addition, the availability of non-bonding electrons which may enter in to transitions also contributes greatly to the effect of an auxochrome.

Steric Effects:

Steric hindrance will also affect the influence of an auxochrome on a chromophore. Electron systems conjugate best when the molecule is planar in configuration. If the presence of an auxochrome prevents the molecule from being planar then large effects will be noticed in the spectrum; e.g., m- and p-methyl groups in the diphenyls have predictable but slight effects on the spectra

Compared with that of diphenyls itself. However, methyl groups in the o-position alter the spectrum completely.

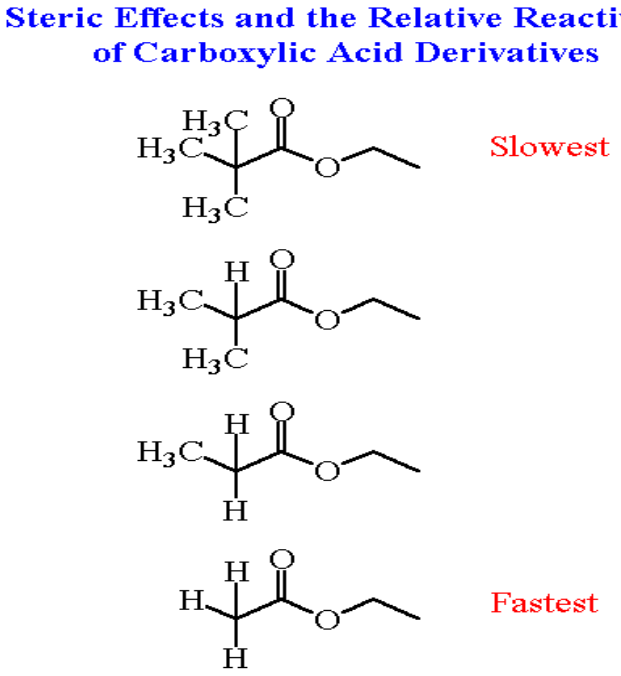


Fig.15: Structures of Steric compounds

Cis and trans isomers of linear polymers also show differences in their spectra. The all-trans isomer has the longer conjugated system. λ_{max} is at a longer wavelength and ε_{max} (molar absorptivity or molar extinction coefficient) is higher than for the all cis or mixed isomer.

Visible Spectra:

In general a compound will absorb in the visible region if it contains at least five conjugated chromophoric and auxochromic groups.

The ability to complex many metals, particularly the transition elements, with complex organic and inorganic molecules which absorb in the visible region provides the basis for their quantitative spectrometric analysis. The absorptions are due to moment of electrons between energy levels of the organo-metal complex. These complexing systems are termed spectrometric reagents. The most common are dithizone, azo reagents (PAN, thoron and zincon), dithiocarbamate, 8-hydroxyquinoline, formaldoxime and thiocynate. In addition, many inorganic ions in solution also absorb in the visible region e.g. salts of Ni, Co, Cu, V etc. and particularly elements with incomplete inner electrons shells whose ions are complexed by hydration e.g. $(Cu(H_2O)_4)_2+$. Such absorptions arise from a charge transfer process where electrons are moved from one part of the system to another due to the energy provided by the visible light.

Solvents

The effect on the absorption spectrum of a compound when diluted in a solvent will vary depending on the chemical structures involved. Generally speaking, non-polar solvents and non-polar molecules show least effect. However, polar molecules exhibit quite dramatic differences when interacted with a polar solvent. Interaction between solute and solvent leads to absorption band

broadening and a consequent reduction in structural resolution and εmax. Ionic forms may also be created in acidic or basic conditions. Thus care must be taken to avoid an interaction between the solute and the solvent.

The effect of iso-octane and ethanol on the spectrum of phenol, a change from hydrocarbon to hydroxylic solvent. The loss of fine structure in the latter is due to broad band h-bonded solvent-solute complexes replacing the fine structure present in the iso-octane. The fine structure in the latter solvent illustrates the principle that non-solvating or non-chelating solvents produce a spectrum much closer to that obtained in the gaseous state.

Commercially available solvents of 'spectroscopic purity' are listed in Table: 3 accompanied by their cut-off wavelengths, based on a 10mm path length. Water and 0.1N solutions of hydrochloric acid and sodium hydroxide are commonly used solvents for absorption spectrometry. Again care has to be taken to avoid interaction. Where methodology requires buffering, solutions have to be non-absorbing and generally both the composition and pH will be specified. However, if this information is not available lists can be found in the literature. For reactions in the 4.2 to 8.8 Ph region, mixtures of 0.1N dihydrogen sodium phosphate and 0.1N hydrogen disodium phosphate are generally used.

Table.4: Various wavelengths for various solvents

Solvent	Cut-Off (nm)
Iso-Octane	202nm
Ethyl alcohol	205nm
Cyclohexane	200nm
Acetone	325nm
Tetrachloroethylene	290nm
Benzene	280nm
Carbon tetrachloride	265nm
Chloroform	245nm
Ethyl ether	220nm
Isopropyl alcohol	210nm
Methyl alcohol	210nm

General Interactions of Light and Matter

When a beam of radiation strikes any object it can be absorbed, transmitted, scattered, reflected or it can excite fluorescence. With scattering it can be considered that the radiation is first absorbed then almost instantaneously completely re-emitted uniformly in all directions, but otherwise unchanged. With fluorescence a photon is first absorbed and excites the molecules to a higher energy state, but the molecule then drops back to an intermediate energy level by re-emitting a photon. Since some of the energy of the incident photon is retained in the molecule (or is lost by a non-radiative process such as

collision with another molecule) the emitted photon has less energy and hence a longer wavelength than the absorbed photon. Like scatter, fluorescent radiation is also emitted uniformly in all directions.

The processes concerned in absorption spectrometry are absorption and transmission. Usually the conditions under which the sample is examined are chosen to keep reflection, scatter and fluorescence to minimum. In the ultraviolet and visible regions of the electromagnetic spectrum, the bands observed are usually not specific enough to allow a positive identification of an unknown sample, although this data may be used to confirm its nature deduced from its infrared spectrum or by other techniques. Ultraviolet and visible spectrometry is almost entirely used for quantitative analysis; that is the estimation of the amount of a compound known to be present in the sample. The sample is usually examined in solutions.

Lambert's (Bouguer's) Law

Lambert's Law states that each layer of equal thickness of an absorbing medium absorbs an equal fraction of the radiant energy that traverses it. The fraction of radiant energy transmitted by a given thickness of the absorbing medium is independent of the intensity of the incident radiation, provided that the radiation does not alter the physical or chemical state of the medium.

If the intensity of the incident radiation is *I*o and that of the transmitted light is *l*, then the fraction transmitted is:

l/lo = T

The percentage transmission is

%T =l/lo X 100

If a series of coloured glass plates of equal thickness are placed in parallel, each sheet of which absorbs one quarter of the light incident upon it, then the amount of the original radiation passed by the first sheet is: (1 – ¼)/1 100 =75% and the second sheet is 56.25%, i.e. 75% of 75%, and by the third sheet is 42.19%, i.e. 75% of 56.25%, and by the nth sheet is (0.75)^n X 100%.

Now imagine a container with parallel glass wall 10mm apart filled with an absorbing solution. If monochromatic light is incident on one face and 75% of the light is transmitted, Lambert's Law states that if a similar cell is put next to the first the light transmitted will be reduced to 56.25%.If the contents of two containers are evaporated to half their volume, thereby doubling their concentration, and then measured in a single container, it will be found that the transmission will again be reduced to 56.25%.

It can be immediately seen that to determine the concentration of an unknown sample the percentage transmittance of a series of solutions of known concentration or 'standards' can be plotted and the concentration or the unknown read from the graph. It will be found that the graph is an exponential function which is obviously inconvenient for easy interpolation.

The Beer-Lambert Law

The Beer-Lambert Law states that the concentration of a substance in solution is directly proportional to the 'absorbance', A, of the solution.

Absorbance A = constant X concentration X cell length

The law is only true for monochromatic light, that is light of a single wavelength or narrow band of wavelengths, and provided that the physical or chemical state of the substance does not change with concentration. When monochromatic radiation passes through a homogeneous solution in a cell, the intensity of the emitted radiation depends upon the thickness (1) and the concentration (C) of the solution.

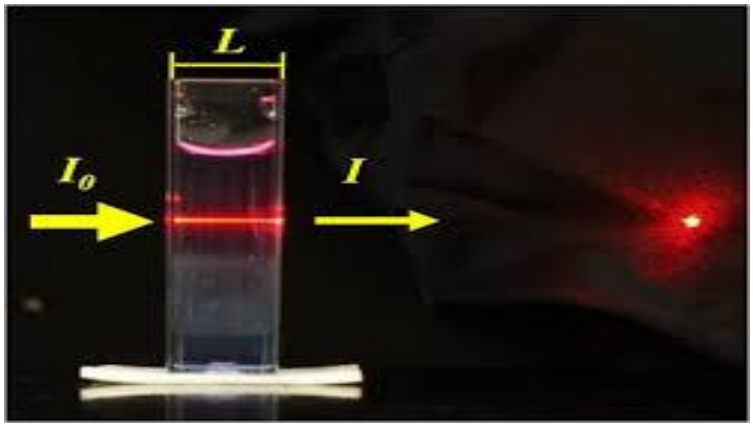


Fig.16: Transmission of light through drug in Cuvette

$\mathbf{I}_0$ is the intensity of the incident radiation and *I* is the intensity of the transmitted radiation. The ratio *I/Io* is called transmittance. This is sometimes expressed as a percentage and referred to as % transmittance.

Mathematically, absorbance is related to percentage transmittance T by the expression:

A = log10 (Io/I) = log10(100/T) = kcl

Where L is the length of the radiation path through the sample, c is the concentration of absorbing molecules in that path, and k is the extinction coefficient – a constant dependent only on the nature of the molecule and the wavelength of the radiation. Now, in the example above, the transmittance of our sample fell from 75 to 56.26% when the concentration doubled. What happens to the absorbance in the same circumstance?

A =log10 (100/T) = log10 (100) – log10 (T) =2 – log10 (T)

When T = 75%, A = 2 – 1.875 = 0.125

When T = 56.25%, A = 2 -1.750 = 0.250

Quite clearly as the absorbance doubles for twice the concentration, it is far more convenient to work in absorbance than transmittance for the purposes of quantitative analysis. It is useful to remember that

0%T = ∞A

0.1% = 3.0A

1.05T = 2.0A

10%T = 1.0A

100% = 0A

Absorbance in older literature is sometimes referred to as ‘extinction’ or ‘optical density’ (OD).

If, in the expression A = kcl, c is expressed in molar – 1 and 1 in m, then k is replaced by the symbol = and is called the molar absorption coefficient. The units of = are mol-1 m2. = was formerly called the molar extinction coefficient and concentrations were often expressed as mol 1-1, mol dm-3 or M and the cell length in cm to give units mol-11cm-1, mol-1dm3cm-1 and M – 1 cm -1 respectively.

Alternatively, if the relative molecular mass (molecular weight) of the substance is unknown, then a 1% w/v solution is prepared and the absorbance is measured in a 1 cm cell. In this case, k is replaced by E1% Sometimes the wavelength is include: E1% (325 nm).

C Sometimes is expressed in g dm-3(gl-1) and 1 in cm. In this case, k is replaced by A (sometimes E). A is known as the specific absorption coefficient.

INSTRUMENTATION OF UV-SPECTROPHOTOMETER:

Fig.17: UV Spectrophotometer

Introduction:

Have a look at this schematic diagram of a double – beam UV – Vis. Spectrophotometer;

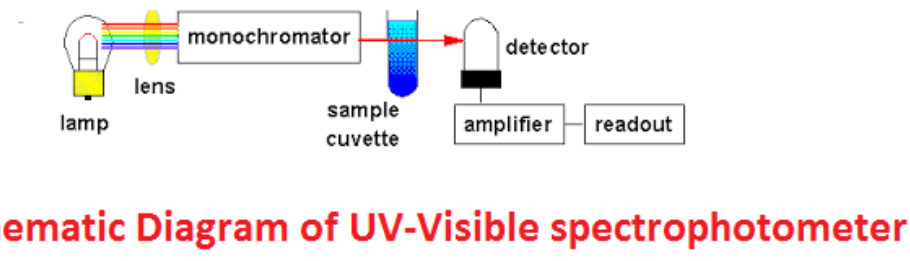


Fig.18: Schematic diagram of UV-Visible spectrophotometer

Instruments for measuring the absorption of U.V. or visible radiation are made up of the following components;

1. Sources (UV and visible)
2. Wavelength selector (monochromator)
3. Sample containers
4. Detectors
5. Single processor and readout

Each of these components will be considered in turn.

Instrumental components:

Sources of UV radiation:

It is important that the power of the radiation source does not change abruptly over it's wavelength range.

The electrical excitation of deuterium or hydrogen at low pressure produces a continuous UV spectrum. The mechanism for this involves formation of an exited molecular species, which breaks up to give two atomic species and an ultraviolet photon. This can be shown as;

D_2 +electrical energy D_2^* D'+ D" + hν

Both deuterium and hydrogen lamps emit radiation in the range 160-375nm Quartz windows must be used in these lamps, and quartz cuvettes must be used, because glass absorbs radiation of wavelengths less than 350 nm.

Sources of visible radiation:

The tungsten filament lamp is commonly employed as a source of visible light. This type of lamp is used in the wavelength range of 350 – 2500 nm. The energy emitted by a tungsten filament lamp is proportional to the fourth power of operating voltage. This means that for the energy output to be stable indeed. Electronic voltage regulators or constant-voltage transformers are used to ensure this stability.

Tungsten/halogen lamps lamps contain a small amount of iodine in a quartz "envelope" which also contains the tungsten filament. The iodine reacts with gaseous tungsten, formed by sublimation, producing the volatile compound WI_2. When molecules of WI_2 hit the filament they decompose, re-depositing tungsten back on the filament. The lifetime of a tungsten/halogen lamp is approximately double that of an ordinary tungsten filament lamp. Tungsten/halogen lamps are very efficient, and their output extends well into the ultra-violet. They are used in many modern spectrophotometers.

Wavelength selector (monochromator):

All monochromators contain the following component parts;

- An entrance slit
- A collimating lens

- A dispersing device (usually a prism or a grating)
- A focusing lens
- An exit slit

Polychromatic radiation (radiation of more than one wavelength) enters the monochromator through the entrance slit. The beam is collimated, and then strikes the dispersing element at an angle. The beam is split into its component wavelengths by the grating or prism. By moving the dispersing element or the exit slit, radiation of only a particular wavelength leaves the monochromator through the exit slit.

Czerney-Turner grating monochromator:

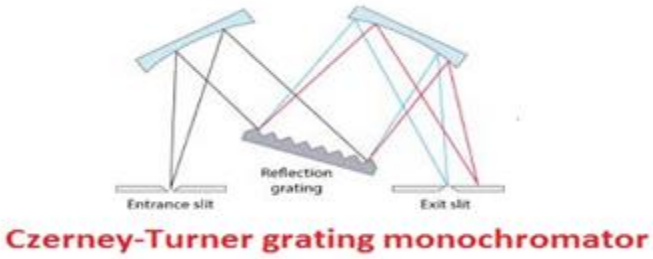


Fig.19: Czerney-Turner grating monochromator

Cuvettes:

The containers for the sample and reference solution must be transparent to the radiation which will pass through them. Quartz or fused silica cuvettes are required for spectroscopy in the UV region. These cells are also transparent in the visible region. Silicate glasses can be used for the manufacture of cuvettes for use between 350 and 2000 nm.

Fig.20: Various types of Cuvettes

Detectors:

The photomultiplier tube is a commonly used detector in UV – V is spectroscopy. It consists of a photo emissive cathode (a cathode which emits electrons when struck by photons of radiation), several dynodes (which emit several electrons for each electron striking them) and an anode.

A photon of radiation entering the tube strikes the cathode, causing the emission of several electrons. These electrons are accelerated towards the first dynode (which is 90V more positive than the cathode). The electrons strike the first dynode, causing the emission of several electrons for each

incident electron. These electrons are then accelerated towards dynode three and so on. Eventually, the electrons are collected at the anode. By this time, each original photon has produced $10^6 – 10^7$ electrons. The resulting current is amplified and measured.

Photomultipliers are very sensitive to UV and visible radiation. They have fast response times. Intense light damages photomultipliers; they are limited to measuring low power radiation.

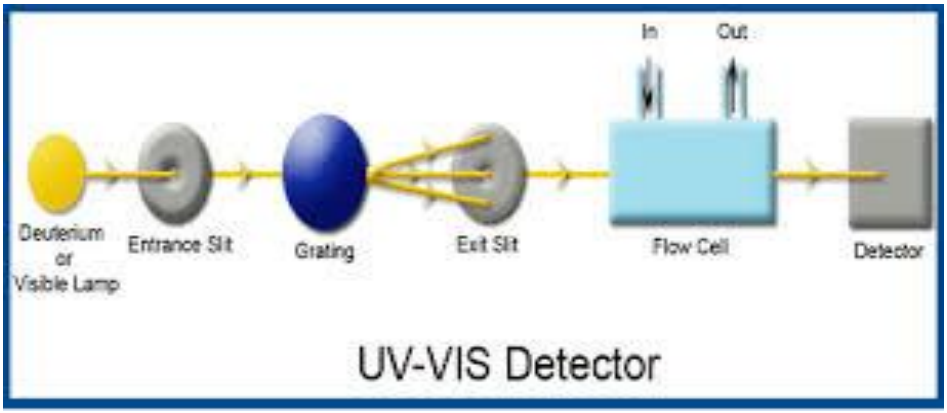


Fig.21: UV-VIS Detector

Cross section of a photomultiplier tube:

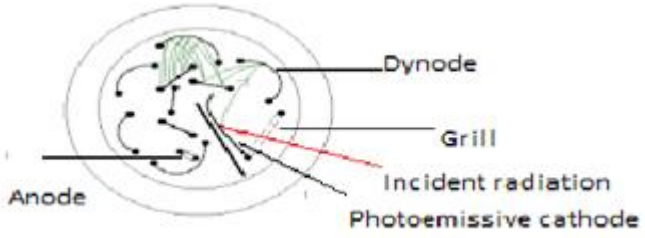


Fig.22: Cross section of photomultiplier tube

The linear photodiode array is an example of a multichannel photon detector. These detectors are capable of measuring all elements of a beam of dispersed radiation simultaneously.

A linear photodiode array comprises many small silicon photodiodes formed on a single silicon chip. There can be between 64 to 4096 sensor elements on a chip, the most common being 1024 photodiodes. For each diode, there is also a storage capacitor and a switch. The individual diode-capacitor circuits can be sequentially scanned.

In use, the photodiode array is positioned at the focal plane of the monochromator (after the dispersing element) such that the spectrum falls on the diode array. They are useful for recording UV-V is absorption spectra of samples that are rapidly passing through a sample flow cell, such as in an HPLC detector.

Charge-Coupled devices (CCDs) are similar to diode array detectors, but instead of diodes, they consist of an array of photo capacitors.

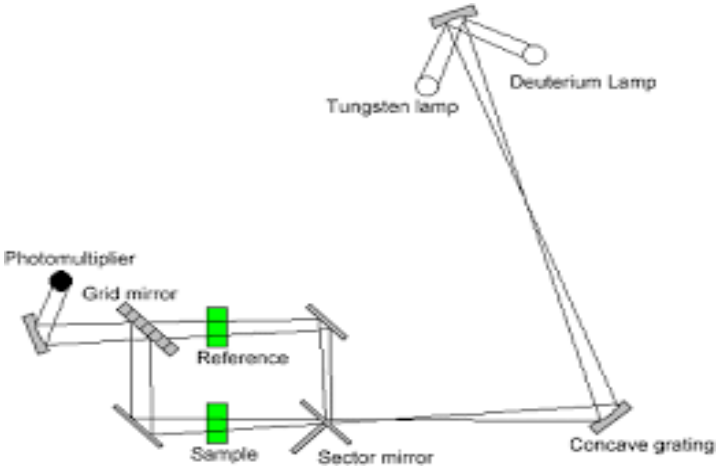


Fig.23: Charge Coupled Device

Applications:

UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules. Spectroscopic analysis is commonly carried out in solutions but solids and gases may also be studied.

- Solutions of transition metal ions can be coloured (i.e., absorb visible light) because d electrons within the metal atoms can be excited from one electronic state to another. The colour of metal ion solutions is strongly affected by the presence of other species, such as certain anions or ligands. For instance, the colour of a dilute solution of copper sulphate is a very light blue; adding ammonia intensifies the colour and changes the wavelength of maximum absorption (λ_{max}).
- Organic compounds, especially those with a high degree of conjugation, also absorb light in the UV or visible regions of the electromagnetic spectrum. The solvents for these determinations are often water for water-soluble compounds, or ethanol for organic-soluble compounds. (Organic solvents may have significant UV absorptions; not all solvents are suitable for use in UV spectroscopy. Ethanol absorbs very weakly at most wavelength.) Solvent polarity and PH can affect the absorption spectrum of an organic compound.
  Tyrosine, for example, increases in absorption maxima and molar extinction coefficient when pH increases from 6 to 13 or when solvent polarity decrease.
- While charge transfer complexes also give rise to colours, the colours are often too intense to be used for quantitative measurement.

The Beer-Lambert's law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a fixed path length, UV/Vis spectroscopy can be used to determine the concentration of the absorber in a solution. It is necessary to know how quickly the absorbance changes with concentration. This can be taken from references (tables of molar extinction coefficients), or more accurately determined from a calibration curve.

A UV/Vis spectrophotometer may be used as a detector for HPLC. The presence of an analyte gives a response assumed to be proportional to the concentration. For accurate results, the instrument's response to the analyte in the unknown should be compared with the response to a standard; this is very similar to the use of calibration curves. The response (e.g., peak height) for a particular concentration is known as the response factor.

The wavelengths of absorption peaks can be correlated with the types of bonds in a given molecule and are valuable in determining the functional groups within a molecule. The Woodward-Fieser rules, for instance, are a set of empirical observations used to predict λ_{max}, the wavelength of the most intense UV/V is absorption, for conjugated organic compounds such as dienes and ketones. The spectrum alone is not, however, a specific test for any given sample. The nature of the solvent, the pH of the solution, temperature, high electrolyte concentrations, and the presence of interfering substances can influence the absorption spectrum. Experimental variations such as the slit width (effective bandwidth) of the spectrophotometer will also alter the spectrum. To apply UV/V is spectroscopy to analyse, these variables must be controlled or accounted for in order to identify the substances present.

UV-Vis spectroscopy is also used in the semiconductor industry to measure the thickness and optical properties of thin films on a water. UV-V is spectrometers are used to measure the reflectance of a light, and can be analyzed via the Forouhi Bloomer despertion equations to determine the Index of Refraction (n) and the Extinction Coefficient (k) of a given film across the measured spectral range.

VALIDATION OF ANALYTICAL ASSAYS AND TEST METHODS FOR THE PHARMACEUTICAL LABORATORY:

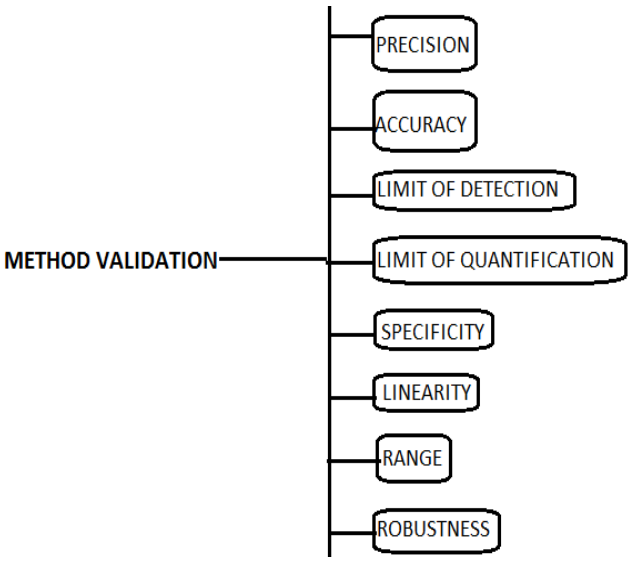


Fig.24: The "Eight Steps Of Assay/Method Validation"

SPECIFICITY:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implications:

- **IDENTIFICATION:** To ensure the identity of an analyte.
- **PURITY TESTS:** To ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e., related substances test, heavy metals, residual solvents content, etc.
- **ASSAY** (Content or potency): To provide an exact result which allows accurate statement on the content or potency of the analyte in a sample.

ACCURACY:

The closeness of agreement between the value which accepted either as a conventional true value or an accepted reference value, and the value found.

Note: When measuring accuracy, it is important to spike placebo preparations with varying amounts of active ingredients(s). If a placebo cannot be obtained, then a sample should be spiked at varying levels. In both cases, acceptable recovery must be demonstrated.

PRECISION:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic (full scale) samples. However, if it is not possible obtain a full-scale sample it may be investigated using a pilot-scale or bench-top scale sample or sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Refer to this month's "The Regulatory Clinic" for a discussion of Accureg's consensual interpretations of the following terms that express precision:

a. **Repeatability:** Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.
b. **Intermediate Precision:** Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipments, etc.
c. **Reproducibility:** Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology).

LOD (limit of detection):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as exact value. Several approaches for determining the detection limit are possible. Based on visual evaluations: The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. Based on Signal-to-Noise Approach: Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope: The CONCENTRATION/SLOPE OF THE CALIBRATION LINE.

LOQ (limit of quantification):

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. Based on visual evaluation: T he detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise Approach: Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1. Based on the Standard Deviation of the Response and the Slope: The quantitation limit (QL) may be expressed as: 10X STANDARD DEVIATION OF LOW CONCENTRATED/SLOPE OF THE CALIBRATION LINE.

LINEARITY:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Note: Measurements using clean standard preparations should be performed to demonstrate detector linearity, while method linearity should be determined concurrently during the accuracy study: Classical linearity acceptance criteria are

1) That the correlation coefficient of the linear regression line is not more than some number close to 1, and
2) That the y-intercept should not differ significantly from zero.

When linear regression analyses are performed, it is important not to force the origin as (0,0) in the calculation. This practice may significantly skew the actual best-fit slope through the physical range of use.

RANGE:

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level precision, accuracy and linearity.

ROBUSTNESS:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage.

Note: Ideally, robustness should be explored during development of the assay method. By far the most efficient way to do this though the use of a designed experiment. Such experimental designs might include a Plackett-Burman matrix approach to investigate first order effects, or a 2k factorial design that will provide information regarding the first (main) and higher order (interaction) effects.

In carrying out such a design, one must first identity variables in the method that may be expressed to influence the result. For instance, consider an HPLC assay which uses an ion-pairing reagent. One might investigate: sample sonication or mixing time; mobile phase organic solvent constituently; mobile phase Ph; column temperature; injection volume; flow rate; modifier concentration; concentration of ion-pairing reagent; etc. It is through this short of a development study that variables with the greatest effects on results may be determined in a minimal number of experiments.

OBJECTIVE OF THE WORK

Method Development

The objective of the present study was to develop simple, sensitive, accurate, precise, reproducible, rugged, and robust and relatively inexpensive analytical method (UV- Spectrophotometric) for the analysis of Efavirenz.

Instrument used

1. UV-Visible spectrophotometer

Validation of the developed methods

1. Accuracy

2. Precision

3. Linearity range

4. Ruggedness

5. Limit of Detection

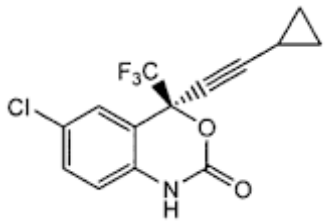
6. Limit of Detection

DRUG PROFILE

DRUG NAME : Efavirenz

IUPAC NAME : (S)-6-Chloro-4-(cyclopropylethynyl) -1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one

STRUCTURE :



MOLECULAR FORMULA: $C_{14}H_9ClF_3NO_2$

MOLECULAR WEIGHT : 315.675g/mol

MELTING POINT : 139-141^{0}C

P^{ka} : 10.2P^{ka}

SOLUBILITY : Practically insoluble in water, soluble in methanol

DESCRIPTION :

Efavirenz is a non-nucleotide reverse transcriptase inhibitor and is used a part of highly activeHuman immune deficiency virus type1. It is a white crystalline powder.Efavirenz is also usedIn combination with other antiretroviral agents as part of an expanded post exposure prophylaxisregimen to reduce the risk of HIV infection.The usual adult dose of efavirenz is 600mg once a day.

MECHANISM OF ACTION :

Efavirenz inhibits the activity of viral RNA-directed DNA polymerase.Antiviral activity of efavirenzis dependent on intracellular conversion to the Active phosphorylated form.The rate of efavirenzphosphorylation varies,depending on cell type.It is believed that inhibition of reverse

transcriptaseinterfers with the generation of DNA copies of viral RNA,which,in turn,are neceessary for synthesis of new virions.Intracellular enzymes subsequently eliminate the HIV particle that previously been uncoated and left unprotected,during entry into the host cell.Thus,reverse transcriptase inhibitors are virustatic and do not eliminate HIV from the body.Even though human DNA polymeraseis less susceptible to the pharmacologic effects of triphosphorylated efavirenz,this action may neveraccount for some of the drug toxicity.

PHARMACOLOGY:

Efavirenz (Adkins et al., 1998) is a non-competitive inhibitor of HIV 1 reverse transcriptase (RT). It has no inhibitory effect on HIV-2 RT or human cellular DNA polymerases alpha, beta, gamma, or delta. Efavirenz binds directly to RT and inhibits viral RNA- and DNA-dependent DNA polymerase activities by disrupting the catalytic site. Although the drug RT template complex may continue to bind deoxynucleoside triphosphate and to catalyze its incorporation into the newly forming viral DNA, it does so at a slower rate.

ADMINISTRATION :

Efavirenz is administered by mouth as capsules or tablets in an adult dose of 600 mg once daily, alternatively, it may be given as an oral solution in an adult dose of 720 mg once daily. Dose at bedtime is recommended during the first 2 to 4 weeks of therapy to improve tolerability. Doses (as capsules) for children over the age of 3 years are based on body weight, children weighing 13 to 14 kg are given 200 mg once daily, those weighing 15 to 19 kg, 250 mg once daily, those weighing 20 to 24 kg, 300 mg once daily, those weighing 25 to 32.4 kg, 350 mg once daily, those weighing 32.5 to 39 kg, 400 mg once daily and those weighing 40 kg or more, 600 mg once daily. Bioavailability of Efavirenz from the oral solution is less than that from the capsule and so proportionately higher doses are used, the dose ranges which are again calculated in terms of body weight depend on the range.

USES :

Efavirenz is a non-nucleoside reverse transcriptase inhibitor with activity against HIV. It is used with other anti-retrovirals for combination therapy of HIV infection.

SINGLE DRUG DOSAGE FORMULATIONS :

Table 4: Single Drug dosage formulation

NAME	DOSAGE FORM	STRENGTH	ROUTE OF ADMINISTRATION	MANUFACTURING COMPANY
Efavir	Capsule	200mg	Oral	Cipla Limited
Efferven	Tablet	200mg	Oral	Ranbaxy Laboratories
Evirenz	Tablet	600mg	Oral	Alkem Laboratories
Revenz	Tablet	600mg	Oral	Sain Medicaments Pvt Ltd.
Viranz	Tablet	200mg	Oral	Aurobindo Pharma Ltd.
Viraday	Tablet		Oral	Cipla

COMBINATION OF DRUGS IN FORMULATIONS

Table.6: Combination of drugs in formulations

NAME	DOSAGE FORM	STRENGTH	ROUTE OF ADMINISTRATION	MANUFACTURING COMPANY
Vonavir	Tablet	**Tenofovir Disproxil Fumerate300mg+Efavirenz600mg**	Oral	Emcure
Zidolrocm-N	Tablet	**Zidovudine300mg+Lamivudine150mg+Efavirenz200mg**	Oral	HeteroHC
Virolis-E Kit	Kit	**Efavirenz,Lamivudine,Stavudine**	-	Ranbaxy Laboratories
Virocmb-E	Kit	**Efavirenz, Lamivudine, Zidovudine**	-	Ranbaxy Laboratories Ltd
Lazid-N	Tablet	**Zidovudine300mg+Lamivudine150mg+Efavirenz300mg**	Oral	Emcure

LITERATURE REVIEW

1. Dogan-Topal *et al.* **[01]** have developed a reverse phase high performance liquid chromatographic method with diode array detection procedure for the simultaneous determination of abacavir, Efavirenz and valganciclovir in spiked human serum. Separation was performed on a Waters spherisorb column with acetonitrile:methanol: KH2PO4 (pH-5.0) (40:20:40v/v/v) isocratic elution at a flow rate of 1.0mL/min. Calibration curves were constructed in the range of 50 - 30,000ng/mL for abacavir and Efavirenz, and 10- 30,000ng/mL for valganciclovir in serum samples. The limit of detection and limit of quantification were 3.80 and 12.68ng/mL for abacavir, 2.61 and 8.69ng/mL Efavirenz, and 1.30 and 4.32ng/mL for valganciclovir.

2. Viana *et al.* **[02]** explained a simple assay method by HPLC for Efavirenz in tablet. Analyses were performed by UV detector at 252 nm, on a reverse phase column C18, 250 mm x 3.9 mm, 10µm using isocratic mobile phase containing acetonitrile/water/orthophosphoric acid (70:30:0.1).

3. Hamrapurkar *et al.* **[03]** have reported a stability-indicating high performance liquid chromatographic (HPLC) method for analysis of Efavirenz in the presence of the degradation products generated in the stress degradation study. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal decomposition. Extensive degradation was found to occur in alkaline medium and under thermal stress. Minimum degradation was observed under acidic medium, in the photolytic conditions and oxidative stress. Separation of drug from degradation products formed under stress conditions was achieved on a C-8 column using acetonitrile:potassium dihydrogen phosphate (pH 2.9, 25 mM) - (60:40, v/v) as the mobile phase. The flow rate was 1 mL/min and the detector was set at in a range of wavelength between 220 nm to 390 nm.

4. Potale *et al*. **[04]** have described a stability indicating high-performance thin layer chromatographic method for analysis of Efavirenz. The method employed TLC aluminium plate's pre coated with silica gel 60 F 254 as the stationary phase. The solvent system consisted of dichloromethane:methanol (5:0.3, v/v). This system was found to give compact spot for Efavirenz (Rf value 0.72 ± 0.03). Efavirenz was subjected to stress test conditions like acid, alkali, neutral hydrolysis, oxidation, and dry heat and photo degradation. The spot for product of degradation were well
resolved from the drug. Densitometric analysis of drug was carried out in the absorbance mode at 247 nm. The linear regression data for the calibration plots showed good linear relationship with r2 was 0.998 in the concentration range of 400-2000ng/spot.

5. Reverse phase high performance liquid chromatography methods have been developed for the simultaneous estimation of Efavirenz, lamivudine and zidovudine in tablet dosage form **[05]**. In reverse phase high performance liquid chromatography analysis is carried out using acetonitrile, methanol and 0.05 M dipotassium hydrogenorthophosphate in the ratio of 40:40:20 (v/v/v) as the mobile phase and Luna C18(4.6 x 250 mm) column as stationary phase with detection wavelength of 259 nm. Linearity was obtained in the concentration range of 100 - 200, 15 - 45 and 40 - 120µg/mL for Efavirenz, lamivudine and zidovudine, respectively.

6. An isocratic HPLC method for the assay of Efavirenz (EFA) in bulk and pharmaceutical dosage forms was reported **[06].** The chromatographic conditions comprise of a Novapak phenyl column. A mixture of phosphate buffer and acetonitrile was used as mobile phase. Quantitation was achieved by UV-detection at 247 nm. A linear response (r2 = 0.999) was observed in the range of 0.05-0.15 mg/mL.

7. Pradeep Kumar *et al.* **[07]** have developed high performance thin layer chromatographic method for the estimation of Efavirenz in tablet dosage forms. The method employed TLC aluminium plate's pre coated with silica gel 60 F 254 as the stationary phase. The mobile phase used was a mixture of toluene: ethyl, acetate: formicacid (10:3:1, v/v). The detection of spot was carried out at 254 nm. The calibration curve was found to be linear in the range 300-1800 ng/mL with regression coefficient of 0.9991.

8. Kwon-Bok Kim and co-workers **[08]** have developed a method for
determining Efavirenz, 8-hydroxyefavirenz, and 8,14 dihydroxyefavirenz in human plasma simultaneously using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Three compounds and ritonavir, an internal standard, were extracted from plasma using ethyl acetate in the presence of 0.1 M sodium carbonate after incubation of *β*-glucuronidase (500 U). After drying the organic layer, the residue was reconstituted in mobile phase (acetonitrile:20mM ammonium acetate, 90:10 v/v) and injected onto a reversed-phase C18 column. The isocratic mobile phase was eluted at 0.2mL/min. The retention time is 1.93, 1.70, 1.52, and 1.82 min for Efavirenz, 8-hydroxyefavirenz, 8,14-dihydroxyefavirenz, and ritonavir, respectively.

9. A simple RP-HPLC method for simultaneous estimation of emtricitabine, tenofovir and Efavirenz was described in the literature **[09].** Chromatography was carried on a column X-terra RP-18 using gradient composition of ammonium acetate buffer as mobile phase A and acetonitrile as mobile phase B at a flow rate of 1.0mL/min, detection at 260 nm. The retention times of the emtricitabine, tenofovir

disoproxilfumerate and Efavirenz was about 4.61, 7.52, 9.10 min, respectively. The linearity was found to be in the range of 50 - 150µg/mL for emtricitabine, tenofovir and Efavirenz.

10. A reverse phase-high performance liquid chromatographic method for the simultaneous determination of lamivudine and Efavirenz in tablet dosage form is developed and validated **[10]**. The chromatographic analysis was performed on a Thermo BDS hypersil C 18 column (250×4.6 mm, 5µm) in isocratic mode, the mobile phase consisted of methanol, acetonitrile and 0.05 M phosphate buffer (adjusted to pH 4.5 with ortho-phosphoric acid) at a ratio of 60:20:20 (v/v/v), and a flow rate of 1mL/min. The elements were monitored at 254 nm. The retention time of lamivudine and Efavirenz were found to be 2.50 and 4.25 min, respectively. The line arranges were found to be 10 – 60.22µg/mL for lamivudine and 10-60 µg/mL for Efavirenz.

11. Bedor *et al.* **[11]** have developed a LC-MS/MS method for the estimation of Efavirenz with hydrochlorothiazide used as an internal standard. Chromatographic separation was performed on an OnixC18 column (50 x 4.6 mm I.D., monolithic). Isocratic elution mode of the analytes from the column was achieved with a mobile phase consisting of acetonitrile/water (50:50 v/v+5% of isopropyl alcohol) at a flow rate of 1.5mL/min. The linear concentration range was 100-5000ng/mL.

12. A validated isocratic HPLC method **[12]** was utilized for the assay of Efavirenz. The method employs a Zodiac C18 column with methanol and acetonitrile (80:20 v/v) as the mobile phase and UV detection at 280 nm. A linear response (r >0.998) was observed over the concentration range of 15-45µg/mL.

13. A high performance liquid chromatography was developed and validated for Efavirenz in tablets by Osnir de Sá Viana *et al.* **[13]**. The physical chemical characteristics of Efavirenz were investigated to developing the method. Analyses were performed by an ultraviolet detector at 252 nm wavelength, on a reverse-phase column (C18, 250 mm x 3.9 mm, 10µm), using an isocratic mobile phase containing acetonitrile/water/orthophosphoric acid (70:30:0.1, v/v/v).

14. Monic Inbaraj **[14]** has developed a LC-MS/MS method for Efavirenz using nevirapine as an internal standard. The analyte extracted from solid phase extraction(SPE). Chromatographic separation was achieved from RP C18. The linear range of this method was 50-5000ng/mL.

15. Suhas *et al.* **[15]** have reported a ultra-violet spectrophotometric method for the estimation of Efavirenz (EFA) in pure drug and in pharmaceutical formulation. Linear response obtained was in the concentration range of 5 - 40µg/mL with correlation coefficient of 0.9993 and 0.9989 in solvent and urine, respectively.

16. Kumaraswamy and coworkers **[16]** have described a UV– Spectrophotometric method for the analysis of Efavirenz in blend and in tablets formulations. The method was based on simple UV estimation in cost effective manner for regular laboratory analysis. The instrument used was Perkin Elmer, UV-spectrophotometer and using 0.1N NaOH as solvent system. Sample was analyzed using UV

Win Lab 5.2.0 Software and matched quartz cells 1 cm and was monitored at 302 nm. Linearity was obtained in the concentration range of 2 - 10mg/mL for Efavirenz.

17. Vishnu Vardan *et al.* **[17]** have described the development of an UV-Spectrophotometric method for the simultaneous determination of Efavirenz, lamivudine and tenofovir disoproxil fumerate. The absorption maxima of the drugs were found to be 247, 273 and 258 nm for Efavirenz, lamivudine and tenofovir disoproxil fumerate respectively in acetonitrile: water (50:50, v/v) solvent system. Linearity was observed over a range of 1-20µg/mL for Efavirenz, 1-10µg/mL for lamivudine and tenofovir disoproxil fumerate.

18. In another Spectrophotometric method **[18],** a quantitative estimation of Efavirenz in bulk and tablets was described, in methanol: water (80:20, v/v), Efavirenz exhibits an absorption maximum at 245 nm and method obeys Beer's law.

19. Sailaja *et al.* **[19]** have reported a liquid chromatographic method for the analysis of Efavirenz in human plasma, chromatography was performed with C18 analytical column and 50:50 acetonitrile-phosphate buffer (pH 3.5) was used as mobile phase. Compounds were monitored by UV-detection at 247 nm. The retention time for Efavirenz was 6.45 min and that of internal standard nelfinavir was 2.04 min.Response was a linear over the concentration range of 0.1 - 10 µg/mL in human plasma.

MATERIAL AND METHOD

Instrumentation

A Double-beam spectrophotometer shimadzu was used for the detection of absorbance, Mettler Toledo as weighing balance and Misonix sonicator, borosil glass apparatus were used for experimental purpose.

Chemical and Reagents

Efavirenz working standard was supplied by Aurobindo pharmaceutical Pvt. Ltd, Hyderabad.

PROCEDURE

Preparation of stock solution

100mg of pure drug efavirenz was weighed and transferred to 100ml volumetric flask, and dissolved in methanol and water (60:40). The flask was shaken and volume was made up to the mark with methanol and water (60:40) to give solution of 1000µg/ml. From this solution, 10ml solution was pipette out and transferred into 100ml volumetric flask. The volume was made up to the mark with methanol and water to give solution 100µg/ml. The standard dilutions were prepared by proper dilutions of the stock standard solution with methanol and water to reach the concentration range of 10-50µg/ml.

Preparation of sample solution

One brand of tablet EFAVIR-600mg was used for the analytical study. The average weight of tablets was determined by weighing 20 tablets and these were powdered. Tablet powder equivalent to 100mg efavirenz was accurately weighed and transferred to a volumetric flask. Made the volume of the solution with methanol and water up to the mark. The stock solution of efavirenz was scanned in the wavelength of 291nm. A calibration curve was constructed over a range 10-50µg/ml. The calibration curve was constructed for efavirenz by plotting absorbance versus concentration at 291nm wavelength.

METHOD OF VALIDATION

For UV spectrometry method the following validation parameters are analyzed

- Accuracy
- Precision
- Ruggedness
- Linearity
- Limit of detection
- Limit of quantification

ACCURACY:

The accuracy of the method was established by adding the Efavirenz test standard solution of the tablet formulation. The analysis at each level was performed in triplicate and the mean recovery of Efavirenz was measured. The percent (%) recovery at each level was found to be well within the range of 96.4% to 99.3% , indicating insignificant interference from the excipients.

Table.7: Recovery Study

S.No.	Amount of drug taken(µg/ml)	Amount of drug added(µg/ml)	Total amount of drug(µg/ml)	Total amount of drug found(µg/ml)	%Recovery	%RSD
1	20	10	30	28.92	96.40%	0.62%
2	40	20	60	57.89	96.48%	0.21%
3	60	30	90	89.40	99.30%	0.20%

PRECISION:

Precision is the degree of repeatability of analytical method under normal conditions. The precision and were determined with standard quality control samples prepared in triplicate at different concentration levels covering the entire linearity range. The precision of the assay was determined by repeatability (intra- day) and intermediate precision (inter-day) and reported as RSD% for a statistically

significant number of replicate measurements. The intermediate precision was completely studied by comparing the assays on three different days and the results are documented as the standard deviation and RSD%.

Table.8: Results of Intraday Precision

Concentration(µg/ml)	Amount Found	% RSD
20	19.90±0.03	0.103
40	38.24±0.04	0.065
60	58.06±0.19	0.125

Table.9: Results of Interday Precision

Concentration(µg/ml)	Amount Found	% RSD
20	19.87±0.03	0.103
40	38.20±0.04	0.065
60	57.74±0.19	0.125

RUGGEDNESS:

Ruggedness of the method was established by having the precision study performed on another analyst. The cumulative %RSD for content of Efavirenz for the samples of precision and ruggedness study were found to be not more than 2%.

Table.10: Results of Ruggedness

	Analyst 1		Analyst 2	
Drug	**%Amount**	**%RSD**	**% Amount**	**%RSD**
EFAVIR 600mg	95%	1.155	94.8%	1.911

LINEARITY:

The method was validated according to ICH guidelines for validation of analytical procedures in order to determine the linearity, sensitivity, precision and of the analysis for Efavirenz , five point calibration curves were generated with the appropriate volumes of the working standard solutions for UV methods. The linearity method is evaluated by least-square regression method using data.

Table.11: Concentration Vs Absorption

Concentration(µg/ml)	Absorption
10	1.25
20	1.43
30	1.62
40	1.80
50	1.97

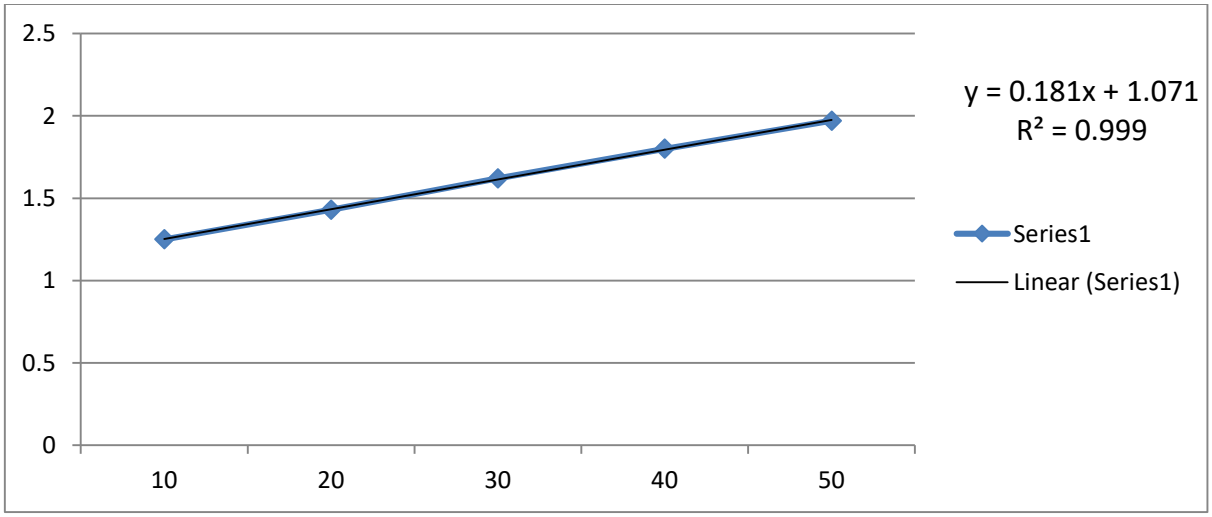


Fig.25: Linearity Graph

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

The limit of detection (LOD) is defined as the lowest concentration of an analyte that an analytical process can reliably differentiate from background levels. In this study ,LOD and LOQ were determined based on the standard deviation of the response and the slope of the corresponding curve using the following equations:

$LOD=3.3\sigma/s$

$LOQ=10\sigma/s$

Where s, the noise estimate σ is the standard deviation of the absorbance of the sample and S is the slope of the calibrations graphs.

The limit of quantification (LOQ) is defined as the lowest concentration of the standard curve that can be measured with an acceptable accuracy , precision and the variability. (Table 12).

SUMMARY OF VALIDATION PARAMETERS

Table.12: Summary and validation parameters

S.No.	Parameters		Results
1	λ_{max}		291nm
2	Regression line equation		y=0.181x+1.071
3	Slope		0.181
4	Inrecept		1.071
5	Correlation coefficient(R^2)		0.999
6	Coefficient variation		33.56
7	Precision	Intraday Precision	0.06-0.12
		Interday Precision	0.065-0.125
8	Ruggedness	Analyst-1	1.155
		Analyst-2	1.911
9	LOD		39.74
10	LOQ		120.44

RESULTS AND DISCUSSIONS:

The method developed for the determination of Efavirenz in tablet dosage form was found to be simple, sensitivity, precise, selective, rapid and economical. Efavirenz exhibited maximum absorption at 291nm and obeyed Beer's law in the concentration range of 10-50µg/ml. The proposed method for determination of Efavirenz showed linear regression y=0.181x+1.071 with correlation coefficient (R^2) of 0.999. Interday and Intraday studies showed high degree of repeatability of an analytical method under normal operating conditions. The %RSD for precision, which was less than 2%.It indicates that the method is precise. Recoveries obtained do not differ significantly from 100% showed that there was no interference from the common excipients used in tablet formulation indicating accuracy and reliability of the method. The proposed method can be used for drug analysis in routine quality control & method proves to be more economical than the published standard methods.

CONCLUSION:

The developed and validated UV spectrophotometry method reported here is rapid, simple, accurate, sensitive and specific. This method was validated as per ICH guidelines and results of accuracy, precision, ruggedness, was in the limit. There was no any interference of excipients in the recovery study. The method was also successfully used quantitative estimation and analysis of Efavirenz from formulation. Thus, the reported method is consider by importance and has great industrial applicability for quality control and analysis of Efavirenz from bulk drug and formulation.

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