Faculty of Health & Life Sciences
Leicester School of Pharmacy

APS500

Pharmacy Research

Title: Spectrokinetic studies of the photodegradation and photostability of Diethylstilbestrol.

Leonie Hough

July 2013

Abstract

A spectrokinetic study has been carried out for research purposes on the photodegradation and photostability of the photoactive Diethylstilbestrol compound (DES) a synthetic oestrogen sometimes used in the treatment of specific cancers. Both photochemical and photostability studies were carried out in order to contribute new knowledge to the research on DES. Due to the drug's use in treatment of diseases it is important to find a way of inhibiting or reducing problems of photosensitivity in the compound. In order to do this a set of sugars capable of forming inclusion complexes with other molecules, known as cyclodextrins were studied in order to determine their ability in changing the photostability of DES.

Studies were carried out incorporating both the CD monomers and the CD polymers with the DES compound in order to determine their effect on DES photostability and whether the monomers or the polymers would result in a better photostabilisation. A number of methods were employed in the studies including spectrophotometry, fluorescence spectroscopy, ATR-FTIR spectroscopy and SEM.

The initial investigations confirmed that DES is photoactive and that the reaction is solely photochemical. It was determined that no thermal activity was involved in the reactions taking place. Other studies carried out on the effect of wavelength provided an insight into the wavelengths at which DES is most sensitive and fluorescence studies allowed a proposal of the reaction mechanism of DES.

On analysing the findings of the cyclodextrin studies it can be determined that all of the cyclodextrins had an effect on the behaviour of DES however the β-P-CD had a substantial

effect on reducing the photodegradation of DES compared with the other CDs studied so was more favourable for a complex. The formation of an inclusion complex with DES was successfully obtained using both the β polymer (β-P-CD) and the β monomer (HP-β-CD). The complex also enabled the preparation of a safe formulation of the CD/drug complex by fully dissolving the complex in water resulting in the solubilisation of water insoluble DES. This was followed by analysis of the complex using ATR-FTIR and SEM methods. The results indicated a strong possibility of the formation of a complex.

Two novel formulations were made using the optimised complexes. These were tested in order to determine their photostability in comparison to DES alone. Both formulations were successful in significantly reducing the photodegradation of DES.

Acknowledgements

I would like to thank my supervisor Dr M. Maafi for all the support and advice throughout the course of this study. I would also like to thank the technicians for all their help and assistance.

Contents

Abbreviations.. 8

Chapter one: Review of literature

1. Introduction... 11
1.1 Nature of light... 11
1.1.1 Electromagnetic spectrum.. 11
1.2 Basics of photochemistry... 13
1.3 The Jablonski diagram.. 14
1.4 Electronic absorption.. 17
1.4.1 The beer Lambert law... 18
1.5 Bohr's theory.. 20
1.6 Fluorescence.. 22
1.6.1 Stokes shift... 24
1.7 Photoreactivity of drugs.. 25
1.7.1 The benefits.. 28
1.8 The ICH guidelines.. 28
1.8.1 Forced degradation... 29
1.8.2 Confirmatory studies... 30
1.8.3 Sequential testing... 30
1.9 Chemical kinetics.. 30
1.9.1 Rates of reaction... 31
1.9.1.1 Half-life.. 32
1.9.2 Reaction mechanisms... 32
1.9.3 Orders of reaction... 33
2. Excipients.. 35

3. Cyclodextrins... 36
3.1 Cyclodextrins and toxicity.. 38
3.2 The principle of cyclodextrins and formation of inclusion complexes............... 39
3.3 Cyclodextrin derivatives.. 42
3.3.1 Cyclodextrin polymers.. 43
4. Photo degradation of oestrogens and synthetic hormones................................ 45
4.1 Stilbene literature and its structure.. 45
4.1.1 The Mallory reaction... 48
4.2 Diethylstilbestrol (DES) literature and its structure.. 49
4.3 Objective... 52

Chapter two: Experimentals

2.1. Methods and equipment.. 54
2.2 The photolysis process.. 54
2.2.1 Photodegradation setup.. 55
2.3 Fluorimetry... 56
2.4 Rotary evaporation.. 57
2.5 ATR-FTIR.. 57
2.6 SEM.. 58
2.7 Chemicals.. 59
2.7.1 Drugs and solvents.. 59

Chapter three: Results and discussion

3.1 Introduction.. 61
3.2 Some spectroscopic and stability characteristics of DES.................................. 61
3.3 Compound solubility and irradiation conditions.. 63
3.4 Photodegradation properties of DES... 63
3.4.1 Spectrophotometric study.. 63
3.4.2 Fluorometric study.. 65

3.5 Spectral characteristics and photodegradation of DES in different media……. 66
3.5.1 UV/Vis spectra of DES….. 66
3.5.2 Thermal stability……….. 67
3.5.3 Photodegradation of DES in different media……………………………… 67
3.5.3.1 Spectra after irradiation.......................................…………… 67
3.5.3.2 Evolutions of DES spectra and traces with irradiation time 70
3.5.3.3 Proposal of DES photodegradation mechanism………………. 75
3.6 Effect of irradiation wavelength... 80
3.7 Liquid formulation of DES……………………………………………………………………….. 86
3.7.1 Formulation of DES in water/ethanol…………………………………………… 87
3.7.2 Solubility of DES/CD in water…………………………………………………………. 87
3.7.3 Minimum water volume required to solubilise DES/CD formulation.. 88
3.8 Characteristics of DES/CD complex………………………………………………………….. 89
3.8.1 ATR-FTIR spectra……………………………………………………………………….. 90
3.8.2 SEM image comparison……………………………………………………………. 92
3.8.3 Spectrophotometric characterisation of the complex………………… 97
3.8.4 Fluorescence…………………………………………………………………………….. 98
3.9 Degradation of DES in the presence of CD.…………………………………………….. 99
3.9.1 Thermal stability……………………………………………………………………….. 99
3.9.2 Traces of DES by spectrophotometry……………………………………………. 100
3.9.3 Fluorimetry time drive traces…………………………………………………….. 102
3.10 Photostability of the novel formulation…………………………………………………. 108

Chapter four: Conclusion and future work

Conclusion…………………………………………………………………………………………………… 114

References…………………………………………………………………………………………………… 118

Appendix A. Calculations……………………………………………………………………………….. 126

Abbreviations

A	Absorbance
α-CD	Alpha cyclodextrin
ATR-FTIR	Attenuated total reflectance Fourier-transform infrared
β-CD	Beta cyclodextrin
β-P-CD	Beta cyclodextrin polymer
c	The concentration of the solution (mol dm^{-3})
CDs	Cyclodextrins
CGT-ase	Cycloglycosyl transferase amylase
DES	Diethylstilboestrol
ε	Epsilon is the molar extinction, which is constant for a particular substance at a particular wavelength (dm^2mol^{-1}).
γ-CD	Gamma cyclodextrin
γ-P-CD	Gamma cyclodextrin polymer
HOMO	Highest occupied molecular orbital
HP-β-CD	Hydroxypropylated beta cyclodextrin
ICH	International conference on harmonisation
k	the rate constant
l	Optical path length (cm)
LUMO	Lowest unoccupied molecular orbital

µl	microlitres
mg	milligrams
mg/mL	milligrams per millilitre
mL	millilitres
min	minutes
NTS BSD	Back scatter detector (used in SEM)
nm	nanometres
r^2	The coefficient value of how well the data fits a linear relationship. A value of 1 means the correlation of the data is exact.
SEM	Scanning electron microscope
S_0	Stock solution
S_1	Solution intermediate (1)
S_2	Solution intermediate (2)
S_{cuv}	Cuvette solution
t	Time
UV	Ultraviolet
UV/Vis	Ultraviolet/Visible
V_0	Initial velocity
VPSE G3	Variable pressure secondary electron (used in SEM)
y=mx+c	The equation of a straight line
λ	Wavelength

Chapter one.

Review of Literature

1. Introduction

1.1 Nature of light

1.1.1 Electromagnetic spectrum

There are two types of waves. These are known as mechanical waves and electromagnetic waves. Examples of mechanical waves include sound waves or ocean waves, they require a medium or an object to travel through which in the two examples is either air or water. Electromagnetic waves are formed through changes in both electric and magnetic fields and are able to travel through solid medium, air and vacuum [7]. The two fields in an electromagnetic wave are perpendicular to each other in the direction at which the wave is travelling. Electromagnetic waves travel at the speed of light which is approximately one foot per nanosecond [22]. This will change when it comes into contact with matter.

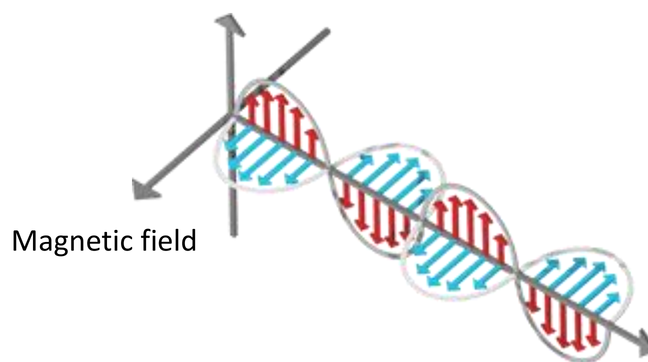


Fig 1.1 The electromagnetic wave [55]

The waves are crucial for providing the energy for everyday needs of the world. These waves are known as electromagnetic energy.

This is a natural phenomenon and can be referred to as light, electromagnetic waves and electromagnetic radiation. The electromagnetic spectrum is made up of a range of frequencies of electromagnetic radiation beginning at very low frequencies to very high frequencies.

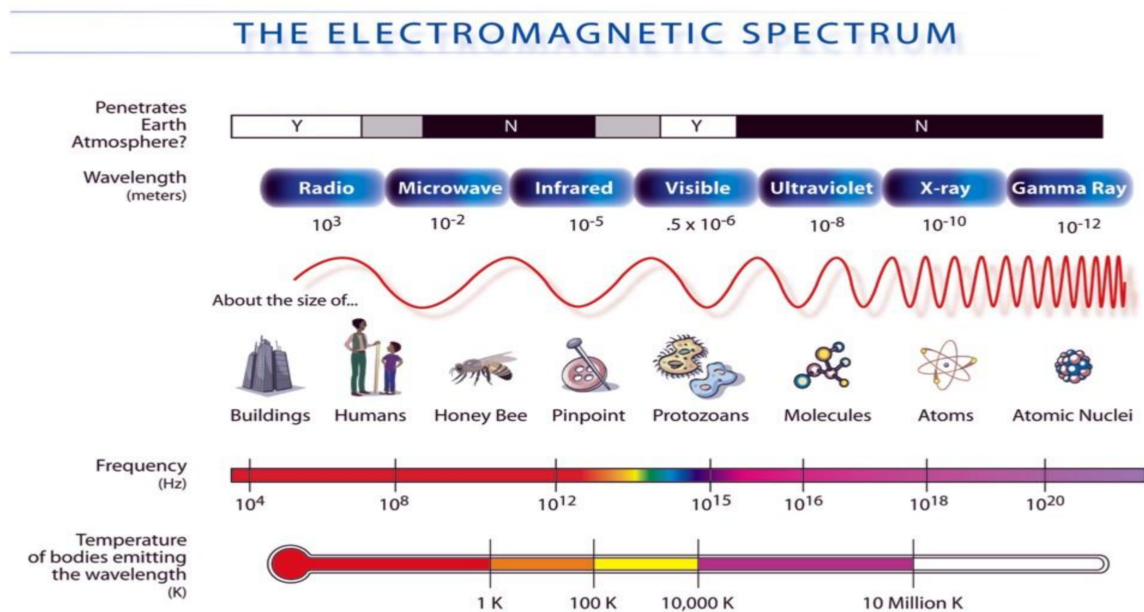


Fig 1.2 A detailed diagram of the electromagnetic spectrum [54]

The range in frequencies can be shown as electromagnetic waves forming a spectrum. Although represented as waves they can be described as a stream of particles known as photons travelling around through the air and objects. Each photon contains a particular amount of energy. The amount of energy increases throughout the spectrum as the wavelength shortens. Only a small section of the electromagnetic spectrum can be detected by the human eye this being the visible part of the spectrum [7]. Although only visible light can be seen by humans many other areas of the spectrum can be absorbed. Some of the more potentially harmful and dangerous radiation levels from Ultraviolet light to gamma rays are prevented from reaching earth by the protective atmosphere that surrounds the earth and the radiation is emitted. However exposure to small amounts of Ultraviolet radiation (UV-VIS) is still possible.

For an object to absorb at a particular wavelength it will need a specific amount of energy which will depend on its properties. The absorbance of photons at particular wavelengths can result in problems that are not widely known or result in effects that cannot be seen by the human eye such as the degradation of drugs. This is known as photodegradation. This happens through what are known as photochemical reactions.

1.2 Basics of photochemistry

It is a known fact that when light is absorbed by an object or material the effect can result in a change in their properties. Visible changes may include a change in colour or a product becoming colourless [48] and is known as photostability. Photostability of drugs can be described as the way in which a compound responds to light exposure and the reactions that take place including degradation, formation of radicals and energy transfer. This can be a cause for concern in many industries, pharmacy in particular especially as the awareness of the photosensitivity and photostability of drugs is increasing [48]. Tønessen [48] states that the European pharmacopeia prescribes light protection for more than 250 medical drugs and various adjuvants but new drugs are regularly added.

Other changes that may occur are due to photochemical reactions taking place within the drug product itself or within the bloodstream upon absorbance of light through the skin (photosensitivity). Photochemical reactions result in degradation of the drug causing adverse effects or light induced side effects as results of the photoproducts formed through degradation and or loss of therapeutic potency [48].

Photochemistry studies the mechanism, in which the electronic structure of a molecule changes through the absorption of light at a specific wavelength [46]. When an object or substance absorbs light photochemical reactions take place resulting in changes in the resulting molecule or neighbouring molecules. The effected molecule is promoted to a

higher state of excitation through energy from the absorbed light. In order to return to its ground state the molecule must get rid of the energy absorbed either through giving it off as heat or light (fluorescence or phosphorescence). The way in which a molecule gets rid of its energy is totally dependent on that specific molecule at that time. All molecules are different and will return to their ground state through different means [46].

There are three basic laws of photochemistry:

- The first law states that light must be absorbed in order for photochemical reactions to take place. The light absorbed must be of a particular wavelength for a system to work or no effects will be detected.

- The second law states that for each photon of light that is absorbed by a system at normal intensities only a single molecule is activated for a photochemical reaction.

- The third law of photochemistry is the Bunsen-Roscoe law of reciprocity which states that a photochemical effect is directly proportional to the total energy dose irrespective of the time period over which the dose is delivered [46].

1.3 The Jablonski Diagram

The Jablonski diagram which illustrated the chemical processes that occur between absorption and emission of light was created by Professor Alexander Jablonski who was born in the Ukraine and studied atomic physics at university. He carried out research on the polarization of luminescence in solution and gained a doctorate in ‘The influence of the change of wavelengths of excitation light on fluorescence spectra’ in 1930 [22].

When a photon is absorbed by an electron it gains an amount of energy specific to that photon. The energy gained promotes the electron to a higher state of energy known as an excited state [46]. The fate of an excited molecule can be explained using the Jablonski diagram.

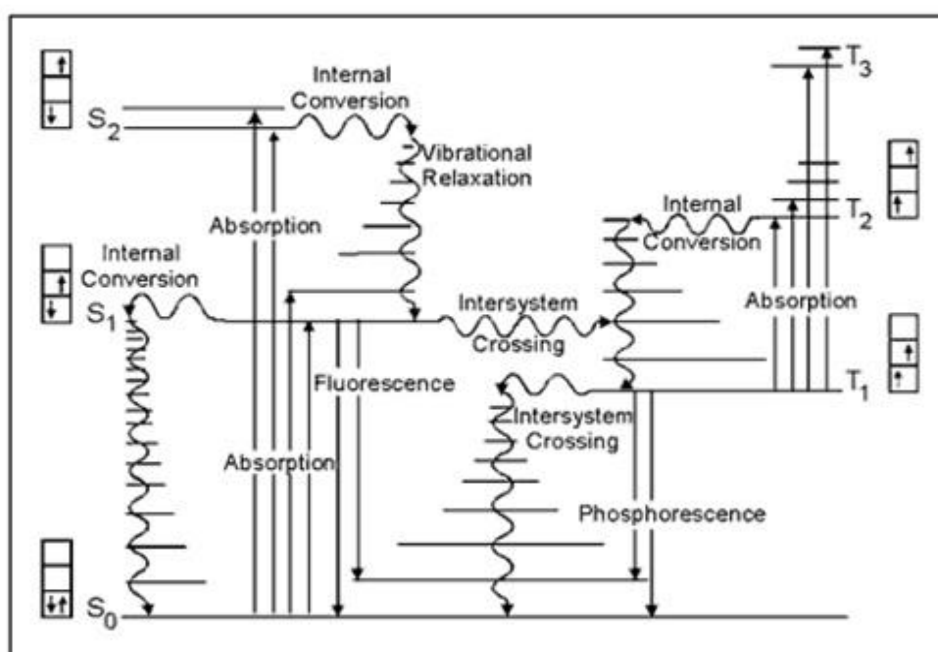


Fig 1.3 The Jablonski diagram [46]

Once an electron has absorbed the energy gained from the photon absorbed and has moved up to another energy level the electron will then vibrate or rotate to the next stable electronic state (S_1 or S_2). This relates to what is known as Kasha's rule in photochemistry. The rule states that an electron will always aspire to reach a semi stable level or ground level (S_0). There are several processes an electron can go through in order to reach its ground state again, these can be both radiative and non radiative. The radiative processes are a result of the emission of energy which can be in the form of a photon when an electron moves to ground state. Those that don't emit are known as non radiative processes. The Jablonski diagram is useful as it shows the connections between electronic states of a molecule and the transitions that take place. The solid lines show the radiative processes taking place and the wavy lines show the non radiative processes. The radiative processes are those that involve absorption.

Internal conversion (IC) is the radiationless transition between energy of the same spin state where S_1 is converted to the isoenergetic state of S_0 which then deactivates into the ground state.

Intersystem crossing (ISC) is the transition between excited states of different multiplicity with a similar quantity of energy. The rate at which intersystem crossing takes place is slower than that of internal conversion as the process is known as spin forbidden.

Vibrational relaxation (VR) is where the remaining energy with particular electronic state is dissipated as heat by collision with neighbouring particles.

Phosphorescence is the radiative decay between energy states of different multiplicity. Light is emitted from the lowest vibrational level of the lowest triplet state through to ground state and is a spin forbidden process so has a long radiative lifetime.

Fluorescence is the radiative decay between energy states of the same multiplicity [48].

Intersystem crossing will result in an electron moving from its singlet state to a triplet state which is also known as the forbidden. The triplet state is an excited state of lower energy. This state is seen as forbidden as forces within an excited state may result in a change in the orbitals resulting in parallel spins. This doesn’t abide by the laws on spin states where spins in an orbit must be antiparallel.

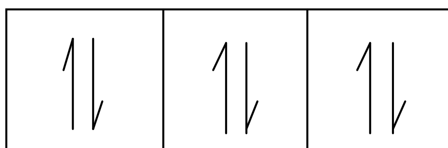


Fig 1.4 Antiparallel spins abide by the spin state laws

1.4 Electronic absorption (UV-VIS)

When electromagnetic radiation interacts with matter there are several different processes that may occur including reflection, fluorescence, phosphorescence and photochemical reactions. On interaction with matter some of the light is absorbed which causes the energy in the absorbing molecule to increase. The total energy a molecule has depends on the electronic vibrational and rotational energies.

$E_{total} = E_{electronics} + E_{vibrational} + E_{rotational}$

In some molecules or atoms the photons of UV or visible light may contain enough energy to excite a molecule resulting in the transition from one state to another. A specific amount of energy is needed to cause a transition from a lower state to a higher state of energy (S_0 – S_1) [35].

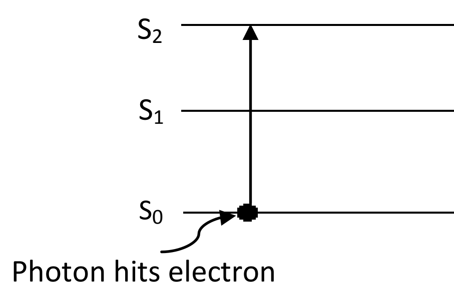


Fig 1.5 schematic diagram of an excited electron changing states of energy

The energy carried by a photon is given by Planck's equation:

$$E = hv = \frac{hc}{\lambda} = hc\bar{\upsilon}$$

Where h = Planck's constant (6.6256 x 10^{-34} Js $photon^{-1}$)

c = Speed of light (2.9979 x 10^{8} ms^{-1})

λ = Wavelength of radiation [m]

v = Frequency of radiation [s^{-1}]

$\bar{\upsilon}$ = Corresponding wave number [m^{-1}]

Before excitation a molecule can be referred to as in its ground state where its energy state is at its lowest. The ground state of a molecule is characterized by the distribution of available electrons in the molecular orbital of the lowest state of energy. This state will contain two electrons with opposing spins at the most. It has been stated that no two electrons within a given orbital must have the same spin state, this is known as the Pauli exclusion principle [57]. Molecules at ground state should have an even number of paired electrons in its electronic configuration. In order to reach a higher state of energy (excited state) a molecule must absorb a photon containing enough energy that is equal to the difference in energy between the high occupied molecular orbital (HOMO) and the lowest occupied molecular orbital (LUMO) of the ground state [57]

1.4.1 The Beer-Lambert Law

The Beer-Lambert law explains the absorption of photons by an object or matter. When a beam of electromagnetic radiation interacts with matter, some of the energy is absorbed, therefore the light that has passed through the matter (I) which is referred to as transmittance will have a smaller intensity than the original incident beam (I_0).

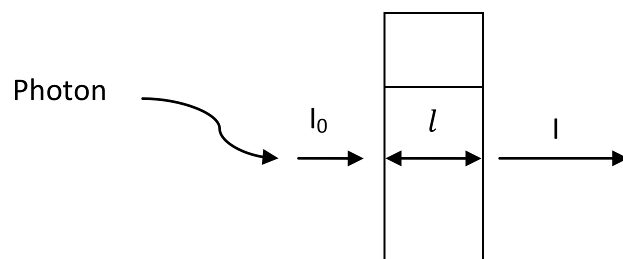


Fig 1.6 the optical path length of light through a cuvette

The incident and transmittance intensities are related to the Beer Lambert Law. The Beer Lambert law illustrates the linear relationship of the absorbance and concentration of an absorbing species. Taking common logarithms using the following equations allows a graph to be produced:

$$Log_{10}\left(\frac{I}{I_0}\right) = -\varepsilon lc$$

Or:

$$A = Log_{10}\left(\frac{I_0}{I}\right) = \varepsilon lc$$

These quantities can be measured using a spectrometer and is known as the absorbance (A). The general equation is often referred to as:

$$A = \varepsilon lc$$

The absorbance against concentration can be plotted. If the Beer Lambert law is obeyed a straight line through the origin should be formed. The Beer Lambert law can be used in the analysis of mixtures to determine a particular substance that may be present and its quantity. It can also be used to determine an unknown concentration of an analyte in a solution.

1.5 Bohr's theory

Niels Bohr was born in Copenhagen in 1885. He studied physics at Copenhagen University from 1903 as well as chemistry, astronomy and mathematics. His first paper was published whilst at University which was printed in the same year that he completed his Master's degree when he became interested in electron theory. Bohr went on to complete a doctorate before arriving at Cambridge to continue studying electron theory [21].

Bohr's theory further contributes and improves on the understanding of Rutherford's original theory on the atom model. Bohr's theory explains the way in which electrons behave within atoms. Borh formed a theory that electrons move in fixed stable orbits around the nucleus of an atom with set energies and set distances.

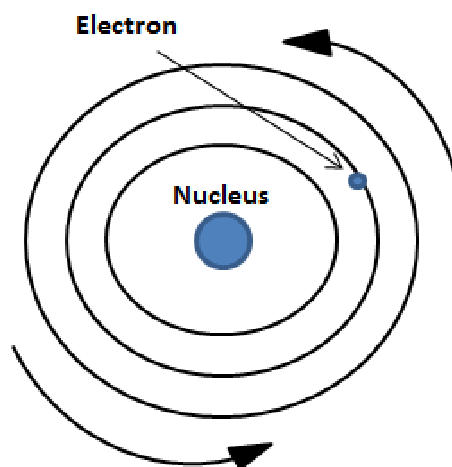


Fig 1.7 Diagram demonstrating Bohr's theory

Bohr formed this idea from his attempt at understanding the phenomena of emission spectra after an experiment where a current was applied to hydrogen gas which resulted in the formation of a number of different coloured lines. Bohr stated that this had to be caused by the behaviour of the electrons within the atom. He also suggested that if a

photon of light colliding with an electron had exactly the same amount of energy as the gap between orbitals, the electron will gain energy from the photon and will be excited allowing it to jump to the next orbit. The amount of energy needs to be specific for the electron to make the transition to the next level and must be equal to the energy between the two orbits. This energy is known as a quantum of energy [21].

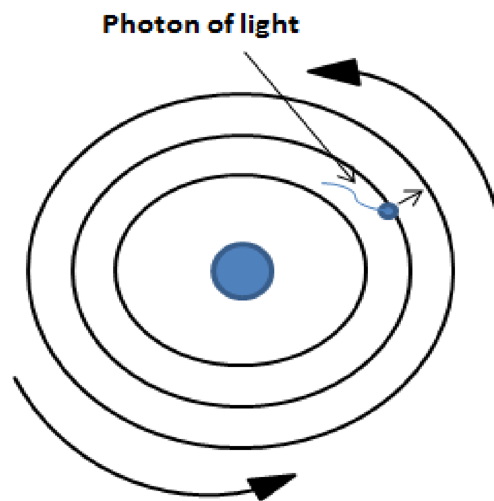


Fig 1.8 Demonstration of electrons jumping from one orbit to another

Bohr explained that when an electron becomes excited it is unstable and will eventually fall back into its stable energy and in doing so will emit the energy it has gained. In the case of the hydrogen gas as the energy is emitted different coloured lines are given off in relation to the different amounts of energy the electrons have.

Bohr also stated that the energy gained to excite an electron obeys Planck's equation of the energy and light relationship, which gives:

$$\text{Energy} = h \times \text{frequency}$$

This means in order for an electron to jump between two orbitals the light must be a specific frequency. When an electron moves down an orbital it will emit the energy it has gained through excitation [21]. Again the light must be of a specific frequency for it to reach a lower orbital.

This model is now presented as an energy level diagram seen in figure 1.5.

1.6 Fluorescence

Luminescence is the emission of light from a substance. This is the result of excited states formed in molecules when the light is absorbed. Luminescence can be formed into two sub categories: Fluorescence and phosphorescence [22]. The result in fluorescence or phosphorescence will depend on the path taken by the excited states.

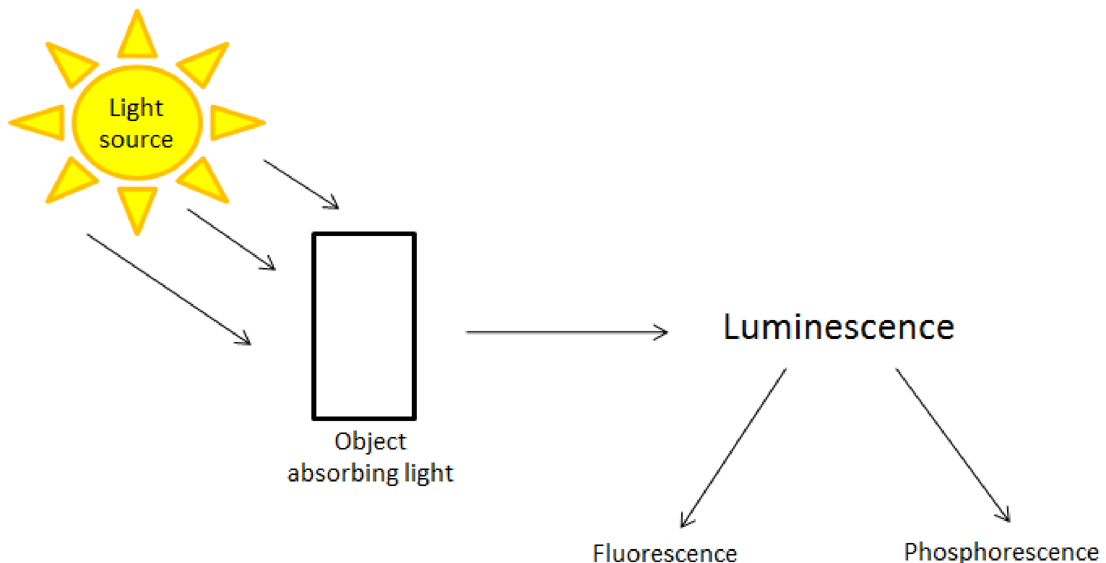


Fig 1.9 Luminescence

Fluorescence is where a photonically excited molecule is capable of deactivation from the singlet excited state to ground state by emitting a photon [5]. Some samples may be fluorescent or may become fluorescent when irradiated by light and will emit light [35]. A molecule that emits this type of light is known as a fluorophore. Fluorophores are responsible for the absorption and emission of specific wavelengths by a molecule. The wavelength and energy depends on the structure of the fluorophore and the chemical environment it's in [44]. A well-known example of a fluorophore described by Lakowicz [22] is quinine. Quinine is one of the first known fluorophores and is present is tonic water. Its fluorescence can be observed by exposing tonic water to light and by doing so a faint blue colouring on the surface can be distinguished. The energy of an emitted photon is always less than that of the absorbed photon [48] as some of the energy is absorbed by the sample. Fluorescence spectra are usually a mirror image of their absorption spectra.

The use of fluorescence measurements in drug analysis provides important information about the excited singlet state of a drug. The emission takes place from the lowest vibrational level of the lowest excited singlet state (Kasha's rule) therefore the maximum emission is the measure of the energy level of the excited singlet state [48]. For an electronically excited atom or molecule to return to its ground state once excited it must lose the energy it has gained through excitation either through emission of radiation or by deactivation through collision with other molecules. In some cases fluorescent emission will occur. Most atoms will emit fluorescence in low pressure conditions. However not all atoms will exhibit fluorescence and others may only be very weakly fluorescent [52].

Fluorescence will also fail to occur if an unstable state of absorption is reached and any intermolecular energy transfer that takes place must be slower than that of the rate of radiation. An example of this is when intersystem crossing occurs from S_1-T_1. It has been found that aromatic hydrocarbons are often fluorescent in comparison with the simple carbonyl compounds which are known to very rarely exhibit any fluorescent. Wayne [52] states that geometrical factors' including the rigidity of a molecule also affects the occurrence of fluorescence. It is found to be common in organic photochemistry that fluorescence emission occurs only from S_1 and not from any of the above singlet states [52].

Kasha's rule states that the emission of energy by electronically excited molecules either by fluorescence or phosphorescence takes place from the lowest excited state of a given multiplicity. However azulene is a known exception to Kasha's rule.

Spectrofluorimetry is a very sensitive technique so can be useful in photochemistry, for the analysis of electronically excited molecules. However the technique is not specific enough for the use of molecular identification [52].

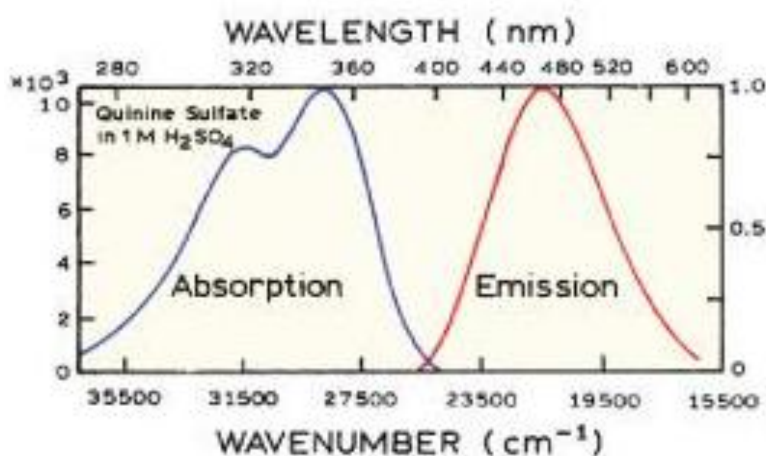


Fig 1.10 Electronic excitation and emission spectra of an organic molecule [22]

1.6.1 Stokes shift

Fluorescence emission spectra generally occurs in the longer wavelengths than the absorption spectra [36] and was first noted by Sir Stokes in 1852 [22]. The Stokes shift is the difference between the maximum of the emission peak and the absorption peak. Energy loses occur between excitation and the emission which is known to be common amongst molecules that fluoresce once excited. There are many situations and conditions that may result in Stokes shift taking place including solvent effect, formation of complexes, energy

transfers and reactions in excited states. The most known and common circumstance resulting in Stokes shift is the rapid decay to the lowest vibrational level of S_1 [22].

1.7 Photo reactivity of drugs

Absorption of light will take place in most therapeutic drugs as well as excipients. For absorption to take place an absorption band in the compound must exist that is similar to that of the incident light energy that the drug is being exposed to [32]. The absorption of light by a compound will indicate that photochemical processes may result from the absorption. Exposure to light can result in changes in a drug's properties and therefore its overall stability [47]. The photochemical processes that may take place include decomposition of the drug into photoproducts or may result in the decomposition of other components within the formulation [47]. Toxic reactants may result from the interaction of pharmaceuticals and light [10] and can also result in the loss of potency of a drug or induce adverse side effects which ultimately generate a huge risk to a patient's health. Other processes may come from photosensitivity where phototoxic or photoallergic reactions take place in body [47].

Most compounds are affected by the UVB and UVA region of the spectrum and therefore the UVB and UVA wavelengths are more greatly studied [32]. UVB is able to pass through the top layers of skin mostly dead cells so may react with topically applied pharmaceutical substances. UVA light is able to penetrate deeper and passes through the skin reaching the blood stream. In some cases where pharmaceutical products are passing through, the light will be absorbed by the drug [10]. If interaction takes place these types of reaction may be induced. All drugs are sensitive to light but not all of them will need to be dealt with under the same levels of precautions as others [47]. There are several classes of drugs well known for their sensitivity to light including antibacterial drugs, non-steroidal anti-inflammatory drugs and tricyclic antidepressants [34].

Both drug substances and drug products are at risk of exposure to either natural or artificial light at some point during synthesis, transport, storage and use. The majority will absorb either UV radiation or potentially visible radiation. It is important to monitor photoreactivity of drugs as the decomposition can result in loss of potency, reduce the quality of a drug and result in inadverse effects in patients. Recognition of photoreactivity of drugs is increasing and is largely a result of the increase in UV radiation reaching the earth [34] therefore research and assessment on drug phototoxicity is consistently ongoing. It is important for the safety and efficiency of drug therapy that the reaction pathways of photoreactive drug substances are known and understood so that assessments on their toxiciology can be carried out before they are made available for treatment [49]. The ICH guidelines were put into place in November of 1996 in order to gain more knowledge and insight into how problems surrounding photoreactivity of drugs can be reduced or even be prevented [3]. Studies carried out allow the documentation of findings therefore providing essential information needed to reduce or prevent photoreactivity [50].

There are many situations in which drugs can be exposed to light and through different light sources including natural light, UV radiation [32] or by artificial means (Fluorescent lighting). Intravenous medication has to be stored in transparent packaging to ensure that any problems with a drug or tampering with medication is visible. In hospital environments in particular drugs are often exposed to irradiation for long periods of time particularly artificial light as well as natural light when products are left close to windows [47]. Drug delivery device pumps are also manufactured using clear plastic pumps. It has also been found that solid dosage forms are often removed from their protective packaging and the capsule or tablet is placed into a unit dose container which is also made of transparent plastic [47].

Some photoreactive drugs can results in photoallergic reactions in the body through UV radiation after administration of the drug. The UV range can be categorised into UVA, UVB and UVC.

UVC – 200-280 nm

UVB – 280-320 nm

UVA – 320-400 nm

Visible – 400-800 nm [48]

UVB is able to reach the top layers of skin made up of dead cells and UVA is capable of reaching the blood stream [10].

These types of light are able to penetrate through the skin at different levels, resulting in a reaction between the drug and the penetrating light within the body [47].

Tønnesen [48] states that Adverse effects have also been reported from administered drugs that have been found to form minor degradation products in storage therefore photostability testing should be carried out on drugs both in vivo and in vitro. It is important that all areas are considered to ensure safety including manufacture, handling, storage, transport and administration. It is important to take into account that not all drugs degrade within the same time period. Some may only take hours and some may take days or weeks. Heat, moisture and oxidation are other factors that are capable of effecting the degradation of a compound [17] and may work alongside light. Thermal and oxidation tests should be carried out to determine whether the drug is affected by such conditions and their involvement in the degradation process. There are several factors that affect the time period over which degradation occurs, including light intensity, wavelength, exposure time as well as the material of the sample holder, its shape and orientation [17]. Photostability tests are often more exaggerated than those that a compound would usually be exposed to in order to ensure that if a drug is stable under the exaggerated conditions it will not be affected by those conditions it would normally be exposed to [17]. By gaining knowledge on

the photostability of a drug the correct labelling can be applied to medication and the correct instructions provided on the storage of the medicinal product as well as the correct administration for when the drug is made available to anyone who may be handling it [48].

1.7.1 The benefits

Although there are a huge number of disadvantages to drug photoreactivity there are also a number of benefits. Photoreactivity has been used in advanced drug delivery for treatment of psoriasis in PUVA therapy where psoralen is applied to skin, used as a bath soak or taken in tablet form and the skin is exposed to UVA light which helps to heal the skin. It is also used in the treatment of cancer using photodynamic therapy (PDT). The use of PDT in the treatment of cancer, skin cancer in particular is increasing. It is also possible that PDT may be of use in the treatment of microbial pathogens reducing the levels of death from infection [49].

Another use of photoactive compounds is there application for the development of novel drug delivery systems. The method can be used for to control release rates of the active ingredient from a dosage form to activate an inactive drug molecule present at the site of action [49].

1.8 International conference on harmonization guidelines (ICH)

The ICH guideline was published in November 1996 and has been put into practice in the United States of America, Europe and Japan. These parameters are mandatory and were put into place for the manufacturing, storage and distribution of drug substances under the testing of new drug substances and any related drug products. Although the guidelines cover synthesis of the drug through to its storage and suitable packaging it does not cover the photostability of drug substances in the conditions under use by patients [3].

The guidelines recommend that photostability testing is carried out on both the drug substance and drug product. Tests are carried out in order to document how environmental factors such as humidity, temperature and UV-Vis radiation may alter the quality of a drug [50]. The information obtained is used to determine photosensitivity and the appropriate packaging and labelling to ensure that the drug is of a satisfactory quality for use [50].

There are particular tests involved in the photostability testing of drugs and the guidelines recommend specific radiation sources, the amount of exposure and the nature of light (λ) that is needed in order to assess photostability [50].

1.8.1 Forced degradation

Forced degradation studies also known as stress testing involves placing the testing substance under forcing conditions [3] and is carried out early in the formulation development [50]. This is done to determine the stability characteristics of the drug, the degradation products, reaction mechanisms and overall photosensitivity. This information then aids in developing the correct system for detecting and quantifying a drug substance [3].

1.8.2 Confirmatory studies

Confirmatory tests are carried out where by the substance is placed under standardised conditions that will enable the prediction of the likely outcome or effects during storage [3]. These tests are carried out later on in the development [50]. The information gained from confirmatory studies is used to help decide on any precautionary measures that may be necessary in a drugs formulation, production and storage [3].

1.8.3 Sequential testing

Sequential testing means that all tests on drug products must be carried out in the specified sequence in the guidelines. The drug product must first be tested in full exposure to light. For those samples that are liquid or semi-solid transparent containers can be used. Unstable products must then be tested further. Tests should first be carried out in an immediate packaging (primary) and market packaging (secondary). Tests should continue until it is possible to fully demonstrate that the drug product is suitably protected from exposure to light. Exposure during testing must follow the conditions set out in the guidelines [48]

1.9 Chemical kinetics

Chemical kinetics studies the rates of chemical processes such as the rates of reactions [59] and the conditions that affect the ways and the rates at which a reaction occurs. As drugs are susceptible to degradation chemical kinetics can therefore be used to study the degradation of drugs [60].

There are a variety of factors and conditions that may affect reactions including concentrations [58], temperature, pressure, surface area, the nature of the reactants involved, light sources and catalysts [60]. By gaining knowledge and understanding on the reaction kinetics and conditions that affect stabilization, strategies can be implemented in drugs formulations that may delay or prevents its degradation [60].

1.9.1 Rates of reaction

The rate of reaction is the rate at which reactants degrade or the rate at which products are formed as the reactants degrade in a given time [60]. The rate of reaction can be described by a rate equation.

A and B are the reactants and C and D are the products. a,b,c and d are the stoichiometric coefficients for the corresponding reactants and products or the number of moles that are contributing to the reaction [60].

The rate equation can be expressed either as the degradation of the reactants (indicated with a negative sign) or the formation of the products (indicated with a positive sign). The rate equation for this can be expressed as:

In terms of reactants:

$$rate = -\frac{1}{a}\frac{dC_A}{dt} = -\frac{1}{b}\ \frac{dC_B}{dt}$$

In terms of products:

$$rate = \frac{1}{c}\frac{dC_C}{dt} = \frac{1}{d}\ \frac{dC_D}{dt}$$

For a reaction to occur the particles of the reactants must collide with each other, however for a reaction to take place there must be enough kinetic energy present in the collision to break the chemical bonds. This kinetic energy is known as the activation energy which is the minimum energy required to start a chemical reaction. Therefore there must be enough energy within the molecules to overcome this energy barrier. If there is enough activation energy in the collision of the reactant particles a reaction will commence, if the minimum energy required is absent the particles will only bounce of one another and will remain the same [68].

1.9.1.1 Half-life

The half-life of a reaction is of importance in stability testing of drugs. The half-life ($t_{1/2}$) in relation to drug degradation can be described as the time required to degrade a starting drug concentration by half [60].

1.9.2 Reaction mechanisms

The reaction mechanism is the sequence of elementary processes [59] or reactions that take place that demonstrate how the overall reaction progresses and leads to the overall chemical change. The overall chemical change that occurs is directly observable but in some cases reactions can be complicated to understand and experiments can be designed to determine the individual steps that may take place within the reaction. By determining the processes that take place the orders of each elementary step can be established. The slowest elementary step is known as the rate determining step.

There are several types of elementary processes. These can be described as follows:

Unimolecular A $\longrightarrow$ P

Bimolecular A + B $\longrightarrow$ P

Alternatively a reaction can be classified in terms of its order [58].

1.9.3 Orders of a reaction

Orders of reaction are the rates determined for each reactant involved. The reactants will be raised to a power which is specific to the order for that particular reactant. Addition of these powers forms the overall rate of the reaction. This information is determined from experimental data.

For **zero order** reaction (n = m = 0) the reaction rate is independent of the concentration of the reactant therefore it is not affected by the specified reactant. A **first order** reaction (e.g. n = 1 and m = 0) is proportional to one concentration and a **second order** reaction (e.g., n = 2 and m = 0) is proportional to the product of two concentrations or the squared of one concentration [58]. Orders of reaction are raised to a power and can be expressed as:

$$\text{Rate} = k\,[A]^n[B]^m$$

[A] and [B] are the concentrations of the reactants

^{n} and ^{m} are the orders of reaction with respect to [A] and [B]

Experimental data obtained can be used to produce a concentration against time plot. If the reaction is a **zero order** the rate will not vary with α. If the reaction is **first order** with respect to A then the rate is directly proportional to its concentration (α). If the overall reaction is a **second order** the rate of reaction is proportional to $α^2$ (Fig.1.11).

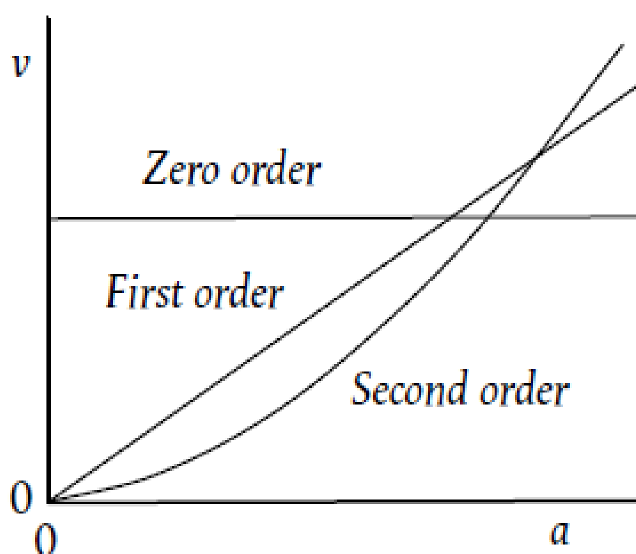


Fig.1.11 Examples of orders of reaction with respect to A [58]

In systems involving temperature or pressure increasing these conditions results in an increase in the rate of reaction. Increasing the pressure (for systems involving a gas state) works the same way as increasing the concentration. Increasing the temperature causes an increase in the collision of particles therefore increasing the rate of reaction.

It has however has been proven in our laboratory [69] that such kinetic treatments are not suitable to describe photoreactions. For drugs' photodegradation reactions it is necessary to develop new strategies for treatment of the kinetic data.

2. Excipients

Excipients are widely used in the food industry as well as pharmaceuticals. In terms of weight a drug formulation consists mainly of excipients. Excipients are pharmacologically inert substances included in a dosage form along with pharmacologically active compounds to aid in its formulation, manufacture, administration to patients and absorption properties [11]. Examples of excipients include sweetening agents, tablet binders, antioxidants, antimicrobial agents (alcohol), buffers, lubricants, anticaking agents, emollients and antiseptics [41]. The properties of the final dosage form are highly dependent on the excipients chosen including the concentration and the interaction with other excipients and the active compounds involved. The types of excipients added to a dosage form will depend on their scientific function. They must also be safe for consumption or application before administration to patients [37].

In the past the safety of pharmaceutical excipients has been overlooked and they were described as 'any more or less inert substance added to a prescription in order to confer a suitable consistency or form to the drug: A vehicle' [37]. Novel forms of drug delivery are continuously rediscovered resulting in the rise in the use of excipients along with excipient mixtures. Their use is important to improve properties in drug delivery systems [41], so are of major importance in the pharmaceutical industry. Although excipients are considered as pharmacologically inert or rarely active like pharmaceutical drugs, excipients have thermal dynamic activity. Although this tends to be very low activity [37] they are still able to initiate, propagate and participate in chemical and physical interactions resulting in the compromise of both the quality and the performance of a medication. This in turn can result in the degradation of drugs and drug products as well as places the effects and safety

of the drug into question [11]. Crowley and Martini [11] state that some excipient functional groups interact directly with active pharmaceutical ingredients resulting in degradation. Some excipients may also contain residues or impurities. There are several ways in which decomposition can take place including hydrolysis, oxidation, isomerization, photolysis and polymerisation [11]. Hydrolysis is the most frequent reaction that is formed from interactions due to the fact that water is often used as a solvent for solutions. Photolysis, isomerisation and polymerisation are less common reactions and only occur from particular excipients. These types of reaction are known for reducing the active ingredient in a formulation as well as giving rise to the formation of hazardous impurities [37]. Sources of excipients include plants, animals, synthesis and minerals however there quality is not always ensured to meet the requirements of safety for the pharmaceutical industry [37]. Due to such problems in drug-excipient interactions recent studies have been carried out in order to guarantee their safety for the use in pharmaceutics. The now realised dangers of excipients, adds to the importance of gaining the information in order to label packaging correctly and provide the correct instruction of ingredients and directions of use. Pifferi and Restini [37] state that it is now required that any new product whose effects on man are not known must pass all the toxicological tests before it can be accepted.

3. Cyclodextrins

Cyclodextrins (CDs) are a group of sugar molecules known as cyclic oligosaccharides connected in a ring. These naturally occurring molecules are made of starch [27] as well as several glucose units [26]. The natural (parent) cyclodextrins (α-CD, β-CD and γ-CD) can be composed of six, seven or eight of these glucopyranose units [70] which are linked by glycosidic bonds in a cylinder structure. Cyclodextrins are formed through the breakdown of the starch by an enzyme called glucosyltransferase in a reaction known as intramolecular transglycosylation [31] which through mechanisms of chain splitting and intramolecular rearrangement generates primary products such as CDs [12]. In order to form CDs the CGT-ase or cycloglycosyl transferase amylases [16] (cyclodextrin glucosyl transferase enzyme) must be produced [2]. This is done so by culturing a microorganism that results in its

formation, the CGT-ase is then separated and purified before the starch is degraded using enzymes. Intramolecular reactions commence from this process resulting in the formation of cyclic and acyclic dextrin products. The CDs result from the formation in links between α (1,4) units of gylcosidic oxygen bridges. The CDs are separated from the mixture before being purified and crystallised [2].

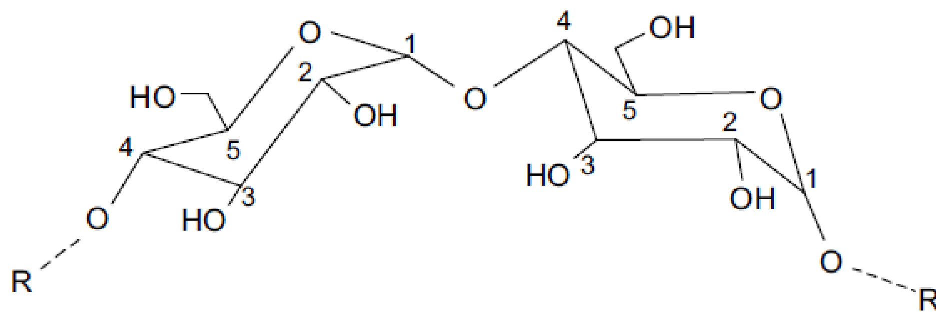


Fig 1.12 α (1,4) glycosidic oxygen bridge bond between two glycopryranose molecules [2]

The number of glucose units in which the molecule is composed of will determine the type of cyclodextrin, these being alpha, beta or gamma cyclodextrins (Fig.1.13) [27].

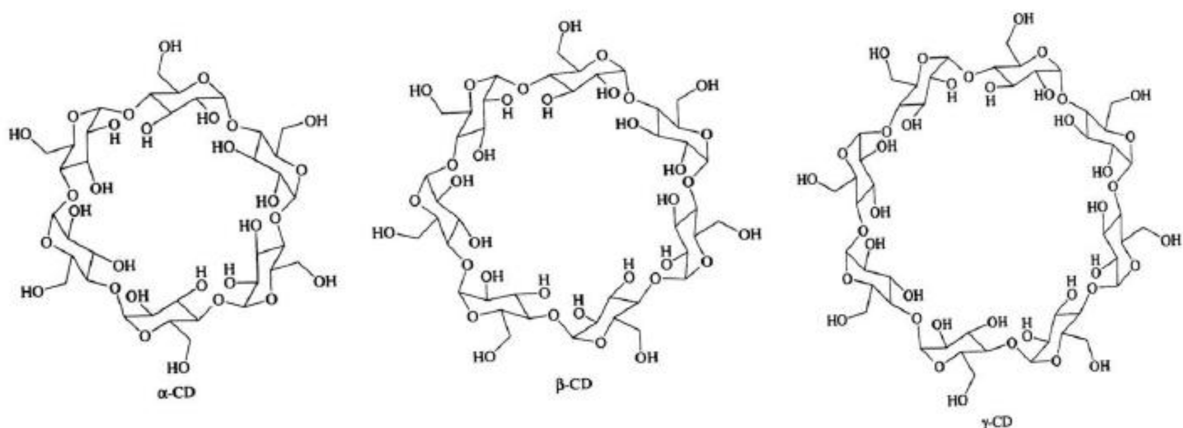


Fig 1.13 Chemical structures of the three parent cyclodextrins (α-CD, β-CD and γ-CD) [2]

α-CD and γ-CD are expensive particularly in there purification during production therefore β-CD is the most preferred and is more commonly used in industry [2], however β-CD has the lowest water solubility. This may be because of the formation of an intramolecular hydrogen bond between the hydroxyl groups [16] which provides the β-cyclodextrin with a more ridged structure than the other CDs. This however prevents any hydrogen bonding with the cyclodextrin and any outside water molecules, this in turn reduces its ability to solubilise in water and therefore the β-CD has a very poor water solubility [16]. γ-CD is the most water soluble due to its flexible structure. The hydrogen bond formation is not fully complete in the α-CD. This is believed to be the result of the position at which one of the glucopyranose units sits within the structure [2].

3.1 Cyclodextrins and toxicity

Cyclodextrins were once believed to be toxic however, research has found that cyclodextrins are harmless [2] and can be extremely useful in many industries particularly in food and pharmaceuticals. They are becoming more and more common due to their advantages [25]. Cyclodextrins do not induce immune responses and there toxicity in humans and animals is low [12]. Martin de Valle [31] states that all studies on cyclodextrins (Natural and CD derivatives) related to their toxicology has shown that through oral administration they are nontoxic and is due to the lack of absorption from the gastrointestinal tract during digestion. However studies on parenteral administration have shown that the α-CD, β-CD and methylated β-CD are unsafe to be administered with this approach [31].

First knowledge of cyclodextrins came to light in 1891 and the chemistry of cyclodextrins was developed between 1903 and 1911 [24]. In 1942 the structures of the α-CD and β-CD was realised using a method known as X-ray crystallography and in 1948 the structure of the γ-CD was discovered [31]. The solubilising and stabilising characteristics of these compounds were eventually discovered in the 1950s. Research on these characteristics determined that

they are capable of solubilising lipophilic compounds and they can also be used as enzyme models [24].

3.2 The principle of cyclodextrins and formation of inclusion complexes

The ring structure can be best described as a conical cylinder [2] made up of glucopyranose units. The central cavity is lined with hydrogen atoms and glycosidic oxygen bridges [2]. The cavity is hydrophobic and the surroundings walls are hydrophilic. There are a large number of hydroxyl groups around the external [16] of the cavity of the CD molecule which gives them there water solubility. The secondary hydroxyl groups are located on the wider edge of the cyclodextrin ring whilst the primary hydroxyl groups are located on the outer edge. This structure makes the outside of the molecule hydrophilic allowing it to dissolve in water and the internal cavity is a hydrophobic matrix. In aqueous solution the hydrophobic cavity allows the formation of inclusion complexes [16] with a spectrum of both organic and inorganic hydrophobic guest molecules. The characteristics of cyclodextrins allows the confining of molecules that are an appropriate dimension for the CD cavity. In the suitable conditions:

Cyclodextrin (CD) + Guest molecule = Inclusion complex

Inclusion complexes will form when an equilibrium is reached between the CD molecule and the guest molecule. Inclusion complexes may take place with several molecules, a single molecule or part of a molecule.

Both the formation and dissociation of inclusion complexes is controlled by the association or equilibrium constant (k). Many factors affect this constant and the end result including the size of the guest molecule, dimensions of the CD cavity and how the molecules fit

together. The better the molecules fit together the more stable the binding is resulting in a stronger complex. The binding constant (k) will be a higher value when the complex is stable therefore the more unstable the complex is the value of k will decrease thus resulting in dissociation of the complex [16].

The formation of inclusion complexes alters the physicochemical behaviour of the guest molecule, this in effect reduces or eliminates any undesirable effect that the guest molecule may produce [27] whilst encapsulated within the host cavity. Cyclodextrin complexes can be employed in an attempt to reduce photoreactivity in drugs and so far many studies have been carried out in an attempt to reduce the problems surrounding adverse reactions from drugs. Inclusion complexes can be formed using the whole drug or only part of the drug [12]. Cyclodextrins are now accepted as pharmaceutical excipients [31].

It has been found that the natural cyclodextrins and the hydrophilic derivatives are only able to permeate specific lipophilic biological membranes such as the eye cornea but with some difficulty. Cyclodextrins have the ability to form solid inclusion complexes with solid, liquid and gas compounds by molecular complexation. The cyclodextrin molecule acts as a host molecule holding the guest molecule within its cavity and a complex is formed. However the complex formation depends on the size of the cyclodextrin molecule to the size of the guest molecule as well as the solvent and any thermodynamic interaction. Water is the most commonly used solvent with the use of cyclodextrins and inclusion complexes. The guest must also be able to displace the solvent from the cyclodextrin cavity in cases where the solvent and the cyclodextrin become complexed. Water is more often the preferred choice as it can be easily displaced. Not all molecules are soluble in water and in some cases other solvents may be used to dissolve the guest molecule before commencing the complexation process. Other solvents may include ethanol and diethyl ether [31]. Binding of the molecules is not permanent and no covalent bonds are formed or broken in the process. The strength of binding in order to form the complex will depend on both how well the complex fits together as well as any interactions between atoms on the surface of the molecules [31].

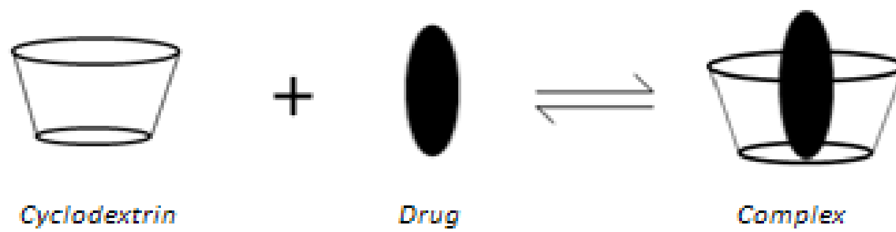


Fig 1.14 Simple diagram of the formation of a cyclodextrin complex

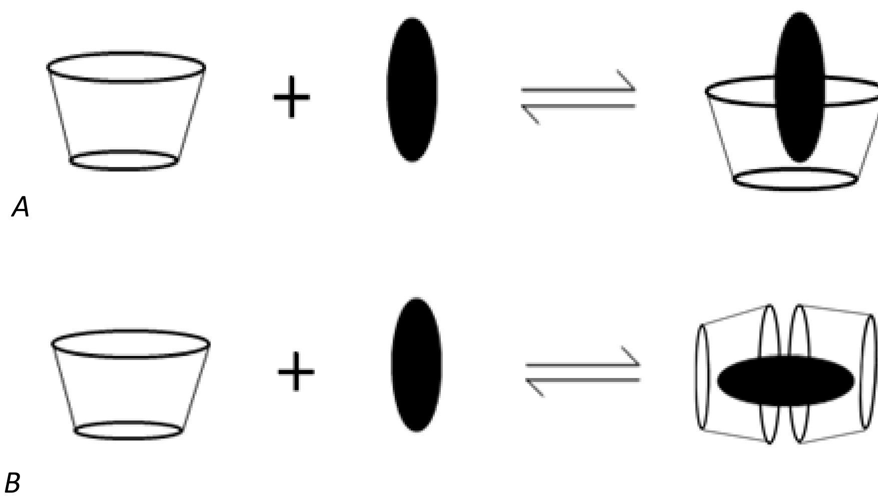


Fig 1.14 Cyclodextrin inclusion complex possibilities (*A* 1:1 complex, *B* 1:2 complex).

Although it is true that the molecule within the CD cavity is apolar or part of the molecule inside the cavity is apolar other types of complexes can be formed. In some cases complexes can be formed which are not inclusion complexes where a guest molecule becomes linked to the external of the cyclodextrin. It is also possible for a guest molecule to interact with one or more cyclodextrins or where one or two guest molecules can interact with one CD [16] these are referred to as complexes 1:1, 1:2 and 2:1 (Fig.14).

3.3 Cyclodextrin derivatives

The α-CD, β-CD and γ-CDs have been found to have a low solubility due to the presence of hydrogen bonds between the hydroxyl groups [16] within the structures of CD molecules so derivatives were later formed in order to overcome this problem [24]. Modifications to CDs have allowed the increase in solubility and still have the ability to form inclusion complexes with other molecules, the CD derivatives however have a higher affinity for water than that of the parent (natural) cyclodextrins [16].

The three main derivatives are known as:

- Methylated CDs (neutral)
- Hydroxylpropylated (neutral)
- Sulfobutylated (negative charge)

These chemical modifications were applied to the basic and less soluble α-CD, β-CD and γ-CDs in order to enhance the aqueous solubility (Fig.15). It was discovered that the substitution of the hydroxyl groups of the cyclodextrins would results in a substantial increase in there aqueous solubility even by introducing hydrophobic moieties such as methoxy functions. More derivatives were later realised including the 2-hydroxypropyl and sulfobutylether derivatives as well as the β-CD glucosyl and maltosyl forms known as branched derivatives [24].

The partly substituted derivatives were found to have a higher solubility than those that were fully substituted which is a result of a decrease in isomers. In the case of the methylated CD derivatives the solubility of β-CD increases as the methylations of the hydroxyl groups increases. The solubilty then begins to decrease once approximately 2/3 of the hydroxyl groups have been methylated [24]. In the substitution of the alkyl derivatives the crystalline parent CDs become on amorphous mixture when chemically manipulated.

Hydroxypropylation will occur randomly on either the primary or secondary hydroxyl groups of the CDs. The only existing sulfobutylated CD is the β-CD derivative [16]. The degree of substitution will also affect the formation of inclusion complexes. The degree of substitution is therefore optimized to a specific pharmaceutical grade based on the solubilizing abilities of the cyclodextrins [24]. Many of these derivatives are now being used within marketed products around the world [12].

Dimethyl-β-cyclodextrin (DMβCD)	$-CH_3$ or -H
Trimethyl-β-cyclodextrin (TMβCD)	$-CH_3$
Randomly methylated-β-cyclodextrin (RMβCD)	$-CH_3$ or -H
Hydroxyethyl-β-cyclodextrin (HEβCD)	$-CH_2CH_2OH$ or -H
2-Hydroxypropyl-β-cyclodextrin (HPβCD)	$-CH_2CHOHCH_3$ or -H
3-Hydroxypropyl-β-cyclodextrin (3HPβCD)	$-CH_2CH_2CH_2OH$ or -H
2,3-Dihydroxypropyl-β-cyclodextrin (DHPβCD)	$-CH_2CHOHCH_2OH$ or -H
2-Hydroxyisobutyl-β-cyclodextrin (HIBβCD)	$-CH_2C(CH_3)_2OH$ or -H
Sulphobutylether-β-cyclodextrin (SBEβCD)	$-(CH_2)_4SO_3Na$ or -H
Glucosyl-β-cyclodextrin (G_1βCD)	-glucosyl or -H
Maltosyl-β-cyclodextrin (G_2βCD)	-maltosyl or -H

Fig 1.15 β-CD chemical derivatives [12].

3.3.1 Cyclodextrin polymers

Cyclodextrin polymers are cyclodextrin derivatives that combine the complex forming properties of cyclodextrins and the high molecular weight and high solubility properties of polymers. Increasing attention has been given to the cyclodextrins polymers over recent years [71] as dramatic enhancements can be obtained from there use with other molecules. They are predominantly used in the pharmaceutical industry to enhance solubility of drugs and are also useful in the protection against unwanted side effects from drug compounds [72].

Cyclodextrin polymers were first formed through crosslinking with ephichlorohydrin and then diepoxides and dissocyanates used as crosslinking agents [71]. Some of the cross-linked polymers are water soluble. They all have higher water solubility's than the parent cyclodextrins. Work has continued on such polymers in order to develop and improve inclusion complexing and drug delivery application [12]. The three common mechanisms by which a drug is released from such systems are through dissolution, diffusion and erosion [73].

Traditional dosage forms can limit physiological barriers, enable undesirable drug properties and drug toxicological issues. Such restrictions may be overcome and improvements in therapeutic effect observed through development of drug delivery systems [73].

The efficiency and applicability of cyclodextrins can be increased if they are incorporated into a polymeric structure [74]. The ability of polymers to modify drug release is well known. Numerous polymer based controlled drug delivery systems have already been realised. One example is Brufen retard known as ibuprofen [73]. By incorporating cyclodextrins into polymeric drug delivery systems the mechanism by which a drug is released can be influenced. They are capable of modifying drug solubility by increasing solubility of poorly water soluble drugs, increasing complexation efficiency, greater binding efficiencies and controlled drug delivery release.

In recent years they have been used in formation of gels, microspheres and nanogels. Gels have been used for slow drug delivery release. Disinfecting drugs have been incorporated into the cyclodextrins polymer beads for use in wound and burns treatment which enables controlled drug delivery release without causing inflammation of surrounding tissue [12].

4. Photodegradation of oestrogens and synthetic hormones

4.1 Stilbene

Stilbenes are a family of naturally occurring compounds which can be found in a wide variety of sources including plants, dietary supplements and aromatherapy products [40].

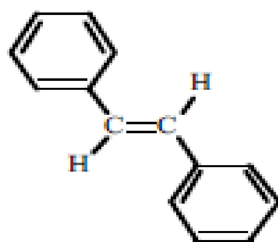


Fig 1.16 Structure of the stilbene trans-isomer [40]

The structure of stilbene is very similar to that of oestrogen and has a small molecular weight averaging around 200-330g/mol.

There are many stilbene compounds which have biological activities in humans [9] including antiallergic, hormonal, antibacterial, anticancer, antimalarial and hypocholesterolemic effects [42]. The most prominent among them are the pinosylvins, pterostilbenes, resveratrols and piceatannols.

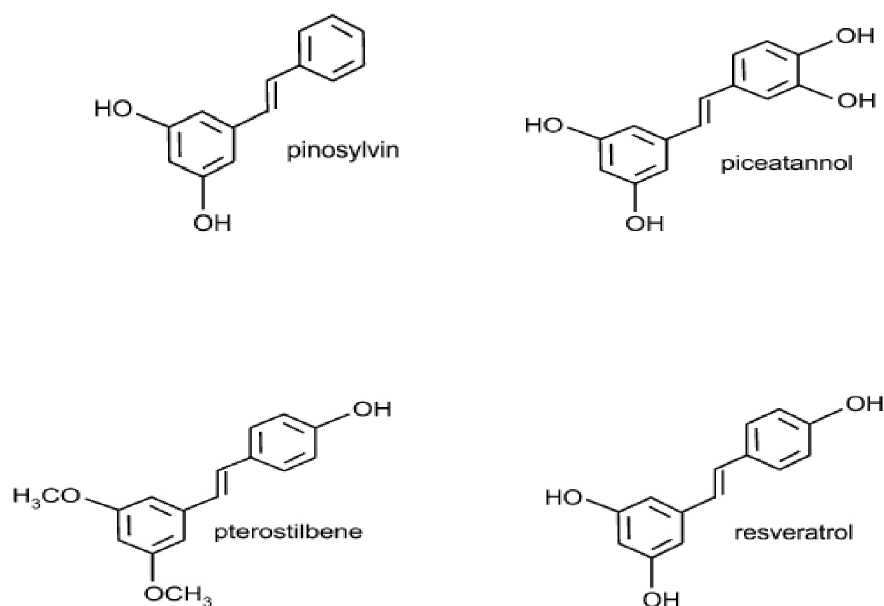


Fig 1.17 Chemical structures of other molecules belonging to the stilbene family [9].

Stilbene derivatives are stress metabolites [39] formed in plants through what is known as the phenylpropanoid pathway when threatened. When a plant is under environmental threat or stress which may occur through damage or disease stilbene is secreted from the plant. This happens naturally by activation of the phenylpropanoid pathway [40]. The production of stilbene is a plants natural defense mechanism and will help in the protection from UV exposure, virus, bacteria and disease.

A lot of research has been carried out on stilbenes to determine and verify their benefits for the pharmaceutical industry which include their antioxidant and anti-inflammatory effects, as well as their ability to fight disease. Other biological activities of stilbenes include their ability to lower cholesterol, increase insulin sensitivity and their anti-ageing effects [39].

Resveratrol is extremely photosensitive [45]. Although resveratrol is highly photosensitive it is one of the stilbene derivatives of great interest to the pharmaceutical industry as it has been found to have many beneficial uses. Resveratrol has demonstrated antioxidant behaviour, antimicrobial activity as well as anti-inflammatory properties [43] hence its formation in damaged plants. There has also been recent speculation in its aid in the prevention of cancer and chemotherapeutic effects [4]. Bonda et al [5] states that it has been found that topical application of resveratrol inhibits UV-B induced tumour initiation, promotion and progression of the skin. It has also been found to inhibit human lung adenocarcinoma cell metastasis. Oxyresveratrol is very similar to resveratrol in structure and is a hydroxystilbene, a natural antioxidant which can be found in mulberry wood [53]. It has been found to have neuroprotective properties that may be useful in the protection of cerebral ischemia. Studies have found that the use of oxyresveratrol with trauma cells results in a significant inhibition of neuronal death [53].

Piceatannol is a naturally occurring compound along with pinosylvin and rhapontigenin. piceatannol is generally found in berries, peanuts, red wine and sugarcane. Recent studies have also found it to be a metabolite of Resveratrol. Roupe et al [40] states that studies conclude it to be a potential therapeutic compound for various diseases. Pinosylvin is found in the wood of several species of pine tree and eucalyptus. It is also induced in pine needles of some trees when exposed to infection or stress. Studies have shown it may have possible anticancer, antifungal and antibacterial activities [40]. It is however less known than other stilbene derivatives. Rhapontigenin occurs in Korean rhubarb rhizomes and has been used for many years by Korea, Japan and China to treat poor circulation and severe inflammation. It has been found to inhibit histamine in anti-allergic activity studies [40].

Stilbene derivatives such as diethylstilbestrol and pinosylvin formed by stilbene synthase are known to have several other biological benefits which include anti-allergic, anti-malarial, anti-bacterial, anti-fungal and hormonal activities [42].

More recent advances have suggested stilbenes as a means of prevention of cancer and cancer chemotherapeutic agents due to studies linking a diet high in fruits and vegetables to the prevention of colon cancer [39]. Roupe et al [40] state that studies have shown that consumption of a diet high in fruit and vegetables is likely to considerably reduce the risk of developing cancer as well as cancer related mortality. Fruit and vegetables are known to be rich in phytochemicals [40] such as stilbene compounds, particularly the smaller fruits such as berries and grapes [39]. These compounds are believed to be associated with the health benefits of consuming a diet high in fruit and vegetables [40].

4.1.1 The Mallory reaction

Stilbene and the stilbene derivatives go through a process known as photocyclization (which is the formation of a ring system through a new bond) when exposed to UV light. This results in the formation of a photoproduct trans-4a, 4b-dihrophenanthrene (DHP) which is generally unstable. This is an intermediate which is oxidatively trapped by hydrogen receptors such as iodine and oxygen which allows the formation of high yields of phenanthrene (PAH). This photoreaction occurs in many of the substituted stilbenes and stilbene related molecules [30]. PAH's a group of polyclic hydrocarbons that are both natural and synthetic in the form of solid crystals which can either be colourless or may in some cases appear yellow. Applications include dyes, plastics, pesticides, explosives and drugs [5]. In 1964 Mallory described the use of Iodine to catalyse the Photocyclization of stilbenes in order to synthesise PAH's [20].

Overall research has shown that stilbene derivatives are important due to their many health benefits with diethystilbestrol being better known for its use in cancer therapy.

4.2 Diethylstilbestrol

The synthetic oestrogen diethylstilbestrol (DES) is an endocrine disruptor [19] and derivative of the stilbene family [14] and is one of the most active oestrogens in the environment [13]. The structure of DES is similar to natural oestrogen E2 (17β-estrodiol) structure. DES has been found to be more beneficial in that it is highly active and therefore more effective and is cheaper than that of E2 [13]. However studies carried out on derivatives of oestrogen have produced evidence demonstrating that their excited states and the formation of free radicals result in the damage of biological tissue [13].

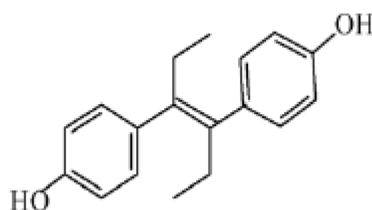


Fig 1.18 Structure of diethylstilbestrol (DES) trans isomer [13].

Studies carried out by Dou et al [13] found that DES becomes excited at 248nm which may lead to the production of oxygen and singlet oxygen this in effect can cause the degradation of DES. It was also confirmed that DES is particularly vulnerable to ·OH which are naturally existing everywhere in the environment and as a consequence serious biological effects can result from the degradation of DES. However Dou et al [13] found that by placing DES in an acid or a neutral aqueous solution the products are safer and so reduces the risk to human health.

DES was prescribed to millions of women with gynaecological problems and in the prevention of miscarriage in pregnant women between 1940-1971. A paper was written in 1948 which discussed the use of DES in the treatment and prevention of the complications

of pregnancy. Very little was mentioned on the dangers. The power of DES was eventually realised and the drug was banned, however its effects have been passed on through generations of family of those who were once prescribed the drug. It was prescribed until 1971 to treat miscarriage in pregnant women in their first trimester often as a result of progesterone deficiency, to treat prostate cancer and breast cancer in post-menopausal women, to control abnormal gynaecological bleeding and stunt the growth of young girls [19].

It was first realised in 1953 that DES had no effect on what it had originally been prescribed for and may be a danger to people's health. However it wasn't banned until 1971 when a paper by Herbst et al was published demonstrating that DES was resulting in an increase in vaginal clear cell adenocarcinoma (CCA) in the daughters of the women who had been prescribed the drug. In the same year the United States food and drug administration (FDA) issued a bulletin advising the prescription of DES to be stopped and withdrew the approval of the use of DES [19]. The use of DES in cattle was banned in 1979.

Studies carried out show that women who are exposed to DES during their pregnancy have an increased risk of breast cancer mortality and the risk of the development of other cancers. In Utero exposure it has been found there is an increased risk of the development of CCA vaginal and cervical adnenosis from birth to the age of 39 as well a risk of squamous cervical cell dysplasia [19]. These risks were found to be due to exposure in the early stages of pregnancy within the first trimester. A study in testicular cancer in men was found to be inconclusive. DES is also potentially dangerous resulting in cardiovascular illness such as deep vein thrombosis and pulmonary embolism [33].

Harris and Waring [19] explain that DES is highly carcinogenic because DES is metabolised to 3'-hydroxy-DES which is then oxidised further becoming DES-3'4'-quinone. Oestrodiol also forms DES-3'4'-quinone. This metabolite then reacts with DNA resulting in the generation of mutations in the critical genes that initiate cancers.

Dose related side effects may include nausea, fluid retention, withdrawal bleeding in women, venous and arterial thrombosis, Impotence and gynaecomastia in men and hypercalcaemia and painful bones may also occur in those with breast cancer as well as jaundice and sodium retention with oedema [8]

In the first trimester of pregnancy high doses of diethylstilbestrol may be associated with vaginal carcinoma, urogenital abnormalities as well as fertility problems in any female offspring and a risk of hyperspadias in male offspring. Caution must be taken with those who have cardiovascular disease and shouldn't be used in those with hepatic impairment.

Although there are potentially dangerous side effects and risks with DES there are also some benefits. Diethylstilbestrol (Stilboestrol) is sometimes still used in the treatment of specific cancers, particularly prostate and may also occasionally be used in postmenopausal women with breast cancer. However it isn't usually the first route of treatment due to its side effects and toxicity with the use of DES is common. DES is now used as hormonal therapy for the treatment of advanced prostate cancer where the cancer has spread beyond the prostate gland to other parts of the body [28]. It works by reducing the levels of testosterone in the body which reduces the rate of growth in cancer cells in order to reduce to size of the cancer. By administering DES to patients with prostate cancer the drug results in the switch off of testosterone as the brain is made to believe there is too much of the hormone in the body. This reduces the growth of cancer cells and may result in the shrinking of tumours. DES is also used in vetinary field for the treatment of urinary retention and incontinence [56]. Dosage is generally one tablet daily, preferably at the same time each day. The drug may be taken for several months or years [28].

Little has been reported on the photostability of DES however Doyle et al [14] reported that when exposed to near ultraviolet radiation the synthetic oestrogen resulted in the isolation

of a stable photoproduct 4a, 4b-dihydrophenanthrene (DHP). The study also reported that DHPs are normally very unstable and it was found that in this case the stability of the DHP photoproduct was due to double tautomerism of dienol to a diketone structure where rearomatization is lost.

Due to the many dangerous side effects reported on the drug, studies are being carried out in order to find a way of reducing the potentially dangerous effects of such drugs including the use of cyclodextrins in a hope they may be useful in reducing or inhibiting these effects [33].

Objective

The objective of the study is to investigate the photodegradation behaviour of DES in solution and to determine the compounds photostability and photodegradation characteristics.

Interaction of DES with cyclodextrins and the possible application of cyclodextrin polymers in photostability studies will be studied.

The possibility of developing a safer liquid formulation of DES will be explored.

Chapter two.

Experimental

2.1. Methods and equipment

2.2 The photolysis process

A UV-VIS spectrophotometer with photo diode array detector was used along with a 1000 W mercury lamp for irradiation. This allows data of samples to be obtained and single spectra or kinetic readings can be carried out quickly (in less than 0.5 s) providing data over the whole UV and visible spectrum with high sensitivity. There are two methods in which samples can be irradiated depending on how reactive they are to light.

Steady state irradiation:

Direct irradiation is used for those drugs (DES) that degrade over very long periods of time (hours) to speed up the degradation process. Steady-state (Direct) irradiation can be carried out by placing the cuvette containing the drug in solution (at a distance of 5 to 10 cm) directly in front of the light passing through the monochromator. The sample is irradiated at a specific wavelength chosen and timed at intervals using a stop watch. Then, the sample is placed in the diode array spectrophotometer for a reading. These results are used to produce absorbance against time plots. Excel is used for this purpose.

Continuous irradiation:

Before commencing irradiation a reading is taken of the DES sample in order to obtain the spectrophotometric characteristics of DES in solution. This will provide information on both the irradiation and observational wavelengths at which the sample should be studied at. The desired wavelengths can then be input into the kinetic software settings which is part of the diode array work station. Other information such as runtime and cycle time must also be added and the irradiation wavelength chosen. An optical fibre is then connected from the light window of the monochromator. The sample is placed into the sample holder where it

will remain for the specified run time. The optical fibre is placed on top of the sample holder where the light is continuously passed through the optical fibre into the sample and readings are automatically taken at set time intervals.

2.2.1 Photodegradation setup

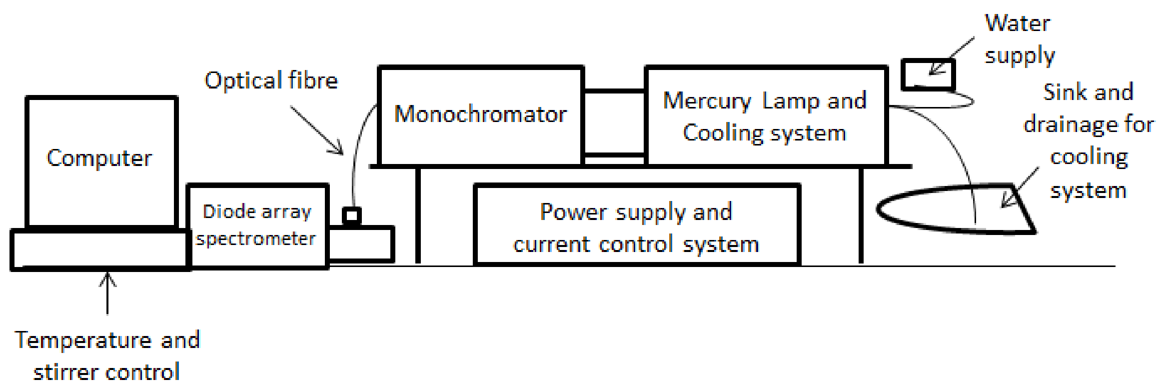


Fig.2.1 Photodegradation setup

Radiant power meter and light intensity measurements

Light intensity measurements were taken using a radiant power meter and probe. This equipment is used to measure energy and power emitted by a light source. Readings can be taken directly from the emitting light source or surfaces and objects that are exposed to a light source. Light intensity may fluctuate throughout the experiment. Intensity may affect the stability of a drug and the results obtained therefore it is important to take intensity readings before and after experiments.

Model used: 70260

2.3 Fluorimetry

A fluorimeter can be used in the analysis of the photodegradation of drugs in order to analyse the fluorescence of either or both the mother compound and photoproducts in a sample. Fluorescence is monitored during sample exposure to light.

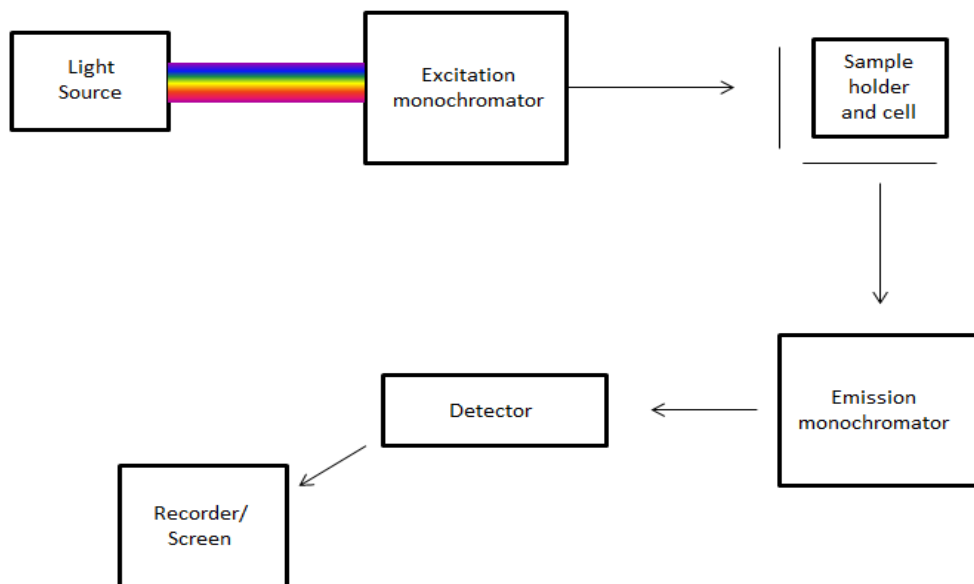


Fig.2.2 Diagram of the setup of fluorescence spectroscopy equipment

2.4 Rotary evaporation

Rotary evaporation is an efficient technique used to gently remove solvents from samples.

In the present work, this has been used in the removal of the solvent (water and/or ethanol) from a cyclodextrin complex with a drug leaving the CD complex in powder form for further analysis using techniques such as ATR-FTIR and SEM.

A water bath is connected to the rotary evaporation equipment in order to aid the evaporation using heat. The vapour from this sample is drawn off as the flask is rotated by a motor unit.

2.5 ATR-FTIR

ATR-FTIR is one of the most widely used spectroscopy techniques used in many fields such as biology, forensics, pharmacy, organic chemistry and analytical chemistry. This spectroscopy technique is used in both research and industrial laboratories throughout these fields [38]. The ATR enables quick sampling whilst the FTIR allows fast and stable collection of spectra. The technique is non-destructive technique that is able to analyse a wide variety of samples allowing there characterization, identification and quantification.

Only small quantities of the sample are needed and samples can be analysed directly either in solid form or liquid form. The technique works by passing an Infrared beam through a crystal made up of materials such as diamond with a high refractive index which results in the production of internal reflections known as an evanescent wave. The ray is then passed through the sample and onto a detector producing a spectrum of the sample.

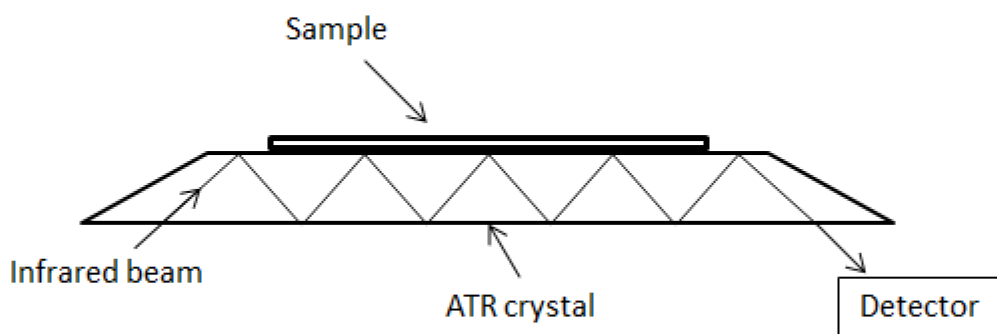


Fig.2.3 Diagram of an ATR system

2.6 SEM

The scanning electron microscope (SEM) allows the observation and characterisation of organic and inorganic materials. A specimen is hit with a fine electron beam which sweeps across the surface of the sample and reflects back [23]. The electrons interact with the electrons in the sample which results in the formation of signals that can be detected. SEM can be combined with energy dispersive X-ray analysis which allows elemental identification of samples by providing information on their composition as well as the topography of sample surfaces. SEM/EDX can be used to characterise a range of materials including glass, powders and fibres [23]. There are ranges of signals that can be detected in the analysis of samples with the SEM. The secondary and back scattered electrons are the most important due to their variation. The variation in these signals is a result of the differences of the surface topography of samples. Characteristic X-rays are also emitted in SEM analysis as a result of electron bombardment and can provide both qualitative and quantitative information [18].

Although the analysis is expensive and complex there are many advantages. Preparation time of samples is fast and very small quantities can be analysed. The SEM allows a high

resolution to be obtained in analysis of bulk objects and large depth of field allows 3D imaging. Higher resolutions result in sharper images. Samples can also be examined at low magnifications and can therefore be used alongside other types of analysis. This particular feature is useful in many fields including forensic science where images produced in SEM analysis complement those found using the light microscope.

Over time the SEM has been found to have uses in many fields of research due to advantages of high resolution and low beam energy capabilities [18].

2.7 Chemicals

2.7.1 Drugs and solvents

DES was purchased from Sigma Aldrich, was used without any further purification and was dissolved in ethanol for analysis. A stock solution was prepared and intermediates were made based on the sensitivity of the methods used for analysis of the compound.

Water/ethanol solutions were made using double distilled water and experiments were carried out at room temperature unless stated otherwise. DES was protected from light at all times when irradiation of the compound was not taking place.

Cyclodextrins are fully soluble in water. All CDs used were dissolved in double distilled water and the monomers were dissolved to their solubility limits.

Chapter three.

Results and discussion

3.1 Introduction

This chapter will discuss characteristics of the DES compound and the results obtained from a range of photodegradation and photostability studies such as effect of solvent, irradiation wavelength and cyclodextrins.

3.2 Some spectroscopic and stability characteristics of DES

The UV/Vis spectrum of trans-DES (Fig.3.1) in pure ethanol before irradiation is shown on Fig.3.2. The compound absorbs exclusively within the UV range below 300 nm with maxima at 240 nm, in addition to a shoulder at 274 nm. The compound is colourless in solution.

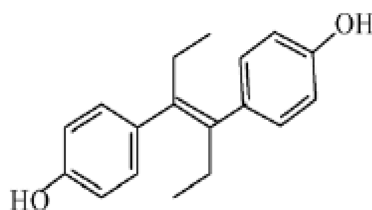


Fig.3.1 Chemical structure of trans-DES isomer [13]

DES analysis in fluorescence has shown that this compound is characterised by a very low fluorescence signal. In addition, the fluorescence spectra were found difficult to record because of the photoreactivity of DES when subjected to the excitation irradiation beam coming from the fluorimeter (see section **3.4.2**).

The compound is thermally stable while maintained in the dark. Fig.3.3 demonstrates that DES is thermally stable in the range of temperatures between 15 to 37°C.

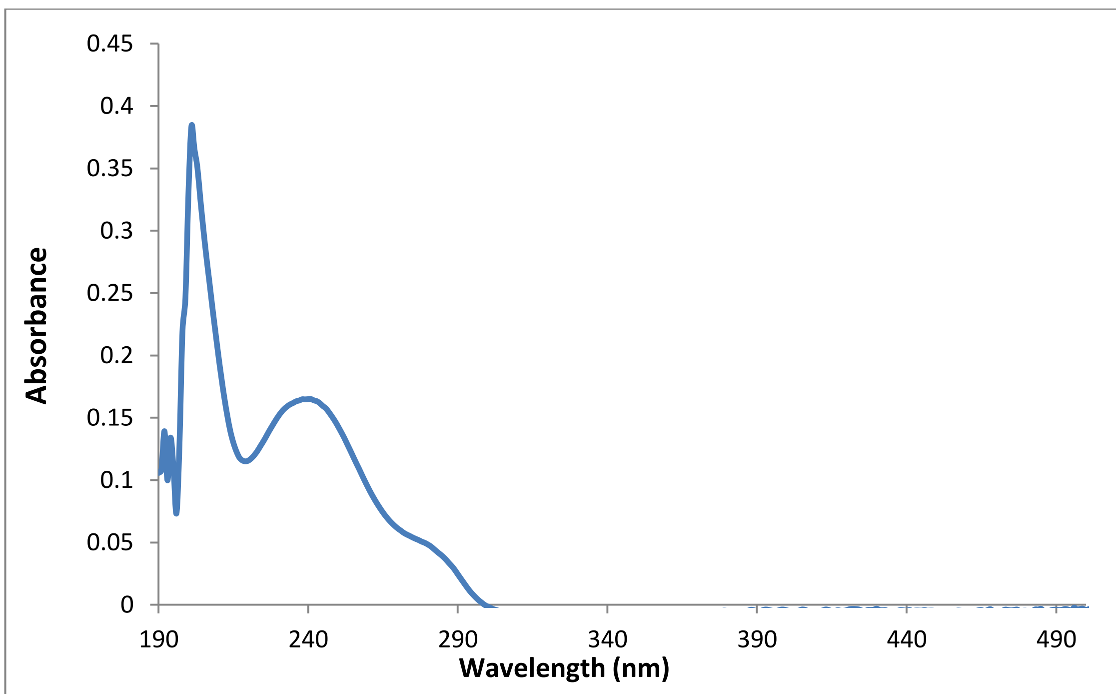


Fig.3.2 UV/Vis spectrum of DES (7.890 x 10^{-6}M) at t=0 in ethanol.

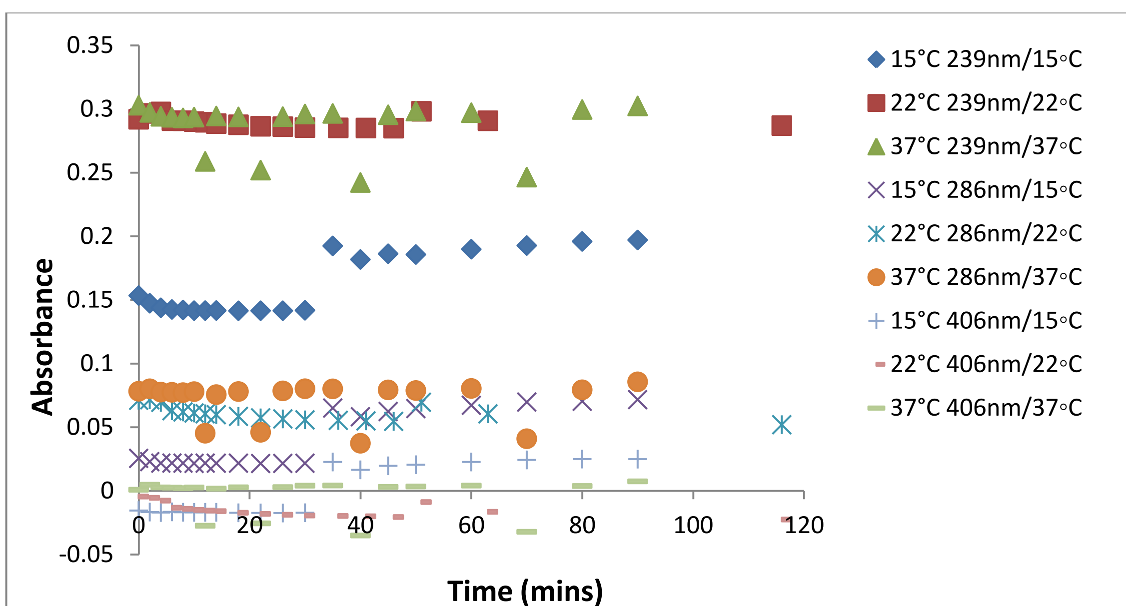


Fig.3.3 Traces of a thermal study of DES (7.890 x 10^{-6}M) in ethanol.

3.3 Compound solubility and irradiation conditions

DES is an organic non-polar species that is readily soluble in organic media (e.g. ethanol). However, it is almost not soluble in water [6]. In order to study the effect of water on the stability/photostability of DES and offer an opportunity to develop safe liquid formulations of DES, it has been solubilised in water with a small amount (2 %) of ethanol as a co-solvent. For the range of concentrations required for spectrophotometric, fluorometric and kinetic studies (which corresponds to a molarity of 10^{-7} to 10^{-6} M for DES), a 98/2, volume/volume ratio of water/ethanol has been adopted and worked fine. This ratio of the water/ethanol medium has been used throughout the following chapter.

DES solutions were generally irradiated at 285 nm as the longest wavelength transition that belongs to both media (pure ethanol, and water/ethanol, v/v, 98/2). In section 3.6, a study of the wavelength effect is described.

3.4 Photodegradation properties of DES

3.4.1 Spectrophotometric study

A transformation of DES is observed when DES solutions are exposed to light over a period of time. As seen in Fig.3.2, before irradiation, DES has 2 characteristic maxima and a shoulder in the UV region. When DES is subjected to irradiation the shoulder (at 274 nm) develops into a well-defined peak, the maximum at 240 flattens out, in addition to a new peak formed in the visible region between 320 nm and 454 nm with a maximum absorbance at 403 nm (Fig.3.4). This new peak results in the formation of a yellow colouration within the solution. Both the change in colour and the considerable change in the electronic spectrum of the reactive medium are proof of the occurrence of a photoreaction.

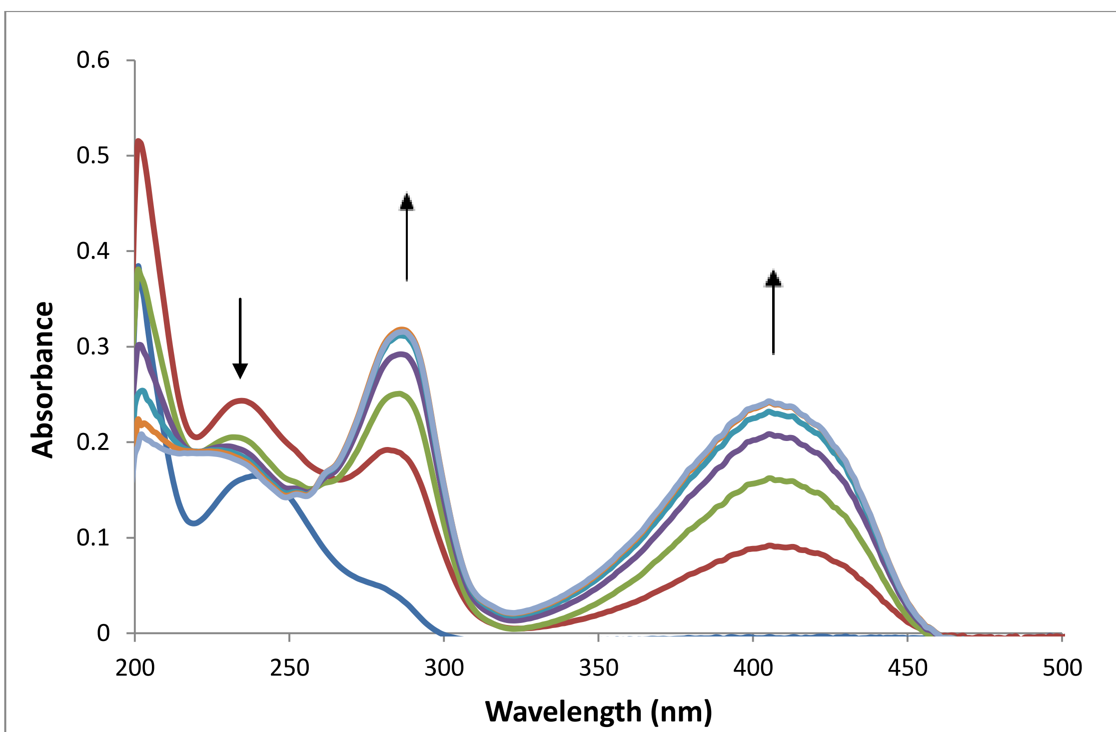


Fig.3.4 Evolution of UV/Vis spectra of DES (7.890 x 10^{-6}M) irradiated in pure ethanol at 285nm at 22◦C.

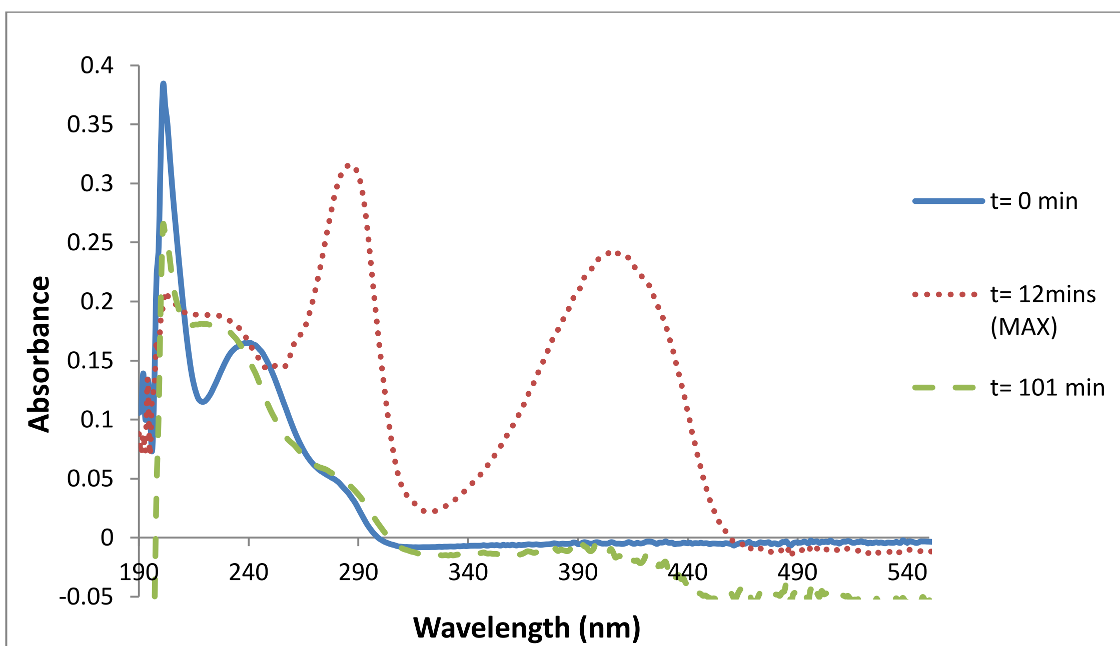


Fig.3.5 Comparison of the UV/Vis spectra of DES (7.890 x 10^{-6}M) irradiated in ethanol at 285nm at 22◦C at different times: initial time (—), after 12 min irradiation (- - -) and after 100 min (…).

3.4.2 Fluorometric study

An example of the fluorescence spectrum evolution of DES solution irradiated at 285 nm is shown in Fig.3.6. At initial time, the fluorescence of the compound is very low. As the irradiation time increases, a distinctive peak appears at around 362 nm. The intensity of this fluorescence band reaches relatively high values. This high fluorescence quantum yield is also a proof of DES photodegradation occurring within the sample.

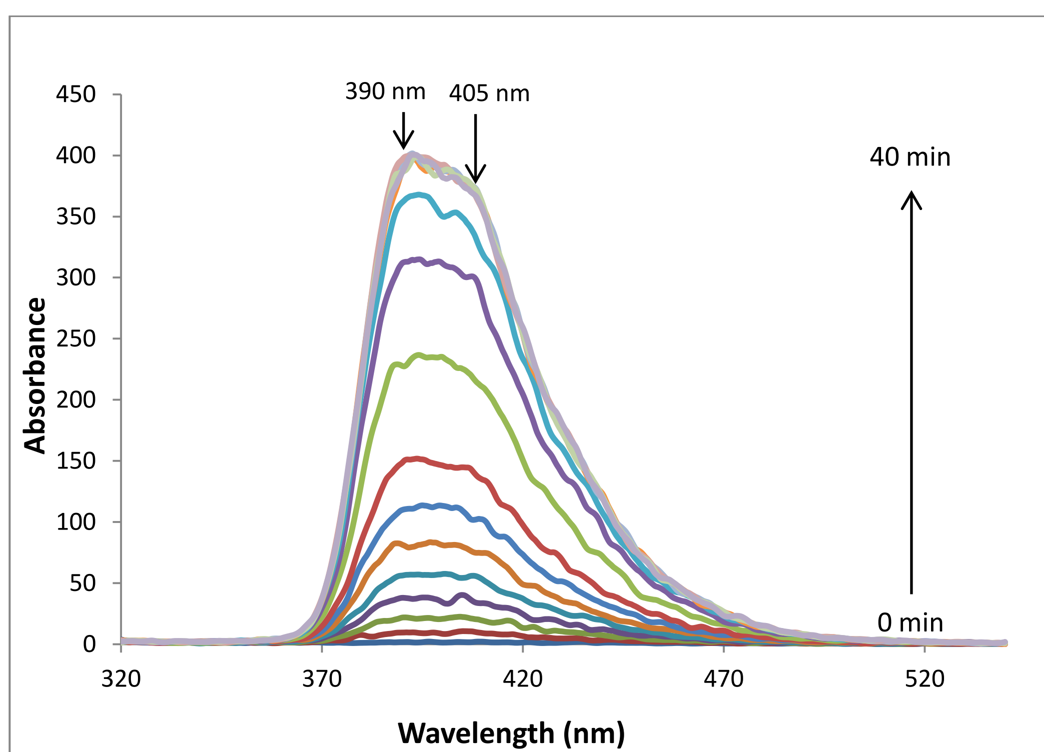


Fig.3.6 Fluorescence evolution spectra of DES compound (7.890 x 10^{-6}M) irradiated in water/ethanol (v/v,98/2) for 40 min at 285nm at 22◦C to its max intensity (filter on).

3.5 Spectral characteristics and photodegradation of DES in different media

3.5.1 UV/Vis spectra of DES

Comparison of the compound spectra in different media show small differences at initial time Fig.3.7.

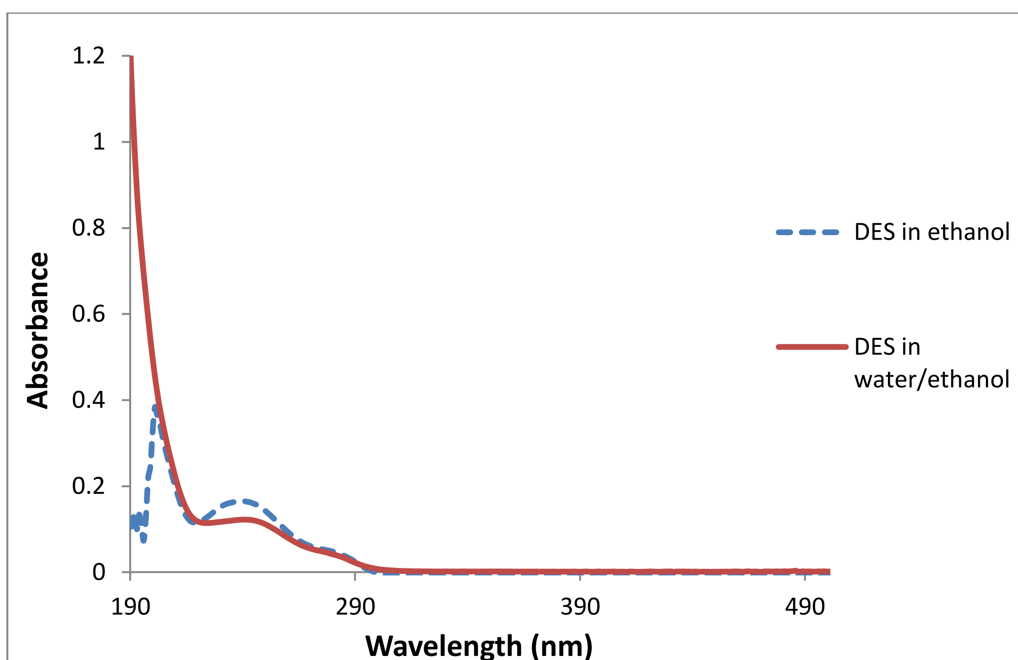


Fig.3.7 UV/Vis spectrum of DES (7.890 x 10^{-6}M) at t=0 in ethanol and water/ethanol (v/v 98/2) in the dark.

3.5.2 Thermal stability

Thermal stability studies of DES were performed in both media. As well as Fig.3.3, the Fig.3.8 shows no changes in the DES spectra under any selected temperatures (25 – 37°C) proving the compound is thermally stable in water/ethanol medium.

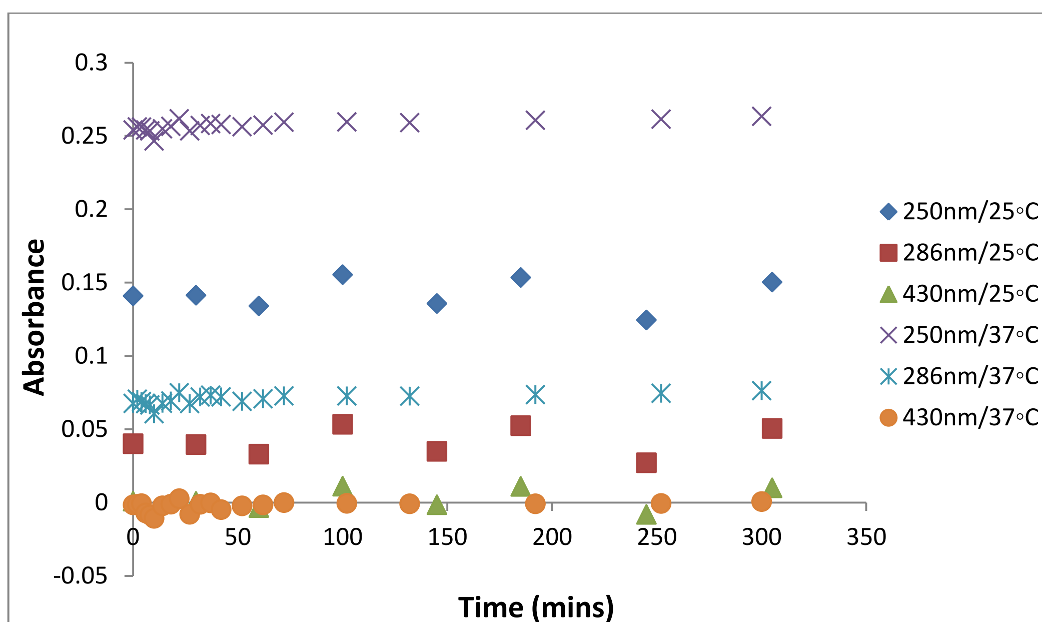


Fig.3.8 Traces of a thermal study of DES (7.890 x 10^{-6}M) in water/ethanol (v/v, 98/2) at several temperatures.

3.5.3 Photodegradation of DES in different media

3.5.3.1 Spectra after irradiation

When DES is subjected to irradiation the spectra for both media are significantly different from one another. When DES is irradiated in ethanol (Fig.3.4) the maxima becomes a

shoulder and an isosbestic point begins to form at 215 nm. The shoulder then forms into a peak. As the irradiation continues a new peak begins to form between 320 nm and 460 nm and becomes very intense.

The behaviour and shape of DES in water/ethanol (Fig.3.9) is unlike DES in pure ethanol. The absorbance band of DES that is situated below 210 nm decreases whereas the rest of the spectrum beyond the isosbestic point formed at around 211 nm, increases in intensity. The evolution continues until very distinctive peaks are formed. The shoulder at 280 nm also becomes a peak whilst a new peak appears in the visible region.

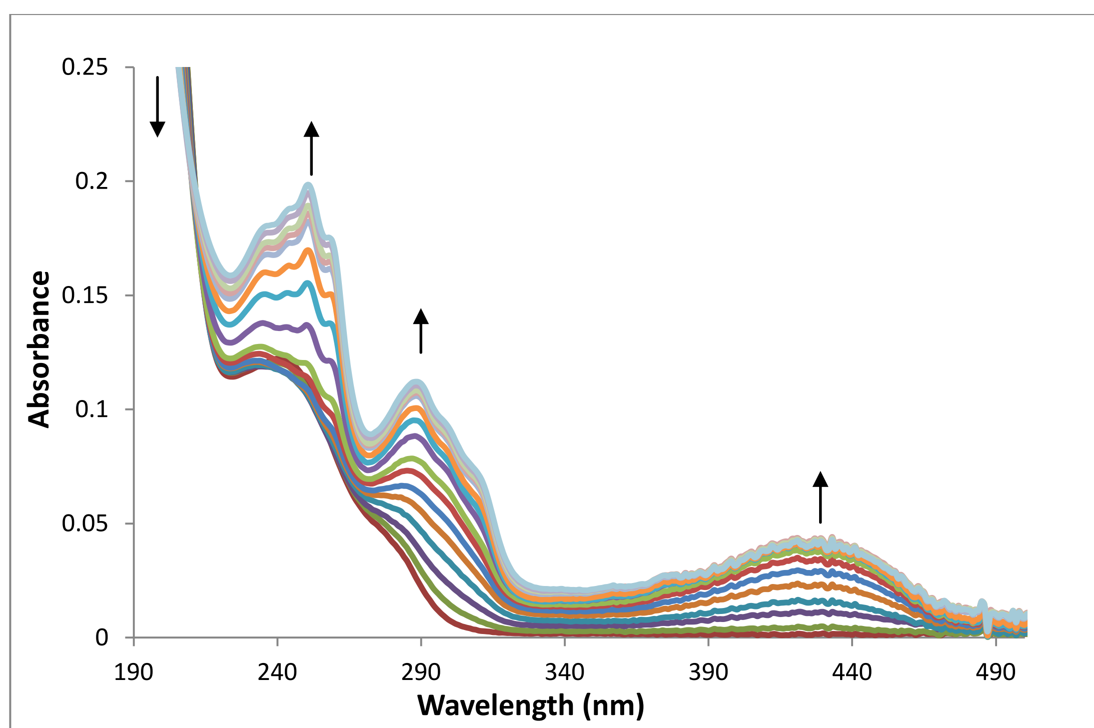


Fig.3.9 Evolution of UV/Vis spectra of DES (7.890 x 10^{-6}M) irradiated in water/ethanol (v/v,98/2) at 285nm at 22◦C.

By comparing the spectra of DES in both media, a spectral shift can be observed (Fig.3.4 and Fig.3.9). This spectral shift is evident as the new peak formed in the visible has a max of 403 nm in ethanol however in water/ethanol the max of this peak sits at 426 nm.

Although this entirely new peak forms in both media its intensity is much greater in pure ethanol than in the water/ethanol medium (Table 1). The yellow coloration is also more intense. This is proof of a new compound forming in the solution. The shapes of the peaks in Fig.3.9 start off smooth and over time become pointed and more defined.

<u>Table 1: Comparison of peak maxima of DES in ethanol and DES in water/ethanol</u>

Ethanol (after 12 minutes of irradiation)			Water/Ethanol, v/v, 98/2 (after of irradiation 40 minutes)		
Max wavelength and (shoulders)					
202 nm (226 nm) 285 nm 403 nm			198 nm (232 nm) 247 nm (256) nm 285 nm (310 nm) 426 nm		
202 = 202/403	285 = 285/403	403 = 403/403	247 = 247/426	285 = 285/426	426 = 403/426
0.501	0.707	1	0.58	0.669	0.95

It is apparent that by comparing the ratios of the peaks formed in the UV in both media (Table 1) that the difference between the peak maxima in ethanol and the peak maxima in water/ethanol is very small.

3.5.3.2 Evolutions of DES spectra and traces with irradiation time

Comparisons of the spectra at intervals shows how the compound changes in both media at different times of irradiation (Fig.3.5 and Fig.3.10).

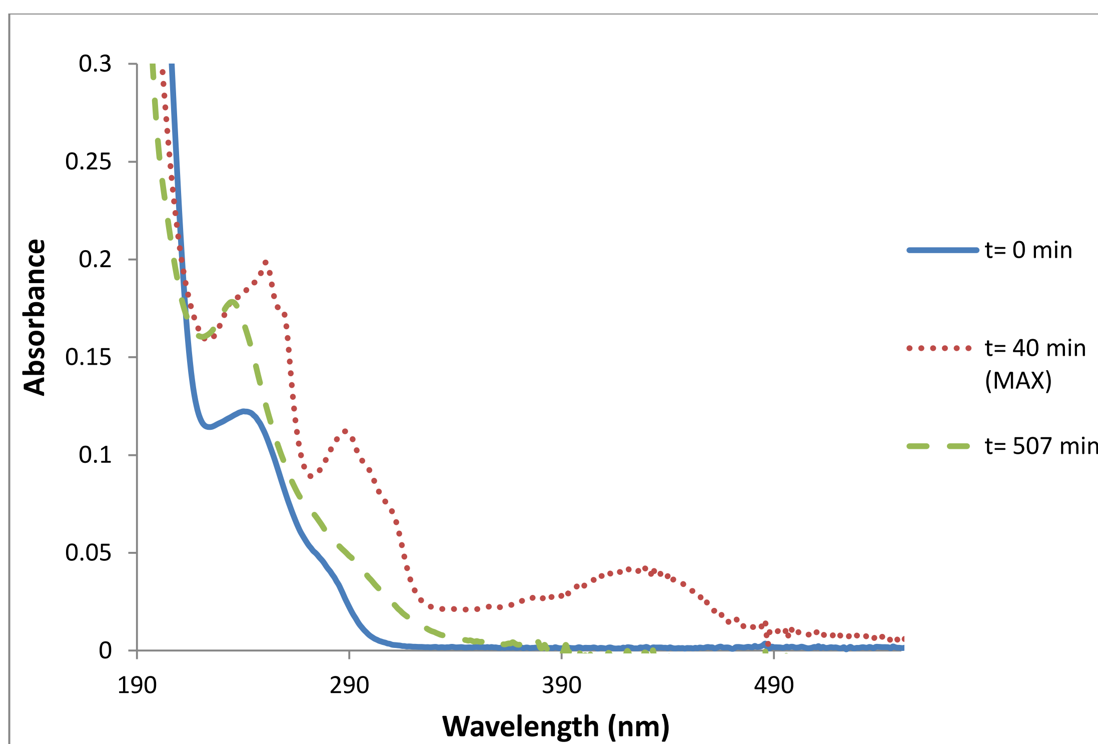


Fig.3.10 Comparison of the evolution of UV/Vis spectra of DES (7.890 x 10^{-6}M) irradiated in water/ethanol (v/v,98/2) at 285nm at 22◦C at different intervals for *ca*. 500 min.

A comparison of the spectra of DES in different media at their max absorbance [13][14] during irradiation can be seen in Fig.3.11.

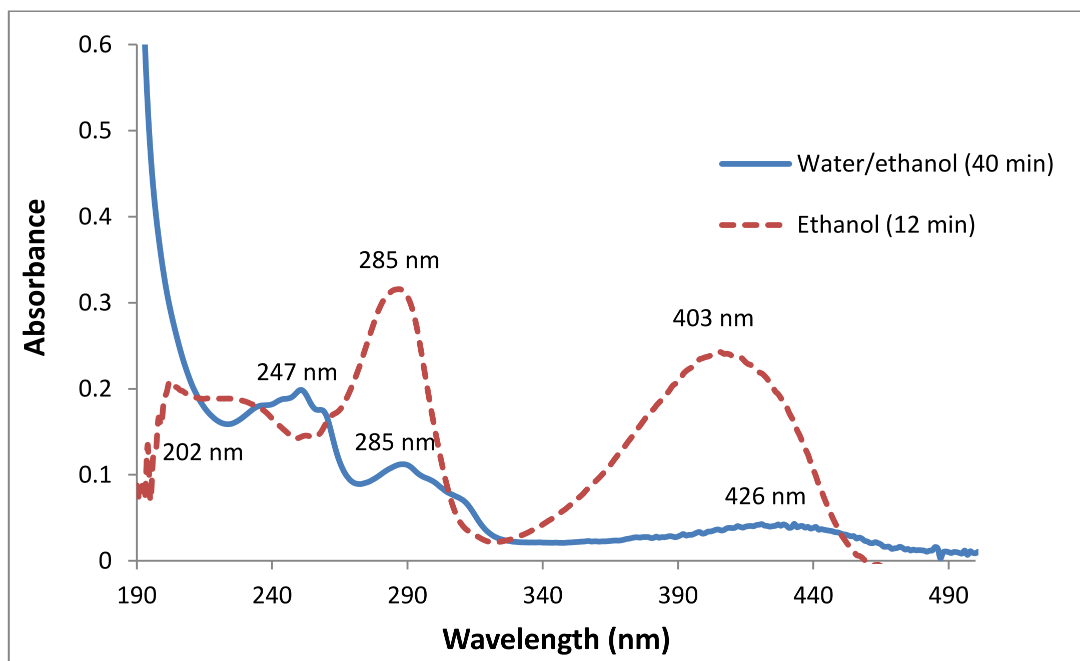


Fig.3.11 UV/Vis traces of DES (7.890 x 10^{-6}M) in both media at max absorbance at irradiated at 285 nm

UV/Vis traces of DES in both media throughout irradiation also show a difference in behaviour. DES in pure ethanol (Fig.3.12) increases to higher intensities than DES in water/ethanol (Fig.3.13). The traces in the higher observation wavelengths increases steadily to high intensities before peaking. The absorbances at the maxima increase sharply before starting a steady and slower decrease up to a lower absorbance similar to the starting one.

The traces of DES in water/ethanol develop slowly and at lower absorbances. The maxima of these traces were less defined than those recorded in ethanol.

In both media, the traces recorded at 239 nm begin by moving down in absorbance for a period 10 – 15 min, before starting up a slow curved-shape. Both traces form a lot slower than those at the higher wavelengths.

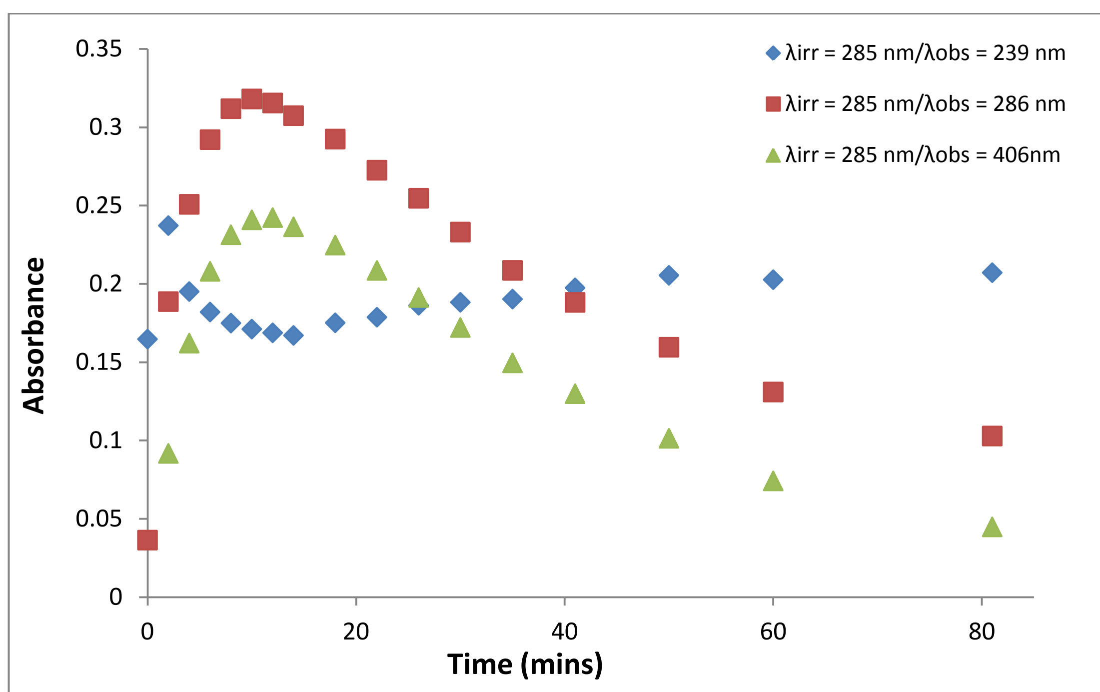


Fig.3.12 UV/Vis spectral evolution traces of DES (7.890 x 10^{-6}M) irradiated in ethanol at 285nm at 22◦C

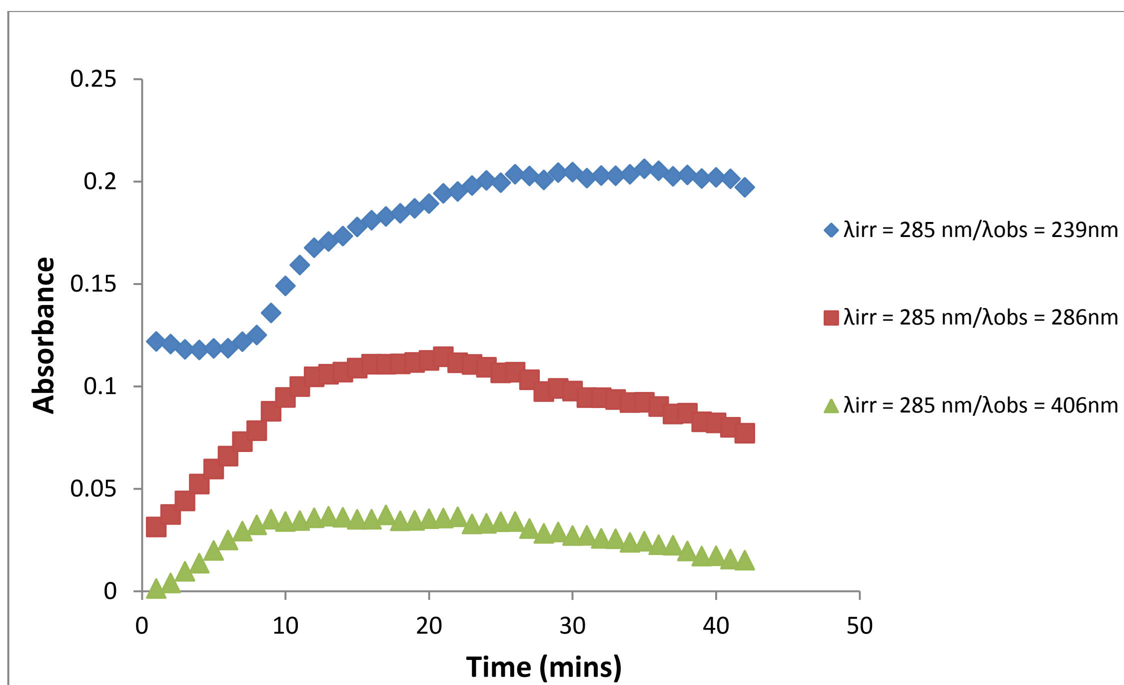


Fig.3.13 UV/Vis spectral evolution traces of DES (7.890 x 10^{-6}M) irradiated in water/ethanol (v/v, 98/2) at 285nm at 22◦C

Overall it is evident in the UV traces that the media has no effect on the speed of the photoreaction of the original compound (DES).

Another study of DES in both media can be seen in Fig.3.14. DES was irradiated in both media at interval value of 0.15 min for a period of 540 min (9 hours) and time drive traces of the photoreaction were recorded. Fluorescence studies Fig.3.6 and Fig.3.14 show the characteristics of DES during irradiation. DES fluorescence begins very low. It then continues to increase forming a peak at a higher intensity and then decreases back to where it started.

A comparison of the time drive traces formed shows that DES in both media begins at a very low intensity. DES in water/ethanol steadily increases to higher fluorescence intensity before peaking at a maximum intensity of 359 at approximately 190 min of irradiation. The trace then decreases very slowly reaching an intensity of 170 at 540 min. DES in ethanol

increases slowly and at much lower intensities reaching its maximum value of 71 after 251 min of irradiation.

The gradient of DES in water/ethanol is a lot steeper than DES in ethanol. The peak is a lot less defined than DES is in water/ethanol reaching a similar intensity it started with after an irradiation time of 468 min and reaching zero at 530 min.

It is interesting to notice that the intensities of the UV spectra of water/ethanol and ethanol are reversed when compared to those recorded in fluorescence.

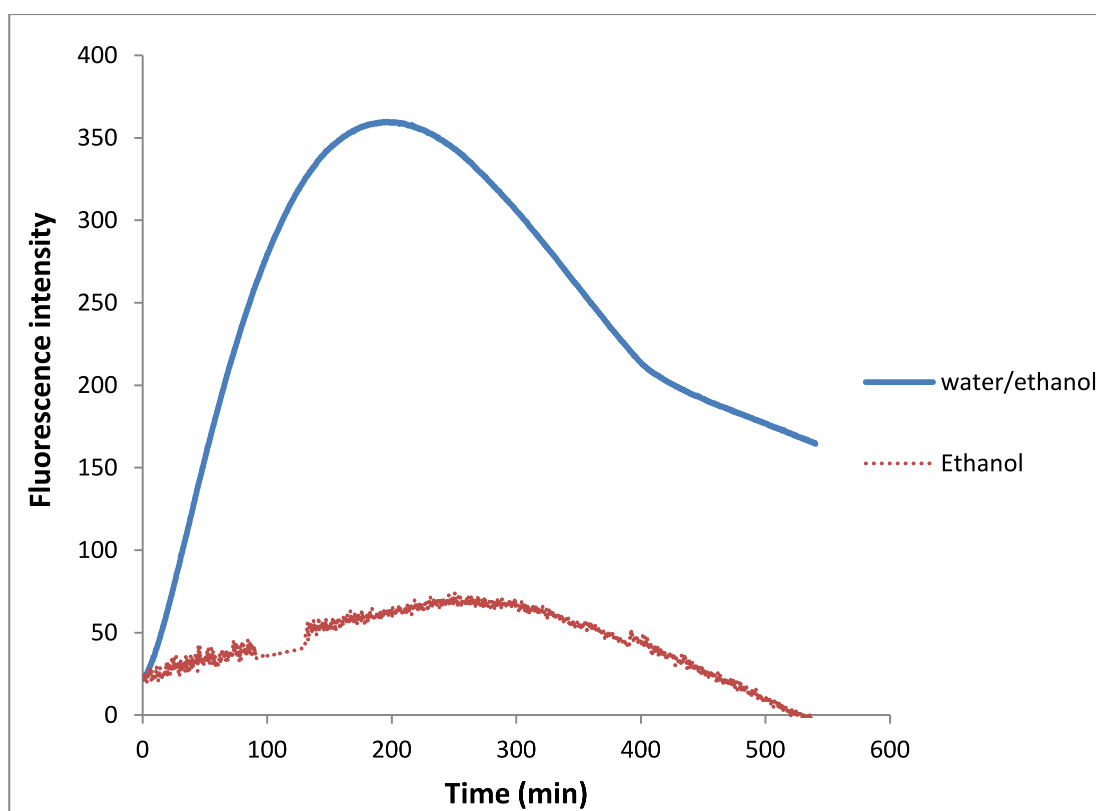


Fig.3.14 Fluorescence time drive traces of DES irradiated in water/ethanol and pure ethanol (1.003 x 10^{-7}).

3.5.3.3 Proposal of DES photodegradation mechanism

A photodegradation mechanism for the compound can be proposed as Scheme 1. At initial time the original compound fluorescence intensity is very low (**DES**). It begins a steady increase in intensity where trans DES transforms to the cis isomer (**A**). This steady increase becomes a rapid increase in intensity reaching a max intensity value of 400 at around 40 min of irradiation. This behaviour indicates clearly the formation of a new compound (labelled **B**). Once the max intensity is reached the intensity decreases in a much slower pace compared to the first regime and lasts approximately 470 min. The final fluorescence intensity recorded is as low as that observed for DES. This kinetic section is as well indicative of the formation of another compound (**C**). This is clearer in the UV traces at the lower wavelengths (Fig.3.13 at λobs 239 nm). Another possible mechanism in regards to Fig.3.15 can be suggested. The original compound **DES** (trans isomer) becomes the cis isomer (**A**) which then becomes a new compound (**B**) as the fluorescence intensity increases to its maximum. The decrease back to a very low intensity could be indicative of compound **B** returning to the **DES** compound as the fluorescence intensity is almost the same as the original compound before irradiation. However if the latter mechanism was true then one should expect that the reaction will reach an equilibrium so will not reach a value of zero meaning that the sample will contain some of **DES** and some of compound **B**. Compound **C** is therefore something different to **DES**. It can be determined that **DES, A** and **C** have a low fluorescence and one of the compounds (**B**) has a very high fluorescence.

It has been demonstrated that the reaction of DES shown within the steady state fluorescence study are purely photochemical (Fig.3.15).

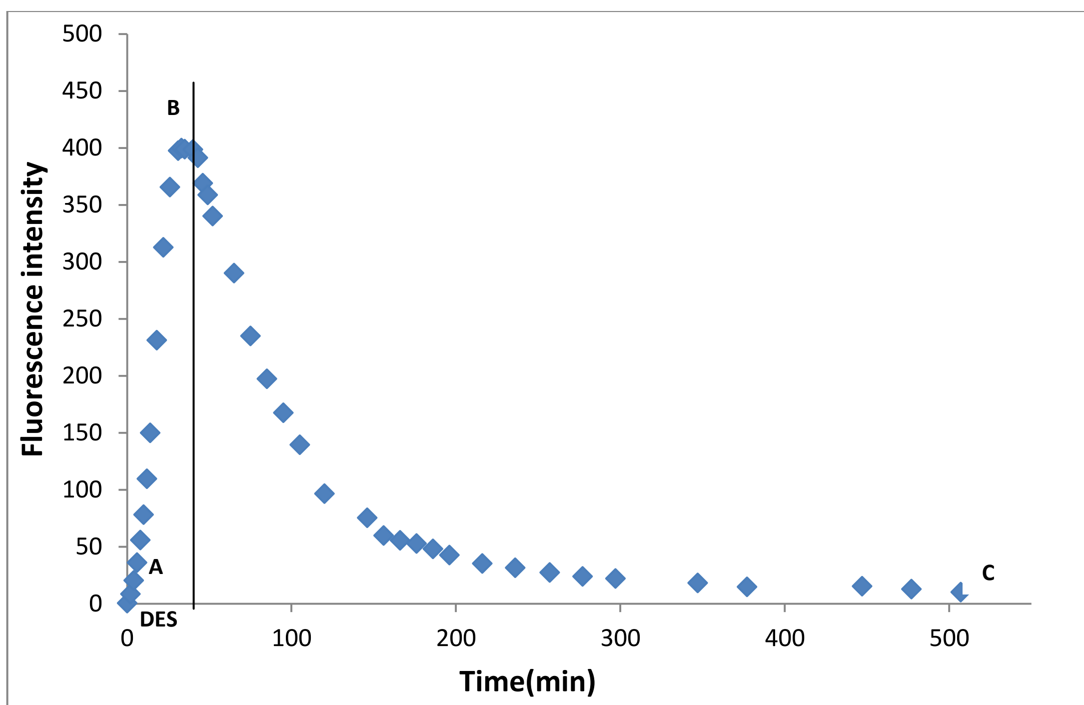


Fig.3.15 Fluorescence intensity evolution spectra of DES (7.890 x 10^{-6}M) irradiated at 285nm in water/ethanol (v/v,98/2) at 22◦C (filter on)

The compound has been proved to be thermally stable when the compound was irradiated to its maxima before irradiation was stopped (Fig.3.16). During irradiation, a steady increase in the fluorescence intensity can be observed. The irradiation was discontinued once the fluorescence maximum was reached (~ 40 min) and readings of the sample were continued at timed intervals. The point at which the irradiation was discontinued is indicated by an arrow on Fig.3.16. These findings imply that the photoproduct of DES is thermally stable [14].

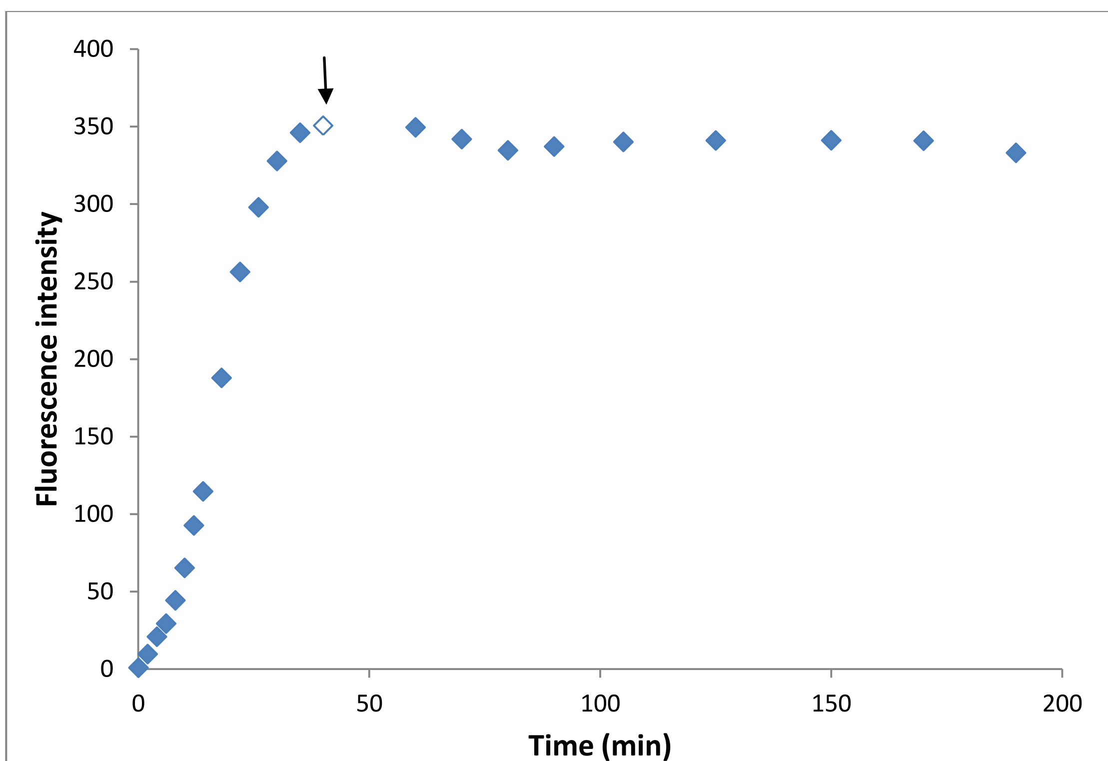


Fig.3.16 Fluorescence trace of a thermal study of DES (7.890 x 10^{-6}M) irradiated in water/ethanol (v/v 98/2) at 285nm for 40 min (filter on)

An additional experiment was performed to demonstrate the photochemical activity of the photoproduct. After irradiating DES with a 285 nm light beam for a period of 40 min, the time required for the spectra reach a maximum, the irradiation was stopped. Then the irradiation wavelength was changed to 430 nm, which correspond to the absorption of the photoproduct only (not DES or other possible photoproducts present in the medium, e.g. compound B). Then the reaction medium was subjected to irradiation with this light beam. The evolution of the spectra is given in Fig.3.17

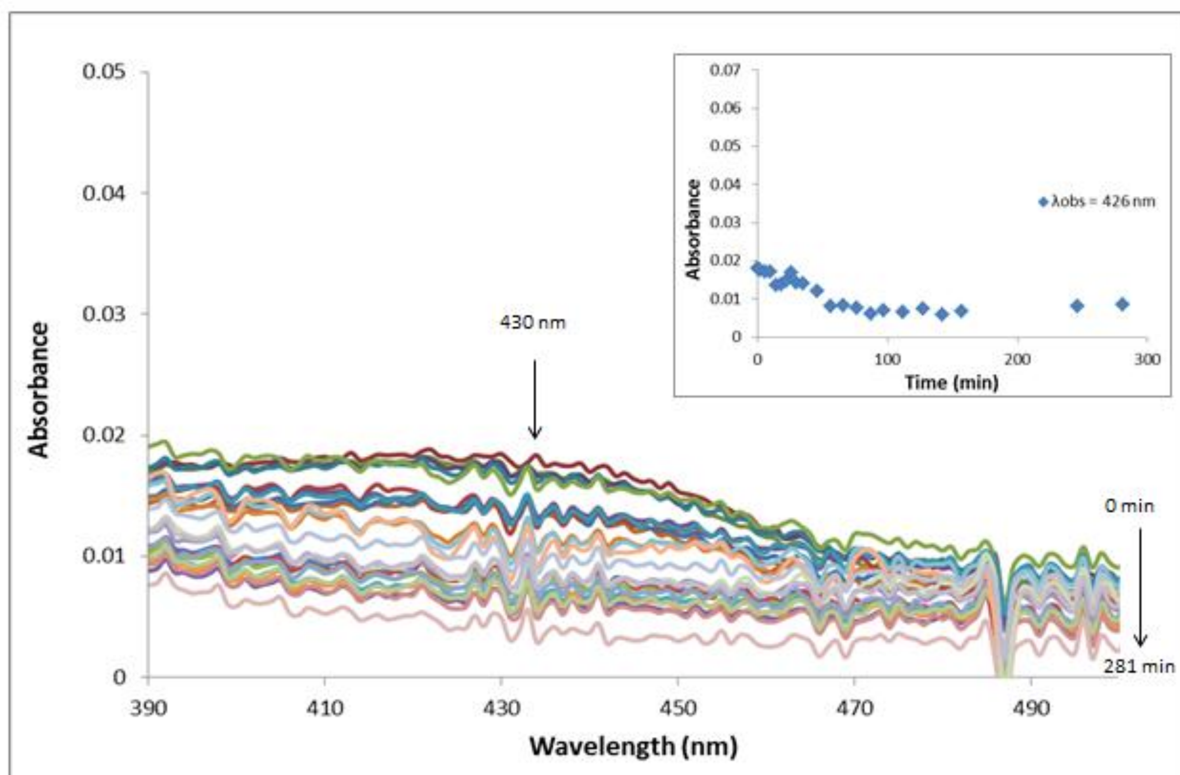
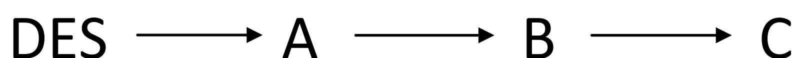


Fig.3.17 UV spectra evolution of continued irradiation (281 min) of DES photoproduct (7.890 x 10^{-6}M) in water/ethanol (v/v 98/2) at 430 nm at 22◦C

It is interesting to underline the fact that the degradation of DES is characterised by a change in colour from transparent to yellow at the maximum of the trace of compound B (*ca*.40 min, Fig.3.15). Continuing the irradiation further (40 – 400 min, Fig.3.15, where compound B is transformed into C) causes a bleaching of the solution to finally go back to a transparent colour. This colouration did not occur when DES was kept in the dark.

According to the kinetic and spectroscopic results gathered in the above sections, the following mechanism (involving the minimum number of species) can be proposed for DES photodegradation:



Scheme 1. DES photodegradation mechanism

The colour changes during the photoreaction of DES are confirmed by earlier studies carried out by Doyle et al [14]. They have isolated and identified the intermediate product as dihydrophenanthrene (DHP) which crystallises as a golden yellow colour. This is also confirmed by Doyle et al [15] in spectrophotometric studies carried out on Dienestrol another synthetic estrogen where a yellow colouration takes place during irradiation which is the result of an intermediate of DHP formation. The study found that further irradiation led to the loss of this colouration resulting in the formation of phenanthrenediol (PDOL).

The mechanism given in the literature [14] (Fig.18) demonstrates that the suggested mechanism in this study of DES is suitable. Irradiation of the trans-DES (1) results in cis-trans photoisomerisation where the trans-isomer becomes a cis-isomer (2). Ring closure of the cis-isomer results in the formation of Dihyrophenanthrenediol (DHP) (3). This immediately tautomerises to give the isolable dihydrophenanthrene diol (4) and is a process which takes place very quickly and cannot be identified in the spectra. This can result in either a ring opening reaction where the result is dienone (5) or phenanthrenediol (6). Process 5 is the result of irradiation and is a non coloured product whereas process 6 is coloured and is the result of the application of heat therefore the suggested possible mechanism would involve processes 1-5.

The different steps indentified for the photodegradation of DES (Fig.18) correspond closely to the proposed mechanism (scheme 1).

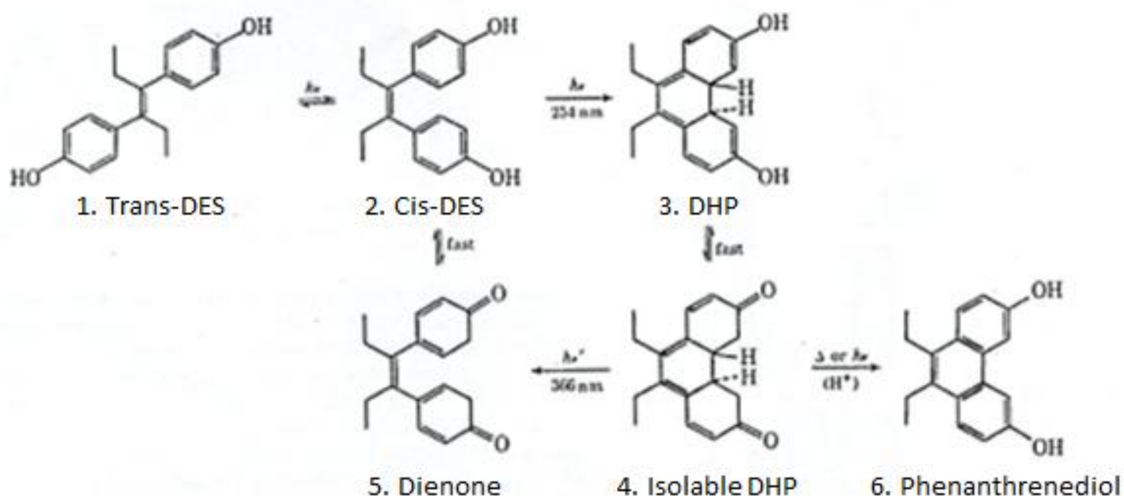


Fig.18 Suggested mechanism of DES photoreaction [14]

3.6 Effect of irradiation wavelength

An effect of irradiation was carried out by irradiating DES in both media at a range of different wavelengths. The irradiation wavelengths chosen for the study were based on the compounds characteristics. The specific wavelengths were chosen where DES absorbs in the spectrum including the maxima and any shoulders formed in the spectra as well as isosbestic points. An isosbestic point can be observed in the spectrum of DES in water/ethanol at 211nm (Fig.3.9). The maxima is formed around 247 nm [13] and the shoulder around 285nm therefore the study was carried out at these irradiation wavelengths. This method was used for both media. On comparison of the fluorescence intensity traces in Fig.3.19 it can be determined that the observed trace for DES photoreaction in water/ethanol is much slower at 211 nm than the rest of the wavelengths

studied. In fact, the observed rate of the reaction clearly increases with the irradiation wavelength which can be seen in table 2 where the initial velocity value increases as the wavelength increases. The initial velocity traces can be seen in Fig.3.20.

This was also found to be the case when DES was irradiated in pure ethanol. It can be established that the mechanism of the photodegradation of DES is the same however the wavelength has a big effect on the overall velocity of the reaction. Observations of the data suggest that the more the wavelength increases through the UV region the faster the reaction takes place. Overall 270nm and 285nm were found to show a higher velocity of degradation than the other wavelengths studied.

These findings clearly indicate that the effective wavelength range for the degradation of DES is situated between 250 and 290 nm. This means that DES is very sensitive to UVB and UVC [48].

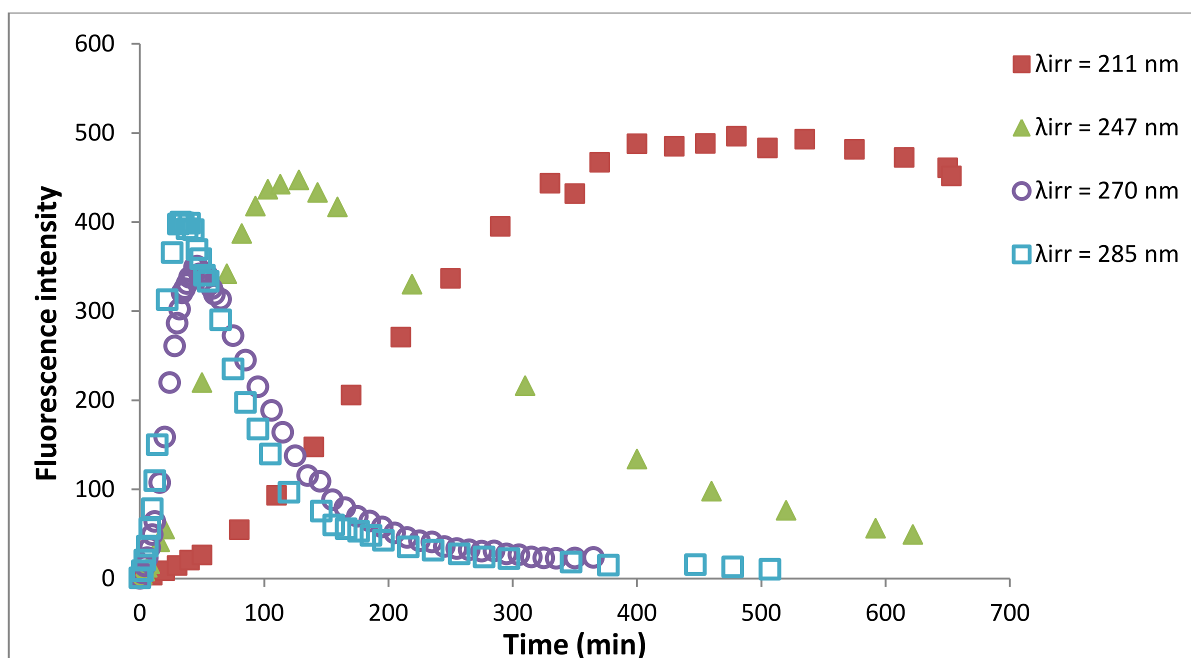


Fig 3.19 Comparison of the fluorescence intensity evolution over time of DES (7.890 x 10^{-6}M) in water/ethanol (98%:2%) irradiated at different wavelengths (filter on).

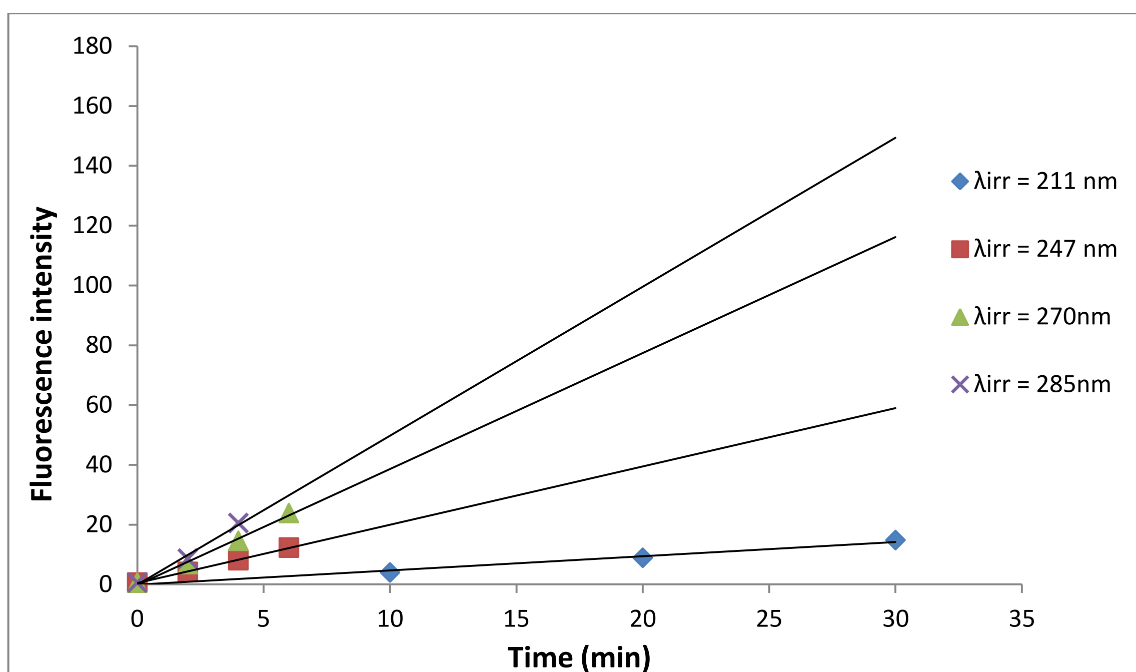


Fig.3.20 Fluorescence intensity comparison (filter on) of DES (7.890 x 10^{-6}M) in water/ethanol irradiated at different irradiation wavelengths

Table 2. Initial velocities of the fluorescence of DES in water/ethanol at different irradiation wavelengths

λirr (nm)	V_0	Linear equation (y=mx+c)	r^2
211	0.4754	y = 0.4754x - 0.066	0.986
247	1.9525	y = 1.9525x + 0.425	0.9986
270	3.8775	y = 3.8775x - 0.185	0.9921
285	4.98	y = 4.98x - 0.06	0.9874

The UV wavelength studies Fig.3.21 and Fig.3.23 also demonstrate that DES is very sensitive to this range of wavelengths this is particularly clear for the traces at 277 nm and 285 nm in ethanol (Fig.3.21) and at 270 nm and 285 nm in water/ethanol (Fig.3.23) in which DES degrades faster than at the lower wavelengths recorded. The traces reach a maximum

absorbance within a faster time period than the other traces. This can be seen in Fig.3.22 and Fig.3.24 in the initial velocity traces of DES in water/ethanol and DES in ethanol. A comparison of the initial velocity values for each wavelength are represented in table.4 for DES in ethanol and table.5 for DES in water/ethanol. As the wavelengths increase the value of the initial velocity increases.

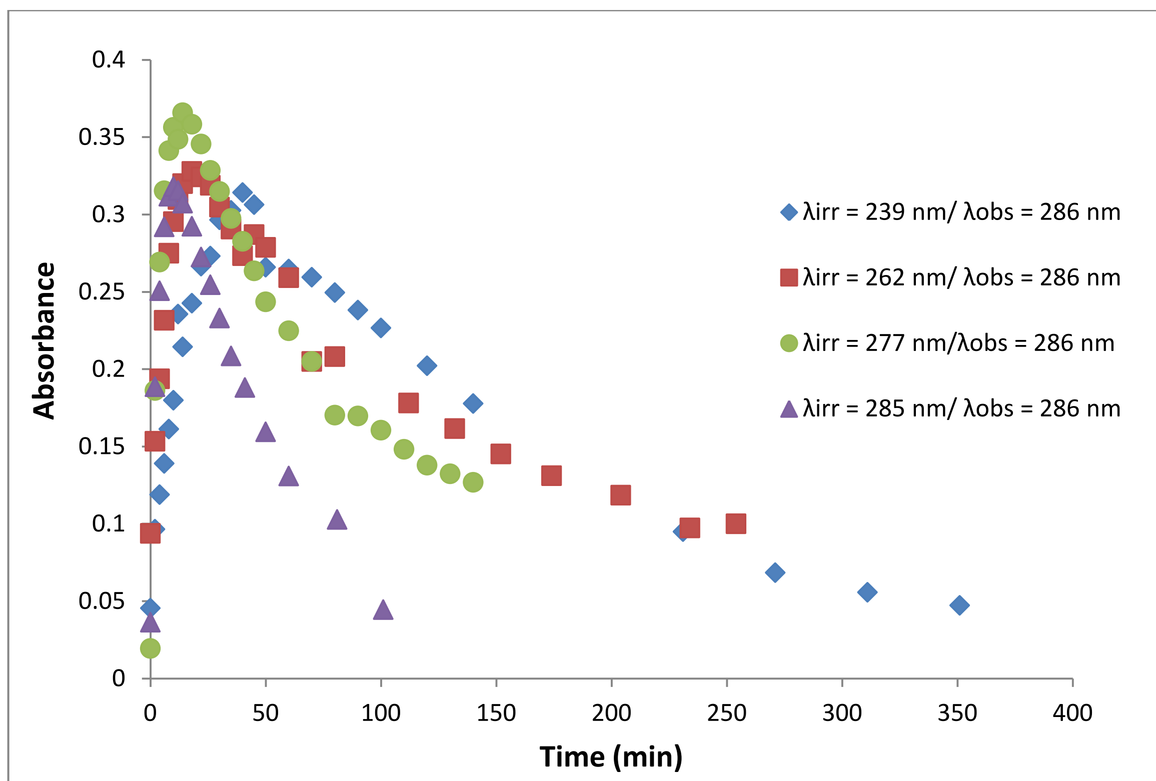


Fig 3.21 Comparison of the UV/Vis spectra evolution of DES (7.890 x 10^{-6}M) in ethanol irradiated at different wavelengths

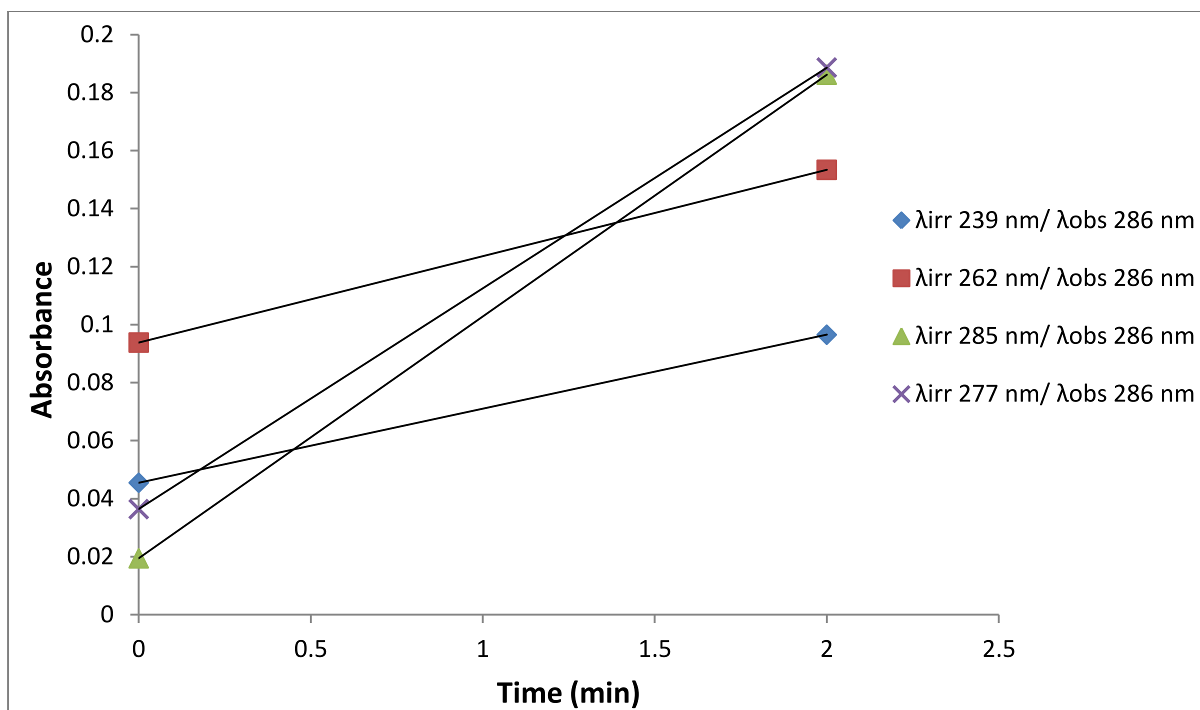


Fig.3.22 UV/Vis initial velocity comparison of DES in ethanol at different irradiation wavelengths

Table 4. Initial velocities of DES in ethanol at different irradiation wavelengths

λirr/λobs (nm)	**V_0 (Initial velocity)**	**Linear equation (y=mx+c)**	**r^2**
239/286	0.0255	y = 0.0255x + 0.0455	1
262/ 286	0.0298	y = 0.0298x + 0.0938	1
277/ 286	0.0761	y = 0.0761x + 0.0364	1
285/286	0.0834	y = 0.0834x + 0.0194	1

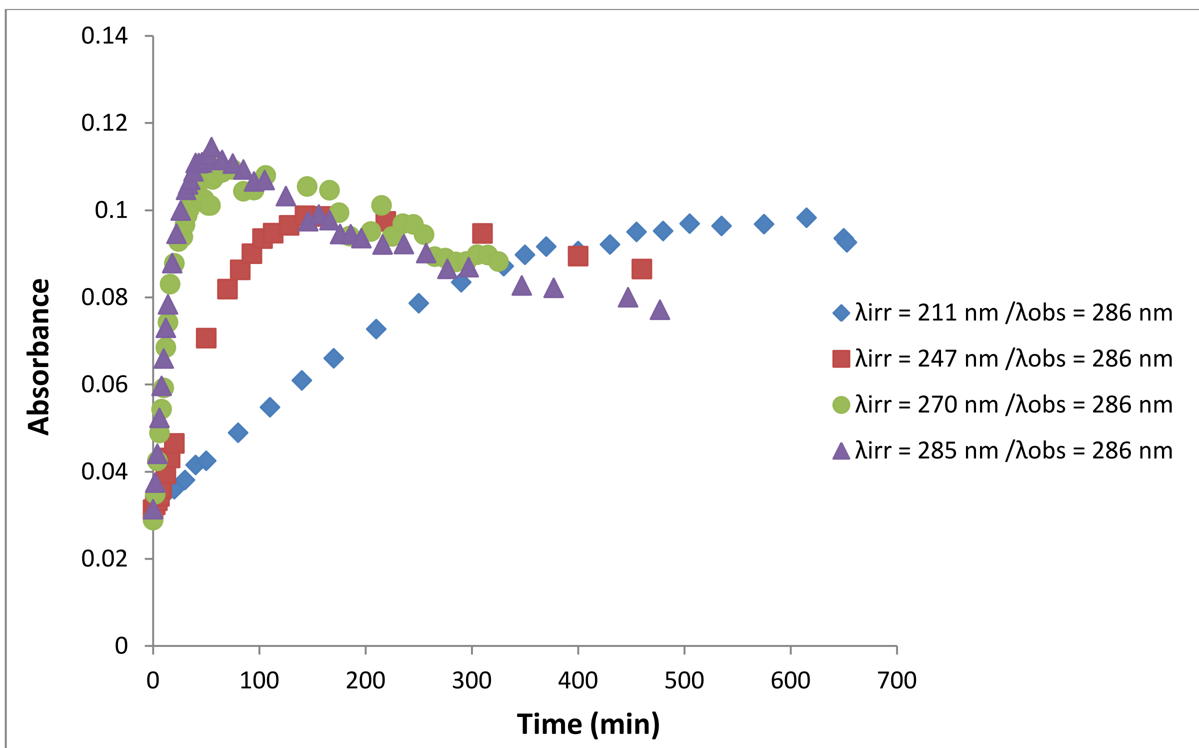


Fig 3.23 Comparison of the UV/Vis spectra evolution of DES (7.890 x 10^{-6}M) in Water/ethanol (92/2,v/v) irradiated at different wavelengths.

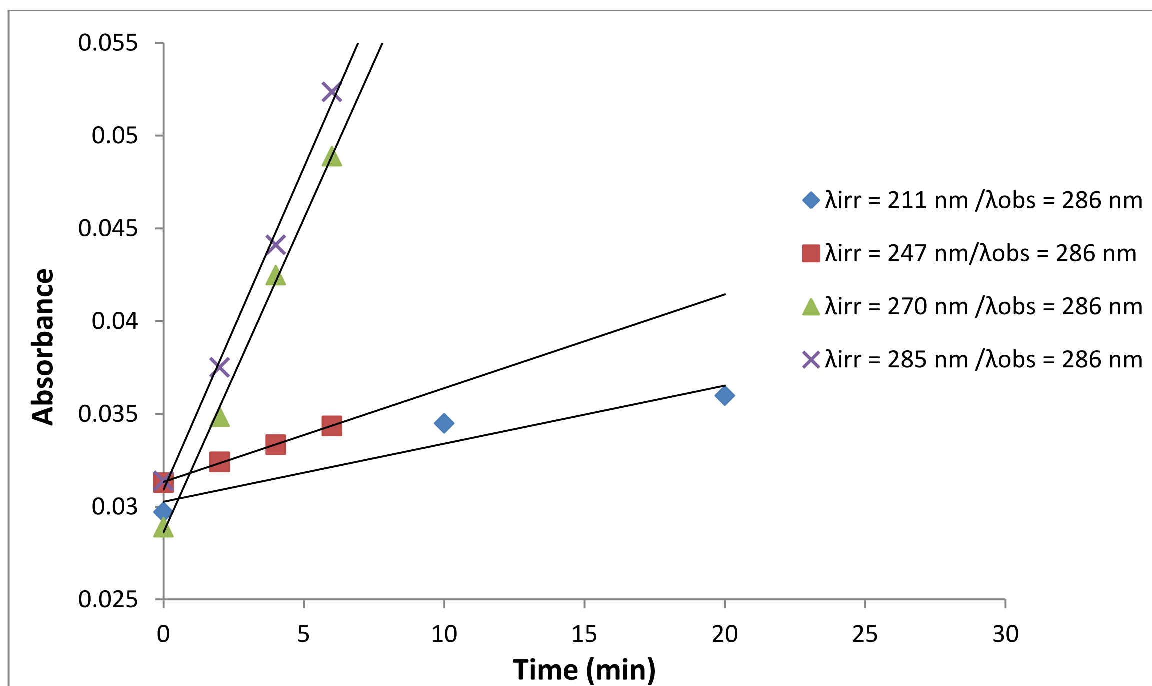


Fig.3.24 UV/Vis initial velocity comparison of DES (7.890 x 10^{-6}M) in water/ethanol at different irradiation wavelengths

Table 5: Initial velocities of DES in water/ethanol at different irradiation wavelengths

λirr/λobs (nm)	V_0 (Initial velocity)	Linear equation (y=mx+c)	r^2
211/286	0.0003	y = 0.0003x + 0.0303	0.916
247/286	0.0005	y = 0.0005x + 0.0313	0.9986
270/286	0.0034	y = 0.0034x + 0.0286	0.9979
285/286	0.0035	y = 0.0035x + 0.0309	0.9951

3.7 Liquid formulation of DES

It is important to determine if it is possible to formulate a safe liquid formulation of DES if the drug was to be provided for administration to a patient for a number of reasons including consideration for the percentage of ethanol within the formulation, consideration for the quantity of liquid and the patient involved i.e. children, the elderly and patients with swallowing difficulties [67]. Establishing the amount of water needed in order to complete a safe formulation of DES is important because the amount of liquid the drug is prepared in must be appropriate for the administration to a patient. If the drug can be prepared in a small amount of water, a few mL, this would be suitable. For patients including children and babies as well as those with swallowing difficulties small quantities can be administered by syringe or intravenously. However if the drug must be prepared in a large volume of water it would not be suitable for administration as the liquid would be to higher quantity for an individual to consume on each occasion the drug is administered.

It is particularly important to determine if a water/ethanol formulation can be prepared as the compound is dissolved in pure ethanol and is insoluble in water. It is not safe to give a patient a high percentage of ethanol and therefore must be prepared in water after the compound has been fully dissolved in ethanol.

3.7.1 Formulation of DES in water/ethanol

In order to determine the possibility of the formulation in water/ethanol, 5 mg of DES was dissolved in 1 mL of pure ethanol. 100µl (0.1 mL) of distilled water was added to the drug each time in order to determine the quantity of water needed before the compound became insoluble.

After 1300 µl (1.3 mL) of water was added to the solution it became cloudy resulting in the drug becoming insoluble however the solution remained clear at 1200 µL.

Therefore in this formulation 1 mL of ethanol and 1.2 mL of water are required. This represents a formulation with 45.45% ethanol. This amount is too high to be safely considered.

3.7.2 Solubility of CD/DES in water

CDs are generally used in pharmaceutical formulations to solubilise non water soluble drugs [24]. Several β-P-CD/DES mixtures were made at different concentrations/ratios. Because there is no indication of such ratios in the literature it is important to optimise the CD/DES ratio that satisfies the complete solubility in water.

Initially 20 mg β-P-CD in water was added to 4 mg DES in ethanol. In this case, a cloudy solution was formed when the components in the different media were added to each other. Therefore, it was evident at this stage that the concentration of β-P-CD was too low in order to completely solubilise the available amount of DES.

An 80 mg β-P-CD/ 4 mg DES was then made up initially forming a clear mixture when added to each other. The solvent was then evaporated using a rotatory evaporator. Once the solvent had been evaporated an amount of distilled water was added to the remaining dry sample and a clear solution was formed providing proof of a complex. Further analysis by ATR-FTIR determined that the concentration of β-P-CD was too high and therefore a 60 mg β-P-CD/4 mg DES was made up. The same procedure was carried out and proof of a complex was determined by adding distilled water to the β-P-CD/DES powder.

In order to optimise the formulation as well as follow the stated dosage (1 mg or 5 mg) 5 mg of DES was used to determine the least amount of β-P-CD needed to form an inclusion complex. A 30 mg β-P-CD/5 mg and a 40 mg β-CD-P/5 mg DES mixture were found to be too low in concentration and precipitated as a result. A 50 mg β-P-CD/5 mg DES mixture was made and evaporated. The addition of water to the powder formed a clear solution providing proof of a complex as well as the optimised ratio for the complex formulation (1/10, w/w, DES/β-P-CD).

The same procedures were carried out in order to determine a possible complex between the HP-β-CD and DES. A complex formed in the same conditions using the HP-β-CD. A complex was formed at an optimised ratio of 40 mg CD/5 mg drug.

3.7.3 Minimum water volume required to solubilise the CD/DES Formulations

5 mg of DES was dissolved in 3 mL ethanol and was placed in the ultrasonic bath for 5 min to ensure the drug was fully dissolved. 50 mg of β-P-CD was then dissolved in 5 mL water and also placed in the ultrasonic bath. Once the samples were fully dissolved the samples were added to each other and a clear solution was formed. The mixture was evaporated using a rotary evaporator. Once the sample was dried another 2 mL of water was added resulting in a clear solution and providing proof of a complex. This was then dried again.

In order to establish the formulation of DES in pure water the dry sample taken from the 50 mg β-P-CD/ 5 mg DES complex was weighed. 50 µL quantities of distilled water were then added to the dry sample until the complex was fully dissolved. This was carried out in order to determine the smallest amount of water needed to prepare the formulation.

28.5 mg of complex was formed between of 50 mg of β-P-CD and 5 mg of DES which required a minimum of 250 µL (0.25 mL) distilled water to be completely dissolved. This would correspond to 482 µL (0.482 mL) for the dose of DES (5 mg) usually used in tablet formulations, i.e. 5 mg of DES in 50 mg of β-P-CD.

14.6 mg of the complex of 40mg HP-β-CD/ 5mg DES required a minimum amount of 2000µl (2 mL) distilled water to dissolve. This would correspond to 6164 µL (6.164 mL) for the dose of DES (5 mg).

In conclusion, two water soluble formulations of DES have been established. They require small volumes of water (0.5 mL - 6 mL).

3.8 Characteristics of CD/DES complex

In order to attempt to characterise the complex and make a comparison between the samples of DES and the complex a number of methods were employed. This section investigates the findings of the complex characterisation.

3.8.1 ATR- FTIR spectra

The ATR-FTIR results can be observed in Fig.3.25 and Fig.3.26. The spectra in Fig.3.25 show the characteristics of DES, β-P-CD, a physical mixture of β-P-CD and DES as well as the complex. The spectra in Fig.3.26 show a comparison between the characteristics of DES, HP-β-CD, a physical mixture of HP-β-CD and DES and the HP-β-CD/DES complex. The spectra can be used to make a comparison between the samples to determine if the optimised complex can be characterised. This method is often used to successfully characterise CD/drug complexes [61][62].

The spectra of the pure β-P-CD and the physical mixture seem to have very similar spectral features (Fig.3.25). This clearly indicates that the spectrum of the physical mixture is dominated by the excess β-P-CD (where the characteristics of DES are missing).

However, the spectrum of the 50 mg/5 mg β-P-CD/DES formulation show some features that do not appear in any of the other spectra. Mainly, the two strong peaks located at 1022 and 1425 cm^{-1} can be interpreted as due to the complex formation.

It is also clear that some displacement of peaks has taken place. The peaks between 400cm^{-1} and 499cm^{-1} as well as a large peak at 1012 cm^{-1} and 1400 cm^{-1} may correspond to displaced DES peaks recorded between 819 cm^{-1} and 1585 cm^{-1}.

Bands shift and the intensity increases of peaks has been observed in the literature by Al-Marzouqi et al [1] and was interpreted as the presence of a complex.

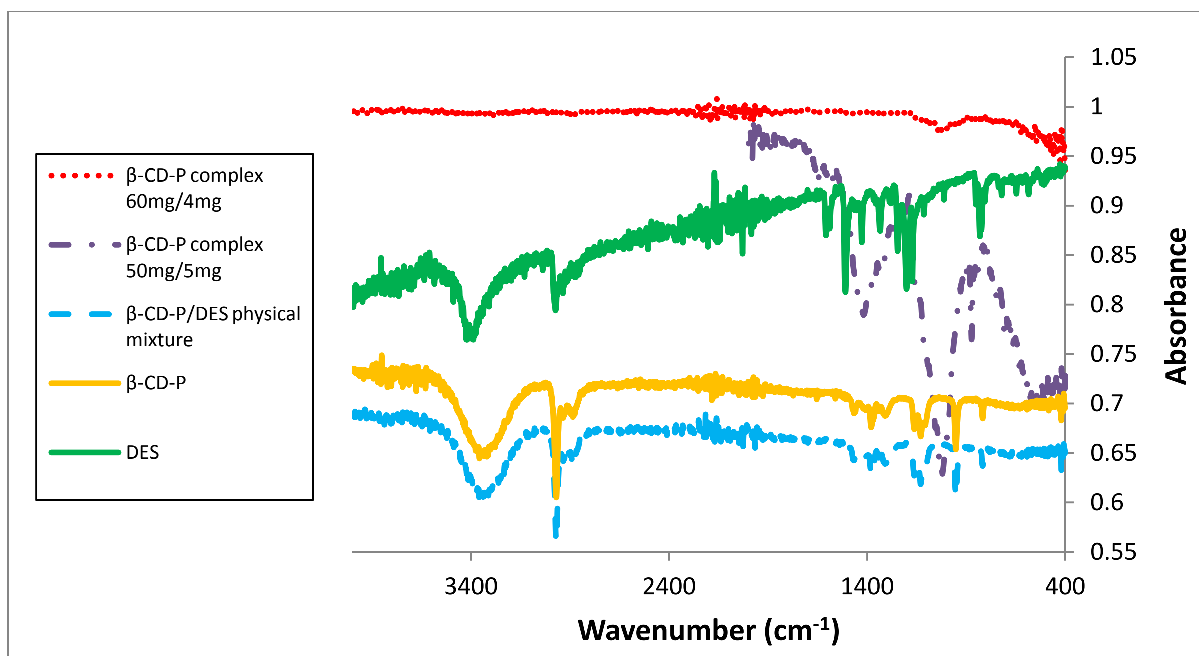


Fig.3.25 Comparison of ATR-FTIR spectra of DES and β-CD-P with the complex

A comparison of the ATR spectra of the HP-β-CD/DES complex, HP-β-CD/DES physical mixture, HP-β-CD and DES can be seen in Fig.3.26. The HP-β-CD/DES complex, HP-β-CD/DES physical mixture and HP-β-CD are all very similar which implies that the HP-β-CD/DES complex and the HP-β-CD/DES physical mixture are dominated by excess HP-β-CD therefore it is difficult to differentiate between the samples.

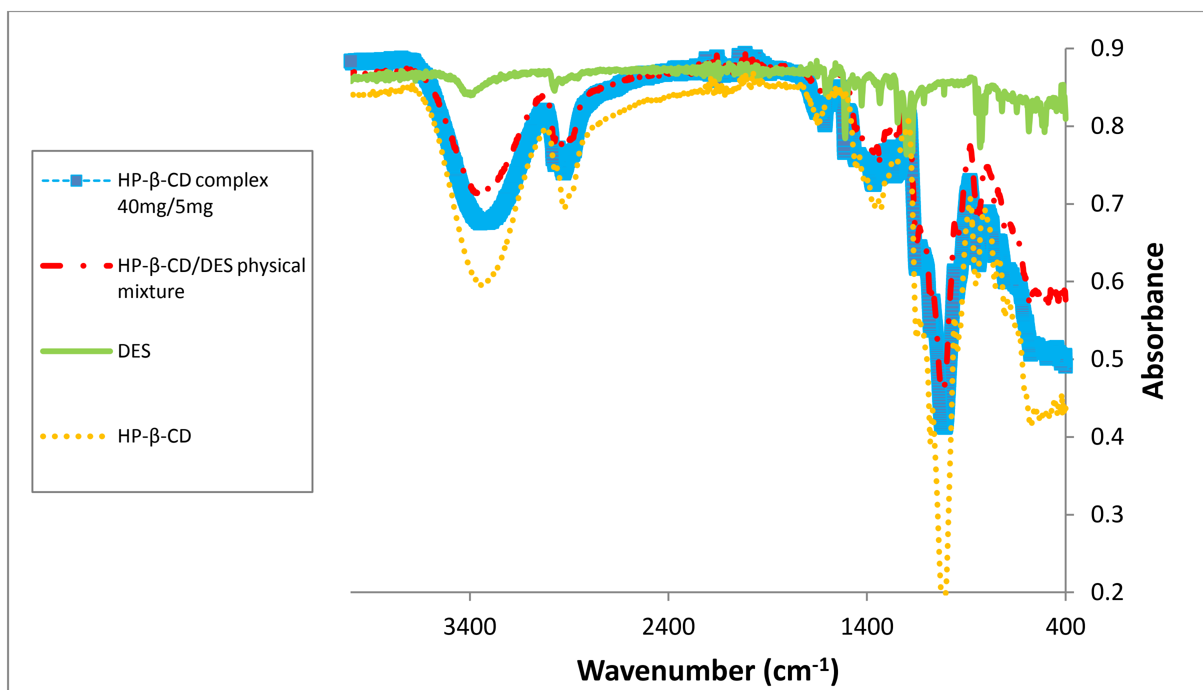


Fig.3.26 Comparison of ATR-FTIR spectra of DES and HP-β-CD with the complex

3.8.2 SEM image comparison

SEM is often used to characterise CD/drug complexes [63][64]. A SEM image comparison of the β-P-CD/DES complex (as for the formulation), β-P-CD, β-P-CD/DES physical mixture and DES samples can be seen in Fig.3.27 and Fig.3.28. The samples were taken from an NTS BSD detector and a VPSE G3 detector. The NTS BSD detector results in an image where the electrons are reflected from the sample when excited by a beam that hits the sample. The VPSE G3 detector picks up electrons that are emitted onto the detector from the sample when the beam excite the atoms within the sample.

On comparing the images A-H (Fig.3.27) it is clear that all four samples can be distinguished from one another. The β-P-CD/DES complex (**A**) appears 3D and uniform. The sample pieces

are fragmented and quite large in size and have a sharper and more defined appearance. The sample appears almost like cracked glass.

The β-P-CD (**B**) sample looks silky and flatter in appearance and the pieces are very close together and are built up in fine layers on top of one another. The sample appearance is smooth and uniform. The particles of the β-P-CD/DES (**C**) physical mixture particles appear 3D and fine in texture. The pieces are uniform in shape and size but are a lot smaller in size compared to the other three samples. The DES compound (**D**) forms a more spongy looking appearance and the particles are squashed closer together.

The characteristics of the samples images taken with the NTS BSD detector (E-H) are clearer and more defined. The pieces of the complex (**E**) are very large and sharp with much more defined shapes than the other samples. The cyclodextrin sample (**F**) fragments appear softer with smoother edges and are more compact. Although the physical mixture (**G**) and the DES compound (**H**) are in some ways more similar than the other samples the fragments of the physical mixture are overall a lot smaller whereas the majority of the fragments in the DES samples are larger.

On analysing the images in Fig.3.28 the differences are obvious. The shapes and sizes of the complex (**I**) pieces are uniform with sharp edges. The sample appears like small 3D cubes. The β-P-CD (**J**) appears very fine and leafy looking although with sharper edges. The cyclodextrin sample appears very flat rather than 3D and looks almost opaque. The physical mixture (**K**) appears 3D and looks like a group of fine, compact fibres with a soft texture. The DES (**L**) sample fragments appear chunky and give the impression of a soft, spongy texture which is consistent throughout the sample.

These findings show clearly that a significant modification of the solid has occurred for the complex compared to the other samples. This supports the conclusion about a possible complex formation between DES and CD.

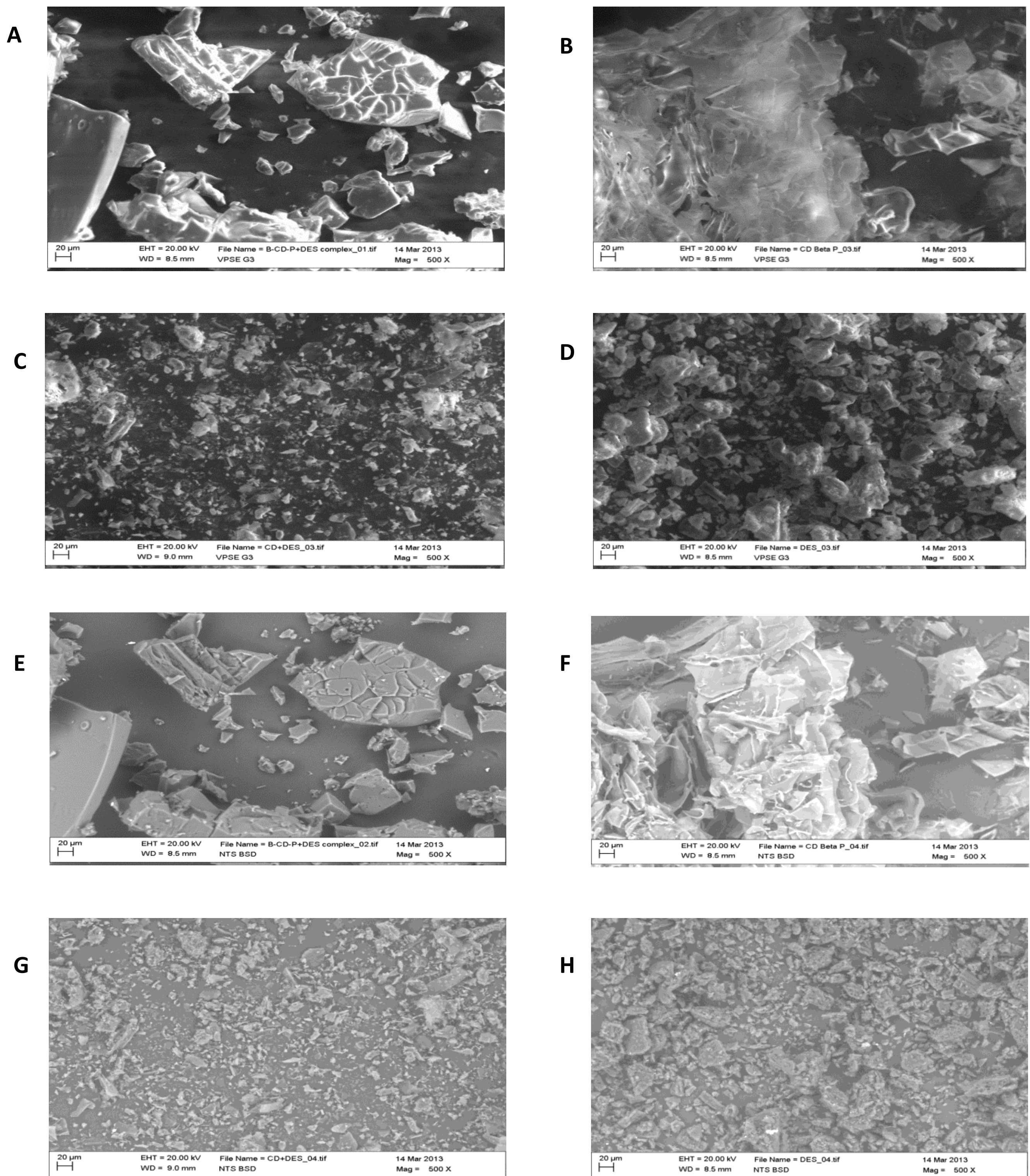


Fig.3.27 SEM images 500x magnification

Fig.3.27 Images **A-D** were taken using VPSE G3 detector at 500x magnification. **A**: β-P-CD/DES complex, **B**: β-P-CD, **C**: β-P-CD/DES physical mixture, **D**: DES compound

Images **E-H** taken using NTS BSD detector at 500x magnification. **E**: β-P-CD/DES complex, **F**: β-P-CD, **G**: β-P-CD/DES physical mixture, **H**: DES compound

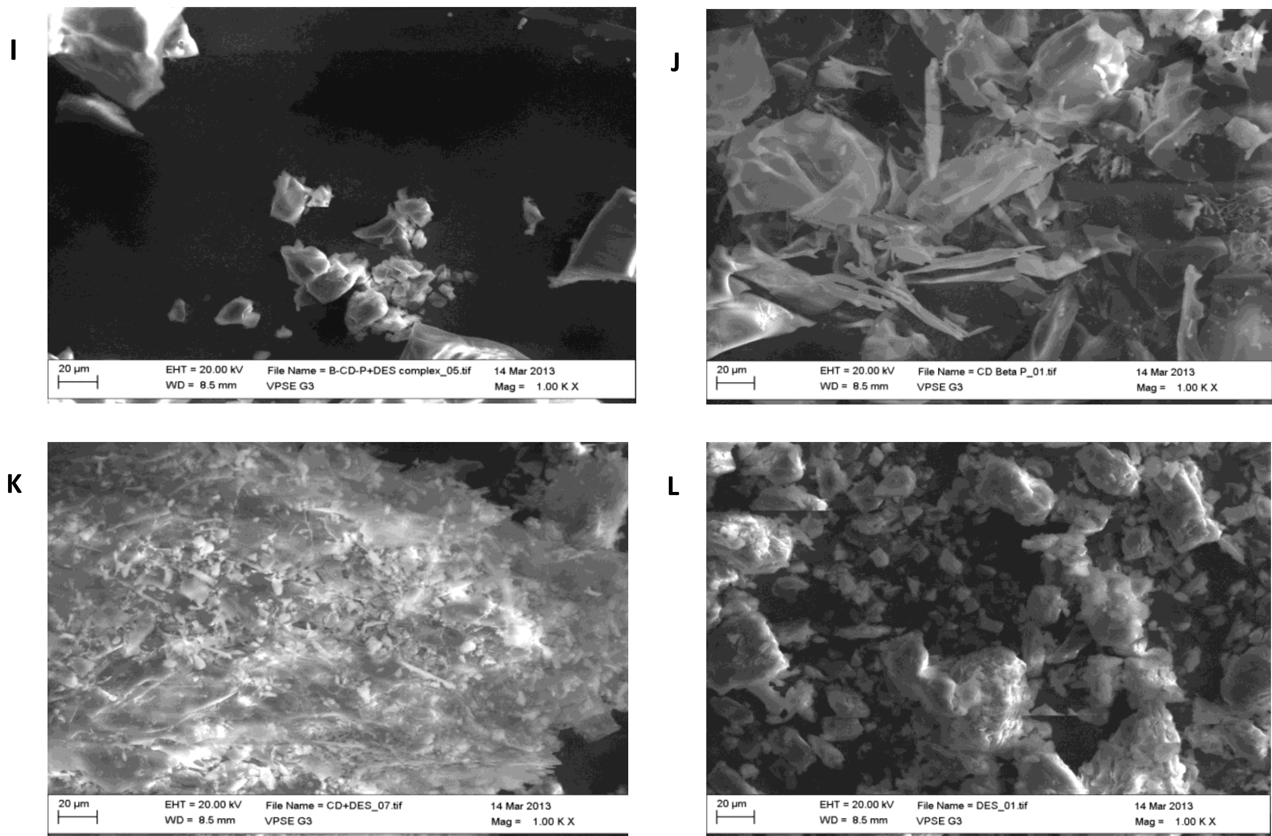


Fig.3.28 SEM images 1000x magnification

Fig.3.28 Images **I-L** taken using VPSE G3 detector at 1000x magnification. **I**: β-P-CD/DES complex, **J**: β-P-CD, **K**: β-P-CD/DES physical mixture, **L**: DES compound

3.8.3 Spectrophotometric characterisation of the complex

A comparison of the UV spectra of DES at initial time in the presence of CDs can be seen in Fig.3.29. DES in ethanol and DES in the presence of β-P-CD are similar with a peak at 203 (ethanol) and 197 (β-CD-P) which doesn't appear in any of the other spectra. This may be proof of a complex as ethanol and CD interior have similar polarities.

DES in water/ethanol and DES in HP-β-CD show very similar spectral characteristics below 250 nm; they both show a well-defined peak with a maximum at ~ 230 nm. However, this feature is not observed for the remaining media which are rather behaving as the water/ethanol solution with no maximum in that region (the peak may be below 200 nm). This observation may indicate that the HP-β-CD does form a complex with DES by offering it an environment similar to that of ethanol but at the same time this proves that if complexes are formed with the other cyclodextrins these complexes may well be different in structure to that of the complex formed with the HP-β-CD.

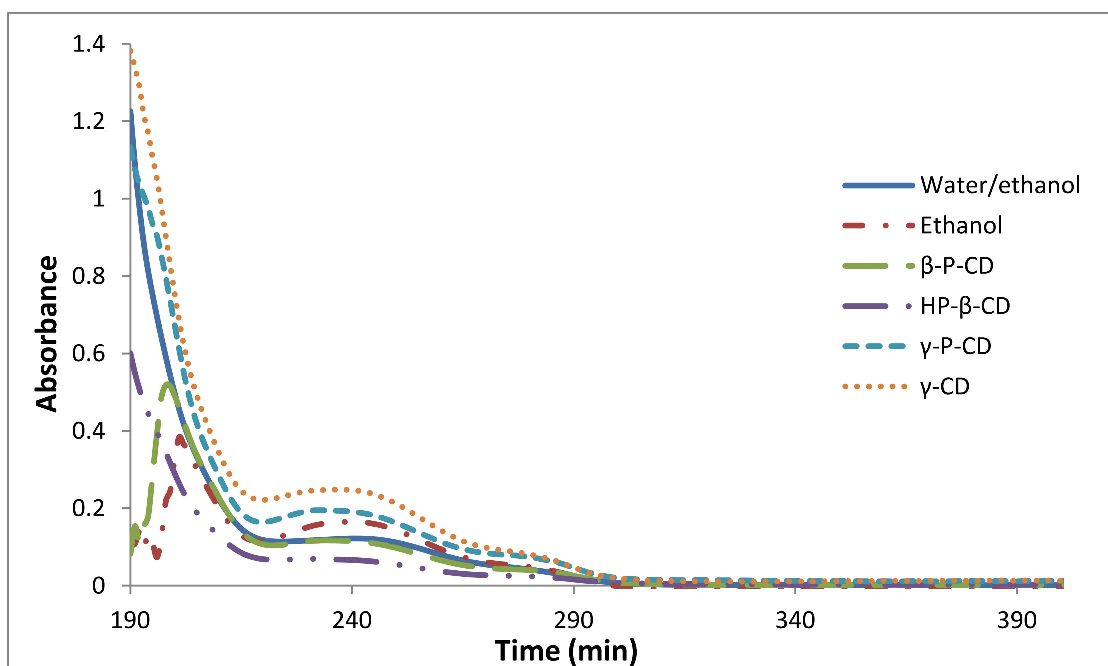


Fig.3.29 Overlay of UV spectra of DES (7.890 x 10^{-6}M) in different media at initial time

3.8.4 Fluorescence

A CD concentration effect on DES was carried out by adding increasing concentrations of CD to DES. The results are shown in Fig.3.30.

The results show that by increasing the concentration of CD the fluorescence intensity of DES will eventually plateaux. This is proof of a complex formation. This can also be seen in Maafi et al [29] where fluorescence has been used a means of identifying cyclodextrin complexes. A comparison of the CD ratio's that correspond to Fig.3.30 can be seen in table 6.

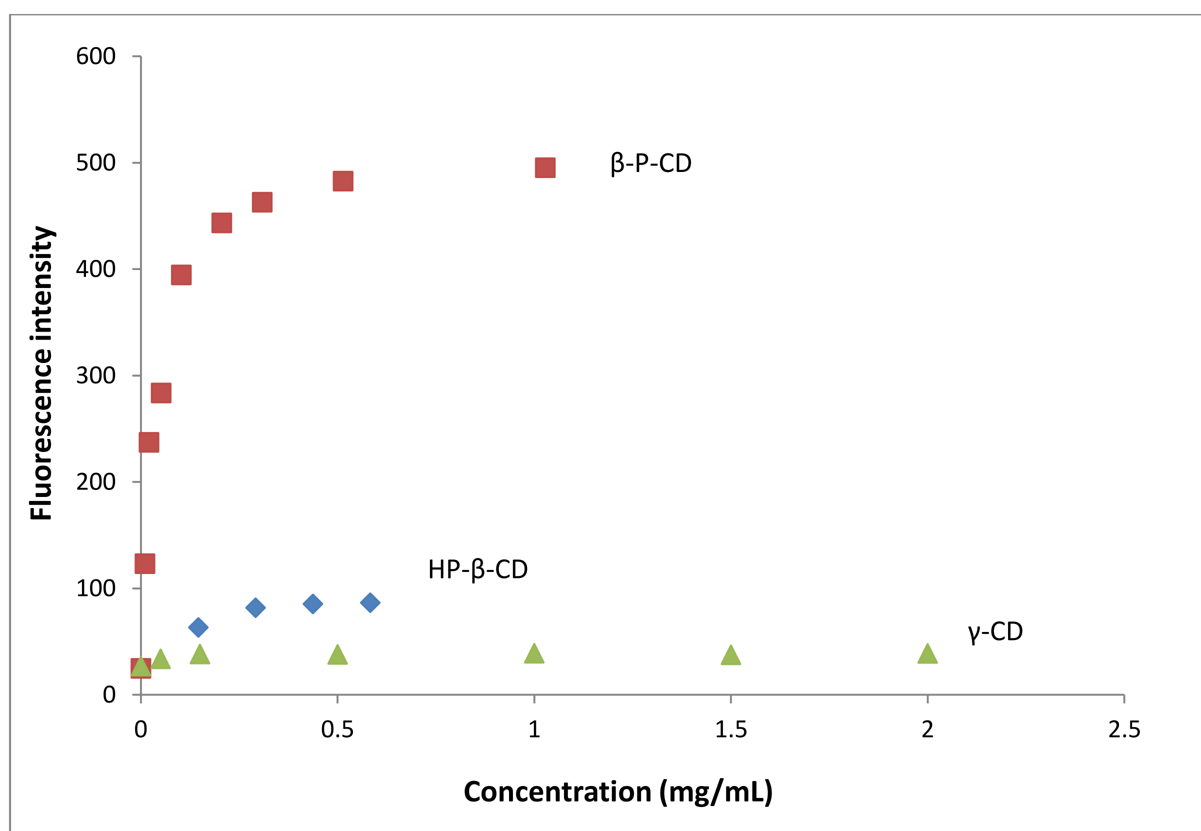


Fig.3.30 Emission fluorescence intensity comparison of the effect of concentration of CD in purified water on DES (7.890 x 10^{-6}M)

Table 6. CD ratio comparison using max fluorescence intensity

	β-P-CD	HP-β-CD	γ-CD
Fluorescence intensity (AU)	495.38	89.07	38.91
Ratio F(β-P-CD)/ F(CD)	1	5.56	12.73

Section 3.8 provides proof of a complex formation in solution. The UV traces provide very simple proof of a possible complex and the ATR-FTIR and fluorescence results are further, more substantial proof of a complex. The SEM is not 100% solid proof however the images show some clear, defined differences between the complex and the other samples.

Nunez Delicado et al [33] were able to form complexes with DES and a number of cyclodextrins including HP-β-CD and β-P-CD which confirms the complexation of HP-β-CD with DES in this study.

3.9 Degradation of DES in the presence of CD

3.9.1 Thermal stability

A number of Cyclodextrins were studied at varying temperatures and results were studied at several observational wavelengths taken from the UV spectra obtained (Fig.3.31). The study shows that there is very little or no change in absorbance for all results obtained demonstrating that temperature has no effect on the use of Cyclodextrins with the DES compound.

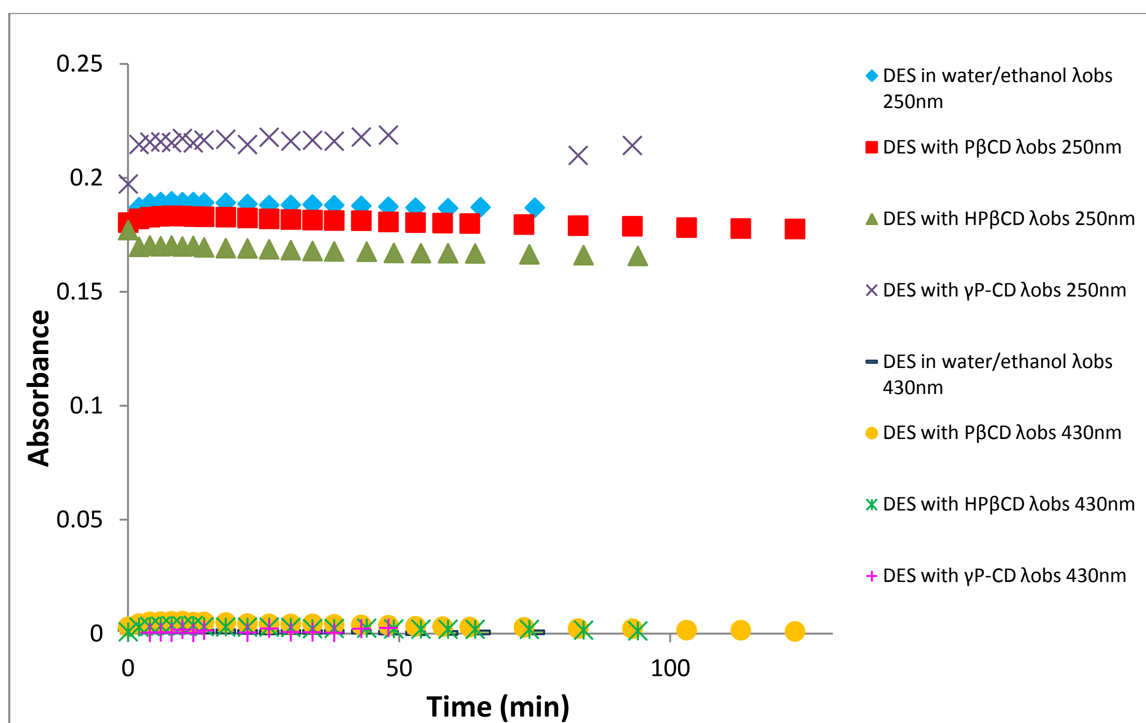


Fig.3.31 Comparison of traces produced in the thermal stability study of DES (7.890×10^{-6}M) at 37°C in water/ethanol (v/v, 98/2) and with cyclodextrins

3.9.2 Traces of DES by spectrophotometry

The UV traces of DES in the presence of cyclodextrins were recorded. A comparison of DES in water/ethanol and in the presence of the cyclodextrins monomers can be seen in Fig.3.32. A comparison with the cyclodextrins polymers can be seen in Fig.3.33.

It can be determined from both figures that DES in the presence of CDs continues its characteristic behaviour when in water/ethanol media, however the CDs tested have clearly had an effect on the photodegradation of DES. A comparison of the UV trace of DES in water/ethanol media in both figures clearly shows the traces of DES in the presence of the CD's reach their max absorbance later than DES in water/ethanol.

The effect of the CDs is shown in more detail in the fluorescence time drive traces recorded in section 3.9.3.

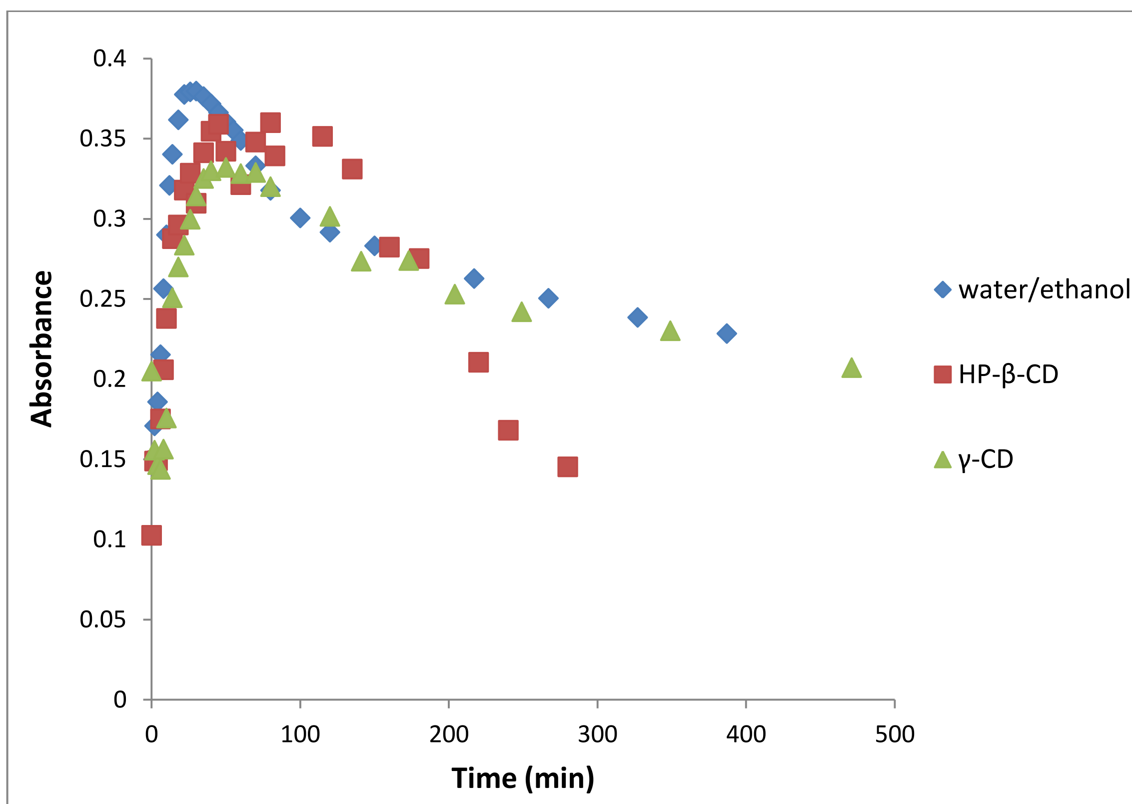


Fig.3.32 Comparison UV/Vis traces of DES (7.890 x 10^{-6} M) in the presence of the CD monomers

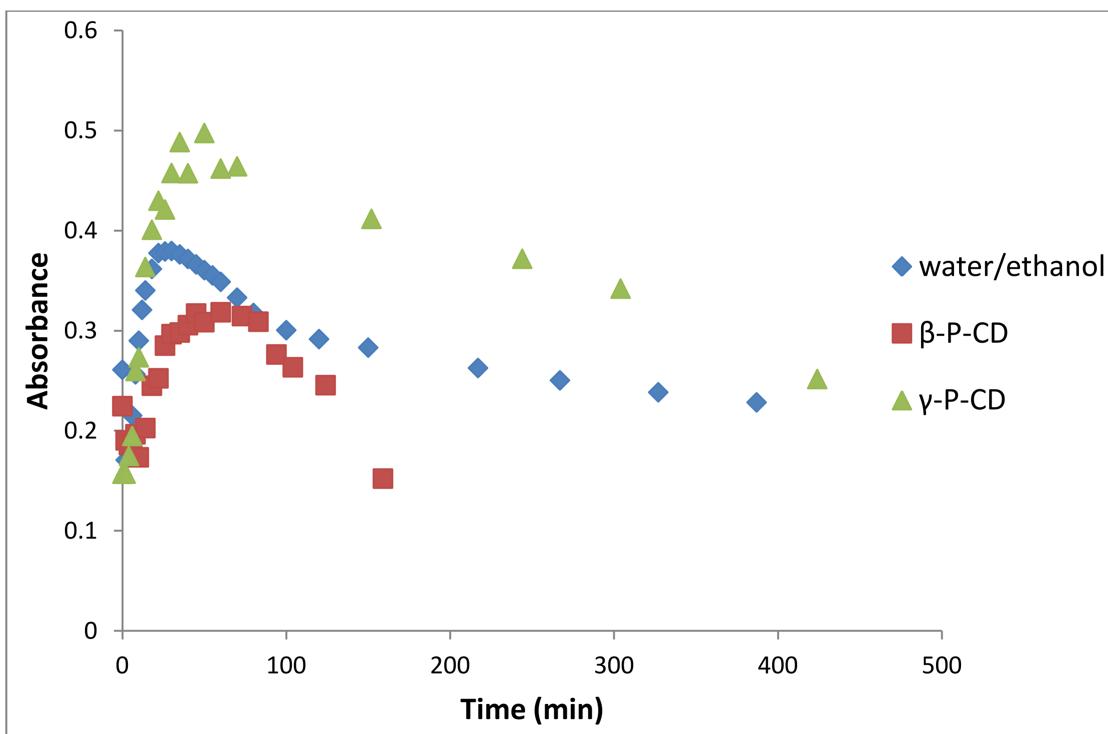


Fig.3.33 Comparison of UV/Vis traces of DES (7.890 x 10^{-6}M) in the presence of the CD polymers

3.9.3 Fluorimetry time drive traces

A fluorescence time drive study was carried out in order to determine the effect of different CDs on DES. Several experiments were carried out on DES in both ethanol and water/ethanol solvents, and in water/ethanol in the presence of various cyclodextrins. Time drive traces (Fluorescence intensity against time) of DES in the presence of different CDs were recorded and were compared with DES in water/ethanol in order to determine any variation in the behaviour of DES in the presence of CDs.

The observed characteristic behaviour of DES in water/ethanol and ethanol media can be seen in section 3.5.3.2 (Fig.3.14). The trace begins at a low intensity and increases quickly to

a high intensity before decreasing steadily. The fluorescence intensity of the photoproduct (B, scheme 1.) in ethanol only increases slightly and doesn't reach fluorescence intensity as high as in water/ethanol. DES in the ethanol media increases steadily until it reaches its max intensity before decreasing to zero.

Comparison of the photostabilities of DES in the presence of CD monomers (Fig.3.34) demonstrates that the fluorescence intensity of the photoproduct in the presence of γ-CD reaches a higher relative intensity (484) than that recorded in water/ethanol (358) but reaches its max after a longer period of irradiation time (308 min compared to the 180 min recorded in water/ethanol). HP-β-CD reaches its max intensity of only 198 at approximately 353 min compared to water/ethanol at 358 at around 180 min. The fluorescence intensity in HP-β-CD shows a plateau up to 500 min where it starts decreasing slightly towards the end of the irradiation period. This is clearer in Fig.3.35 where the traces are cut off at the max fluorescence intensity of DES in water/ethanol.

The HP-β-CD is more effective in slowing down the photoreaction than the γ-CD as the γ-CD seems to be following the more characteristic behaviour of DES in water/ethanol in the region 0 – 150 min but not in the remaining reaction period (150 – 550 min); here the trace obtained in the presence of γ-CD seems to considerably hamper the transformation B -> C (scheme 1). The reactivity (initial rate constants) in water/ethanol is comparable to that in the γ-CD which might be due the large cavity of γ-CD that offers enough space for the species to structurally rearrange.

The shapes of the traces between HP-β-CD and the β-P-CD may confirm a difference in complexation of the photoproduct corroborating what was found earlier in UV. The formation of the fluorescent photoproduct and its deletion seem very sensitive to the different CDs. The β-P-CD, γ-P-CD and the HP-β-CD seem to cause much more slowdown of the reaction than the γ-CD. This would indicate that the reactive species have less room to move. This may also indicate that the complexes in the former three CDs are very similar as

all reduce not only the degradation of DES but also the reaction of the fluorescent photoproduct.

Comparison of the polymers (Fig.3.36) shows that the γ-P-CD reaches a max intensity of 316 at 426 min compared to water/ethanol reaching its max (358) at 180 min. The β-P-CD reaches a max fluorescence of approximately 105 at 488 min and continues around this fluorescence intensity value. Therefore compared with DES in water/ethanol both CDs have somewhat slowed the degradation of DES however the β-P-CD seems to have a better effect than the γ-P-CD.

This study demonstrates a different behaviour of DES in the all of the recorded traces during the irradiation time period. Most of the traces begin with a very low fluorescence and continue at a much lower fluorescence than water/ethanol although γ-CD continues to increase significantly following the characteristic behaviour of the compound. The velocities of each trace however are different. It could not be determined during the irradiation time period if all traces will decrease to a lower intensity or not. Although the characteristic behaviour demonstrates that DES is degrading and the difference in velocity shows that the CDs are effecting the time of the degradation. It is clear by comparing the traces that the second reaction is slowed in all CD traces compared to water/ethanol. The γ-P-CD is slowed more than the γ-CD monomer where the conversion of B-C is a faster system than any other CD system. A comparison of the CD concentrations can be seen in table 7.

Overall the HP-β-CD and the β-P-CD seem to be more effective as they are almost the same with the fluorescence at much lower intensities than the other CD's studied. The β-P-CD is consistently the lowest reactivity throughout therefore an inclusion with the β-P-CD is more favourable. This is clearer in Fig.3.37 which demonstrates a comparison of the time drive traces at initial velocity. Table 8 also demonstrates this where the initial velocity is the lowest for the β-P-CD and the HP-β-CD traces.

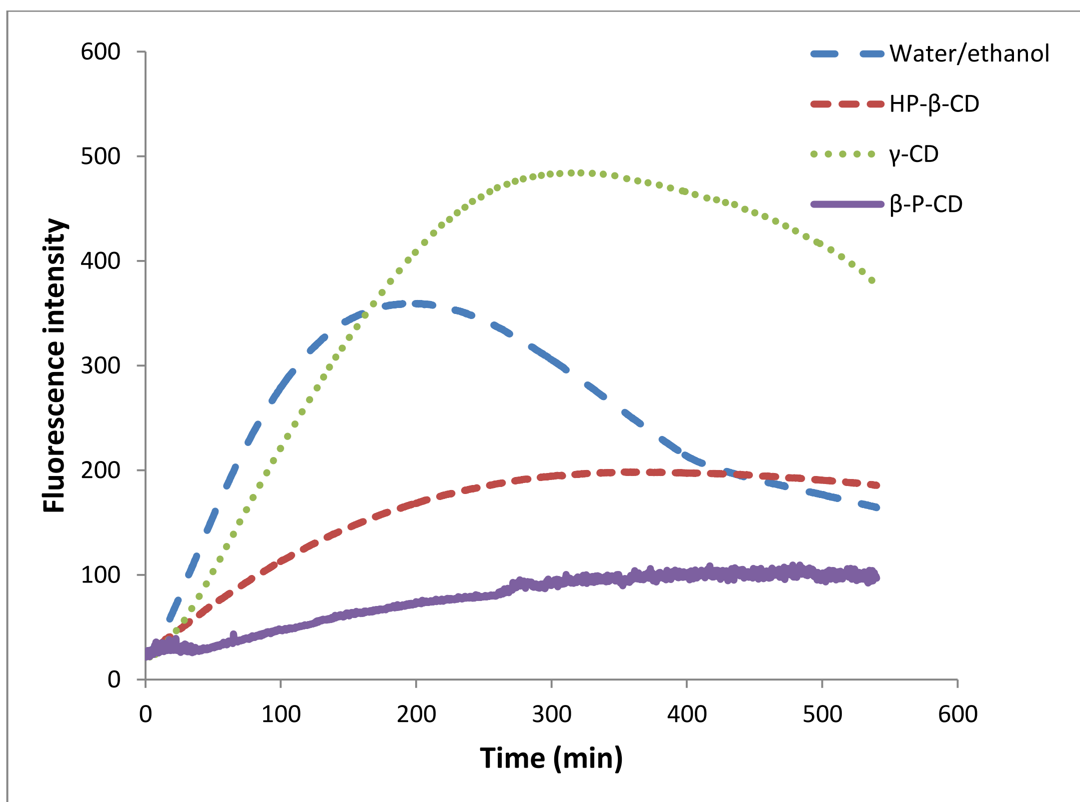


Fig.3.34 Comparison of time drive traces of DES (1.003 x 10^{-7} M) in the presence of the CD monomers (99.75% CD in H_2O:0.25% DES in ETOH)

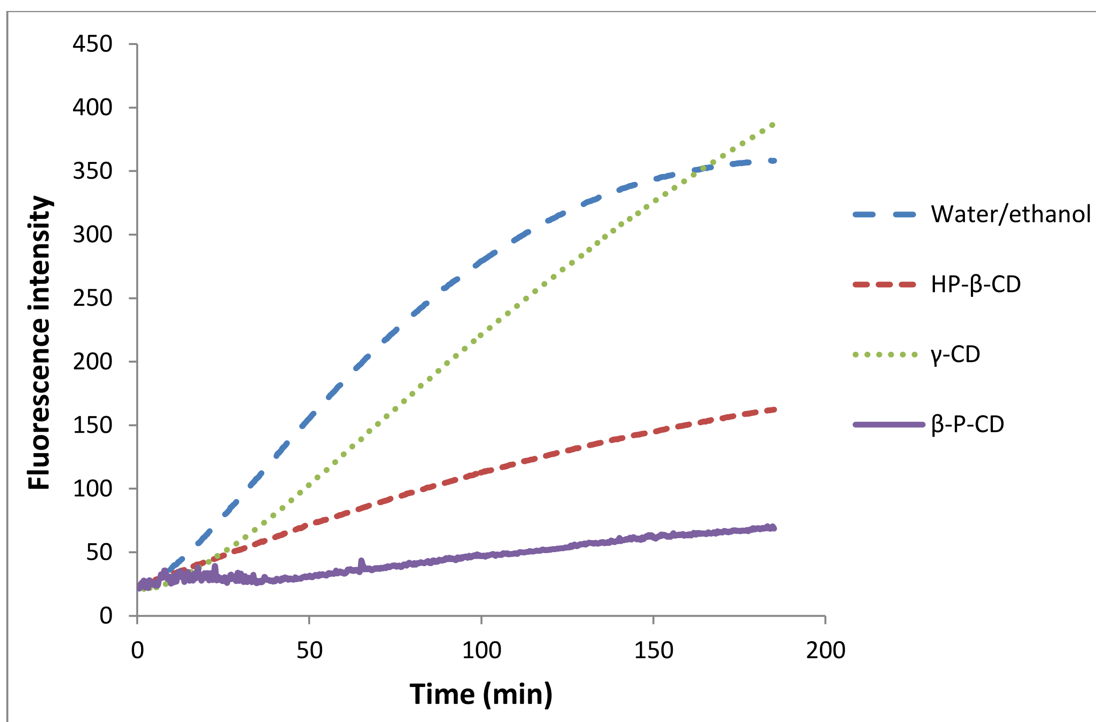


Fig.3.35 Comparison of CDs with DES (1.003×10^{-7} M) in water/ethanol trace at its max fluorescence intensity.

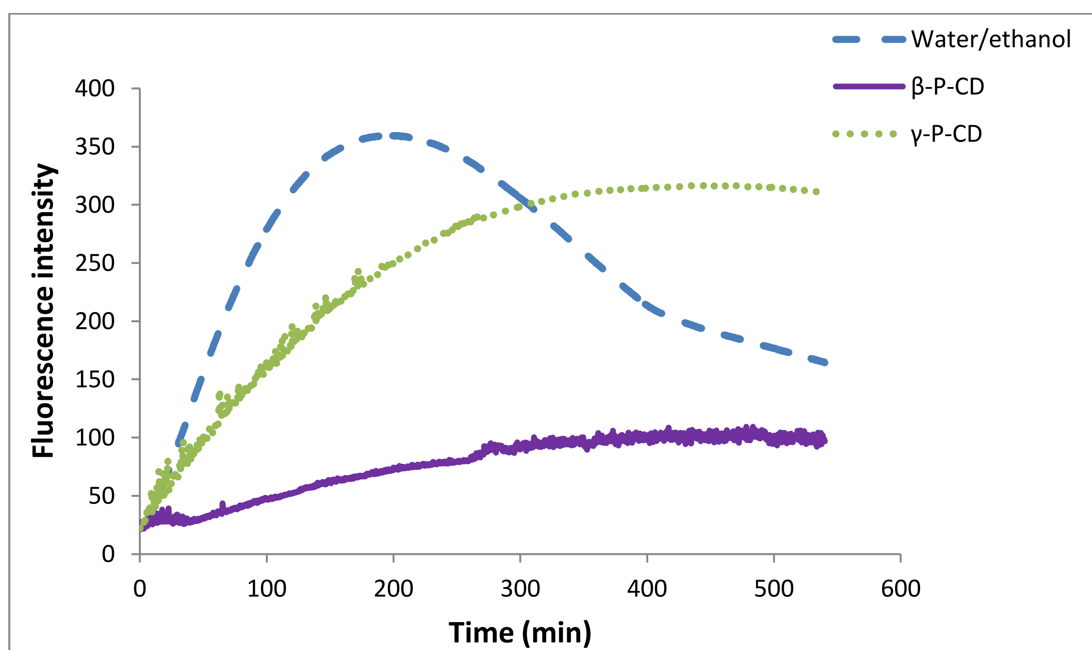


Fig.3.36 Comparison of time drive traces of DES (1.003×10^{-7} M) in the presence of the CD polymers (99.75% CD in H_2O:0.25% DES in ETOH).

Table 7: Comparison of the CD mass in the solutions [70]

Cyclodextrin	Mass (mg/mL)	Volume (mL)	Limit
β-P-CD	0.5	2	-
HP-β-CD	2.004	2	>600mg/L
γ-P-CD	4.979	2	-
γ-CD	0.232	2	232 mg/L

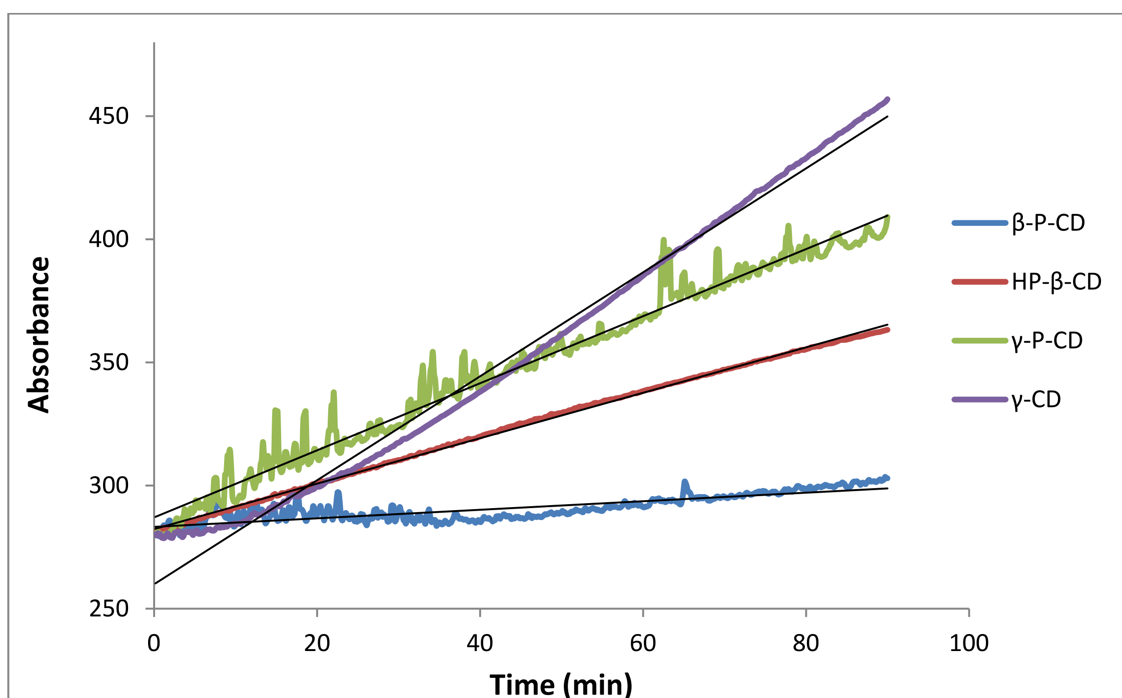


Fig.3.37 Initial velocity comparison of time drive traces of DES (1.003×10^{-7} M) in the presence of the CDs.

Table 8: Initial velocity comparison of DES in the presence of CDs

Cyclodextrin	V_0 (Initial velocity)	Linear equation (y=mx+c)	r^2
β-P-CD	0.1739	y = 0.1739x + 283.14	0.7035
HP-β-CD	0.9212	y = 0.9212x + 282.33	0.9986
γ-P-CD	1.3615	y = 1.3615x + 287.07	0.9769
γ-CD	2.1106	y = 2.1106x + 259.91	0.9897

3.10 Photostability of the novel formulation

The photostability of the optimized β-P-CD/DES formulation and the HP-β-CD/DES formulation were irradiated and their effects on the degradation of DES measured.

The formulations were prepared by forming the optimised complexes of 50 mg B-P-CD/5 mg DES and 40 mg HP-β-CD/5 mg DES. Solutions were prepared from the complexes ready for analysis and were irradiated within the range at which DES is most sensitive at 285 nm. A comparison of the formulation with DES in water/ethanol can be seen in Fig.3.38. The results demonstrate that although the formulations have not completely inhibited the degradation of DES they have both however slowed the degradation significantly.

DES in water/ethanol reaches its max fluorescence intensity (398) at approximately 40 minutes of irradiation whereas the β-P-CD/DES formulation hadn't reached its maximum fluorescence intensity after almost 4 hours of irradiation. A comparison can be made between DES in water/ethanol irradiated at 211 nm (Fig.3.19) and the β-P-CD/DES formulation irradiated at 285 nm (Fig.3.38). This clearly shows that the fluorescence intensity of the trace of the formulation is similar to that of the DES in water/ethanol trace irradiated at 211 nm (Fig.3.19) and demonstrates a substantial alteration from its behaviour when irradiated at 285 nm. The HP-β-CD/DES formulation has also had an effect in reducing

the fluorescence intensity of DES reaching its max fluorescence intensity after approximately 130 min of irradiation compared with the 40 min in its absence.

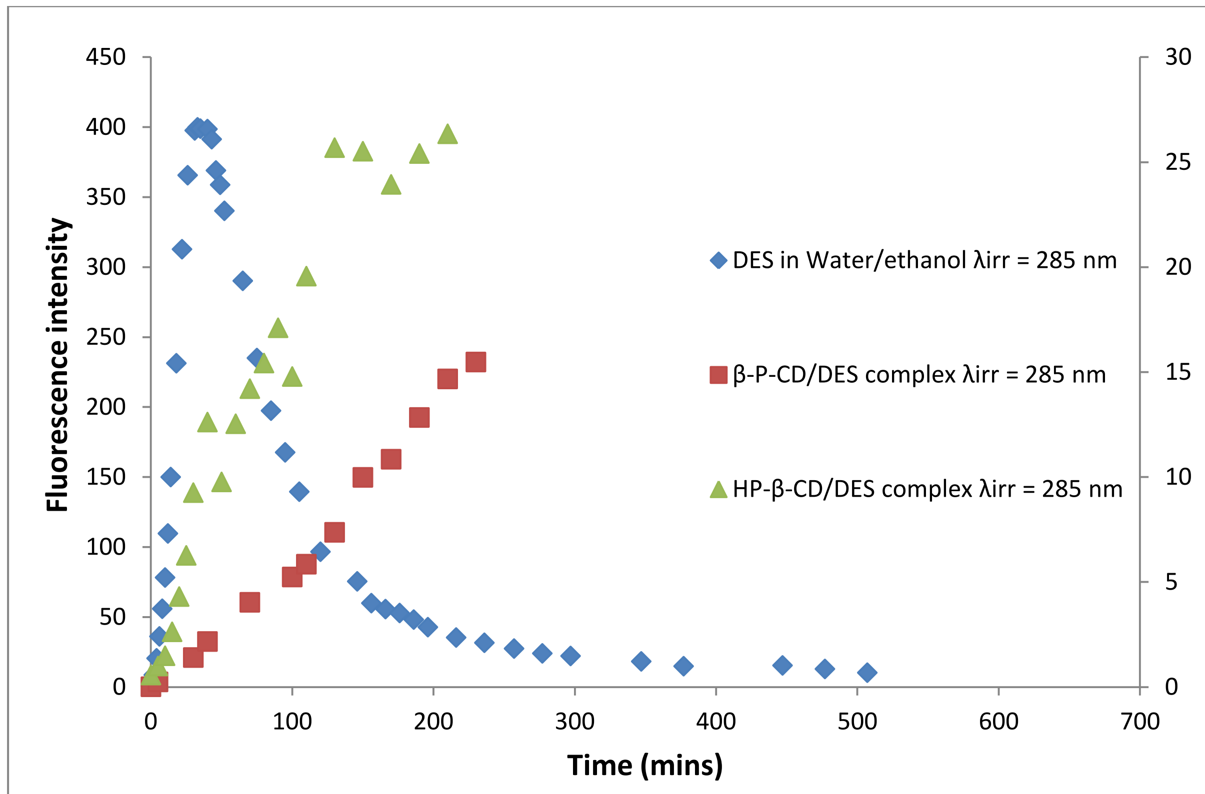


Fig.3.38 Comparison of the fluorescence spectra (filter on) of DES in water/ethanol and the optimized formulations irradiated at 285 nm

The UV traces (Fig.3.39 and Fig.3.40) show similarities to that of DES in water/ethanol. An increase in the absorbance during the irradiation time period takes place and curving of the trace can be observed before the absorbance decreases. However a lot of fluctuation is present, therefore the UV data isn't as evident as the fluorescence data.

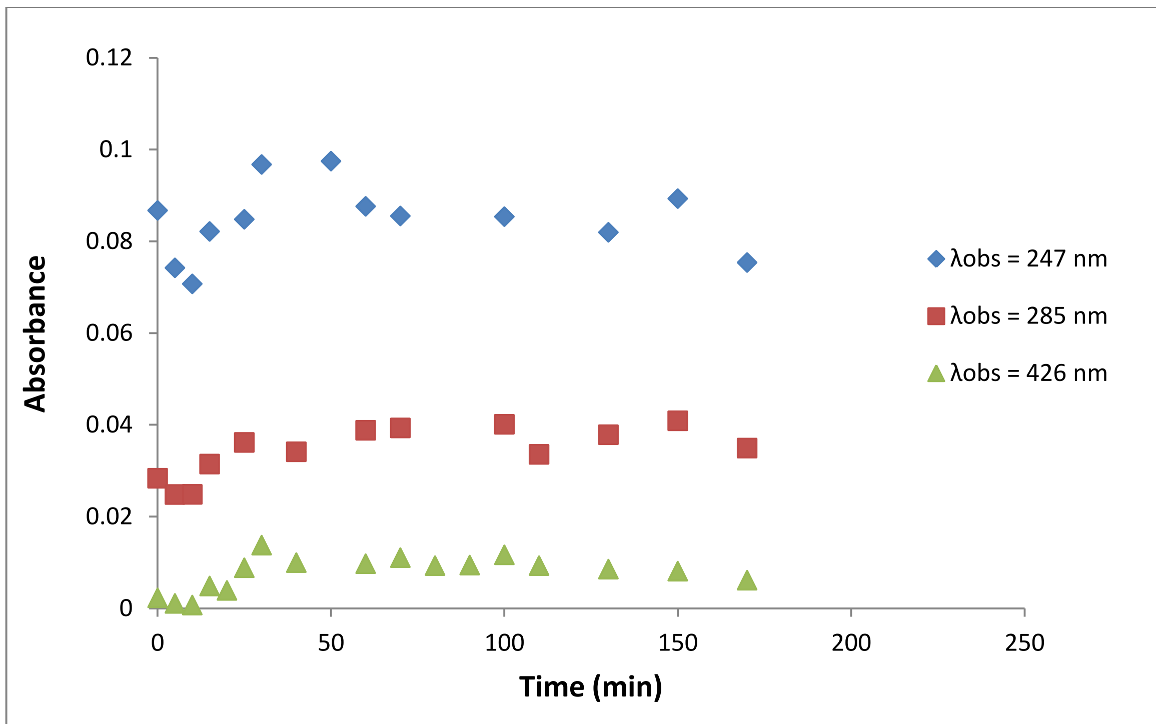


Fig.3.39 UV/Vis results of the β-P-CD/DES formulation irradiated at 285 nm

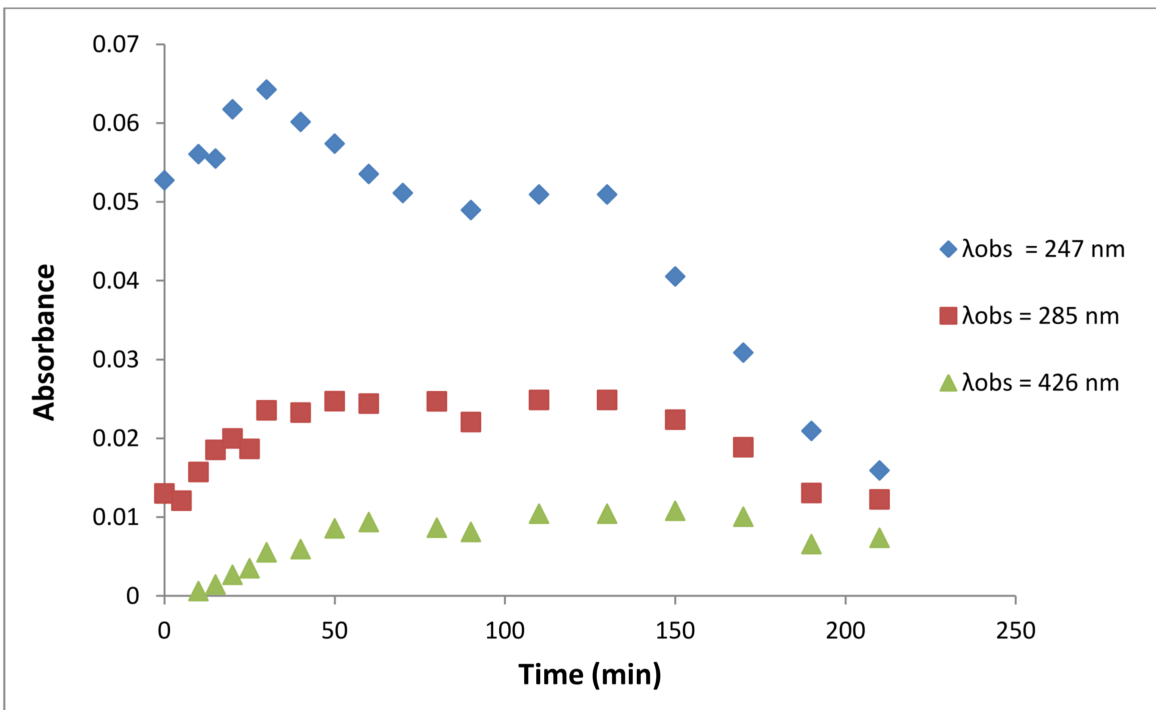


Fig.3.40 UV/Vis results of the HP-β-CD/DES formulation irradiated at 285 nm

The fluorescence spectra evolution of the β-P-CD/DES formulation (Fig.3.41) and the HP-β-CD/DES formulation (Fig.3.42) show the same behaviour of DES in water/ethanol (Fig.3.6).

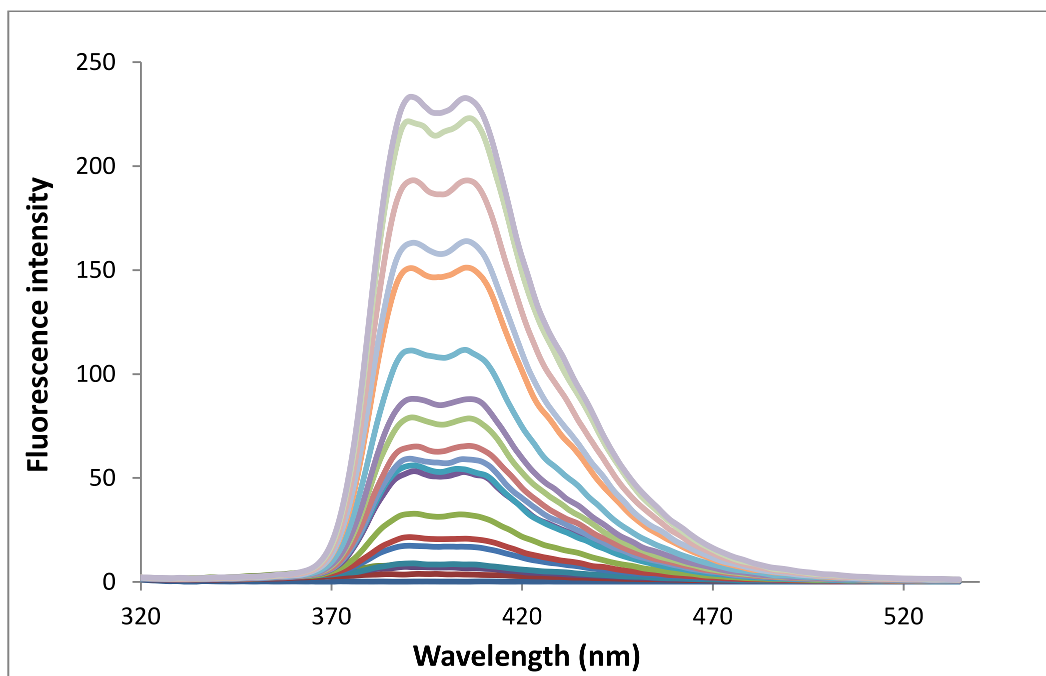


Fig.3.41 Fluorescence spectra evolution of the β-P-CD/DES formulation (filter on)

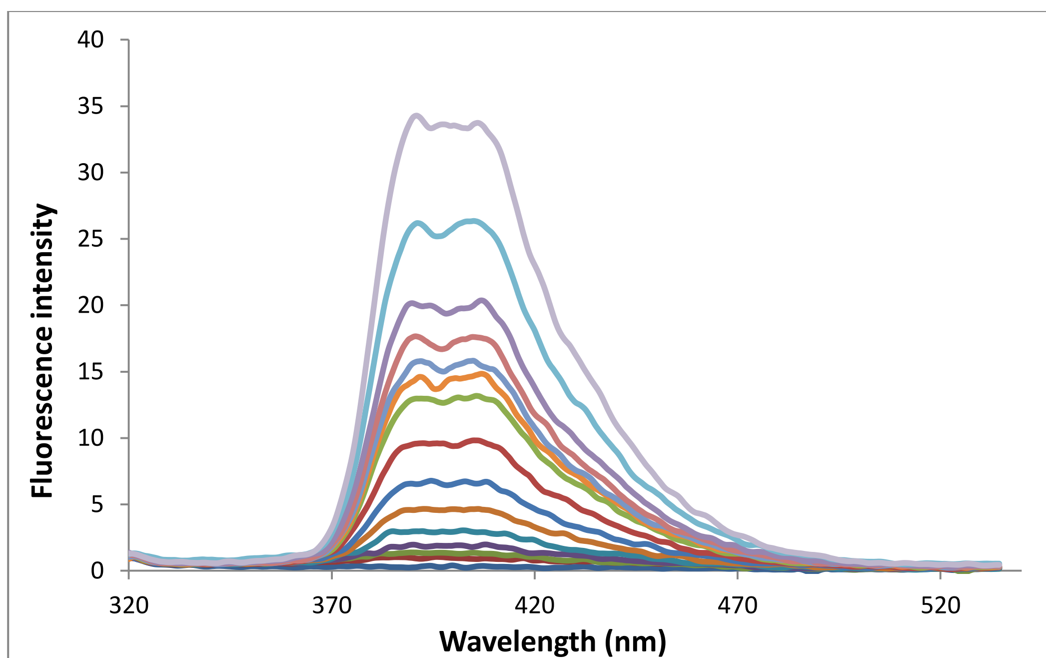


Fig.3.42 Fluorescence spectra evolution of the HP-β-CD/DES formulation (filter on)

A comparison of the fluorescence traces of the two formulations can be seen in Fig.3.43. Overall it can be determined that a comparison of the two optimised formulations demonstrates that the β-P-CD/DES formulation has had a greater effect in reducing the degradation of DES and is more photostable.

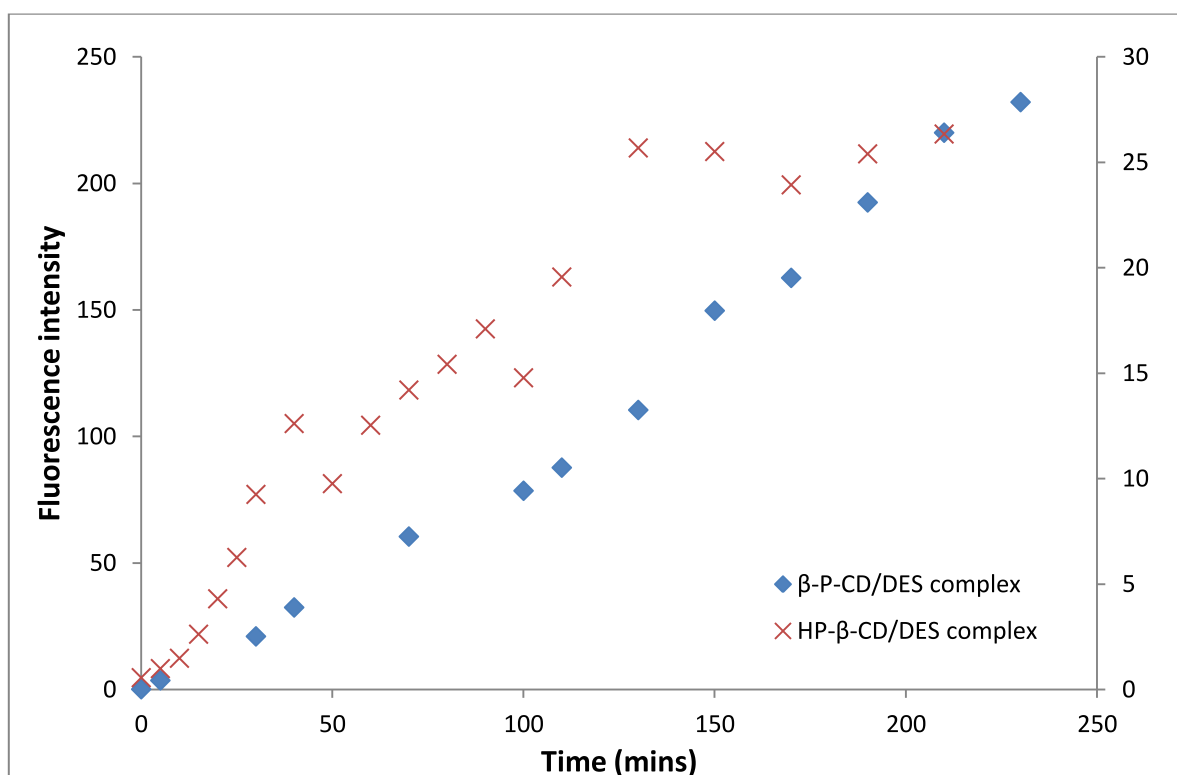


Fig.3.43 Fluorescence intensity comparison of the β-P-CD/DES formulation and the HP-β-CD formulation (filter on)

It can be concluded from photostability testing of the formulations that the data demonstrates that the behaviour of formulations are following the characteristic behaviour of DES, however the speed at which the photodegradation process of the compound takes place has been significantly reduced by the use of inclusion complexes with β-P-CD and the HP-β-CD. This proves that both inclusion complexes have had a photo protective effect on DES [65][66].

Chapter four.

Conclusion and future work

Conclusion

Finding ways to reduce such problems as photosensitivity in drugs is of high importance as such problems can result in toxicity, adverse effects and loss of potency which potentially limits their use in the pharmaceutical industry. Therefore carrying out research on such problems and working on methods of reducing or inhibiting such side effects will allow the use of drugs that are potentially life changing as well as provides knowledge to the research community that may lead to further discoveries in the future.

It is clear from reviewing the studies already completed that cyclodextrins and their uses are of major importance for the pharmaceutical industry. Although many studies have been carried out on the use of cyclodextrins in the pharmaceutical industry and the need for them there is very little on their uses in photostability of drugs and the polymers have yet to be studied. It is clear that photosensitivity of drugs is increasingly becoming a recognized problem therefore the demand for ways to deal with it is becoming a necessity. As well as the need for such inclusion complexes, once a complex has been obtained it is then important to provide a safe formulation of the drug. It is essential that the CD/drug complex can be formulated safely with the required amount of liquid that will prevent the drug escaping the complex in solution and a dosage form that is nontoxic to a patient.

This research study was carried out in order to determine the effect of both cyclodextrin monomers and cyclodextrin polymers on the photostability of DES and to investigate possible inclusion complex formation with DES. The results of this study have found that photodegradation and photostability studies carried out on the stilbene derivative DES have proved that the photoreaction taking place is purely photochemical.

The initial investigation proves that DES is photoactive and that the reaction is solely photochemical. There is no thermal activity involved in the degradation of the compound. Further investigation of the compound into the effect of solvent also shows that the photochemical reaction will take place in a mixture of water/ethanol (v, v, 98/2) as well as pure ethanol. Studies on the effect of wavelength has provided information into the wavelength range at which the drug is most sensitive to and the time period of degradation, providing more up to date information on the drug therefore aiding in safety and storage of the compound. Studies using fluorescence spectroscopy gave information on the fluorescence of DES, the intermediate and the photoproduct allowing the proposal of the photoreaction mechanism of DES.

The investigation found that all of the cyclodextrins studied showed some change in the behaviour of DES however not all had a substantial effect on the degradation process. By comparing the cyclodextrins studied, the γ-P-CD, β-P-CD and HP-β-CD had a better effect on the photostabilisation of DES compared with the γ-CD. Overall the fluorescence spectroscopy studies showed that the most effective cyclodextrin on reducing the photodegradation of DES was found to be β-P-CD resulting in a slowing down of the degradation process over time.

The studies carried out on the compound have allowed the successful formation of an inclusion complex with DES using β-CD-P at an optimised 50mg CD: 5mg drug ratio which is efficient as this uses the prescribed dosage of 5mg of DES and 50mg of β-CD-P. Complexation studies using HP-β-CD was found to only need 40mg CD: 5mg of drug in order to complex compared to the 50mg of β-CD-P needed. On comparing prices (Sigma Aldrich UK) 5g of β-CD-P is £115.50 however for 5g of the HP-β-CD it is considerably cheaper at £28.40 therefore the HP-β-CD is both efficient and cost effective. However the fluorescence studies on the CD effect on the compound show that β-P-CD would be a more favourable inclusion complex as its inhibitory effect on the degradation process proved to be the most successful. The price would also be reduced if the substance was used more regularly and ordered in bulk.

By forming an inclusion complex with the drug this has also allowed the water insoluble compound to be fully dissolved in a safe medium (purified water) for administration which has in effect enabled the production of the safe formulation of the drug. Analysis of the complex by ATR-FTIR and SEM methods have allowed the samples to be distinguished from one another through their different characteristics. Differentiating the complex specimen from the DES compound and the β-CD-P provides evidence that indicates the strong possibility of a complex formation between DES and β-CD-P or at least a significant interaction between these species.

The photostability of the β-P-CD/DES and the HP-β-CD/DES novel formulations were tested which proved successful resulting in a substantial reduction in the photodegradation of DES. A comparison between them found the HP-β-CD to be the most effective.

The β-P-CD and HP-β-CD are similar in reducing the time period of degradation and the degradation of the compound itself therefore they would be more efficient in terms of the preparation at industrial level. The time period to prepare the powder and water formulation in a hospital would take less than an hour therefore the complex has also created an opportunity for safe administration removing the concern of degradation during preparation of the drug.

Limitations

The SEM samples were not all prepared in the same way therefore they are not 100% conclusive.

Future work

The results from this study can be used as a chemical template for other Stilbene related molecules such as Pinosylvin, Pterostilbene and Oxy-Resveratrol in the future.

Other future work may include the development of kinetic strategies for the treatments of the photodegradation data. Further work on the complexation may be carried out by attempting to complex the photo product. The use of an irradiation chamber can also be incorporated for the formulation powder and liquid.

References

1. AL-Marzouqui, A, Jobe, B, Corti, G, Cirri, M and Mura, P. *Physicochemical characterization of drug-cyclodextrin complexes prepared by supercritical carbon dioxide and by conventional techniques*. J Incl Phenom Macrocycl Chem, **57**, pp.223-231 (2006).

2. Astray, G, Gonzalez-Barreiro, C, Mejuto, J.C, Rial-Otero, Simal-Gándara, J. *A review of the use of cyclodextrins in food. Food Hydrocolloids*, **23**, pp.1631-1640 (2009).

3. Baertschi, S.W, Alsante, K.M and Tønnesen, H.H. *A critical assessment of the ICH guideline on photostability testing of new drug substances and products (Q1B): Recommendation for revision*. Journal of pharmaceutical science, **99**(7), pp.2934-2940 (2009).

4. Bernard, E and Britz-Mckibban, P. *An intergrative guided enquiry experiment*. Journal of chemical education, **84**(7), p.1159 (2007).

5. Bonda, C, Zhang, J, and Pavlovic, A. *The photostability and photostabilization of trans-resveratrol*. Hallstar. Cosmetic magazine, **126**(9) p.652-660 (2011).

6. Budavari, S (ed) (1989) *The Merck index*. Merck & Co. Inc, New Jersey.

7. Butcher, G. *Tour of the electromagnetic spectrum*. NASA Headquarters: Indyne Inc.

8. British National Formulary (BNF 62) September 2011. British medical association Royal pharmaceutical society.

9. Chong, J, Poutaraud, A, Hugueney, P. *Metabolism and roles of Stilbene plants*. Plant Science, **177**, pp.143-155 (2009).

10. Cosa, G. *Photodegradation and photosensitization in pharmaceutical products: Assessing drug phototoxicity*. Pure applied Chemistry, **76**, pp.263-319 (2004).

11. Crowley, P and Martini, L.G. *Drug-excipient interactions* (2001) Pharmaceutical technology.

12. Davis and Brewster. *Cyclodextrin-based pharmaceutics: Past, presence and future*. Nature reviews. Drug discovery, **3**, pp.1023-1035 (2004).

13. Dou, D, Lui, Y, Zhao, H, Kong, L and Yao, S. *Radiolysis and photolysis studies on active transient species of diethylstilboestrol*. Journal of photochemistry and photobiology, **171**(3) pp.209-214 (2005).

14. Doyle, T.D, Benson, W.R and Filipescu, N. *Photocyclization of Diethylstilboestrol. Isolation of a stable, self-trapping Dihydrophenanthrene intermediate*. Journal of the American Chemical Society **9**(11) pp.3262-3267 (1976).

15. Doyle, T.D, Benson, W.R and Filipescu, N. *Spectrophotometric study of Dienestrol photoisomerization*. Photochemistry and Photbiology, **27**, pp.3-8 (1978).

16. Duchêne, D. *Cyclodextrins and their inclusion complexes. Cyclodextrins in pharmaceutics, cosmetics, and biomedicine: Current and future industrial applications,* 1st ed (2011). John Wiley and Sons, Inc.

17. Glass, B.D, Novák CS, Brown, M.E. *The thermal and photostability of solid pharmaceuticals. A review*. Journal of thermal analysis and calorimetry, **77**, pp. 1013-1036 (2004).

18. Goldstein, J. *Scanning electron microscope and x-ray microanalysis*, 3rd ed (2003). Springer Science and business media Inc, New York.

19. Harris, R.M and Waring, R.H. *A Review. Diethylstilboestrol – A long term legacy*. Maturitas, **5746**, pp.1-5 (2012).

20. Jørgessen, K.B. *Photochemical oxidative cyclisation of Stilbenes and Stilbenoids-The Mallory-Reaction*. Molecules, **15**(6) pp.4334-58 (2010).

21. Kragh, H. *Niels Bohr and the quantum atom: The Bohr model of atomic structure* (2012). Oxford University Press, Oxford.

22. Lakowicz, J.R. *Principles in Fluorescence Spectroscopy*, 3rd ed (corr) (2012). Springer Science and Business media Ltd, New York.

23. Langford, A, Dean, J, Reed, R, Holmes, D, Weyers, J, Jones, A. *Practical skills in Forensic Science* (2005). Pearson education Limited, England.

24. Loftsson, T and Duchêne, D. *Cyclodextrins and their pharmaceutical applications*. International Journal of Pharmaceutics, **329** (1-2) pp.1-11 (2007).

25. López-Nicholás, J.M, Núňez-Delicado, E, Pérez-López, A,J, Barrachina, A.C and Cuadrada Crespo, P. *Determination of stoichiometric coefficients and apparent formation constants for β-cyclodextrin complexes of trans-resveratrol using reversed-phase liquid chromatography*. Journal of chromatography A, **1135**, pp.158-165 (2006).

26. Lu, Z, Cheng, B, Hu, Y, Zhang, Y and Zou, G. *Complexation of resveratrol with cyclodextrins: Solubility and antioxidant activity*. Food chemistry, **113**, pp.17-20 (2008).

27. Lucas-Abellán, C, Fortea, I, López-Nicholás, J.M and Núňez-Delicado, E. *Cyclodextrins as resveratrol carrier system*. Food chemistry, **204**, pp.39-44 (2006).

28. Macmillan (2012) *Diethylstilbestrol (Stilboestrol)* [WWW] Macmillan cancer support. Available from: www.**nhs**.uk/ipgmedia/.../Diethylstilboestrol(Stilboestrol)(CB).pdf [Accessed 05/07/12].

29. Maafi, M, Aaron, J.J, Mahendero, M.C and Salinas, F. *Evidence for the formation of a Benzo [a]phenothiazine/2-Hydroxypropyl-β-Cyclodextrin Inclusion Complex. Using a fluorescence method*. Applied spectroscopy, **52**(1) pp.91-95 (1998).

30. Mallory, F.B and Mallory, C.W (2005) *Photocyclization of Stilbenes and Related molecules*. Organic reactions. pp.1-456 (2005).

31. Martin Del Valle, E.M. *Cyclodextrins and their uses. A review*. Process biochemistry, *39*, p.1033-1046 (2004).

32. Moore, D.E. *Principles and practice of drug photo degradation studies*. Journal of pharmaceutical and biomedical analysis, **5**(5) pp.441-453 (1987).

33. Núňez-Delicado, E, Sojo, M, Sánchez-Ferrer, A, García-Carmona, F. Cyclodextrins as Diethylstilbestrol Carrier System. *Characterization of Diethylstilboestrol – Cyclodextrins Complexes*. Pharmaceutical research, **16**(6) pp.854-858 (1999).

34. Onoue, S and Tsuda, Y. *Analytical Studies on the prediction of photosensitive/phototoxic potential of pharmaceutical substances*. Pharmaceutical research, **23**(1) pp.156-164 (2006).

35. Owen, T. *Fundamentals of UV-visible spectroscopy* (2000). Agilent technologies.

36. Parson, W.W. *Modern optical spectroscopy* (2009). Springer, New York.

37. Pifferi, G and Restini, P. *The safety of pharmaceutical excipients*. Il Farmaco, **58**, pp.541-550 (2002).

38. Ramer, G and Lendl, B. *Attentuated total reflection fourier transform spectroscopy* IN: Encyclopaedia of analytical Chemistry (2013).

39. Rimando, A.M and Suh, N. *Biological/chemopreventive activity of Stilbenes and their effects on colon cancer*. Planta Med, **74**, pp.1635-1643 (2008).

40. Roupe, K.A, Remsberg, C.M, Yáñez, J,A and Davies, N.M. *Pharmacometrics of Stilbenes: Seguing towards the clinic*. Current clinical pharamacology, **1**, pp.81-101 (2006).

41. Rowe, R.C, Sheskey, R.J and Weller, P.J (eds.) *Handbook of pharmaceutical excipients*, 4th ed (2003). Pharmaceutical Press, London.

42. Sanoh, S, Kitamura, S, Sugihara, K, Fujimoto, N and Ohta, S. *Estrogenic activity of Stilbene derivatives*. Journal of Health Science, **49**(5) pp.359-367 (2003).

43. Sapino, S, Carlotti, M.E, Caron, G, Ugazio,E and Cavalli, R. *In silico design, photostability and biological properties of the complex resveratrol/hydroxypropyl-β-*

cyclodextrin. Journal of Inclusion phenomenon Macrocyclic Chemistry, **63**, pp.171-180 (2008).

44. Sauer, M, Hofkens, J and Enderlein, J. *Handbook of fluorescence spectroscopy and imaging* (2011). Wiley-Vch, Weinheim.

45. Shi, G, Rao, L, Yu, H, Xiang, H, Yang, H and Ji, R. *Stabilization and encapsulation of photosensitive Resveratrol within yeast cells*. International Journal of Pharmaceutics, **349**, pp.83-93 (2008).

46. Smith, K.C. *Basic photochemistry* (2009) Available from: http://www.photobiosci.info/Photochem.html

47. Tønnesen, H.H. *Formulation and stability testing of photolabile drugs*. International Journal of pharmaceutics, **225**, pp.1-14 (2001).

48. Tønnesen, H.H (ed.) *Photostability of drugs and drug formulations*, 2nd ed (2004). CRC Press LLC, Florida.

49. Tønnesen, H.H. *Photoreactivity of drugs*. In: Solar radiation and Human health (2008).

50. Tønnesen, H.H. *The International conference on harmonization photostability guideline*. In: Piechocki, J.T and Thoma, K (eds.) Pharmaceutical photostability and stabilization technology (2010). USA: Informa Healthcare USA inc. pp.47-59.

51. US environmental protection agency (2012) Phenanthrene fact sheet (PDF).

52. Wayne, R.P. *Principles and applications of photochemistry* (1988). Oxford University Press, New York.

53. Weber, J.T, Lamont, M, Chibrikova, L, Fekkes, D, Vlug, A.S, Lorenz, P, Kreutzmann, P and Slemmer, J.E. *Potential neuroprotective effects of Oxyresveratrol against traumatic injury*. Journal of European Pharmacology, **680**, pp.55-62 (2011).

54. The electromagnetic spectrum (2012) The electromagnetic spectrum [online image] Available from: www.mynasadata.larc.nasa.gov [Accessed 01/07/12].

55. Anatomy of an electromagnetic wave (2011) The electromagnetic wave [Online image] Available from: http://missionscience.nasa.gov/ems/02_anatomy.html Accessed [01/07/12].

56. Bishop, Y (ed) *The Vetinary Formulary*, 6th ed (2004). Pharmaceutical press, London.

57. Braun, A.M, Maurette, M.T,Oliveros, E. *Photochemical technology* (1991). Wiley.

58. Cornish-Bowden, A. *Fundamentals of enzyme kinetics*, 4th ed (2012). Wiley-VCH verlag GmbH and Co. KGaA.

59 Mortimer, M and Taylor P (eds). *Chemical Kinetics and Mechanism* (2012). The Royal society of chemistry, Cambridge.

60 Mahato, R.I and Narang, A.S. *Pharmaceutical dosage forms and drug delivery*, 2nd ed (2012) pp. 123-148. CRC Press, Florida.

61. C, Cannavá, Crupi, V, Ficarra, P, Guardo, M, Majolino, D, Stancanelli, R and Venuti, V. *Physicochemical characterization of coumestrol/β-cyclodextrin inclusion complexes by UV-VIS and FTIR-ATR spectroscopies*. Vibrational spectroscopy, **48**(2) pp.172-78 (2008).

62. Tayade, P.T and Vavia, P.R. Inclusion complexes of Ketoprofen with β-cyclodextrin: *Oral pharmacokinetics of Ketoprofen in human*. Indian Journal of pharmaceutical Sciences, **68**(2) pp.164-70 (2006).

63. Farcas, A, Jarroux, N, Farcas, A.M, Harabagiu and Guegan, P. *Synthesis and charcaterization of Furosemide complex in β-cyclodextrin*. Digest Journal of Nanomaterials and Biostructure, **1**(2) pp.55-60 (2006).

64. Sinha, V.R, Anitha, R, Ghosh, S, Nanda, A and Kumria. R. *Complexation of celecoxib with beta-cyclodextrin: Characterisation of the interaction in solution and in solid state*. J Pharm Sci, **94**(3) pp.676-87 (2005).

65. Ragno, G, Risoli, A, Ioele, G, Cione, E and De Luca, M. *Photostabilization of 1,4-dihydropyridine antihypertensives by incorporation into beta-cyclodextrin and liposomes*. J Nanosci Nanotechnol, **6** (9-10) pp.2979-85 (2006).

66. Glass, B.D, Brown, M.E, Daya, S, Worthington, M.S, Drummond, P, Antunes, E, Lebete, M, Anoopkumar- Dukie, S and Maharaj, D. *Influence of cyclodextrins on the photostability of selected drug molecules in solution and the solid state*. International Journal of photoenergy, **3**, pp.205-211 (2001).

67 Perrie,Y, Singh Badham, R.K, Kirby, D.J, Lowry, D, Mohammed, A.R and Ouyang,D. *The impact of ageing on the barriers to drug delivery*. Journal of controlled release, **161**, pp.389-398 (2012).

68 M.J, Pilling and P.W, Seakins. *Reaction kinetics*, 2nd ed (1996). Oxford University Press, USA.

69 M, Maafi et al. Unpublished data (2013).

70 The European cyclodextrin society (2009) *What are Cyclodextrins* [WWW] Available from:http://www.eurocdsoc.com/index.php?option=com_content&view=article&id=67: [Accessed 28/07/13]

71 Fenyvesi, É. *Cyclodextrin polymers in the pharmaceutical industry*. Journal of Inclusion phenomena, **6**, pp. 537-545 (1988).

72 Loftsson, T and Brewster, M.E. *Pharmaceutical applications of cyclodextrins. 1. Drug solubilisation and stabilization*. Journal of pharmaceutical science, **85** (10) pp.1017-1025 (1996).

73. Bibby, D.C, Davies, N.M and Tucker, I.G. *Mechanisms by which cyclodextrins modify drugs release from polymeric drug delivery systems*. International journal of pharmaceutics, **197**, pp.1-11 (2000).

74 Machín, R, Isasi, J.R and Vélaz, I. *β-cyclodextrin hydrogels as potential drug delivery systems*. Carbohydrate polymers, **87**, pp.2024-2030 (2012).

Appendix A

Calculations

Calculations

DES stock

To make the DES stock solution (S_0), for example, 0.0054g of DES was dissolved in 10ml of ethanol with a concentration of 2.012×10^{-3}M.

DES Intermediates and cuvette solutions

Concentrations were determined using the $C_1V_1 = C_2V_2$ equation where C is the concentration and V is the volume. In order to determine the concentration of intermediates or cuvette solutions the equation was rearranged to give:

$$C_2 = \frac{C_1V_1}{V_2}$$

Intermediate $S_1 = 4.024 \times 10^{-4}$ M

Intermediate $S_2 = 4.024 \times 10^{-5}$ M

S_{cuv} using 40µl S_1 intermediate = 7.890×10^{-6} M

S_{cuv} using 5µl S_2 intermediate = 1.003×10^{-7} M

The novel formulations

The novel formulations were prepared by collecting the dried powder from the optimized complexes (50 mg β-P-CD/ 5 mg DES and 40 mg HP-β-CD/ 5 mg DES). The dried powder was weighed and dissolved in pure water in a volumetric flask.

A volume of 2 mL was taken from the volumetric flask and was added to a cuvette for irradiation. The solution was very concentrated for analysis therefore in order to take a reading of the solution a specific volume was taken from the cuvette using a syringe and was added to another cuvette containing 2 mL of pure water after each period of irradiation.

The volume taken from the irradiation solution was determined by finding a concentration close to that of the DES in water/ethanol solution (7.890 x 10^{-6} M). The calculated volumes to be taken were very small and impractical therefore in order to make the analysis possible and to reduce the margin of error a larger volume was taken.

In order to determine the concentrations the mass of DES in the dried powder was determined:

$$\text{DES} = \frac{\text{Original mass of DES (for example 5 mg) x mass of the dried powder}}{\text{Original total mass (both CD and Drug)}}$$

The $C_1V_1 = C_2V_2$ equation can then be employed to determine the concentrations of novel formulation solutions.

15 µl of the irradiated β-P-CD/ DES formulation gave a concentration of 8.025×10^{-5}.

10 µl of the irradiated HP-β-CD/ DES formulation gave a concentration of 6.95×10^{-4}.