1.1 Vitamins

A vitamin is an organic compound required as a nutrient in tiny amounts by an organism [(1)]. In other words, an organic chemical compound (or related set of compounds) is called a vitamin when it cannot be synthesized in sufficient quantities by an organism, and must be obtained from the diet. Thus, the term is conditional both on the circumstances and on the particular organism. For example, ascorbic acid (vitamin C) is a vitamin for humans, but not for most other animals, and biotin and vitamin D are required in the human diet only in certain circumstances.

By convention, the term vitamin does not include other essential nutrients such as dietary minerals, essential fatty acids, or essential amino acids (which are needed in larger amounts than vitamins), nor does it encompass the large number of other nutrients that promote health but are otherwise required less often[(2)].

Thirteen vitamins are universally recognized at present. They are classified by their biological and chemical activity, not their structure. Thus, each "vitamin" refers to a number of vitamer compounds that all show the biological activity associated with a particular vitamin. Such a set of chemicals is grouped under an alphabetized vitamin "generic descriptor" title, such as "vitamin A", which includes the compounds retinal, retinol, and four known carotenoids. Vitamers by definition are convertible to the active form of the vitamin in the body, and are sometimes inter-convertible to one another, as well.

Vitamins have diverse biochemical functions. Some have hormone-like functions as regulators of mineral metabolism, or regulators of cell and tissue growth and differentiation (e.g., some forms of vitamin A). Others function as antioxidants (e.g., vitamin E and sometimes vitamin C). [(3)]

The largest number of vitamins (e.g., B complex vitamins) function as precursors for enzyme cofactors that help enzymes in their work as catalysts in metabolism. In this role, vitamins may be tightly bound to enzymes as part of prosthetic groups: For example, biotin is part of enzymes involved in making fatty acids. Vitamins may also be less tightly bound to enzyme catalysts as coenzymes, detachable molecules that function to carry chemical groups or electrons between molecules. For example, folic acid carries various forms of carbon group – methyl, formyl, and methylene in the cell. Although these roles in assisting enzyme-substrate reactions are vitamins' best-known function, the other vitamin functions are equally important [(4).]

Until the mid-1930s, when the first commercial yeast-extract and semisynthetic vitamin C supplement tablets were sold, vitamins were obtained solely through food intake, and changes in diet (which, for example, could occur during a particular growing season) can alter the types and amounts of vitamins ingested. Vitamins have been produced as commodity chemicals and made widely available as inexpensive semisynthetic and synthetic-source multivitamin dietary supplements, since the middle of the 20th century. The term vitamin was derived from "vitamine," a combination word made up by Polish scientist Casimir Funk from vital and amine, meaning amine of life, because it was suggested in 1912 that the organic micronutrient food factors that prevent beriberi and perhaps other similar dietary deficiency diseases might be chemical amines. This proved incorrect for the micronutrient class, and the word was shortened to vitamin [(5)]. The value of eating a certain food to maintain health was recognized long before vitamins were identified. The ancient Egyptians knew that feeding liver to a patient would help cure night blindness, an illness now known to be caused by a vitamin A deficiency [(6)].

The advancement of ocean voyages during the Renaissance resulted in prolonged periods without access to fresh fruits and vegetables, and made illnesses from vitamin deficiency common among ships' crews [(7)].

In 1749, the Scottish surgeon James Lind discovered that citrus foods helped prevent scurvy, a particularly deadly disease in which collagen is not properly formed, causing poor wound healing, bleeding of the gums, severe pain, and death[(6)]. In 1753, Lind published his Treatise on the scurvy, which recommended using lemons and limes to avoid scurvy, which was adopted by the British Royal Navy. This led to the nickname Limey for sailors of that organization. Lind's discovery, however, was not widely accepted by individuals in the Royal Navy's Arctic expeditions in the 19th century, where it was widely believed that scurvy could be prevented by practicing good hygiene, regular exercise, and maintaining the morale of the crew while on board, rather than by a diet of fresh food.

As a result, Arctic expeditions continued to be plagued by scurvy and other deficiency diseases. In the early 20th century, when Robert Falcon Scott made his two expeditions to the Antarctic, the prevailing medical theory was that scurvy was caused by "tainted" canned food [(6)].

1.1.1 Vitamins classification

Vitamins are classified as either water-soluble or fat-soluble. In humans there are 13 vitamins: 4 fat-soluble (A, D, E, and K) and 9 water-soluble (8 B vitamins and vitamin C). Water-soluble vitamins dissolve easily in water and, in general, are readily excreted from the body, to the degree that urinary output is a strong predictor of vitamin consumption[(8)]. Many types of water-soluble vitamins are synthesized by bacteria [(9)]. Fat-soluble vitamins are absorbed through the intestinal tract with the help of lipids (fats). Because they are more likely to accumulate in the body, they

are more likely to lead to hypervitaminosis than are water-soluble vitamins. Fat-soluble vitamin regulation is of particular significance in cystic fibrosis [(10)].

1.2 Riboflavin

Riboflavin is chemically defined as 7,8-dimethyl-10-(1′-d-ribityl) isoalloxazine, lactoflavin or vitamin G, has a molecular weight of 376.37g/mol, and melting point of 290 °C [(11)] . The planar isoalloxazine ring provides the basic structure not only for riboflavin (vitamin B2) but for the naturally occurring phosphorylated coenzymes that are derived from riboflavin that shown in Figure (1.1) [(12)].

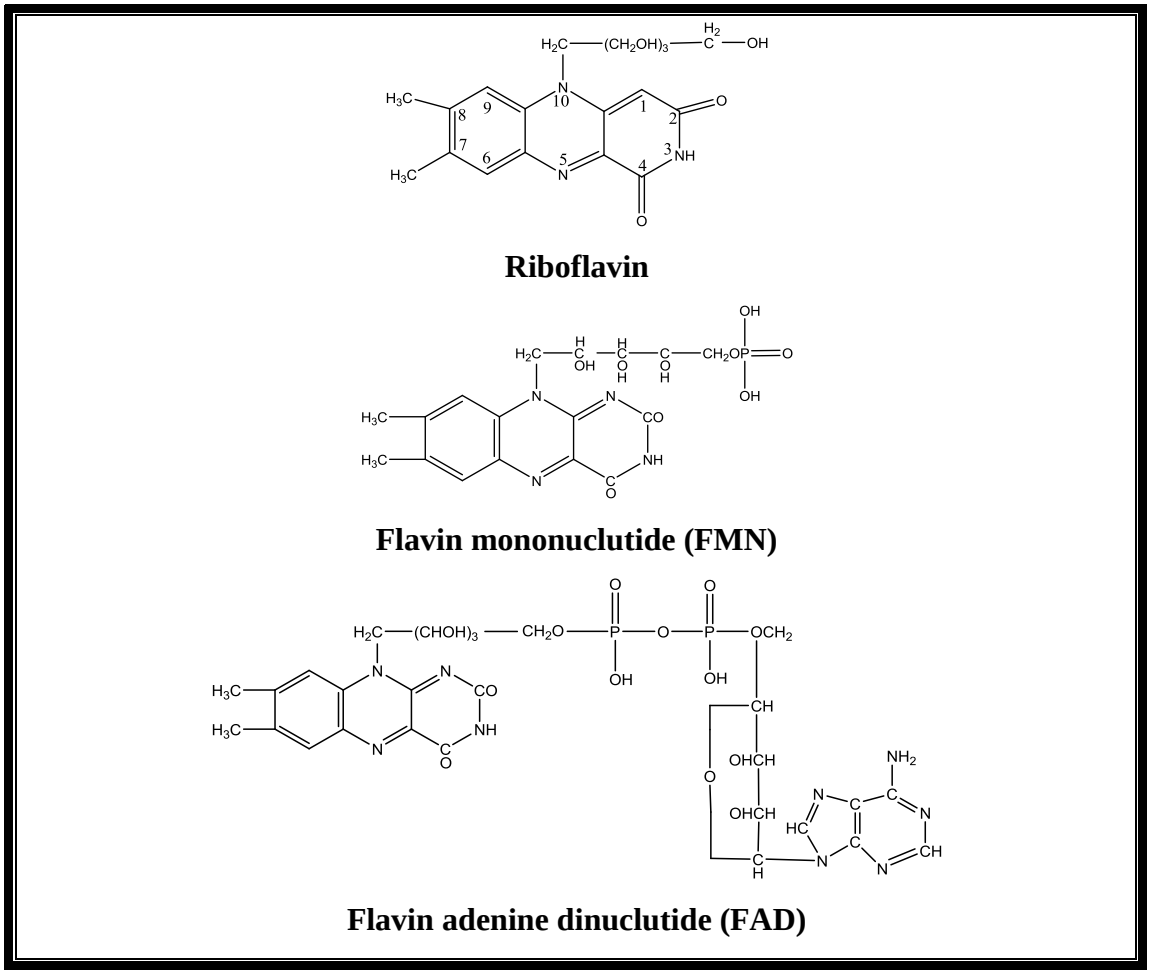


Figure (1.1): Structural formulas of riboflavin, flavin mononucleotide (riboflavin-5'-phosphate, FMN) and flavin adenine dinucleotide (FAD) [(12)]

These coenzymes include flavin mononuclutide (FMN), flavin adenine dinuclutide (FAD), and flavin coenzymes linked covalently to specific tissue proteins, generally at the 8-〈 methyl position of the isoalloxazine ring [12]. Riboflavin was originally recognized as a growth factor in 1879 and named vitamin B2 according to the British nomenclature system. It was first isolated from egg whites in 1934 and synthesized in 1935[13]. The name "riboflavin" comes from "ribose" (the sugar whose reduced form, ribitol, forms part of its structure) and "flavin", the ring-moiety which imparts the yellow color to the oxidized molecule (from Latin flavus, "yellow"). The reduced form, which occurs in metabolism along with the oxidized form, is colorless [14].

Riboflavin is yellow, crystalline powder. Decompose at 278-282. Slightly soluble in water (0.033-0. 606 g/100 ml of water). Soluble in alcohol at 27.5°C (0.0045g/100ml of abs.ethanol). It is also slightly soluble in cyclohexanol , amyl acetate, benzene alcohol and phenol.

Riboflavin gives green fluorescence [15]. The dietary sources include milk, eggs, meats, yogurt, broccoli, almonds, cheese, soy, fortified grains, and dark green vegetables, in descending order of concentration [14].

There are a number of variations in structure in the naturally occurring flavins. Riboflavin and its coenzymes are sensitive to alkali and to acid, particularly in the presence of UV light. Under alkaline conditions, riboflavin is photodegraded to yield lumiflavin (7,8,10-trimethylisoalloxazine), which is inactive biologically. Riboflavin is photodegraded to lumichrome (7, 8-dimethylalloxazine) under acidic conditions, a product that is also biologically inactive [12].

Thus, an important physical property of riboflavin and its derivatives is their sensitivity to UV light, resulting in rapid inactivation [12].

As one of the family of B vitamins, riboflavin contributes to cellular growth, enzyme function, and energy production. FAD is a cofactor in many reactions of intermediary metabolism, such as carbohydrate, fat, and amino acid synthesis; FAD and R5P are also necessary for the activation of other vitamins and enzyme systems. Folate and pyridoxine are vitamins that rely on riboflavin for activation. Clinicall\y, riboflavin has several applications due to its ubiquitous nature in metabolism. It was found to be necessary for healthy skin and eyes, for red blood cell formation, antibody production, and growth. It does also facilitate the use of oxygen by tissues of the skin, nails and hair. Research supports the use of riboflavin in anemia, cataracts, hyperhomocysteinemia, migraine prophylaxis, and alcoholism. A riboflavin deficiency can result in angular stomatitis, seborrhea, glossitis, neuropathy, and anemia.

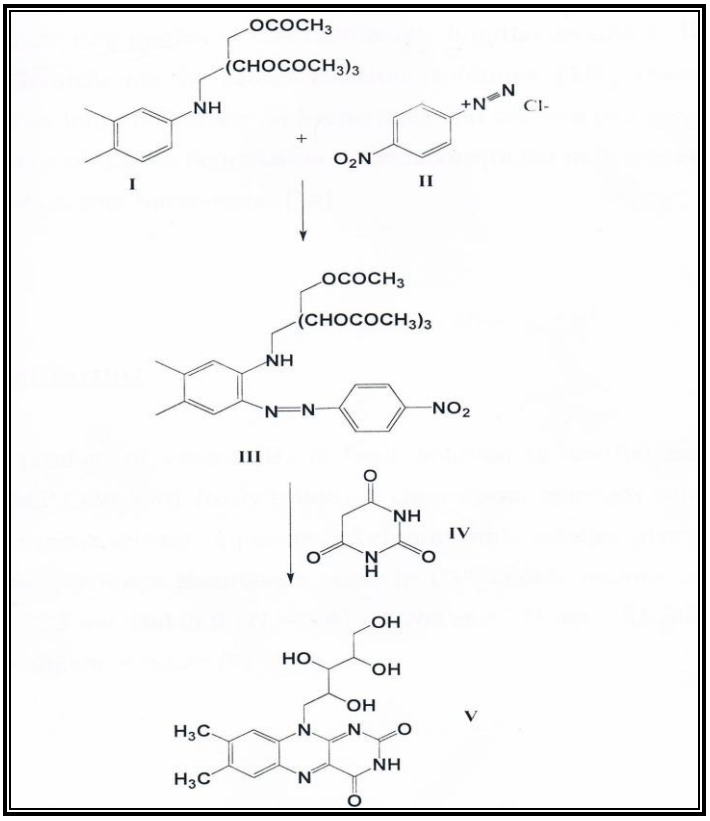
The major function of riboflavin, as noted above, is to serve as the precursor of the flavin coenzymes, FMN and FAD, and of covalently bound flavins. These coenzymes are widely distributed in intermediary metabolism and catalyze numerous oxidation–reduction reactions. Because FAD is part of the respiratory chain, riboflavin is central to energy production. Other major functions of riboflavin include drug and steroid metabolism, in conjunction with the cytochrome P450 enzymes, and lipid metabolism. The redox functions of flavin coenzymes include both one-electron transfers and two electron transfers from substrate to the flavin coenzyme. Flavoproteins catalyze dehydrogenation reactions as well as hydroxylations, oxidative decarboxylations, dioxygenations, and reductions of oxygen to hydrogen peroxide. Thus, many different kinds of oxidative and reductive reactions are catalyzed by flavoproteins [(12)].

The latest (1998) RDA recommendations for vitamin B_2 are similar to the 1989 RDA, which for adults suggested a minimum intake of 1.2 mg for persons whose caloric intake may be > 2,000 Kcal. The

current RDAs for riboflavin for adult men and women are 1.3 mg/day and 1.1 mg/day, respectively; the estimated average requirement for adult men and women are 1.1 mg and 0.9 mg, respectively. Recommendations for daily riboflavin intake increase with pregnancy and lactation to 1.4 mg and 1.6 mg, respectively (1in advanced). For infants, the RDA is 0.3 0.4 mg/day and for children it is 0.6-0.9 mg/day [16].

1.3 chemical synthesis of riboflavin

Vitamin B2 is prepared by the reaction between 4,5-dimethyl aniline tetraacetate (I) and p-nitro-phenyldiazanioum chloride(II) yielding azoderivative (III), which by reacting with barbituric acid(IV), result in riboflavin (V) as shown in scheme (1.1) [17].



Scheme (1.1): Chemical synthesis of riboflavin [17]

1.4 Photophysical and photochemical processes of riboflavin

1.4.1 Photophysical processes

The first step of a physical process induced by light involves absorption of a quantum of light by a molecule, producing an electronically excited state. The molecule is said to go from the ground to an excited state. Once in the excited state, the molecule has several available pathways to release the absorbed energy. Different processes may occur [18]:

(i) Nonradiative decay: the process of vibrational relaxation, in which the excess energy is transferred to the surroundings as thermal motion of the environment (heat) as:

A- Internal conversion: Absorption of a photon (hν A in Figure (1.2)) populates the vibrational levels of S_1 (or S_2). This process is very fast and the molecule relaxes to the lowest vibrational level of S_1.

B- Intersystem crossing (ISC): excitation to a vibrational level of S_2, an intersystem crossing (ISC) to an electronic triplet excited state occurs.

(ii) Fluorescence or phosphorescence in which a photon of the same or lower energy is emitted while the molecule returns to the ground state. Radiative transitions involve light emission radiation is called fluorescence if spin multiplicities both in ground and excited state are the same [18].

Phosphorescence involves excited and ground states with different spin multiplicities. The phosphorescence phenomenon light is emitted over a longer period of time since the excited molecule undergoes a transition to a different excited state; The radiative decay (hν A in Figure

(1.2)) from the triplet state is a slow process:the return to the singlet ground state is spin-forbidden.

(iii) Chemical change, when energy results in changes in bonding structures, or a combination of these. [18]

The photophysical processes occurring from absorption to emission are- often shown in a so-called Jablonski diagram, which is a simplified representation of the relative positions of electronic and vibrational energy levels of a molecule (Figure (1.2)). [18]

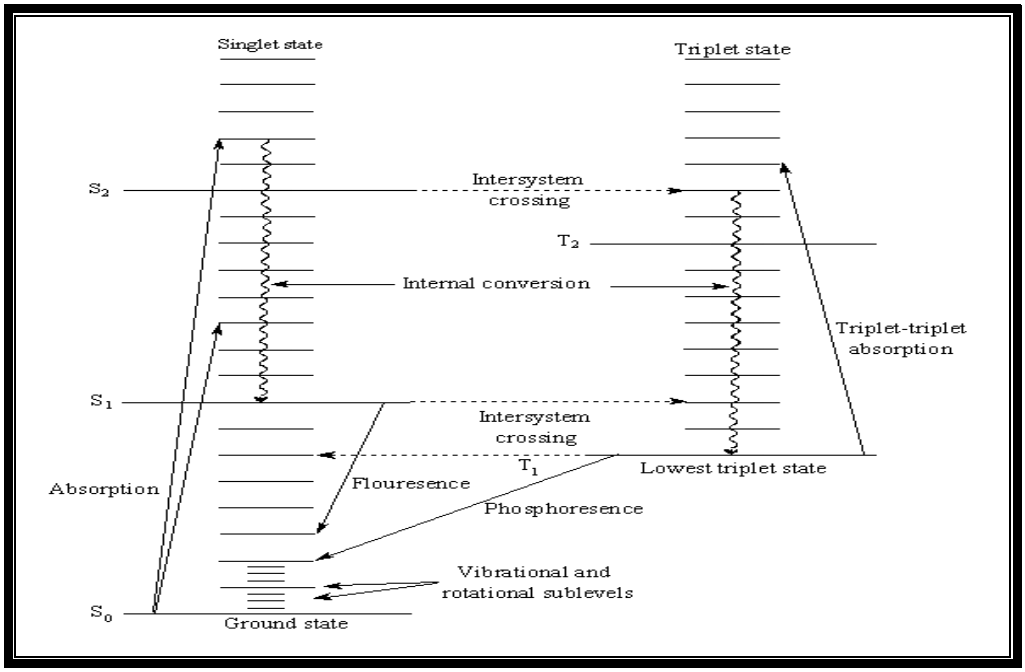


Figure (1.2): Simplified Jablonski diagram.

Where: S_0 is singlet ground state

S_1 and S_2 are the singlet first and second excited states

T_1, T_2 are the first and second triplet state ***(18).***

1.4.2 Photochemical Reactions of Riboflavin

Riboflavin (RF) exhibits strong absorption in the ultraviolet and visible region and undergoes degradation by complex photochemical reactions. These reactions involve intramolecular and intermolecular photoreduction, intramolecular and intermolecular photoaddition and intramolecular photodealkylation. The nature and magnitude of photochemical reactions are influenced by many factors including solvent polarity [19], pH of medium [20], buffer kind and concentration [21], ionic strength [22], oxygen content [23], and light intensities and wavelengths [24].

1.4.2.1 Photoreduction

1.4.2.1.1 Intramolecular photoreduction

This is the main photodegradation reaction undergone by RF and is the basis of its normal photolysis in aqueous solution under anaerobic conditions. Intramolecular photoreduction involves dehydrogenation of the N (10)-ribityl side chain to produce a variety of ketonic or aldehydic functions with simultaneous reduction of the isoalloxazine ring system. The oxidation of the side chain results in the formation of formylmethylflavin (FMF) as the major photoproduct and intermediate in the reaction [25]. FMF is reported to be more sensitive to photolysis than RF [26] and is hydrolysed to lumichrome (LC) and lumiflavin (LF) [27] and oxidized to carboxymethylflavin (CMF) [28]. The chemical structures of the photoproducts of RF are shown in Figure (1.3). It has been suggested that the primary process in RF photolysis involves the abstraction of a hydrogen atom from α- CH group resulting in the formation of a biradical intermediate [29], which subsequently disproportionates to give the reduced 2′ keto flavin (scheme (1.2)). It has been suggested that in phosphate buffer the reaction is catalyzed by $H_2PO_4^-$ species [30].

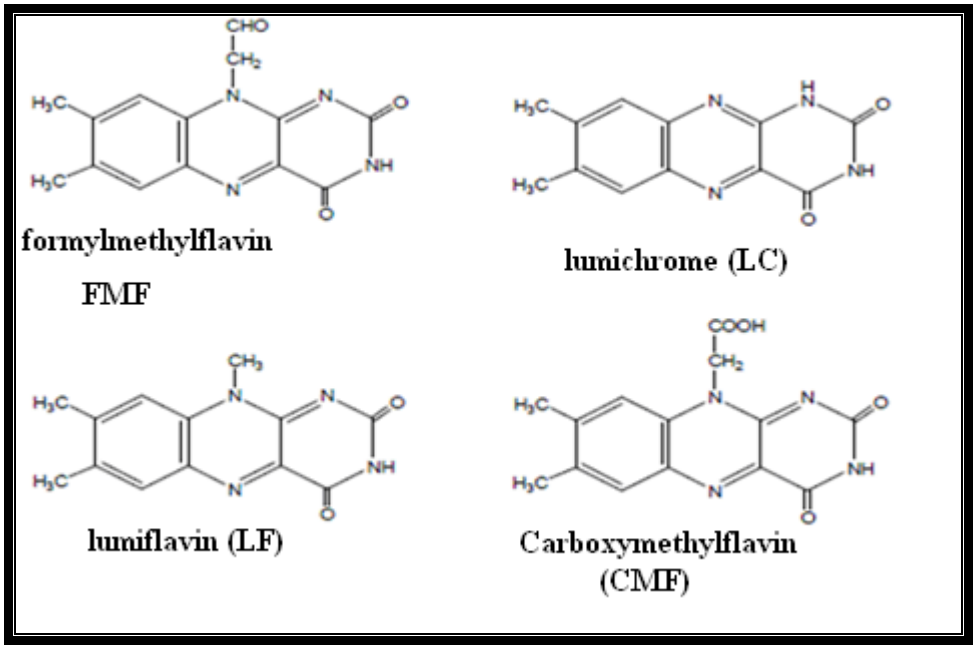
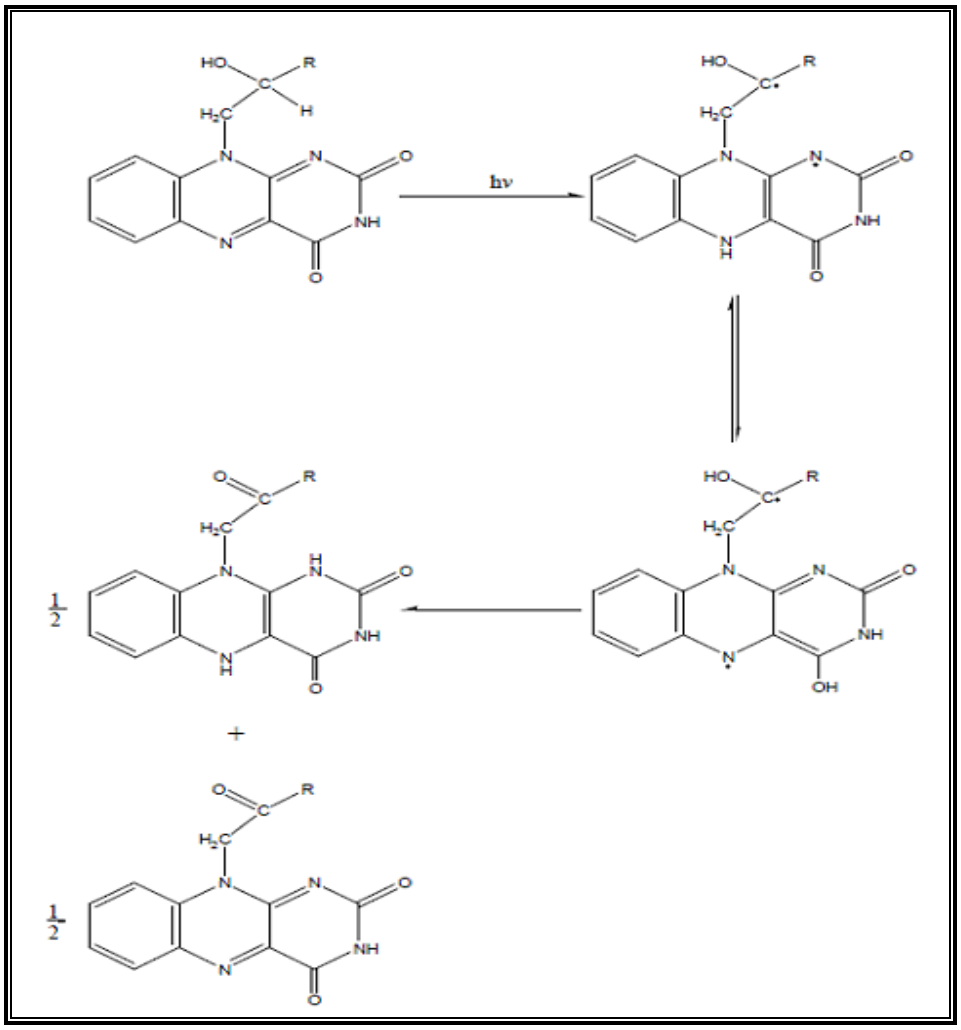


Figure (1.3): photoproducts of riboflavin [27].



Scheme (1.2): Intramolecular photoreduction of flavins via a one-electron transfer from the side chain to the nucleus [30].

1.4.2.1.2 Intermolecular photoreduction

RF or other flavins play an important role as the prosthetic group of a number of enzyme systems (e.g. FMN, FAD) that are involved in biological oxidation-reduction processes. Intermolecular photo reduction occurs by the uptake of two electron equivalents by a flavin (FL) from an external donor to produce a fully reduced flavin (e.g. 1,5-FLH_2).

$$FL + RH \rightarrow FLH_2 + R\text{–products} \qquad (1.1)$$

In this reaction several compounds can act as donors (RH), including amino acids, α- hydroxycarboxylic acids, thiols, aldehydes and unsaturated hydrocarbons. Most of the reduced flavins are oxidized by molecular oxygen under certain conditions.

$$FLH_2 + O_2 \rightarrow H_2O_2 + FL_{ox} \qquad (1.2)$$

It has been suggested that the flavin triplet state is involved in the primary step of intermolecular photoreduction since it is quenched by electron donors [(29)].

The intermolecular photoreduction of flavins proceeds through a one-electron equivalent (either a hydrogen atom, or an electron) transfer from the donor to the flavin triplet state (3F) to form a flavosemiquinone radical ($FLH^{\cdot}$) and subsequent dismutation of the flavosemiquinone radical yielding the fully reduced flavin.

$$3F + RH \rightarrow FLH^{\cdot} + R^{\cdot} \qquad (1.3)$$

$$2\ FLH^{\cdot} \rightarrow FLox + FLH_2 \qquad (1.4)$$

Simultaneous two-electron up take by the flavin triplet state occurs in the presence of powerful hydride donors such as borohydride to give HFL– [(31)]. The various primary reaction schemes involving dimeric triplet states, triplet-ground state reactions or triplet–triplet reactions contributing to the

overall photoreduction reactions of flavins have been discussed by Heelis (29).

1.4.2.2 Photoaddition

1.4.2.2.1 Intramolecular photoaddition

The intramolecular photoaddition reaction of RF involves photoaddition of the C(2′)-OH group at the C(9) position to form cyclodehydroriboflavin (CDRF) as shown in Figure (1.4). It has been stated that for riboflavin reaction occurs at pH >6 in the presence of divalent ions such as HPO^{2-} and SO4 [32]. The involvement of the excited singlet state ($1FL_{ox}$*) in this reaction has been suggested on the basis of the quenching studies. The presence of $HPO4^{2-}$ ions may facilitate the reorientation of C (2′)-OH group to affect photoaddition. The autoxidation of the dihydroflavin intermediate in the reaction results in the formation of CDRF.

$$RF \xrightarrow{HPO_4^{2-}} [RF\text{–}HPO_4^{2-}]\ \text{complex} \xrightarrow{h\nu} [^1FL_{ox}^*] \qquad (1.5)$$

$$[^1FL_{ox}^*] \longrightarrow [\text{Dihydroflavin}]\ \text{intermediate} \xrightarrow{\text{autoxidation}} \text{CDRF} \qquad (1.6)$$

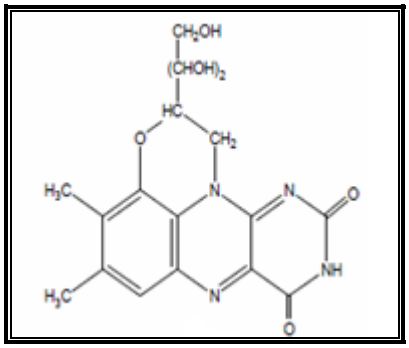


Figure (1.4): Cyclodehydroriboflavin [32].

1.4.2.2.2 Intermolecular photoaddition

Intermolecular photoaddition requires the addition of cyanide, ammonia, or water to the C (9) or C (16) positon of the benzenoid subnucleus of isoalloxazine ring system of flavins. It has been suggested that the cyanide or ammonia addition to the molecule occur through the triplet state in a single-step nucleophilic addition. This is followed by a C (9) → N (5) or C (16) → N (5) shift [(33)].

1.4.2.3 Photodealkylation

The photodealkylation of flavins is an intramolecular reaction and involves the loss of N (10) side chain to form alloxazines (e.g., lumichrome) and an alkene. The reaction occurs through synchronous breakage of the N (10)–C (1′) and C (2′)–H bonds in a cis periplanar confirmation via the excited singlet state [(34)].

1.5 Riboflavin-Sensitized Photoreactions

Riboflavin in the presence of oxygen plays an important role in photosensitized reactions involving a wide range of electron donating substrates [(35)]. In the presence of light, RF can exhibit photosensitizing properties through mixed Type I–Type II mechanisms [(36)].

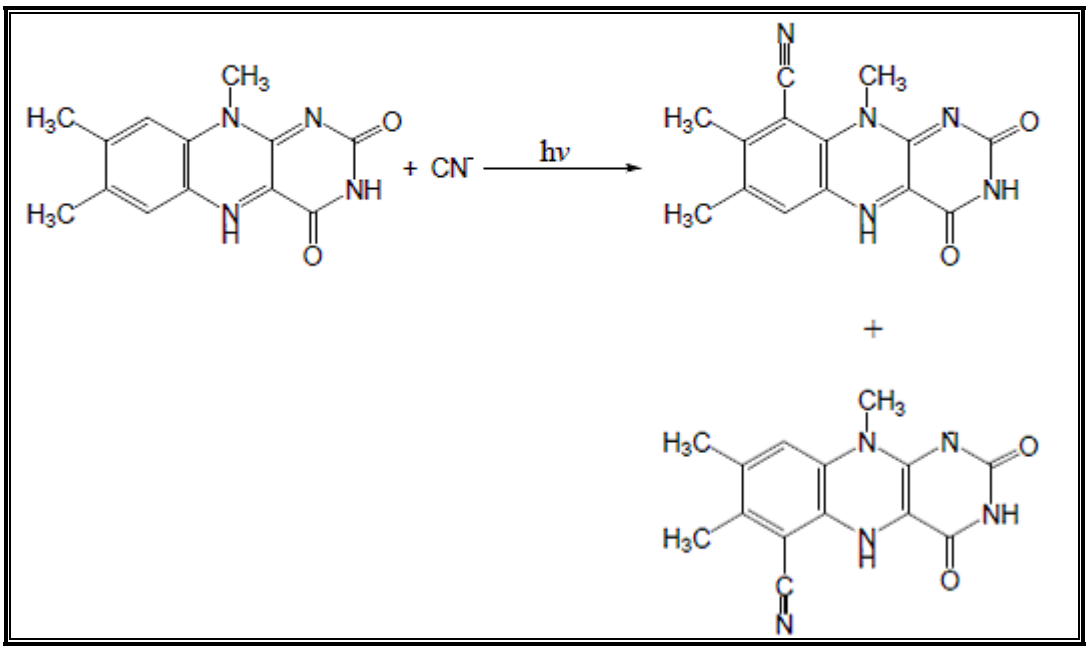


Figure (1.5): Intermolecular addition of cyanide ion to flavin nucleus

(33)

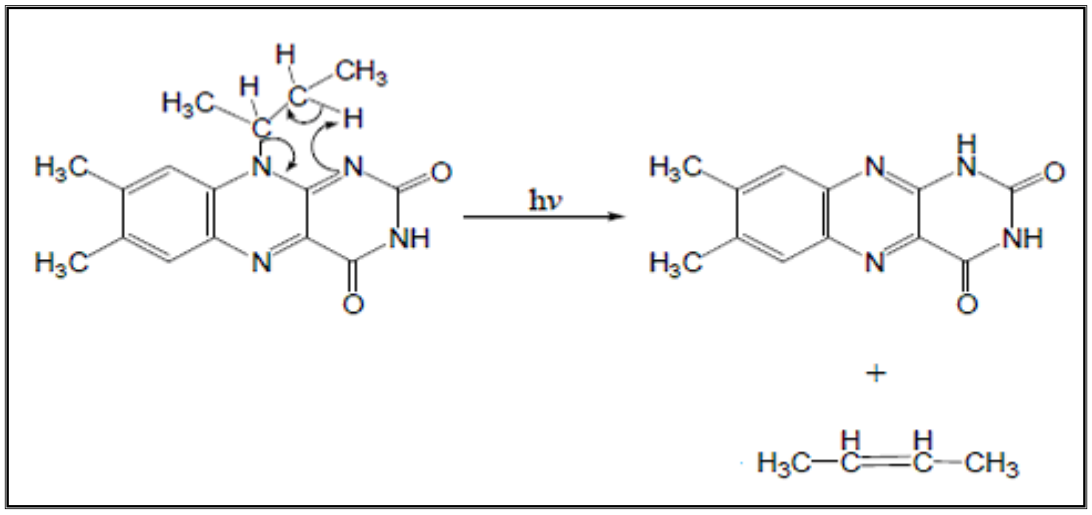


Figure (1.6): Dealkylation of N(10)- isobutyl substituted flavins. [34]

The Type I mechanism is favored at low oxygen concentrations. In the Type I process, substrate reacts with the sensitizer excited state (either singlet or triplet state) to give radicals or radical ions, respectively, by hydrogen atom or electron transfer. Reaction of these radicals with oxygen gives oxygenated products. In the Type II process, the excited sensitizer reacts with oxygen to form singlet molecular oxygen which then reacts with the substrate to form the products. The mechanisms of photosensitized

reactions induced by drugs have been discussed by Quintero and Miranda [37]. The reactions involved in Type I and Type II mechanisms are as follows:

Type I mechanism

$$RF + h\upsilon \rightarrow {}^1RF \rightarrow {}^3RF \quad (1.7)$$

$$^3RF + SH \rightarrow RF^{\cdot -} + SH^{\cdot} + \rightarrow RFH^{\cdot} + S^{\cdot} \quad (1.8)$$

$$RF^{\cdot -} + O_2 \rightarrow RF + O_2^{\cdot -} \quad (1.9)$$

$$2RFH^{\cdot} \rightarrow RF + RFH_2 \quad (1.10)$$

$$RFH_2 + O_2 \rightarrow RF + H_2O_2 \quad (1.11)$$

$$H_2O_2 + O_2^{\cdot} \longrightarrow {}^{-}OH + {}^{\cdot}OH + O_2 \quad (1.12)$$

$$S^{\cdot} \text{ and / or } SH^{\cdot +} + H_2O_2 / O_2^{\cdot -} \rightarrow S_{oxid} \quad (1.13)$$

Type II mechanism

$$RF + h\upsilon \rightarrow {}^1RF \rightarrow {}^3RF \quad (1.14)$$

$$^3RF + O_2 \rightarrow RF + {}^1O_2 \quad (1.15)$$

$$SH + {}^1O_2 \rightarrow S_{oxid} \quad (1.16)$$

In these reactions, RF, 1RF and 3RF represent RF in the ground state and in the excited singlet and triplet states, respectively; $RF^{\cdot -}$, $RFH^{\cdot}$ and RFH_2 are the radical anion, the radical and the reduced form of RF, SH is the reduced substrate and $SH^{\cdot +}$, $S^{\cdot}$ and S_{oxid} represent the intermediate radical cation, the radical and the oxidized form of the substrate, respectively.

The details of riboflavin-sensitized photo-reactions are given in the following sections:

1.5.1 Photooxidation

The RF-sensitized photooxidation reactions involving free-radical or singlet oxygen mediated mechanisms are the most widely investigated reactions [38]. These include the photooxidation of proteins such as eye lens crystallins [39].

1.5.2 Photodecarboxylation

Flavins are involved in the sensitized photodecarboxylation of α substituted acetic acids by a radical mechanism [40].

1.5.3 Photoisomerization

Bilirubin undergoes photoisomerization in the presence of RF. This is followed by photooxidation of bilirubin to products containing tetrapyrrole skeleton via biliverdin as an intermediate [41].

1.5.4 Photomonomerization

The flavin-sensitized photomonomerization of the *cis*, *syn*-cyclobutane dimer of 1,3-dimethylthymine using riboflavin tetraacetate and a 5-deazaflavin derivative has been reported [42].

1.5.5 Photopolymerization

Aliphatic and aromatic amines undergo photopolymerisation in the presence of RF [43].

1.5.6 Photodegradation

Flavins mediate photosensitized degradation of a number of compounds including hydroxyflavones [44], sulpha drugs [45], amines [46], and retinoids[47].

1.5.7 Photoinitiation

Riboflavin acts as a photoinitiator in the polymerization of acrylamide and 2-hydroxyethyl methacrylate [48].

1.5.8 Photoinactivation

Martin *et al*.[49] have reported the action spectrum of RF-sensitized photoinactivation of lambda phage. RF is involved in the inactivation of blood components [50], and stress kinases [51].

1.5.9 Photoinduction

RF has been found to form photoinduced adducts with lens proteins, tryptophan, and indole-3-acetic acid. The photoproducts of indole-3-acetic acid-riboflavin induce tumor cell death by an apoptotic mechanism. The effect produced has been found to be greater than those of the tryptophan–riboflavin photoproducts [52].

1.5.10 Photomodification

RF takes part in the photosensitized modification of lens proteins. The irradiation of these proteins in the presence of RF leads to modification of the proteins with an increase in the proportion of the high molecular weight fraction [53].

1.6 Photostabilization of Riboflavin

The photostabilzation of drug substances in a liquid dosage form may be achieved by the use of UV absorbers, complexation with other drugs and inclusion compounds and other methods [(54)]. The forces involved in complex formation of two molecules include van der Walls forces, dipole-dipole interactions, hydrogen bonding, Coulomb forces and hydrophobic interactions. Considerable interest has been generated in the use of cyclodextrins to form inclusion complexes with a drug to improve its photostability. Attempts have been made to photostabilize riboflavin solutions by the use of UV absorbers [(55)], cyclodextrin inclusion complexes [(56),] and others.

1.7 Determination methods of riboflavin

1.7.1 *High-speed liquid chromatography*

Margherita G. *etal.*, developed and validated An HPLC method for the concurrent detection and quantitation of seven water-soluble vitamins (C, B1, B2, B5, B6, B9, and B12) in biological matrices (plasma and urine). Separation was achieved at 30°C on a reversed-phase C18-A column using combined isocratic and linear gradient elution with a mobile phase consisting of 0.01% TFA aqueous and 100% methanol. Total run time was 35 minutes. Detection was performed with diode array set at 280 nm. Each vitamin was quantitatively determined at its maximum wavelength. Spectral comparison was used for peak identification in real samples (24 plasma and urine samples from abstinent alcohol-dependent males). Interday and intraday precision were <4% and <7%, respectively, for all vitamins. Recovery percentages ranged from 93% to 100%. [(57)].

1.7.2 Isocratic HPLC method:

An isocratic HPLC method has been developed by Ashoor and welty, 1983. To determine riboflavin in eggs, whole milk, 2% fat milk, skim milk, dry milk, yogurt, cottage cheese, and cheddar cheese. The developed method involves acidification, centrifugation, and quantification of riboflavin in the supernatant with HPLC. The HPLC system consisted of a μ Bondapak C18 column, a solvent system of water-methanol-acetic acid (68:32:0.1 v/v), a flow rate of 1.0 ml/min, and a UV detector. The method is simple, rapid, sensitive, and specific for riboflavin. Recoveries of more than 90% were obtained in all samples analyzed [(58)].

1.7.3 gradient HPLC method

Shahnaz. *et al.,* described a rapid method to determine Water soluble vitamins riboflavin (B2), pyridoxine (B6), cyanocobalamin (B12) and folic acid in neutraceutical product simultaneously by using a rapid, precise and time saving new high performance liquid chromatographic method and its validation. The method involves gradient elution of mobile phase through C18 discovery column in a reverse phase chromatography with UV detection at 254 nm at ambient temperature. The ranges for quantification for B2, B6, B12 and folic acid were 0.13 mg g^{-1} (0.57-131 μg g^{-1}), 0.235 mg g^{-1} (3-235 μg g^{-1}), 7.94 x 10^{-2} mg g^{-1} (8-80 μg g^{-1}) and 9.66 x10^{-2} mg g^{-1} (10-97 μg g^{-1}) respectively For the validation of the method, linearity, precision, accuracy and robustness have been performed. The repeatability was measured in terms of RSD value. The RSD for all vitamins was below 1%. Recovery of vitamins ranges from 98.6 to 100.5%. [(59)].

1.7.4 Thin-layer chromatography

Diaz M. *et al.,* [60] mentioned that thiamine (vitamin B1), riboflavin (vitamin B2) and niacin (nicotinic acid) were separated by thin-layer chromatography and fluorimetrically determined by using a commercially available fibre-optic-based instrument. Under Ruorimetric monitoring riboflavin shows native fluorescence, but nicotinic acid and thiamine had to be pre-chromatographically converted to fluorescent derivatives. A new fluorescent tracer, fluoresceinamine, isomer II, was used to label the nicotinic acid. Thiamine was converted to fluorescent thiochrome by oxidizing with potassium ferricyanide solution in aqueous sodium hydroxide. The analytes were separated on HPTLC silica gel plates using methanol-water (70:30, v/v) as mobile phase [60].

1.7.5 Photochemical spectrophotometric method

Photochemical spectrophotometric determination of riboflavin and riboflavin 5′-phosphate by manual and flow injection methods:

The sensitizing effect of riboflavin (RF) and riboflavin 5′-phosphate (FMN) on the photo-oxidation of dianisidine was studied by Thomas. *et al.,* 1994)[61]. The rate of the photochemical reaction was monitored spectrophotometrically at 460 nm. The method allows the determination of RF and FMN in the range ($1 \times 10^{-7} – 5 \times 10^{-6}$) mol dm^{-3} with a relative standard deviation of about 0.68%. The method can be successfully adapted to flow injection. Manual and flow injection methods were satisfactorily applied to the determination of RF or FMN in fortified breads and pharmaceutical preparations. A possible mechanism for the sensitized photoreaction is proposed [61].

1.7.6 Spectrophotometric determination

Shah et al., described a simple, accurate, precise and cost effective UV-Visible spectrophotometric method for the estimation of Riboflavin raw material. The solvent used throughout the experiment was 0.1N NaOH, the absorption maxima of drug was found at 445 nm. Beer's law was obeyed in the range of 5ppm-30ppm. The developed method was successfully validated with respect to linearity, accuracy and precision. The method was validated and shown linearity in mentioned concentration. The correlation coefficient for Riboflavin was 0.999. The percentage relative standard deviation of inter-day precision range 0.66-1.04% and intra-day precision 1.05-1.39% both should be less than 2%. This validated method can be applicable for quantitative determination of the titled drug with respect to assay from or for their solid dosage forms [(62)].

1.7.7 Fluorometric method

Marc D. Friedman, *etal.*, described an advanced system which combined corneal cross-linking with riboflavin with fluorescence dosimetry, the ability to measure riboflavin diffusion within the cornea both before and during UVA treatment. A corneal cross-linking system utilizing a digital micromirror device (DMD) was assembled and used to measure diffusion coefficients of 0.1% riboflavin in 20% dextran in porcine eyes. A value of $(3.3 \pm 0.2) \times 10^{-7}$ cm^2/s was obtained for the stroma. Diffusion coefficients for the transepithelial formulation of 0.1% riboflavin in 0.44% saline and 0.02% BAK were also measured to be $4.7 \pm 0.3 \times 10^{-8}$ cm^2/s for epithelium only and $(4.6 \pm 0.4) \times 10^{-7}$ cm^2/s for

stroma only. Riboflavin consumption during a UVA treatment was also demonstrated [63].

1.7.8 Electrochemical method

Zhang,. *et al.,* 2010 [64] studied the electrochemical behavior of riboflavin (VB2) at a glassy carbon electrode modified with poly (3-methylthiophene) (P3MT) was investigated by cyclic voltammetry (CV) and differential pulse voltammetery (DPV). The poly (3-methylthiophene) modified glassy carbon electrode (P3MT/GCE) can greatly enhance the peak currents and the detection sensitivity of VB2 under optimal conditions.

1.8 Aim of the study

The aim of this work is to determine vitamin B2 (riboflavin) concentration directly and indirectly by its two photoderivatives (lumiflavine) in basic media and (lumichrome) in acidic media, using spectrophotometric and spectrofluorometric analysis methods to compare between the two techniques and establish the better feasibility and the more sensitivity. Study the photochemical properties of riboflavin by the two previous methods to determine the rate of the decomposition of the riboflavin to its two photoderivatives (lumiflavine and lumichrome) by the spectral changes during the irradiation process.

2.1 Chemicals

Vitamin B2 (riboflavin) powder was given from the state company for drugs industries and medical apppliances (samrra/Iraq).

All other chemicals and reagents were obtained from Aldrich chemical company, Inc.

2.2 Instruments and equipment used

1. Ultraviolet –Visible Spectroscopy (UV)

The UV-Visible spectra were measured using Shimadzu UV-VIS model 1650PC A-Ultraviolet Spectrophotometer in the range (200-900) nm (Japan).

2. Spectrofluorophotometer

Spectrofluorophotometer model (RF -1501), Shimadzu, (japan), with internal computer system was used.

3. PH meter

pHmeter, wtw series terminal 740 with muti 740,(Germany).

4. The Photolysis Apparatus

The photolysis apparatus (Iwasaki Electric Co., Ltd.) was used for irradiation of all the prepared riboflavin solutions at different pH.

2.3 Preparations and measurements of riboflavin standard solutions

1. Standard solution of 1.3×10^{-4} M riboflavin was prepared. A 50 mg of riboflavin standard was dissolved in deionized water and diluted to 1.0 L solution. Other solutions were prepared by subsequent dilution of this stock solution. These solutions were kept in cold and dark place until used; they were stable for these conditions.
2. Standard Solution of 3.3×10^{-5}M riboflavin was prepared at different pH solution ranging from 2 to 10 using phosphate buffer solution. The spectra of these solutions were measured between 300 to 700 nm.
3. Standard solutions of 1.32×10^{-4} M to 9×10^{-7}M riboflavin were prepared at pH7 using the same phosphate buffers, the absorbance of these solutions were measured at 445nm as maximum wavelength.
4. Standard solutions of 2.9×10^{-5} M to 2×10^{-7} M riboflavin were prepared at pH7 using a phosphate buffer, the intensity of the fluorescent was scanned at 445nm as excitation wavelength and 520 nm as emission wavelength.

2.4 Preparation of photodegradation standard solutions

1. Standard solutions of 5×10^{-5} M riboflavin were prepared at pH range (6.00-10.00) using phosphate buffer, and were exposed to UV radiation for several hours to measure the degradation of riboflavin at that pH range.

2. Standard solutions of 4.06×10^{-5} M to 5.00×10^{-6} M were prepared from the degradation product of riboflavin at pH9 and measure the absorbance at 359nm.
3. Standard solutions of 2×10^{-5} M to 5.00×10^{-6} M were prepared from the degradation products of riboflavin at pH9 and the fluorescence intensity were measured at 359 nm and 465 nm as excitation and emission wavelength respectively.
4. Standard solutions of 5×10^{-5} M were prepared at pH ranged from (2.00-4.00) using phosphate buffer, and were exposed to UV light for several hours to measure the degradation of riboflavin in acidic media.
5. Standard solutions of 4.8×10^{-5} M to 3.00×10^{-6} M were prepared from the degradation products of riboflavin at pH4 and their absorbance's were measured at 353nm.
6. Standard solutions of 4.8×10^{-5} M to 3.00×10^{-6} M were prepared from the degradation products of riboflavin at pH4 and their absorbance at 353 nm and 422 nm as excitation and emission wavelengths respectively.

2.5 Preparation of Buffer Solution:

A 2.71 g of KH_2PO_4 and 4.87g of K_2HPO_4 was dissolved in 500 ml deionized water to prepare a buffer solution of pH7, and use 0.1 M HCl and 0.1 M KOH to prepare other buffers of pH2,pH3, pH4, pH6, pH7, pH8, pH9, pH10. by using pH meter.

2.6 The Photolysis Apparatus

The photolysis apparatus used in this work is shown in Figure (2.1a). The apparatus is consisted of a high pressure mercury lamp (1000 W. Iwasaki Electric Co., Ltd., Japan) and collecting quartz lens. The lamp was installed in a fixed vertical position, while the lens was adjusted to produce efficiently parallel beams of light. The light was passed through a glass filter. This filter had been calibrated with the aid of Perkin Elmer 1301 UV-visible double beam spectrophotometer. Calibration has shown that the transmitted light was predominately at the wavelength range (230-400 nm). The transmitted spectrum of the filter is shown in Figure (2.1b).

The distilled water bath was kept at room temperature.

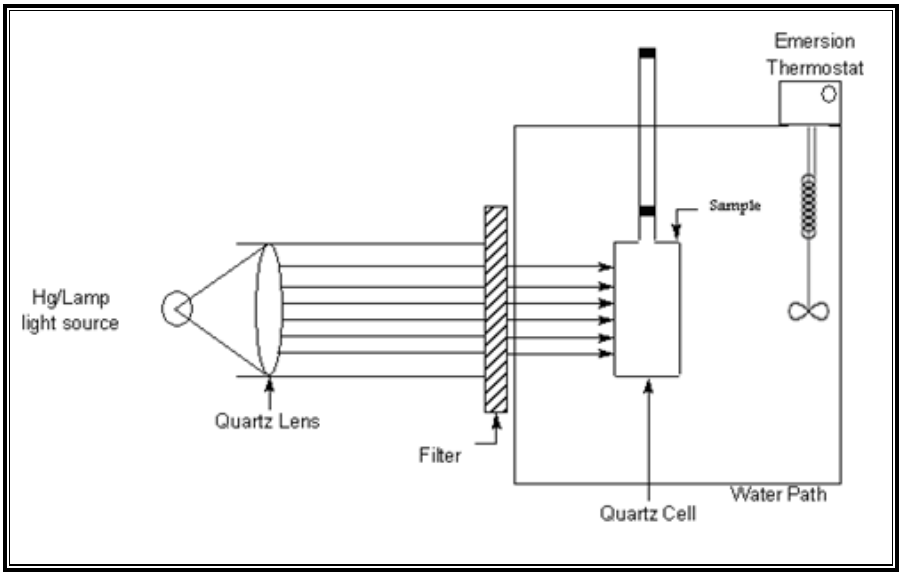


Figure (2.1): (a) Photolysis apparatus set up.

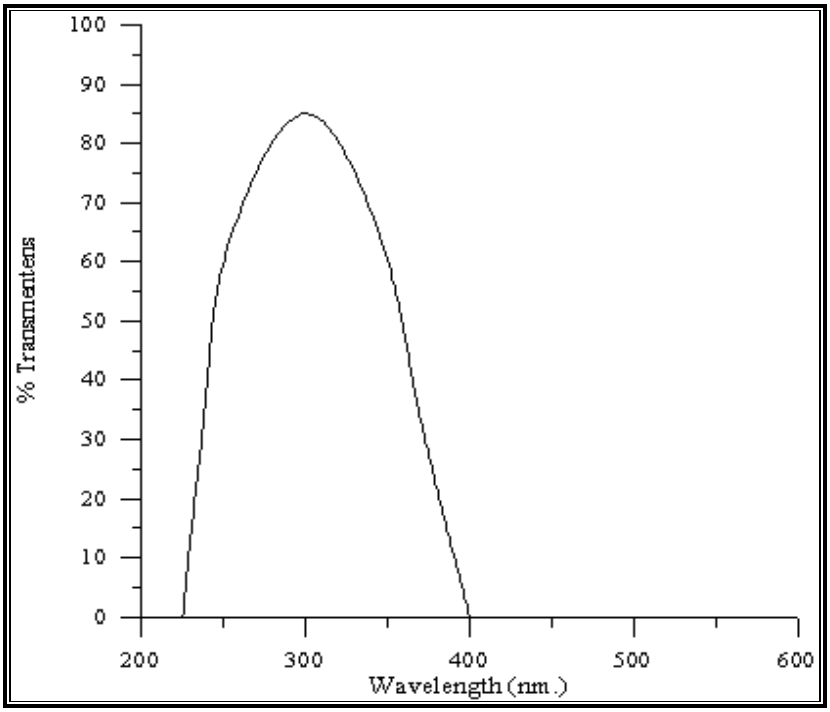


Figure (2.1): (b) Filter transmittance spectrum.

2.7 Incident light intensity measurement

The intensity of the incident light (I_0), was measured by the use of potassium ferrioxalate actinometer method as described by Hatchared and Parker [65].

The actinometer solution (6×10^{-3} mol/l) was prepared by dissolving (3 gm.) of $K_3Fe(C_2O_4)_3.3H_2O$ in 800 ml of distilled water. A 100 ml of 1 N of H_2SO_4 solution was added and the whole solution was diluted to one letter with distilled water. The actionmeter solution absorbs 100% of the incident light at $\lambda = 311$ nm.

The light intensity measurement involves irradiation of the actinometer solution for a known period of time 3 minutes. Ferrous ion concentration was estimated spectrophotometrically using 1,10-phenanthroline (0.1 %) as complexing agent. According to Hatchard and

Parker ferric ion is reduced to ferrouse ion ($Fe^{3+} \longrightarrow Fe^{2+}$) using hydroxyl ammine solution reagent.

A phenanthroline complex is formed with Fe^{2+} which strongly absorbs at 510 nm. For Fe^{2+} formation, the quantum yield was known to be equal to 1.21[(65)].

The intensity of incident light (I_0) was calculated according to the following method [(66)]. A 3 ml of actionmeter solution was irradiated in the irradiation cell. A stream of nitrogen bubbles in the solution was used to remove the dissolved oxygen gas.

After illumination, a 1 ml of irradiation solution was transferred to 25 ml volumetric flask, 0.4ml of hydroxyl ammonium chloride solution, 2 ml of 1, 10-phenathroline solution and 0.5 ml of buffer solution were added to the flask, and then diluted to 25 ml with distilled water. Blank solution was made by mixing 1 ml of unirradiated mixture solution with other components. The solution was left in dark for 30 mins. and then optical density (at λ = 510nm) was measured. The intensity of incident light was then calculated using the relationship [(66)] in equation (2.1) below:

$$I_0 = \frac{A \times V_2 \times N_A}{\phi_\lambda \times \varepsilon \times V_1 \times t \times d} \quad \text{Einstien } l^{-1}.sec^{-1}., \qquad (2.1)$$

Where:

I_0 = Incident light intensity (Einstien $l^{-1}.sec^{-1}$)

A = Absorbance at (λ = 510nm)

V_2 = Final volume (25 ml)

ϕ_λ = Quantum yield =1.21

ε = Molar extension coefficient (slope of the calibration curve),

V_1 = Volume taken from irradiated solution (1 ml)

t = Irradiation time in second

N_A= Avogadro`s number

d=Thickness of the cuvette(1cm)

The cell was irradiated in the same position used for irradiated samples. A calibration curve for Fe^{2+} was obtained using the following solutions:

1- 4×10^{-4} mol/L of $FeSO_4$ in 0.1 N H_2SO_4.

2- 0.1 % w/v phenanthroline monohydrate in water.

3- Hydroxyl ammonium chloride solution was prepared by dissolving 10g of hydroxyl ammonium chloride in 100ml of water.

4- Buffer solution was prepared from mixing 600 ml of 1N sodium acetate with 360 ml. 1 N H_2SO_4 diluted to 1 litter. Solutions of different concentrations of Fe^{2+} (ranged from 5.0×10^{-6} - 1×10^{-4} M) were prepared from solution (1) by taking different amounts in 25 ml volumetric flask, and each of the followings were added:

(a) a 0.4ml of hydroxyl ammonium chloride (b) a 2 ml Phenanthroline solution, (c) a 5 ml of buffer solution, (d) a 0.1 N H_2SO_4 to make the volume equal to 10 ml, diluting the whole solution with distilled water. The volumetric flask was covered with aluminum foil, and was kept in a dark for 30 minutes before the optical densities at λ = 510 nm were measured. A blank solution was used as a reference, which contained all ingredients except the ferrous ion solution.

The plot of optical density versus ferrous ion concentration was a straight line as shown in Figure (2.2). The slope of the line represents the extinction coefficient of $FeSO_4$ solution which was equal to ($\varepsilon=1.1027\times10^4$

$mol^{-1}.L.cm^{-1}$. This value is in good agreement with that reported by Hatchared and Parker [65].

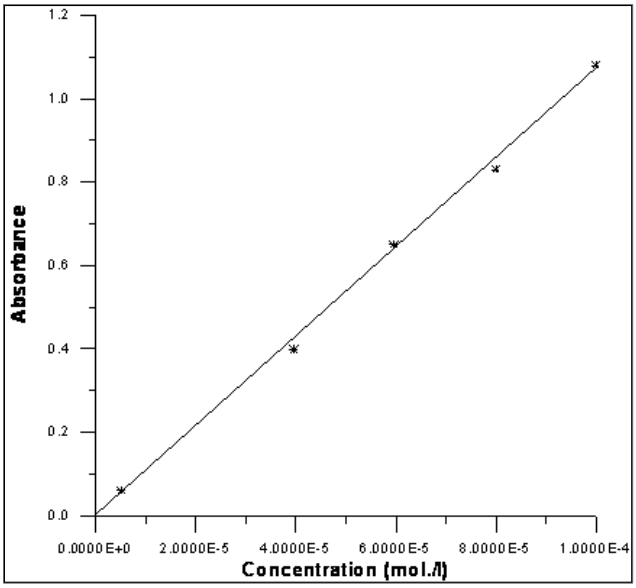


Figure (2.2): Calibration curve for Fe(II) complex at 510 nm.

2.8 The photodecomposition rate of Riboflavin using Ultraviolet –Visible spectrophotometer

The absorption spectrum of riboflavin at each pH (2.00-10.00) was measured in the range of (300-700) nm. The λ_{max} of riboflavin was recorded .The photodecomposition of the riboflavin at (λ_{max}) at room temperature in a proper solvent was followed with time using a covered quartz uv-cell of 1cm path length. A known concentration of riboflavin solution in the proper solvent was introduced in the cell after degassing by nitrogen for 20 minutes. The cell was closed tightly and the absorbance was recorded. Distilled water solvent was used as a reference in order to study the kinetic of photodecay of the riboflavin at different pH. The absorbance at

infinite time (A_∞) was measured after the solution was irradiated for at least 24 hour. The specific rate constant of the decomposition of the riboflavin (K_d) was determined by the following first order equation [67]:

$$\ln(a- x) = \ln a - K_d t \qquad (2.2)$$

Where:

a is the Concentration of riboflavin before irradiation.

(a-x) is he Concentration of riboflavin after t = time of irradiation.

t is the Time of irradiation of riboflavin solution.

A_0 is the Absorbance of riboflavin before irradiation.

A_t is the Absorbance of riboflavin after irradiation time.

ε is the Molar extension coefficient

a is the $(A_0 - A_\infty) / \varepsilon$, $x = (A_0 - A_t) / \varepsilon$, $a - x = (A_t - A_\infty) / \varepsilon$.

By substitution of a and a - x in equation (2.2) and rearrangement

$$\ln (A_t - A_\infty) = \ln(A_0 - A_\infty) - K_d t \qquad (2.3)$$

Thus a plot of $\ln |A_t - A_\infty|$ versus irradiation time (t) gives a line with a slope equal to K_d (S^{-1}). The rate of photodecomposition (R_d) was calculated for riboflavin at each pH using the following equation [67]:

$$R_d = K_d \times [\text{concentration of riboflavin}]$$

2.9 Decomposition Quantum yields (Q_d)

For the determination of the quantum yield, reactions were carried out in closed quartz uv-cell of 1cm path length so that the quantum input could be measured precisely; the absorbance was measured by the technique as described above.

A knowledge of the incident light intensity (determined by actinometry using the method of Hatchard and Packer [65] and the extinction coefficient of the compound enable the quantum input (I_{abs}) to be calculated.

The quantum yield of photodecomposition (Q_d) is defined by:

$$Q_d = \frac{\text{Rate of photodecomposition}}{\text{Quantum input}}$$

For riboflavin at each pH using the following equation (2.4) [68]:

$$Q_d = K_d \times [\text{conc.}] / I_{abs} \qquad (2.4)$$

The reactivity ratio (R_r) which equals (K_2/K_{-1}) was also calculated for riboflavin at each pH using the following equation [68]:

$$R_r = Q_d / 1 - Q_d \qquad (2.5)$$

3.1 UV-VIS spectrum of riboflavin

The UV-VIS spectrum of riboflavin solution has shown a maximum absorption at two different wave lengths 445 nm and 373 nm when scanning with a range of 300 -700 nm as shown in Figure (3.1).

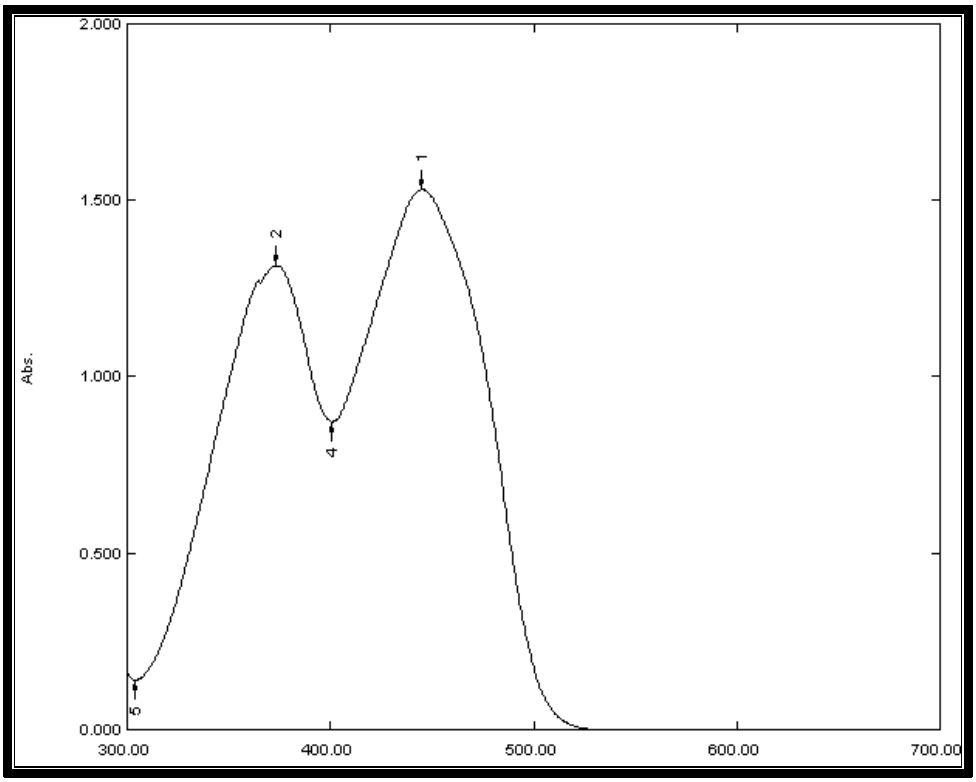


Figure (3.1): UV-VISIBLE spectrum of riboflavin.

From the Figure above, the highest peak is in the visible region of 445 nm. This wavelength was chosen for the determination of riboflavin in different solutions since this wavelength has a maximum sensitivity of measured absorbance (such as that at the absorption maxima) and minimum interference from instrumental factors that have been found to be ideal for the analytical work [(69)]. The molar absorptivity at this wave length was (11671 L/mol cm^{-1}).

The high value of the molar absorptivity of the riboflavin at maximum wavelength (445 nm) (i.e. $>10^4$ L/mol cm^{-1}) indicates that the

molecule is undergoing π–π* electronic transition [70]. Which is due to the high π system conjugation in riboflavin structure that make the π–π* more favorable than the n–π* electronic transition [71]. It has been suggested that the π–π* electronic transition of the near UV band at 375 nm may have some contribution from the n– π* electronic transition involving the N_1 non-bonding electrons. The red shift on moving to the protic solvents may result from the destabilization of the N_1 non-bonding electrons by hydrogen bond [70].

3.2 Effect of pH on riboflavin spectra

Standard solution of (5×10^{-5} M) riboflavin was prepared with different pH using phosphate buffer ranging from (2.00-10.00), the absorbance of these solutions were recorded at 445 nm and plotted versus pH as shown in Figure (3.2).

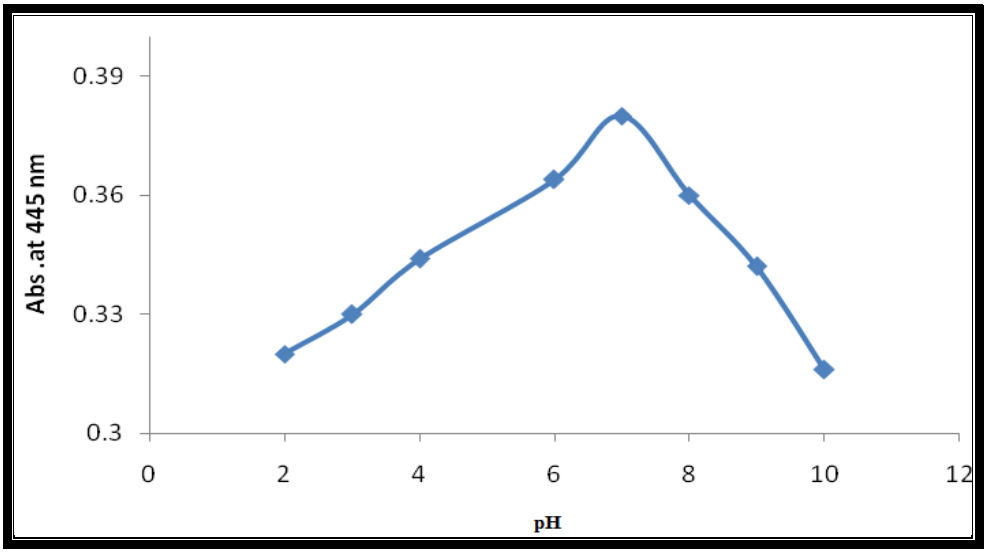


Figure (3.2): Absorbance of riboflavin at 445 nm at different pH.

The maximum absorption of riboflavin at 445 nm occurs at pH 7, then decreased with the increase of pH. This indicates that riboflavin is more suitable in neutral and acidic media than in basic media. Therefore

pH7 solution was selected for the quantitative determination of riboflavin in this work.

A calibration curve was then constructed using the absorbance at 445 nm for standard solutions prepared at pH7. A linear calibration curve was obtained from (9×10^{-7} to 1.32×10^{-4} M as shown in Figure (3.3) with straight line equation of:

$$Y= 11538X +0.0088 \ldots\ldots\ldots\ldots (3.1)$$

Where y represents the absorbance of riboflavin at 445 nm, X represents its molar concentration.

The correlation coefficient (R) for this line was 0.9999. The concentrations of two newly prepared standard solutions of riboflavin were measured using the above equation as shown in Table (3.1). The recovery of this method was found to be 98.68% with relative error of 1.31%.

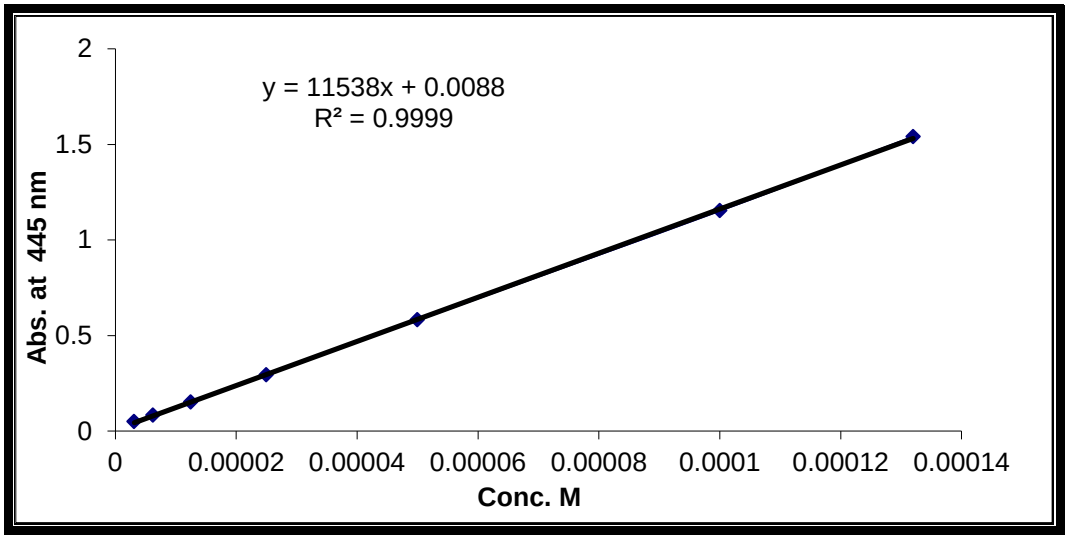


Figure (3.3): Calibration curve of riboflavin using spectrophotometric method

Table (3.1): Concentration of riboflavin in various prepared standard solutions using spectrophotometric method.

Sample	Concentration of vitamin B2		% Recovery	% Error	RSD%
	Prepared	Calculated*			
Stand.1	8×10^{-5}	7.93×10^{-5}	99.12	0.88	0.33
Stand.2	8×10^{-6}	7.86×10^{-6}	98.25	1.75	0.28

***Each concentration represents an average of three measurements**

3.3 Spectrofluorometric study of riboflavin

Spectrofluorometric scanning of (5×10^{-5} M) standard solution of riboflavin with excitation wavelength at 445 nm shows a maximum emission wavelength at 520 nm as shown in Figure (3.4).

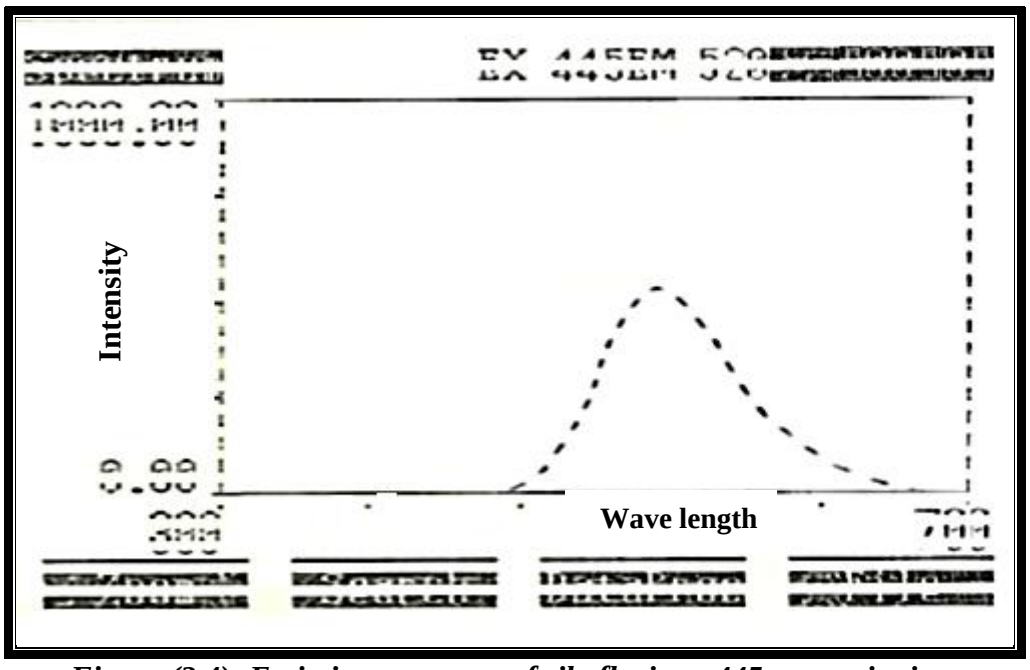


Figure (3.4): Emission spectrum of riboflavin at 445 nm excitation.

A calibration curve was constructed using spectrofluorometric method with excitation wave length of 445 nm and 520 nm as emission wavelength at pH7, as shown in Figure (3.5).

A linear calibration curve was obtained from (2×10^{-7} to 2.9×10^{-5}) M with linear equation of:

$$Y= 2X \times 10^{7}+ 32.581 \ldots\ldots\ldots\ldots (3.2)$$

Where Y represents the intensity of fluorescent light at 520 nm, X represents the riboflavin molar concentration; the correlation coefficient R for this straight line was 0.9997. The concentrations of two newly prepared standard solutions of riboflavin were measured using the above equation as shown in Table (3.2). The recovery of this method was found to be 99.68% with relative error of 0.315%.

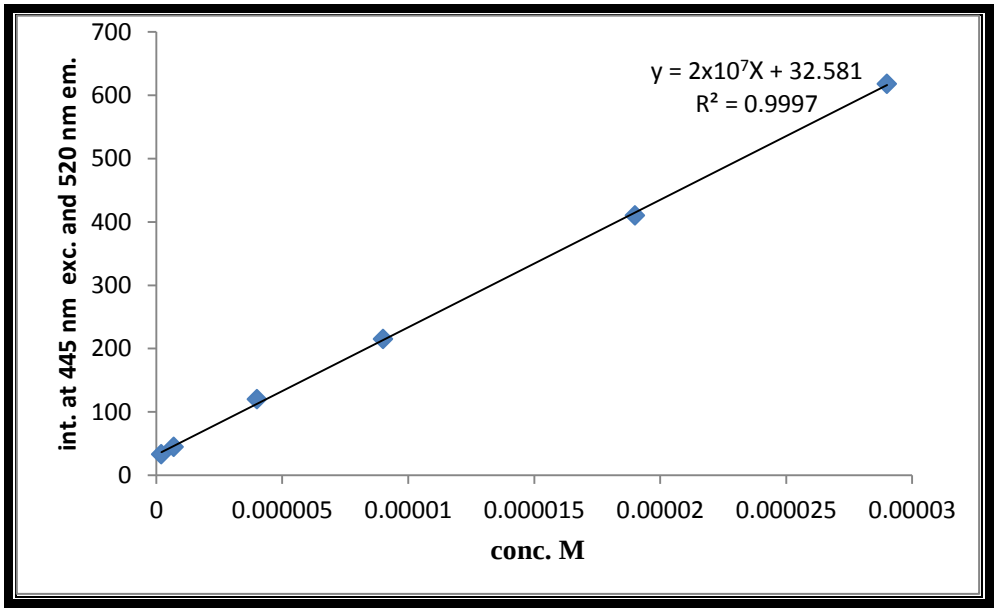


Figure (3.5): Calibration curve of riboflavin using spectroflourometric method.

Table (3.2): Concentration of riboflavin in various prepared standard solutions using spectrofluorometric method.

sample	Concentration of vitamin B2		% Recovery	% Error	RSD%
	prepared	calculated			
Stand.1	8×10^{-5}	7.99×10^{-5}	99.87	0.13	0.88
Stand.2	8×10^{-6}	7.96×10^{-6}	99.5	0.5	0.46

***Each concentration represents an average of three measurements**

3.4 Spectrophotometeric measurements

On irradiation of 5×10^{-5} M of riboflavin in phosphate buffer at different pH (2.00-10.00) at room temperature, the riboflavin absorption spectrum changes with the irradiation time. A decrease in the absorbance intensity was observed at wavelength of its maximum absorbance as shown in Figures (3.6-3.13) for ($\pi\rightarrow\pi^*$) transitions in all prepared solutions. Table (3.3) lists the changes in absorbance (A_t) values with the irradiation time. From these changes in absorbance, one could say that photoreduction and photodealkylation reaction occurs predominantly through the excited triplet state of riboflavin.

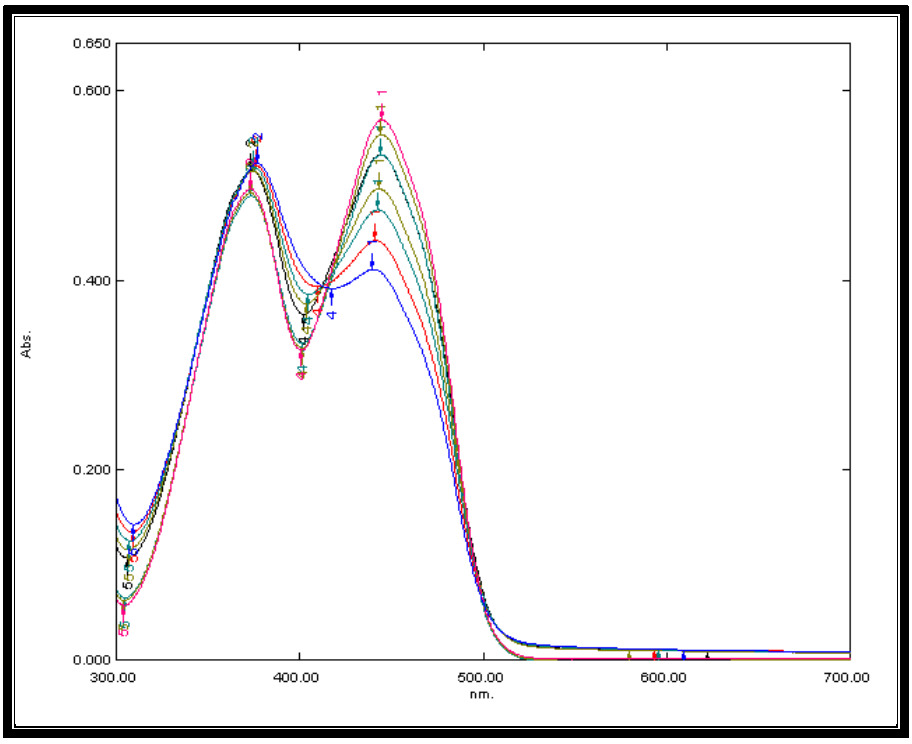


Figure (3.6): Photodegradation spectra of (5×10^{-5} M) riboflavin at pH2 after exposure for (1= 0hr, 2=0.5hr, 3=1 hr, 4=1.5 hr, 5=2hr, 6=2.5 hr, 7=3 hr, 8=3.5 hr).

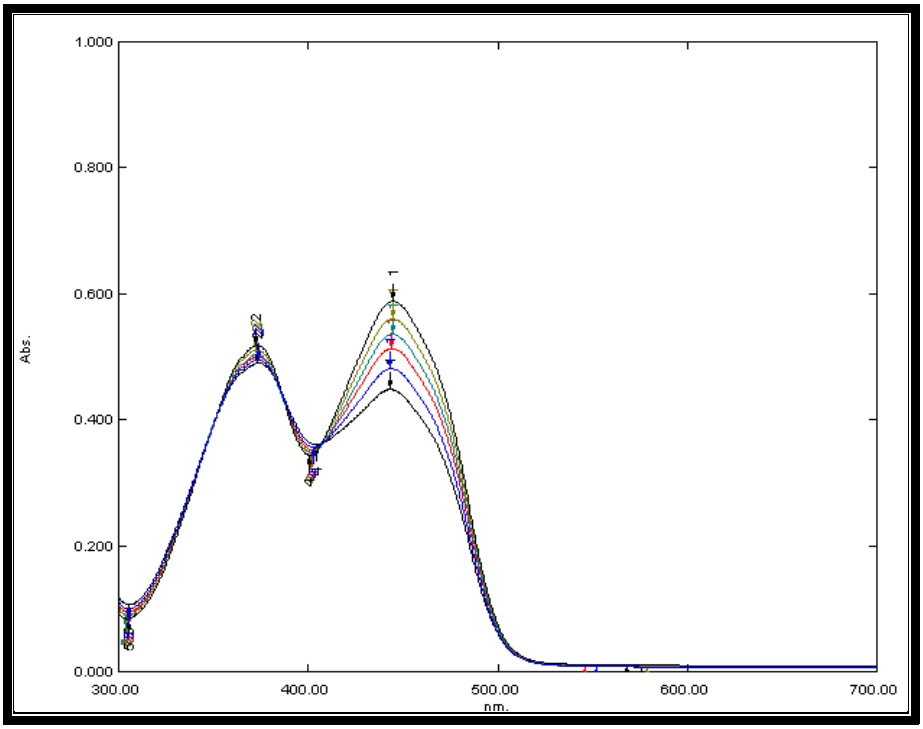


Figure (3.7): Photodegradation spectra of (5 × 10^{-5}M) riboflavin at pH3 after exposure for (1= 0hr, 2=0.5hr, 3=1 hr, 4=1.5 hr, 5=2hr, 6=2.5 hr, 7=3 hr, 8=3.5 hr).

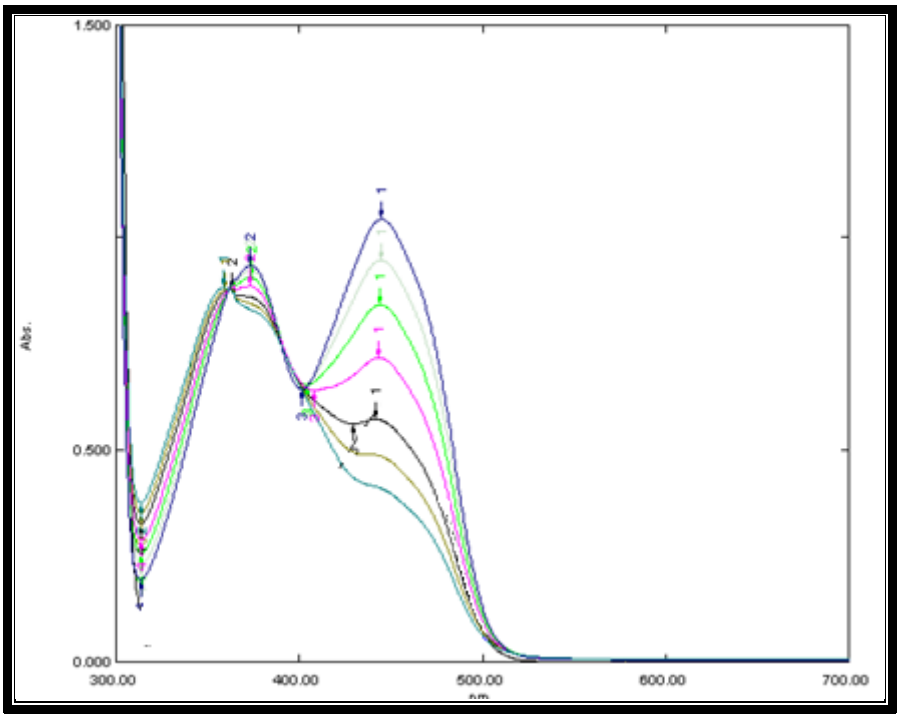


Figure (3.8): Photodegradation spectra of (5 × 10^{-5}M) riboflavin at pH4 after exposure for (1= 0hr, 2=0.5hr, 3=1 hr, 4=1.5 hr, 5=2hr, 6=2.5 hr, 7=3 hr, 8=3.5 hr).

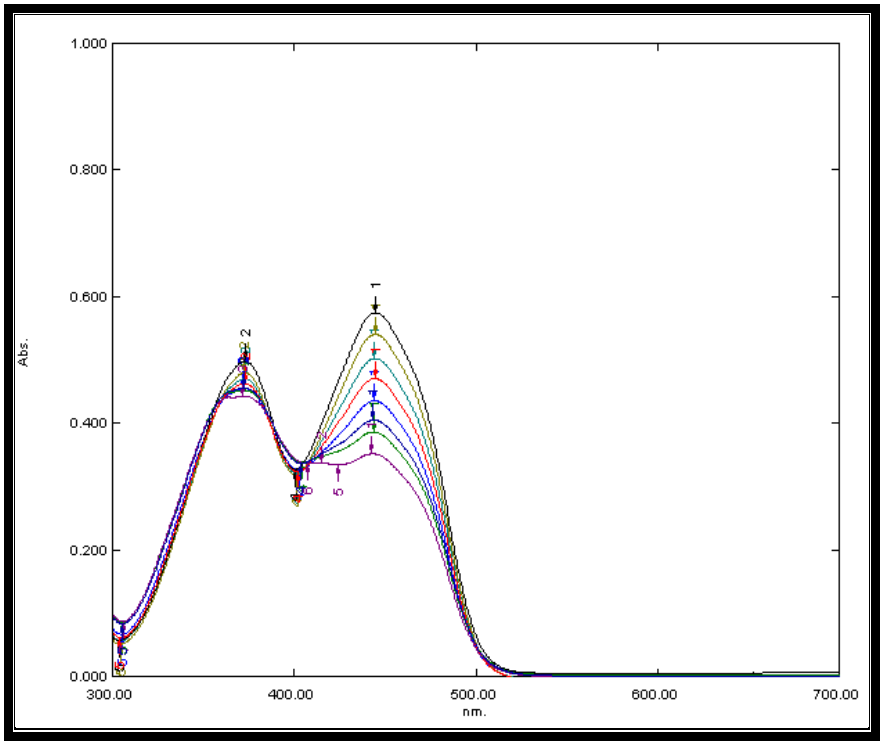


Figure (3.9): Photodegradation spectra of (5 × 10^{-5}M) riboflavin at pH6 after exposure for (1= 0hr, 2=0.5hr, 3=1 hr, 4=1.5 hr, 5=2hr, 6=2.5 hr, 7=3 hr, 8=3.5 hr).

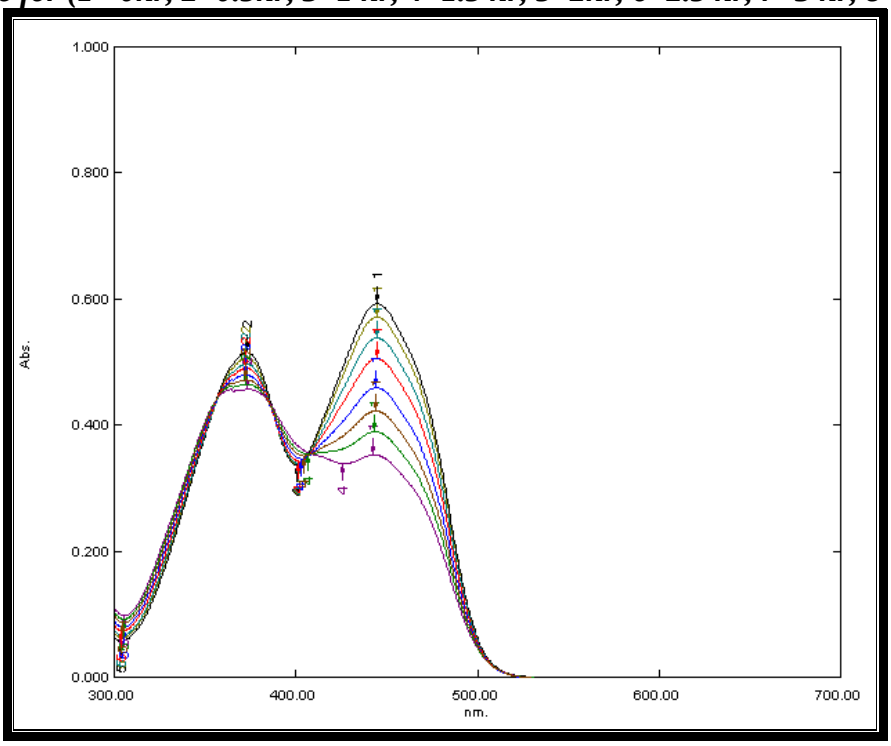


Figure (3.10): Photodegradation spectra of (5 × 10^{-5}M) riboflavin at pH7 after exposure for (1= 0hr, 2=0.5hr, 3=1 hr, 4=1.5 hr, 5=2hr, 6=2.5 hr, 7=3 hr, 8=3.5 hr).

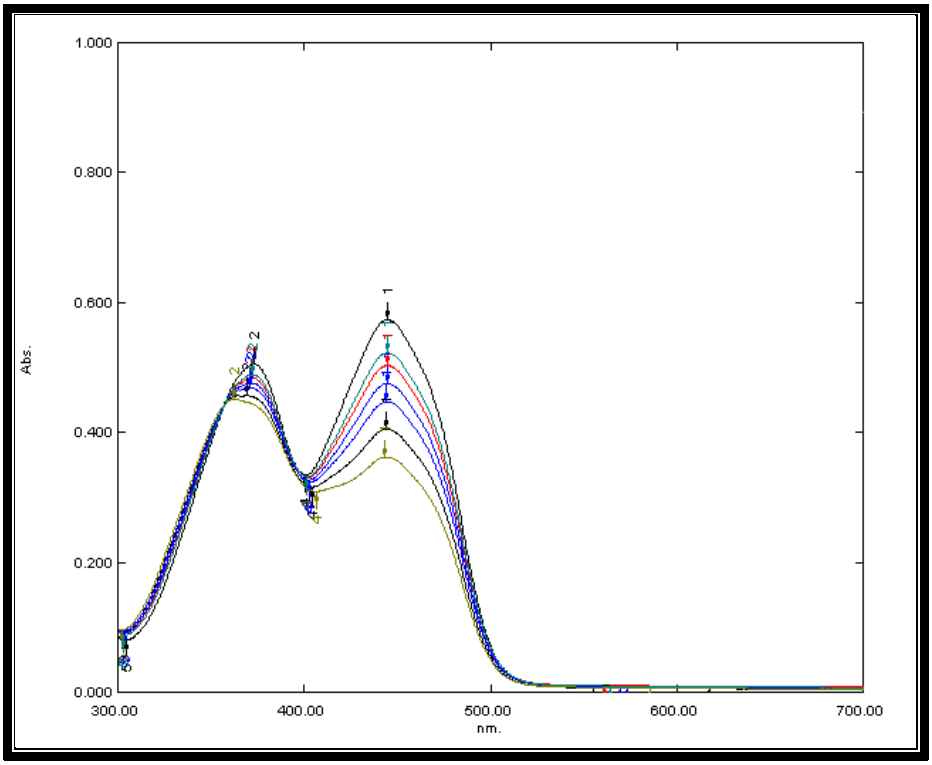


Figure (3.11): Photodegradation spectra of (5 × 10^{-5}M) riboflavin at pH8 after exposure for (1= 0hr, 2=0.5hr, 3=1 hr, 4=1.5 hr, 5=2hr, 6=2.5 hr, 7=3 hr, 8=3.5 hr).

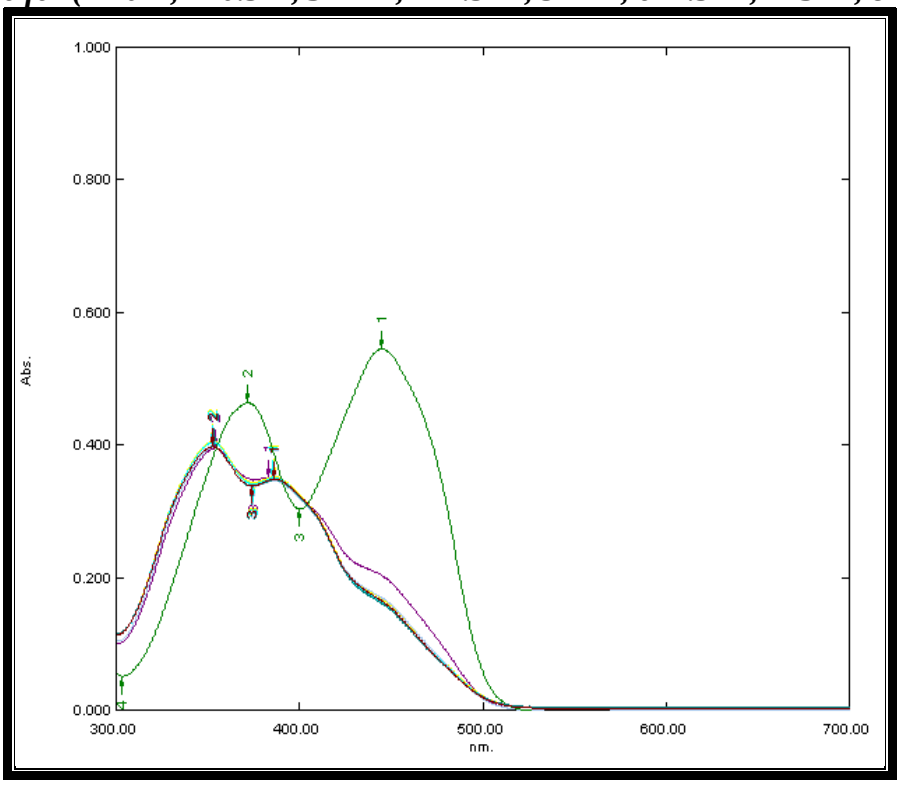


Figure (3.12): Photodegradation spectra of (5 × 10^{-5}M) riboflavin at pH9 after exposure for (1= 0hr, 2=0.5hr, 3=1 hr, 4=1.5 hr, 5=2hr, 6=2.5 hr, 7=3 hr, 8=3.5 hr).

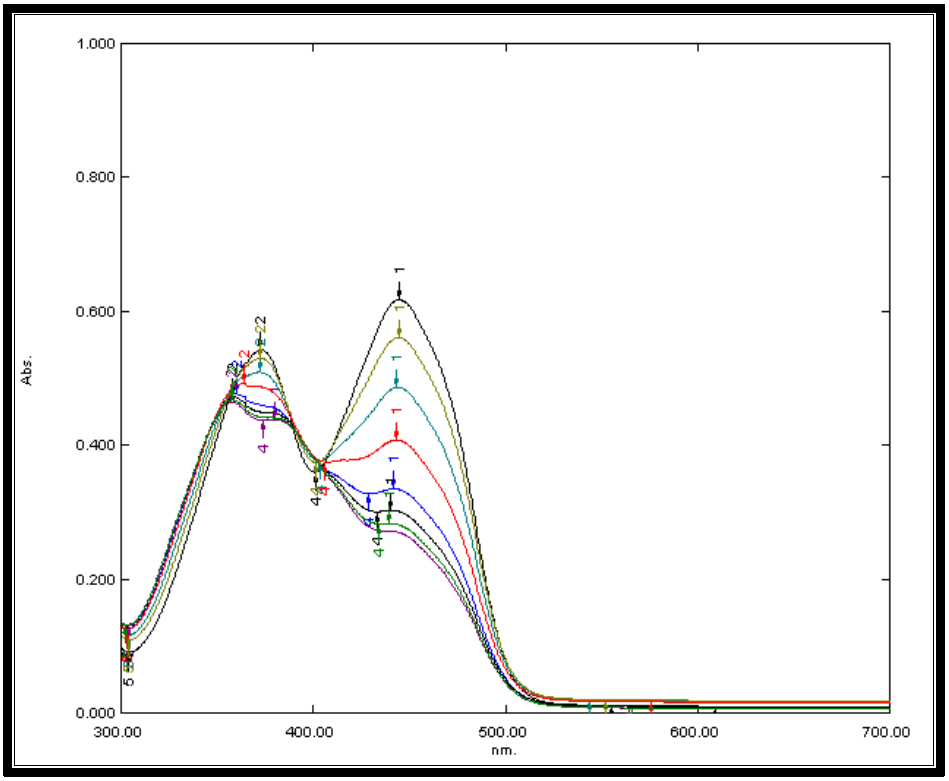


Figure (3.13): Photodegradation spectra of (5 × 10^{-5}M) riboflavin at pH10 after exposure for (1= 0hr, 2=0.5hr, 3=1 hr, 4=1.5 hr, 5=2hr, 6=2.5 hr, 7=3 hr, 8=3.5 hr).

Table (3.3): The absorbance of 5×10⁻⁵ M standard solution of riboflavin at different pH at 445 and 373 nm at each half hour of irradiation.

		Wave Length															
		445nm							373nm								
	Time (Sec)	0	**1800**	3600	5400	7200	9000	10800	0	1800	3600	5400	7200	9000	10800	12600
PH	10	**0.565**	**0.552**	**0.534**	**0.518**	**0.503**	**0.484**	**0.471**	**0.464**	**0.41**	**0.338**	**0.344**	**0.341**	**0.339**	**0.337**	**0.339**
	9	**0.569**	**0.526**	**0.488**	**0.461**	**0.425**	**0.398**	**0.369**	**0.48**	**0.47**	**0.48**	**0.48**	**0.47**	**0.46**	**0.45**	**0.44**
	8	**0.582**	**0.495**	**0.446**	**0.394**	**0.356**	**0.321**	**0.280**	**0.54**	**0.53**	**0.5**	**0.48**	**0.46**	**0.45**	**0.44**	**0.43**
	7	**0.62**	**0.547**	**0.473**	**0.415**	**0.342**	**0.299**	**0.256**	**0.51**	**0.5**	**0.498**	**0.49**	**0.48**	**0.47**	**0.46**	**0.45**
	6	**0.591**	**0.512**	**0.451**	**0.389**	**0.345**	**0.296**	**0.265**	**0.49**	**0.47**	**0.47**	**0.46**	**0.456**	**0.455**	**0.452**	**0.44**
	4	**0.583**	**0.533**	**0.478**	**0.442**	**0.396**	**0.366**	**0.333**	**0.56**	**0.53**	**0.52**	**0.5**	**0.48**	**0.46**	**0.44**	**0.43**
	3	**0.576**	**0.529**	**0.492**	**0.457**	**0.436**	**0.408**	**0.385**	**0.562**	**0.55**	**0.53**	**0.52**	**0.51**	**0.5**	**0.49**	**0.48**
	2	**0.573**	**0.549**	**0.524**	**0.502**	**0.477**	**0.454**	**0.431**	**0.553**	**0.532**	**0.52**	**0.52**	**0.52**	**0.52**	**0.52**	**0.517**

The absorbencies at infinite irradiation (A_∞) of each solution were measured after a period of more than 24 hours at 445 nm. Each absorbance was subtracted from (A_∞) as listed in Tables (3.4 - 3.11). The natural logarithm of each value was taken. To convert the negative values of the resulting logarithm, a value of two was added to each.

Table (3.4): Natural logarithm of absorbance with irradiation time of riboflavin at pH=2

Irradiation time(sec.)	**A_t**	**$(A_t - A_\infty)$**	**$In(A_t - A_\infty)$**	**$2+In\ (A_t - A_\infty)$**
0	**0.573**	**0.561**	**-0.578**	**1.421**
1800	**0.549**	**0.537**	**-0.621**	**1.378**
3600	**0.524**	**0.512**	**-0.669**	**1.330**
5400	**0. 502**	**0.490**	**-0.713**	**1.286**
7200	**0.477**	**0.465**	**-0.765**	**1.234**
9000	**0.454**	**0.442**	**-0.816**	**1.183**
10800	**0.431**	**0.419**	**-0.869**	**1.130**
12600	**0.418**	**0.406**	**-0.901**	**1.098**
	A_∞ = Absorbance at infinite time = 0.012			

Table (3.5): Natural logarithm of absorbance with irradiation time of riboflavin at pH=3

Irradiation time (sec.)	**A_t**	**$(A_t - A_\infty)$**	**$In(A_t - A_\infty)$**	***$2+In\ (A_t - A_\infty)$***
0	**0.576**	**0.562**	**-0.576**	**1.413**
1800	**0.529**	**0.515**	**-0.663**	**1.336**
3600	**0.492**	**0.478**	**-0.738**	**1.261**
5400	**0. 457**	**0.443**	**-0.814**	**1.185**
7200	**0.436**	**0.422**	**-0.862**	**1.137**
9000	**0.408**	**0.394**	**-0.931**	**1.068**
10800	**0.385**	**0.371**	**-0.991**	**1.008**
12600	**0.36**	**0.346**	**-1.061**	**0.938**
	A_∞ = Absorbance at infinite time = 0.014			

Table (3.6): Natural logarithm of absorbance with irradiation time of riboflavin at pH=4

Irradiation time(sec.)	**A_t**	**$(A_t - A_\infty)$**	**$In(A_t - A_\infty)$**	***$2+In\ (A_t - A_\infty)$***
0	**0.583**	**0.571**	**-0.560**	**1.439**
1800	**0.521**	**0.521**	**-0.652**	**1.347**
3600	**0.478**	**0.466**	**-0.763**	**1.236**
5400	**0. 442**	**0.430**	**-0.843**	**1.156**
7200	**0.396**	**0.384**	**-0.957**	**1.042**
9000	**0.366**	**0.354**	**-1.038**	**1.964**
10800	**0.333**	**0.321**	**-1.136**	**0.863**
12600	**0.310**	**0.298**	**-1.210**	**0.789**
	A_∞ = Absorbance at infinite time = 0.012			

Table (3.7): Natural logarithm of absorbance with irradiation time of riboflavin at pH=6

Irradiation time(sec.)	**A_t**	**$(A_t - A_\infty)$**	**$In(A_t - A_\infty)$**	**$2+In\ (A_t - A_\infty)$**
0	**0.591**	**0.578**	**-0.548**	**1.451**
1800	**0.512**	**0.499**	**-0.695**	**1.304**
3600	**0.451**	**0.438**	**-0.82554**	**1.174**
5400	**0.389**	**0.376**	**-0.978**	**1.021**
7200	**0.345**	**0.332**	**-1.102**	**0.897**
9000	**0.296**	**0.283**	**-1.262**	**0.737**
10800	**0.265**	**0.252**	**-1.378**	**0.621**
12600	**0.242**	**0.229**	**-1.474**	**0.525**
	A_∞ = Absorbance at infinite time = 0.013			

Table (3.8): Natural logarithm of absorbance with irradiation time of riboflavin at pH=7

Irradiation time(sec.)	A_t	$(A_t - A_\infty)$	$In(A_t - A_\infty)$	$2+In\ (A_t - A_\infty)$
0	0.62	0.61	-0.494	1.505
1800	0.547	0.537	-0.621	1.378
3600	0.473	0.463	-0.770	1.229
5400	0.415	0.405	-0.903	1.096
7200	0.342	0.332	-1.102	0.897
9000	0.299	0.289	-1.241	0.758
10800	0.256	0.246	-1.402	0.597
12600	0.221	0.211	-1.555	0.444

A_∞ = *Absorbance at infinite time = 0.01*

Table (3.9): Natural logarithm of absorbance with irradiation time of riboflavin at pH=8

Irradiation time(sec.)	A_t	$(A_t - A_\infty)$	$In(A_t - A_\infty)$	$2+In\ (A_t - A_\infty)$
0	0.582	0.57	-0.562	1.437
1800	0.495	0.483	-0.727	1.272
3600	0.446	0.434	-0.834	1.165
5400	0.394	0.382	-0.962	1.037
7200	0.356	0.344	-1.067	0.932
9000	0.321	0.309	-1.174	0.825
10800	0.280	0.268	-1.316	0.683
12600	0.250	0.238	-1.435	0.564

A_∞ = *Absorbance at infinite time = 0.012*

Table (3.10): Natural logarithm of absorbance with irradiation time of riboflavin at pH=9

Irradiation time(sec.)	A_t	$(A_t - A_\infty)$	$In(A_t - A_\infty)$	$2+In\ (A_t - A_\infty)$
0	0.569	0.555	-0.588	1.411
1800	0.526	0.512	-0.669	1.330
3600	0.488	0.474	-0.746	1. 253
5400	0. 461	0.447	-0.805	1.194
7200	0.425	0.411	-0.88916	1.110
9000	0.398	0.384	-0.95711	1.042
10800	0.369	0.355	-1.035	0.964
12600	0.351	0.337	-1.087	0.912
	A_∞ = Absorbance at infinite time = 0.014			

Table (3.11): Natural logarithm of absorbance with irradiation time of riboflavin at pH=10

Irradiation time(sec.)	A_t	$(A_t - A_\infty)$	$In(A_t - A_\infty)$	$2+In\ (A_t - A_\infty)$
0	0.565	0.555	-0.587	1.413
1800	0.552	0.539	-0.618	1.382
3600	0.534	0.521	-0.651	1. 349
5400	0. 518	0.505	-0.682	1.318
7200	0.503	0.49	-0.712	1.288
9000	0.482	0.469	-0.757	1.242
10800	0.471	0.454	-0.789	1.211
12600	0.456	0.443	-0.81419	1.185
	A_∞ = Absorbance at infinite time = 0.013			

These values were then plotted against irradiation time, as shown in Figures (3.14-3.21). The slopes of these plots represent the photodecomposition rate constant (K_d) of riboflavin at each pH.

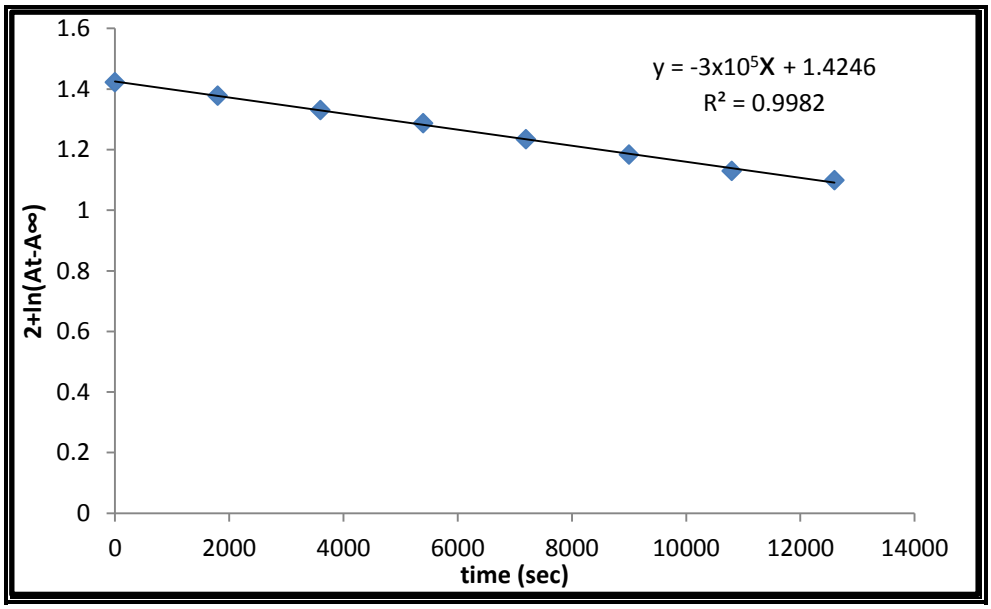


Figure (3.14): Plot of natural logarithmic of absorbance against irradiation time for riboflavin at pH2

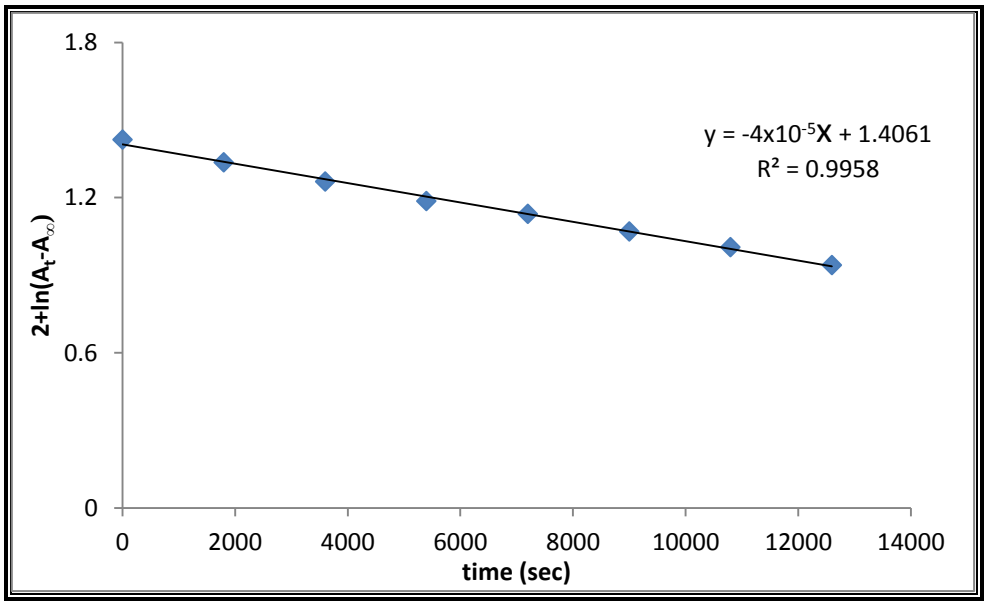


Figure (3.15): Plot of natural logarithmic of absorbance against irradiation time for riboflavin at pH3

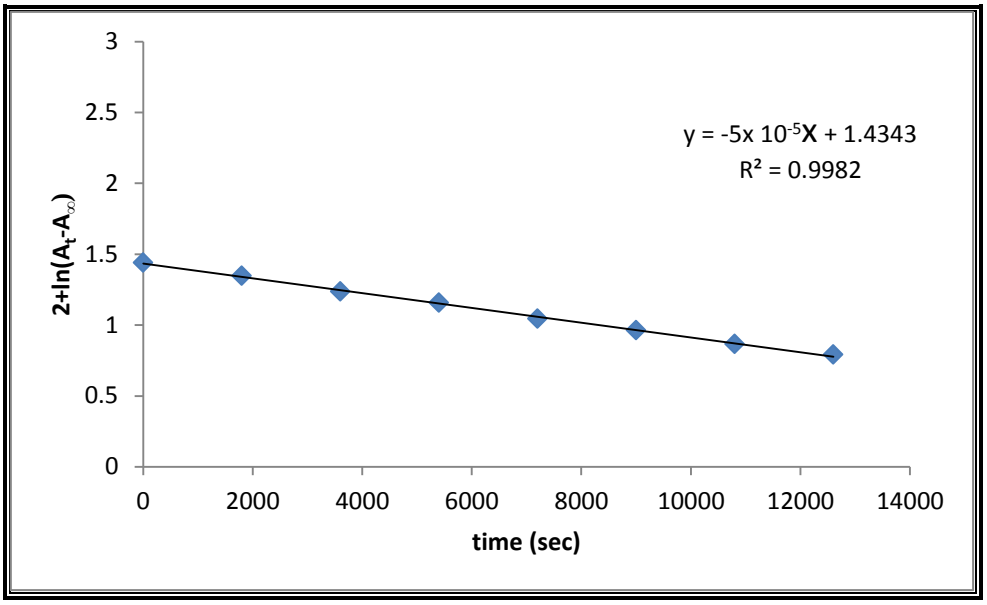


Figure (3.16): Plot of natural logarithmic of absorbance against irradiation time for riboflavin at pH4

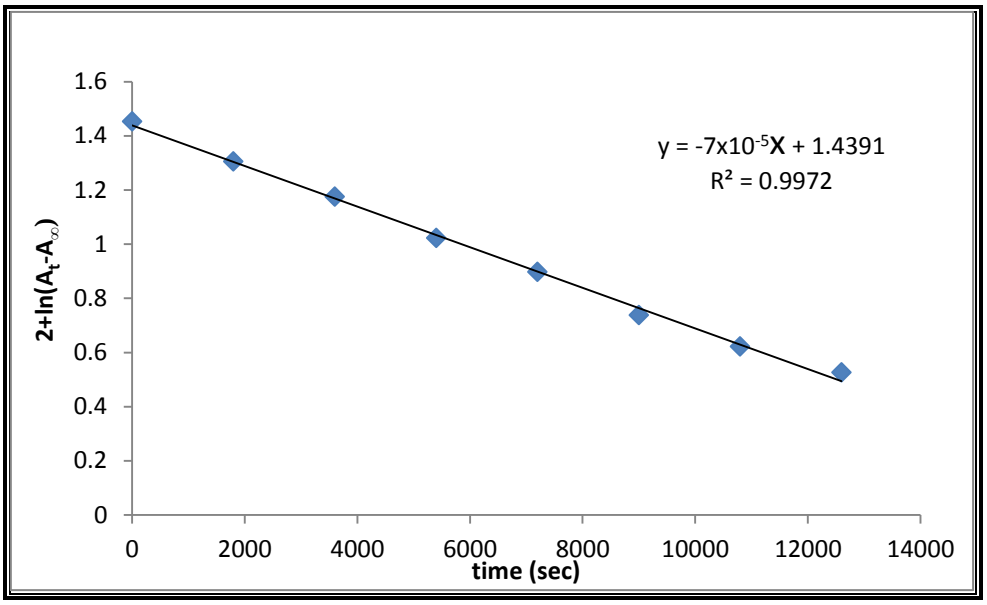


Figure (3.17): Plot of natural logarithmic of absorbance against irradiation time for riboflavin at pH6.

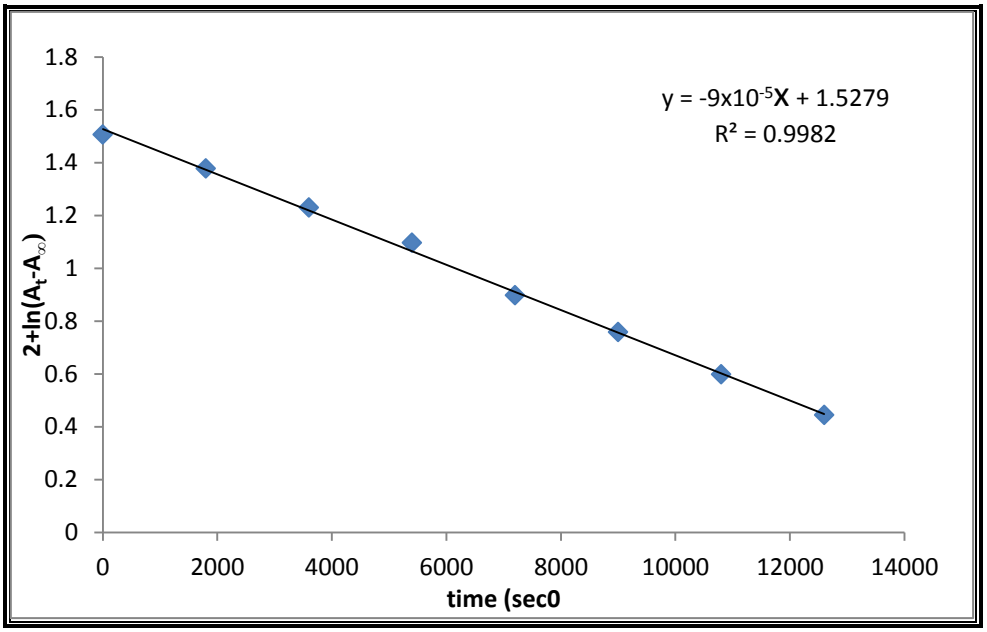


Figure (3.18): Plot of natural logarithmic of absorbance against irradiation time for riboflavin at pH7.

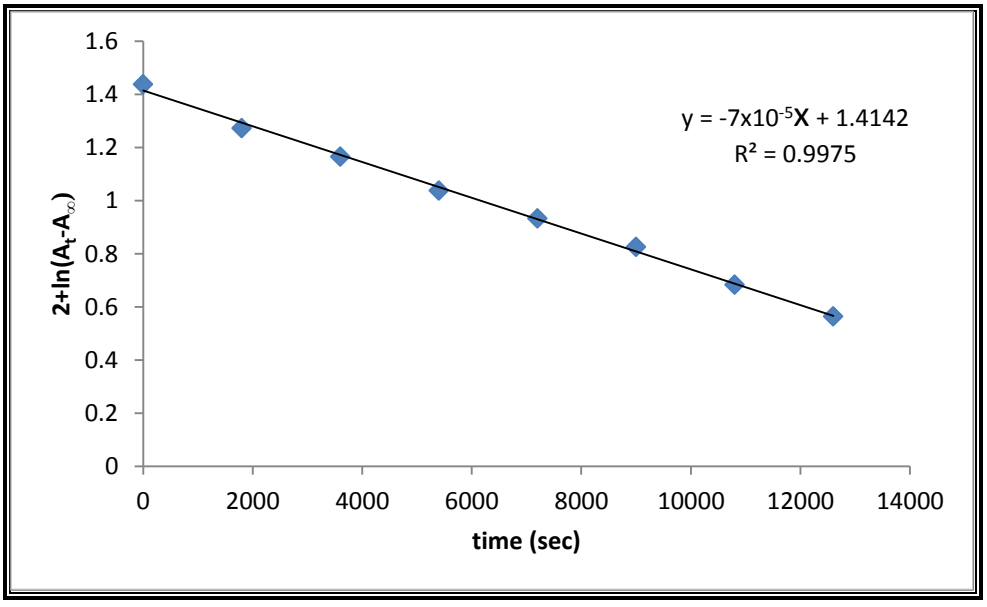


Figure (3.19): Plot of natural logarithmic of absorbance against irradiation time for riboflavin at pH8.

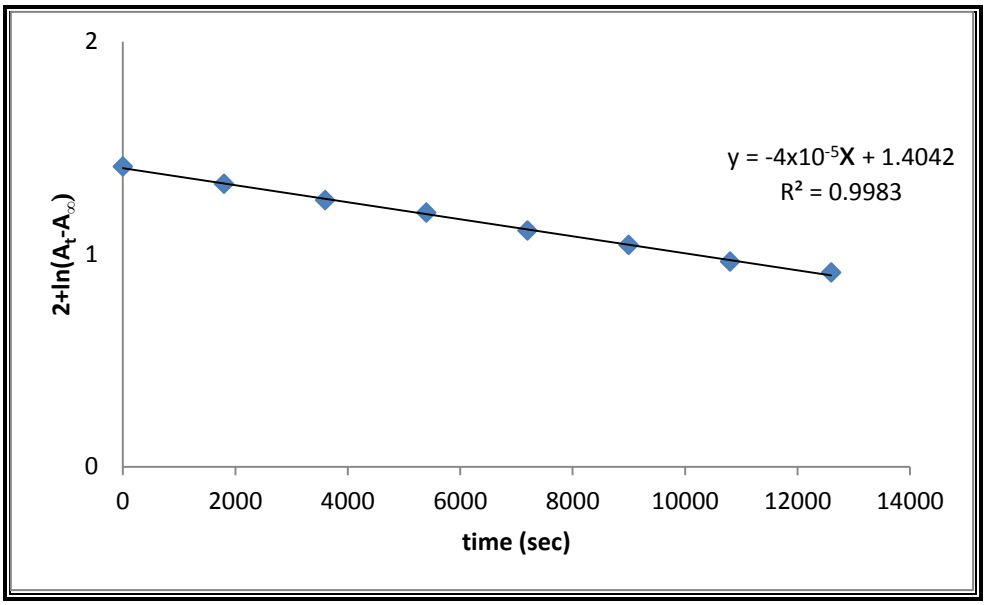


Figure (3.20): Plot of natural logarithmic of absorbance against irradiation time for riboflavin at pH9.

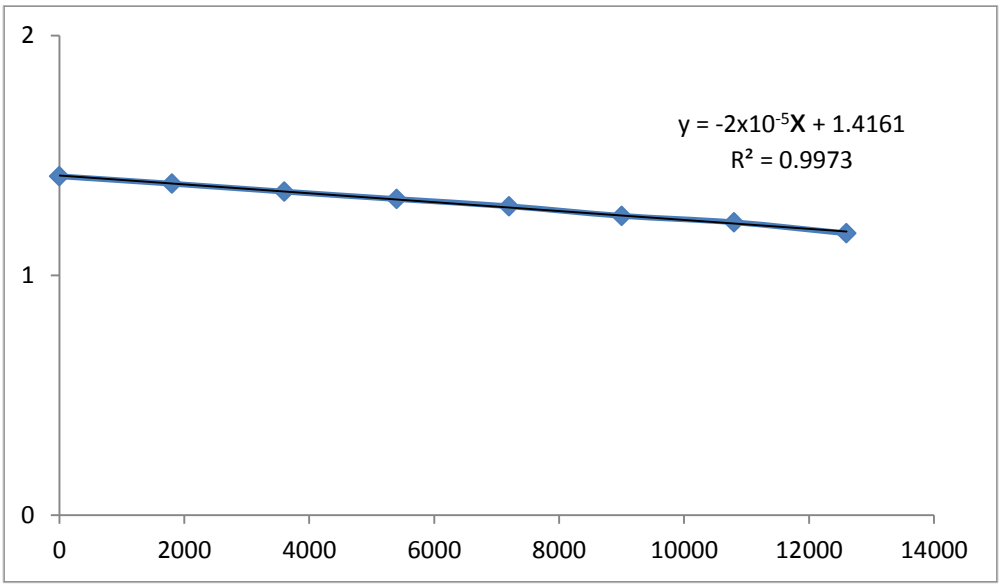
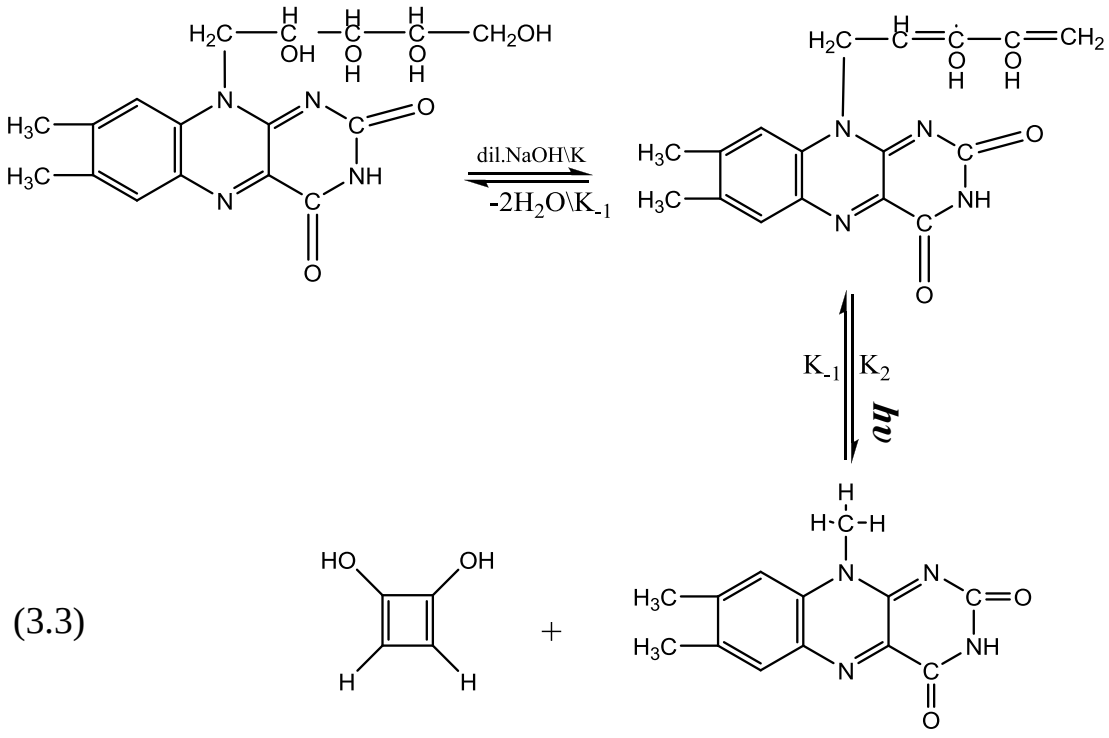


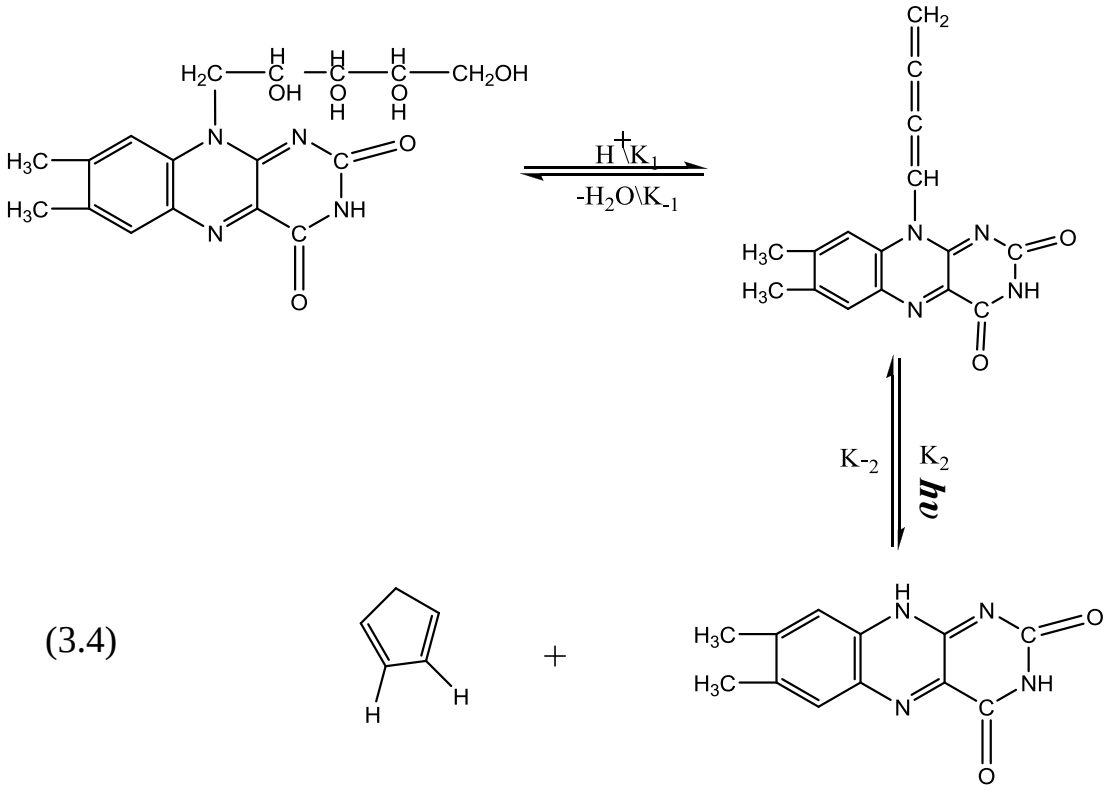
Figure (3.21): Plot of natural logarithmic of absorbance against irradiation time for riboflavin at pH10.

3.5 Kinetic of the photodecomposition reactions using UV-VISIBLE spectrophotemetric measurements

The change in the UV-VISIBLE absorptions spectra during irradiation were monitored through the photolysis experiments. The photolysis of riboflavin during the irradiation at 445±1 nm was followed by the change in the vitamin concentration spectrophotometrically, in order to determine the photodecomposition rate of riboflavin from this change. It was found that the value of (A_t-A_∞) decreased exponentially with irradiation time corresponding to the first order vitamin decomposition and was consistent with first order reaction. (3.3)



(3.3)



(3.4)

The straight lines are consistent with the first order riboflavin decomposition processes. Therefore, from the slopes of these straight lines, the values of specific rate constants (K_d) were evaluated. Using the value of (K_d), the rate of photodecomposition (R_d) were calculated (Rate $=K_d$ [Concentration of riboflavin]) and the quantum yield of this process is deduced. The following kinetic equilibrium [67] might be followed for the reactions in equation (3.3) and equation (3.4).

$$-\frac{d[RF]^*}{dt} = I_{abs} - K_{-1}[RF]^* \qquad (3.5)$$

Where I_{abs} is absorbed intensity radiation. I_0 of water was calculated and found to be equal to 1.3891×10^{-5} E in.l^{-1}.S^{-1}. [67] These values were used in the calculation of the quantum yield according to equation (3.6):

Q_d = rate of photodecomposition / I_{abs} (3.6)

Since the rate of excited state decomposition can be expressed as in equation (3.5) is:

$$-\frac{d[RF]^*}{dt} = I_{abs} - K_2[RF]^* - K_{-1}[RF]^* \quad (3.7)$$

Assuming that the $[RF]^*$ excited state concentration is fixed, then:

$$[RF]^* = \frac{I_{abs}}{K_{-1} + K_2} \quad (3.8)$$

The value of excited state concentration, $[RF]^*$, in equation (3.8) is substituted in equation (3.5), one can get:

$$-\frac{d[RF]}{dt} = I_{abs} - \frac{I_{abs} K_{-1}}{K_{-1} + K_2} = I_{abs}\left(1 - \frac{K_{-1}}{K_{-1} + K_2}\right) \quad (3.9)$$

Then equation (3.6) can take the form:

$$Q_d = \frac{\text{Rate of photodecomposition}}{I_{abs}} = -\frac{d[RF]}{dt} \Big/ I_{abs} \quad (3.10)$$

The value of quantum yield of photodecomposition (Q_d) can then be given by equation (3.11):

$$Q_d = 1 - \frac{K_{-1}}{K_{-1} + K_2} \quad (3.11)$$

Or

$$Q_d = \frac{K_2}{K_{-1} + K_2} \qquad (3.12)$$

By rearranging equation (3.11) and equation (3.12), we can obtain equation (3.13) for the value of reactivity ratio Rr which equals (K_2 / K_{-1}).

$$\frac{K_2}{K_{-1}} = \frac{Q_d}{1 - Q_d} \qquad (3.13)$$

Equation (3.13) was used to calculate the reactivity ratio of the photodecomposition of riboflavin at different pH. These values are listed in Table (3.12). The results of Table (3.12) for the reactivity ratio indicated that these values began to increase from pH (2.00-7.00) then decreased from pH (7.00-10.00) it may be due to the lack of riboflavin-phosphate divalent ions complex break down preferring photoreduction reaction than the photoaddition reaction [(69)]. It may also be due to the presence of HCl with the buffer component (HPO_4^{-2}, $H_2PO_4^{-1}$) that would make the medium to be more acidic (H_3PO_4) and increase the rate of the degradation. While for the photodegradation of riboflavin in the basic medium it may be due to the presence of KOH and that would make salty medium and cause the decrease in the rate of photodegredation rate.

The presence of CH_3- group in riboflavin basic derivatives may also be responsible for the decrease in its photodegradation rate.

Table (3.12): Specific rate constant (K_d), photodecomposition constant (R_d), the quantum yield (Q_d) and the reactivity ratio (R_r) for riboflavin of $5\times10^{-5}M$ at different pH in water (Irradiation wavelength 445 ± 1).

PH	K_d 10^{-5} s^{-1}	R_d $10^{-9}s^{-1}M$	Q_d 10^{-4}	R_r 10^{-4}
pH2	**3**	**1.5**	**1.474**	**1.474**
pH3	**4**	**2**	**1.961**	**1.961**
pH4	**5**	**2.5**	**2.439**	**2.44**
pH6	**7**	**3.5**	**3.391**	**3.392**
pH7	**9**	**4.5**	**4.265**	**4.267**
pH 8	**7**	**3.5**	**3.414**	**3.415**
pH9	**4**	**2**	**1.972**	**1.972**
pH10	**2**	**1**	**1**	**1**

From the results shown in Table (3.12), one could notice that K_d and Q_d values were dependent on the presence of CH_3- group in the compound. The photodecomposition decreased as the CH_3- group existed in the compound.

3.6 Photodegradation products of riboflavin

The photodegradation products of RF formed on irradiation in the presence of phosphate buffer solution at pH 2.00–10.00 were detected by spectrophotometric and spectroflourometric methods.

The two major photoproducts were lumiflavine (LF) in basic medium and lumichrome (LC) in acidic medium. The results of photodegradation showed uniformly decreasing values of RF absorbance with time with inherent increase of LC in acidic medium and LF in basic medium during the reaction.

3.6.1 Photodegradation of riboflavin in basic media

Standard solution of 5×10^{-5} M riboflavin was prepared with different pH using phosphate buffer ranging from (7.00 - 10.00), these solutions were exposed to UV radiation using a system consisted of 1000 watt UV lamp placed in a box with (35, 25, 25 cm) dimension, provided with an electrical fan for cooling. These solutions were placed straight to the lamp; the distance between the lamp and theses solutions was about 15 cm to prevent excessive heating of the sample.

The spectrum of the standard solutions of riboflavin has two peaks as mentioned previously, irradiation of pH9 solutions caused the disappearance of 445 nm and shifting of 372 to 359 nm which refer to the formation of lumiflavine, due to cleavage of ribityl side chain which caused blue shift to shorter wavelength.

The spectrum of each solution was scanned after each half hour of UV irradiation as shown in Figures (3.10- 3.13), the absorbance of these solutions at 445 nm and 373 nm are summarized in Table (3.3). Solution of pH9 gave (which gave the shifting of the 373 nm to the 359 nm which may refer to the formation of lumiflavine)the highest absorbance at 359nm, after exposure for more than 24 hours as shown in Figure (3.22), therefore this pH was chosen for the preparation of standard solution of lumiflavine.

3.6.2 Photodegradation products of riboflavin in acidic media

The earlier mentioned procedure was performed for standard solution of 5×10^{-5} M riboflavin prepared with pH ranging from (2.00-4.00).

The spectra of these solutions have also shown the disappearance of 445 nm band and shifting of 372 nm band to 353 nm band as shown in Figure

(3.8) at pH4. This difference compared to that of lumiflavine may be due to the presence of CH_3 group on the structure of lumiflavine. The spectrum of each solution was scanned after each half hour of UV irradiation as shown in Figures (3.6-3.9) and the absorbance of these solutions at 445, 373 nm is summarized in Table (3.3). Solution of pH4 gave the highest absorbance at 353 nm, therefore this pH was chosen for the preparation of standard solution of lumichrome.

3.7 Lumiflavine

When the polyhydroxy containing ribityl group in riboflavin is easily cleaved under light [(70)], fragmentation between N 10 and C 1' in excited triplet riboflavin produces lumichrome and lumiflavine upon exposure to sunlight and ultraviolet light as shown in Scheme (3.1) [(72)]. The triplet riboflavin transforms to lumiflavin in alkaline solution [(72)]. Lumichrome and lumiflavin are stable under UV irradiation [(70)].Photoreduction and photodealkylation occur.

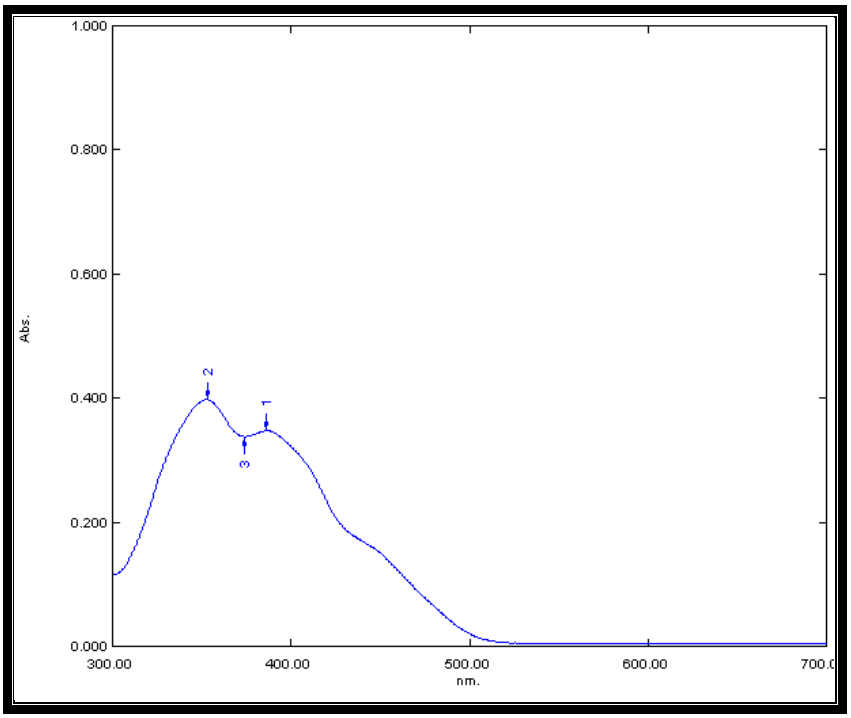
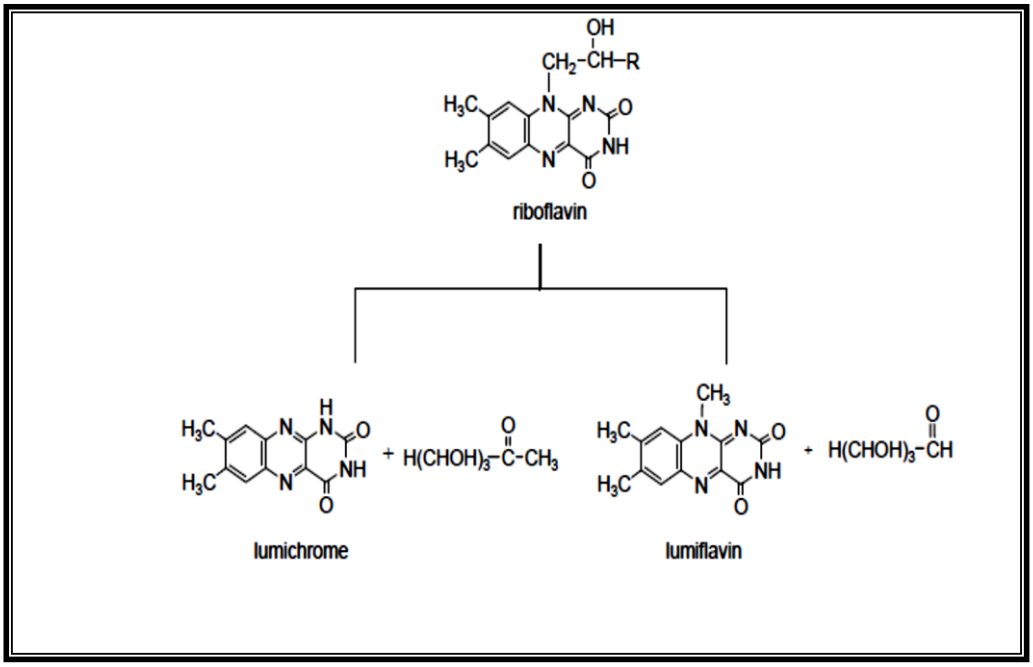


Figure (3.22): UV-VISIBLE spectrum of lumiflavine produced.



Scheme (3.1) Lumichrome and lumiflavine formation from triplet riboflavin by dealkylation.

A standard solution of lumiflavine was prepared by photodegradation of 5.5×10^{-5} M riboflavin for more than 24 hour at pH9 which was used for indirectly measurement of riboflavin concentration. The percent of conversion was calculated using Beer's law as follows:

$$A = \varepsilon bc \quad \ldots\ldots\ldots \quad (3.13)$$

Where:-

A is the absorbance of the product at 359 nm = 0.53

ε is the molar extinction coefficient $\varepsilon_{at\ 359}$ = 11327.27 L/mol cm^{-}

b is the Thickness of the cell=1 cm

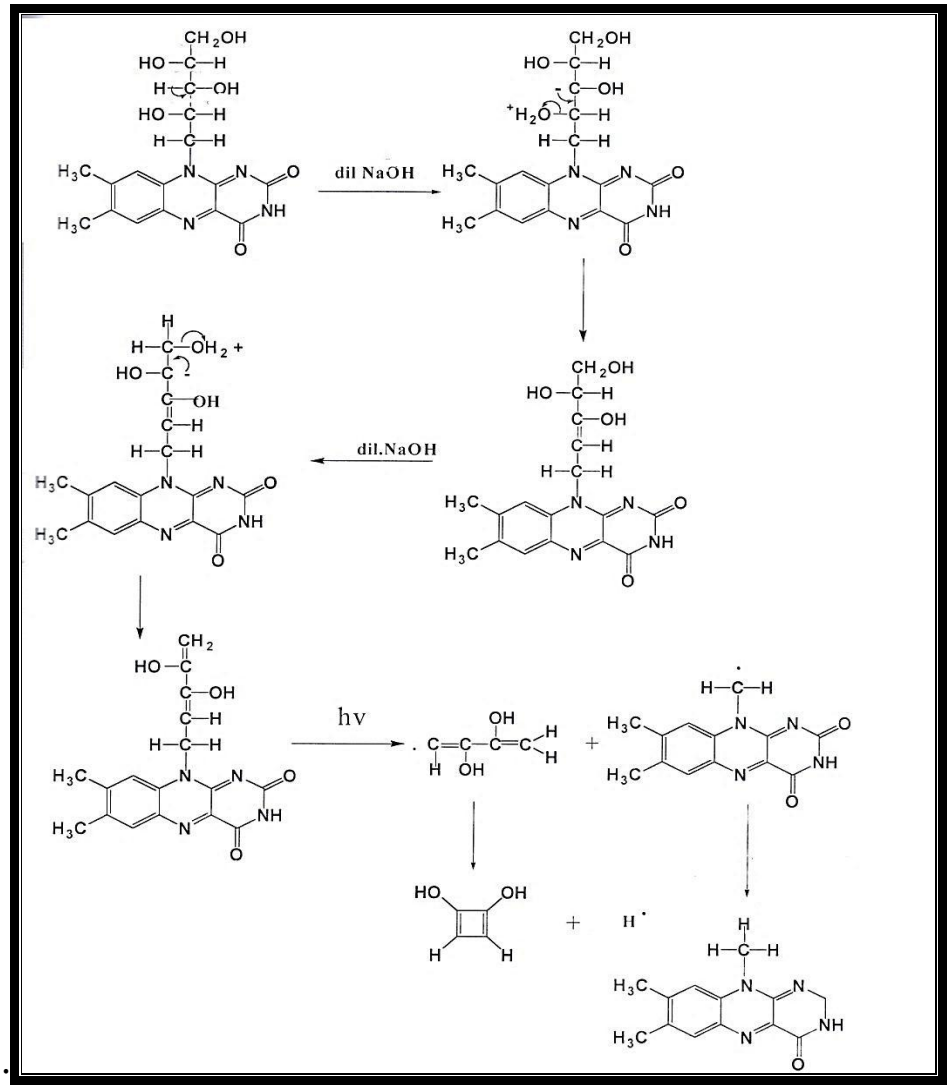
Conc. Of lumiflavine = 0.53 /11327.27=4.6×10^{-5} M

Conversion yield= $4.6\times 10^{-5}\times 100 / 5.5 \times 10^{-5}$ = 85.075% …… (3.14)

The percentage of riboflavin that was converted to lumiflavine by photodegradation at pH9 was found to be 85٪ . This percentage yield was

taken as conversion factor for concentration correction for the determination of riboflavin in the standard samples.

A suggested mechanism for photodegradation of riboflavin to lumiflavine in basic solution may be proposed as in scheme (3.2).



Schemes (3.2): Suggested mechanism for Photodegradation of riboflavin to lumiflavine in basic solution

A calibration curve was then constructed for lumiflavine using the absorbance at 359 nm as a maximum wavelength as shown in Figure (3.23). A linear curve was obtained ranged from (5.00×10^{-6} to 4.06×10^{-5}) M with linear regression curve equation:

$$Y=10934X +0.033 \ldots\ldots\ldots\ldots\ldots (3.15)$$

Where Y represents the absorbance of lumiflavine at 359 nm and X is the concentration of lumiflavine at pH9.

The correlation coefficient of this line was 0.9997. Similar conditions were used to prepare two newly standard solutions. The concentration of riboflavin was calculated using equation (3.15), then multiply by the conversion factor. The recovery of this method was 95.99% with relative errors of 4%.

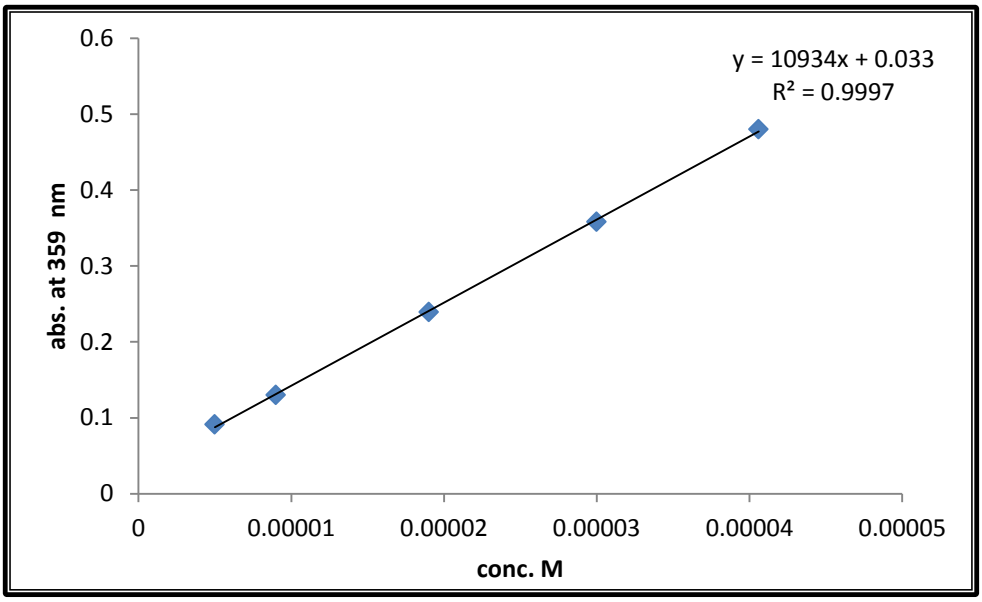


Figure (3.23): Calibration curve of lumiflavine using spectrophotometric method.

***Table (3.13):* Concentration of riboflavin from its photodegredation product lumiflavine by spectrophotometric method.**

sample	Concentration of vitamin B2		% Recovery	% Error	RSD%
	prepared	Calculated*			
Stand.1	8×10^{-5}	7.69×10^{-5}	96.12	3.88	0.18
Stand.2	8×10^{-6}	7.67×10^{-6}	95. 87	4.13	0.4

***Each concentration represents an average of three measurements**

3.8 Spectrofluorometric study of lumiflavine

A standard solution of (5.00×10^{-5} M) riboflavin at pH9 was used after exposure for more than 24 hour. This prepared sample was scanned in the range from (300-900) nm using 359 nm as excitation wavelength as shown in Figure (3.24). This Figure shows a maximum emission wavelength at 465 nm with green fluorescent light. These excitation and emission wavelengths may be attributed to the formation of lumiflavine product. For this reason, these wavelengths were used to measure the concentration of lumiflavine and consequently the riboflavin concentration.

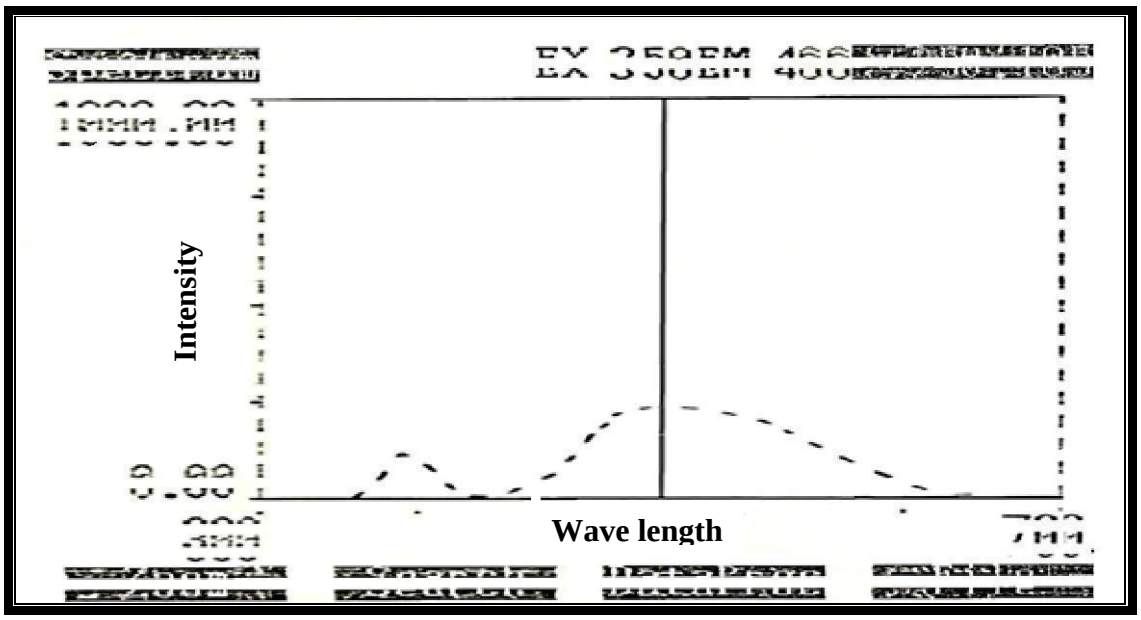


Figure (3.24) fluorescence spectrum of lumiflavine solution

A calibration curve was then constructed using 359 nm and 465 nm as excitation and emission wavelengths respectively. A straight line was obtained from (1×10^{-6} to 2×10^{-5}) M as shown in figure (3.25), with correlation of 0.9997 according to the following equation:

$$I_{lumi} = 1\times10^{7}X + 13.047 \ldots\ldots\ldots\ldots \quad (3.16)$$

Where I_{lumi} is the intensity of fluorescent light, x is concentration of lumiflavine at pH9. Similar conditions were used to prepare two standard solutions. The concentrations of those samples were measured using equation (3.16). The concentration of riboflavin was then measured using equation (3.14). The recovery of this method was 96.42% with a relative error of 3.57%.

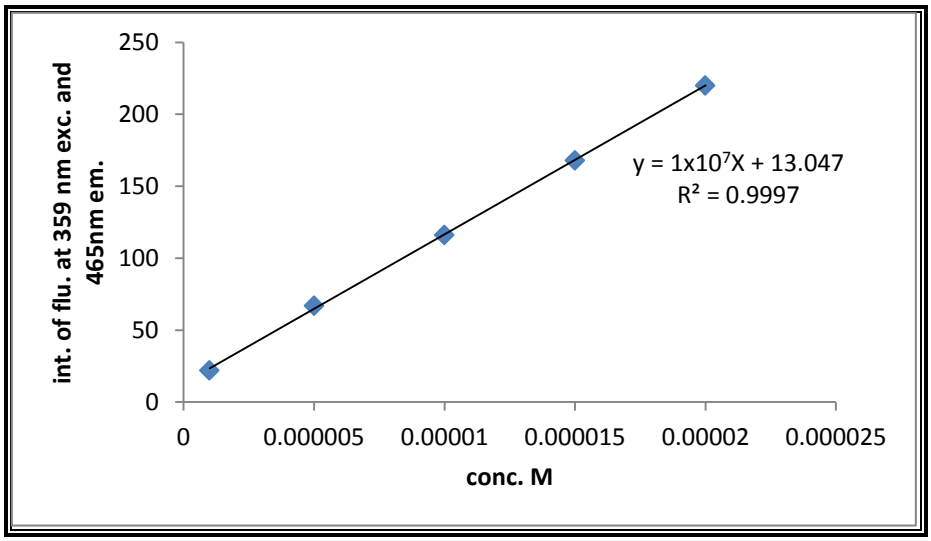


Figure (3.25): Lumiflavine calibration curve in spectrofluorometric method at 359 nm excitation wavelength and 466 nm as emission wavelength.

***Table (3.14):* Concentration of riboflavin from its photodegredation product lumiflavine by spectrofluorometric method.**

sample	Concentration of vitamin B2		% Recovery	% Error	RSD%
	prepared	Calculated*			
Stand.1	8×10^{-5}	7.71×10^{-5}	96. 73	3.27	0.48
Stand.2	8×10^{-6}	7.69×10^{-6}	96.12	3.88	0.5

*Each concentration represents an average of at least three measurements

3.9 Lumichrome

The spectrum of irradiation product of riboflavin solution at pH4 that has been exposed to UV-light for more than 24 hours is shown in Figure (3.26). This Figure shows (as in lumiflavine) the disappearance of two significant peaks of riboflavin (373, 445) nm and appearance of new peak at 353 nm that is probably attributed to the formation of lumichrome. When the polyhydroxy-containing ribityl group in riboflavin is easily cleaved under light [(71)], fragmentation between N (10) and C (1) in excited triplet riboflavin produces lumichrome and lumiflavin upon exposure to sunlight and ultraviolet light as shown in scheme (3.1). Riboflavin transforms mainly to lumichrome through the removal of the ribityl group and reconfiguration of a double bond in the ring structure in neutral and acidic solutions.

A standard solution of lumichrome prepared by degradation of (5.00×10^{-5} M) riboflavin for more than 24 hour at pH4. The percentage conversion was then calculated using Beer's law as follow:

$$A = \varepsilon bc$$

Where:

A is the absorbance of the product (lumichrome at pH4) at 353nm =0.46

$\varepsilon_{at\ 353}$ is the molar extinction coefficient = 11340 L/mol cm^{-}

C = Concentration of lumichrome = 4.05×10^{-5} M

Conversion yield% = $4.05\times10^{-5} \times100 / 5\times10^{-5}$ = 82% ……. (3.17)

The percentage of riboflavin that was converted to lumichrome by photodegradation at pH4 was found to be 82%. This percentage was taken as a conversion factor for the concentration correlation in the determination of riboflavin in unknown and other samples.

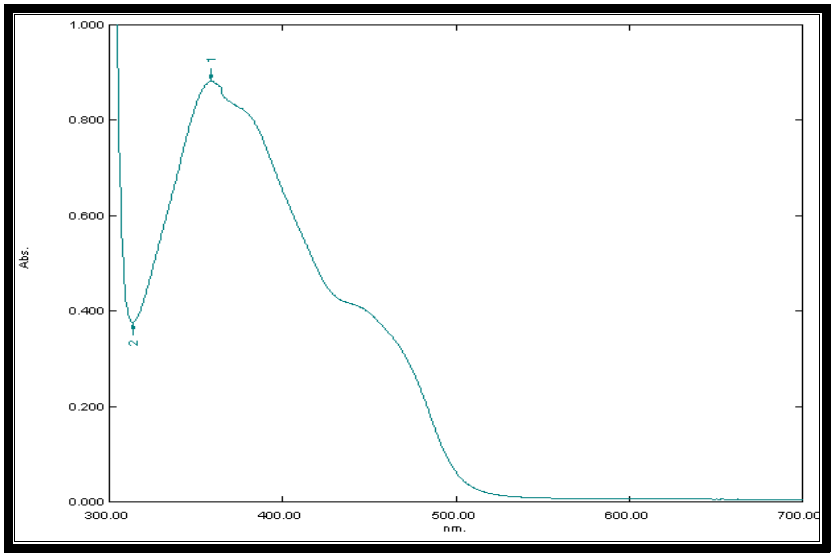


Figure (3.26): UV-VIS spectrum of Lumichrome

A calibration was then constructed for lumichrome using absorbance at 353 nm as a maximum wavelength as shown in Figure (3.27).

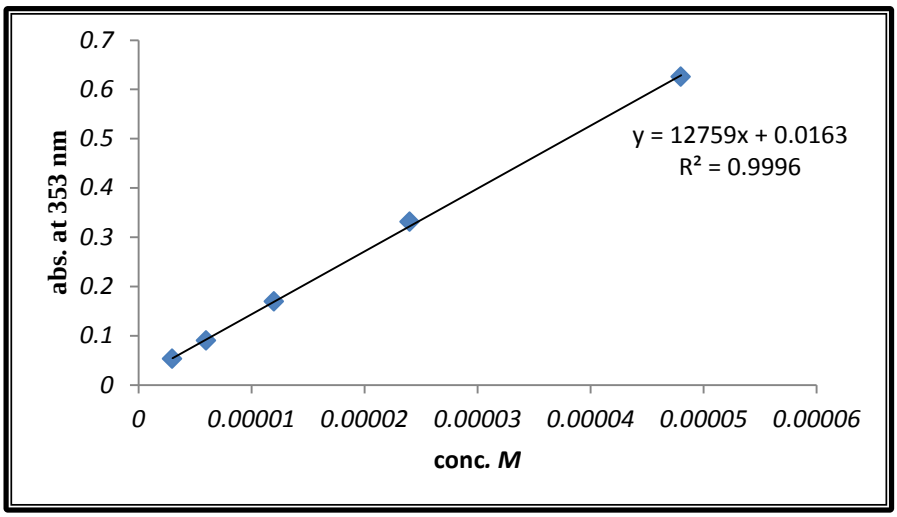
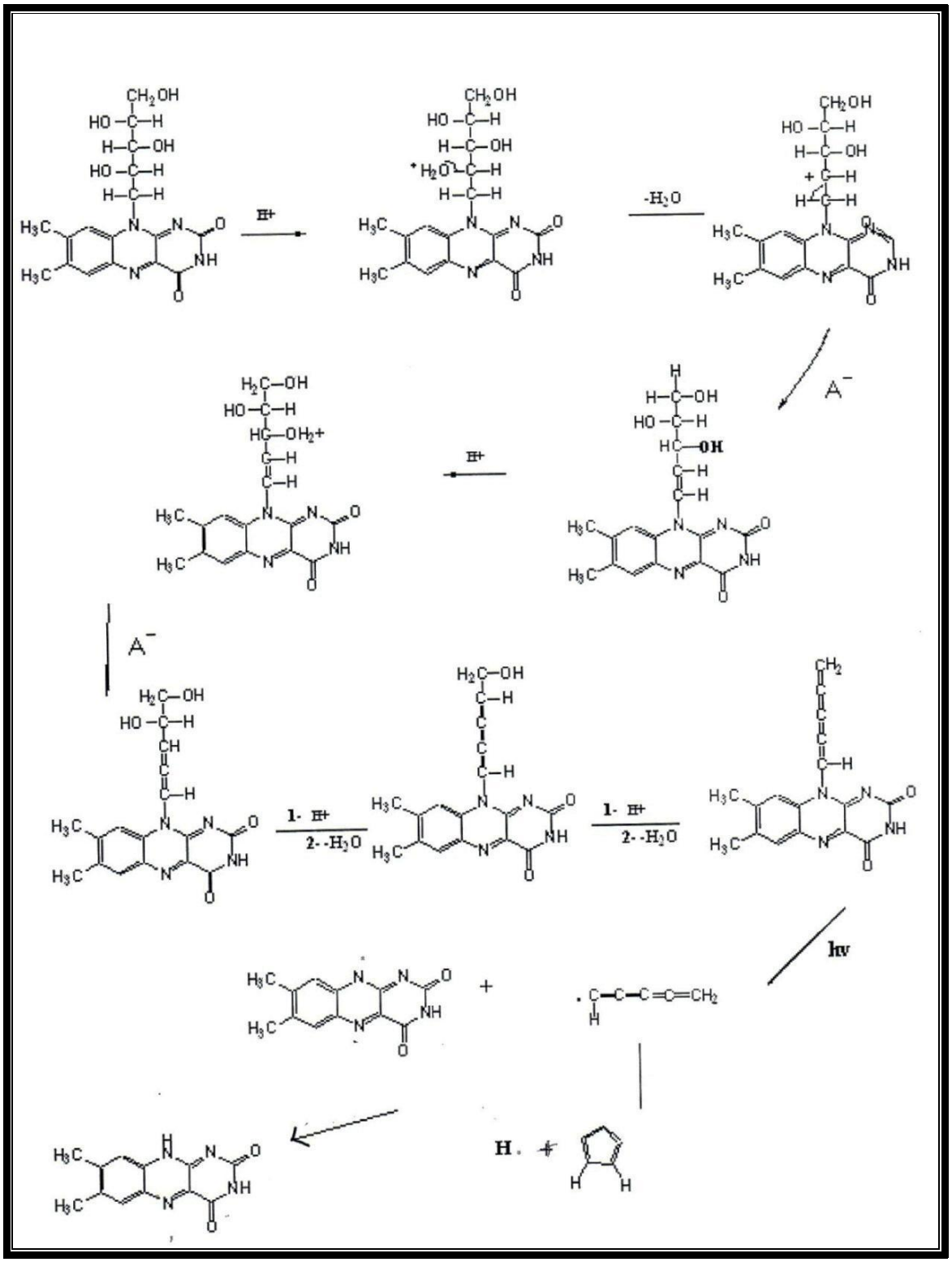


Figure (3.27): Calibration curve of lumichrome using spectrophotometric method.

A calibration curve was optioned from 3.00×10^{-6} to 4.87×10^{-7}M with linear equation:

$$Y= \quad 12759X +0.0163 \qquad (3.18)$$

Where y represents the absorbance of lumichrome at 353 nm and x is the concentration of lumichrome at pH4.



Scheme (3.3): Suggested mechanism for photodegradation of riboflavin to lumichrome in acidic solution.

The correlation coefficient of this line was 0.9996. Similar conditions, were employed to prepare two newly standard solutions UV-light at pH4. The concentration of riboflavin was calculated using equation (3.18), after multiplying by the conversion factor. The recovery of this method was 95.18 % with a relative error of 4.82%.

Table (3.15) **:Concentration of riboflavin from its photodegredation product lumichrome by spectrophotometric method.**

Sample	Concentration of vitamin B2		% Recovery	% Error	RSD%
	prepared	Calculated*			
Stand.1	8×10^{-5}	7.63×10^{-5}	95.37	4.63	0.83
Stand.2	8×10^{-6}	7.6×10^{-5}	95	5	0.67

***Each concentration represents an average of three measurements.**

3.10 Spectrofluorometric study of lumichrome

A standard solution of riboflavin (1.00×10^{-4} M) at pH4 after exposure for more than 24 hour were prepared. These solutions were scanned in the range from (300-700) nm using 353 nm as excitation wavelength as shown in Figure (3.28). This Figure shows a maximum emission wavelength at 422 nm with blue fluorescent light. These excitation and emission wavelengths may be attributed to the lumichrome formation. For this reason, these wavelengths were taken to measure the concentration of lumichrome and consequently the riboflavin concentration.

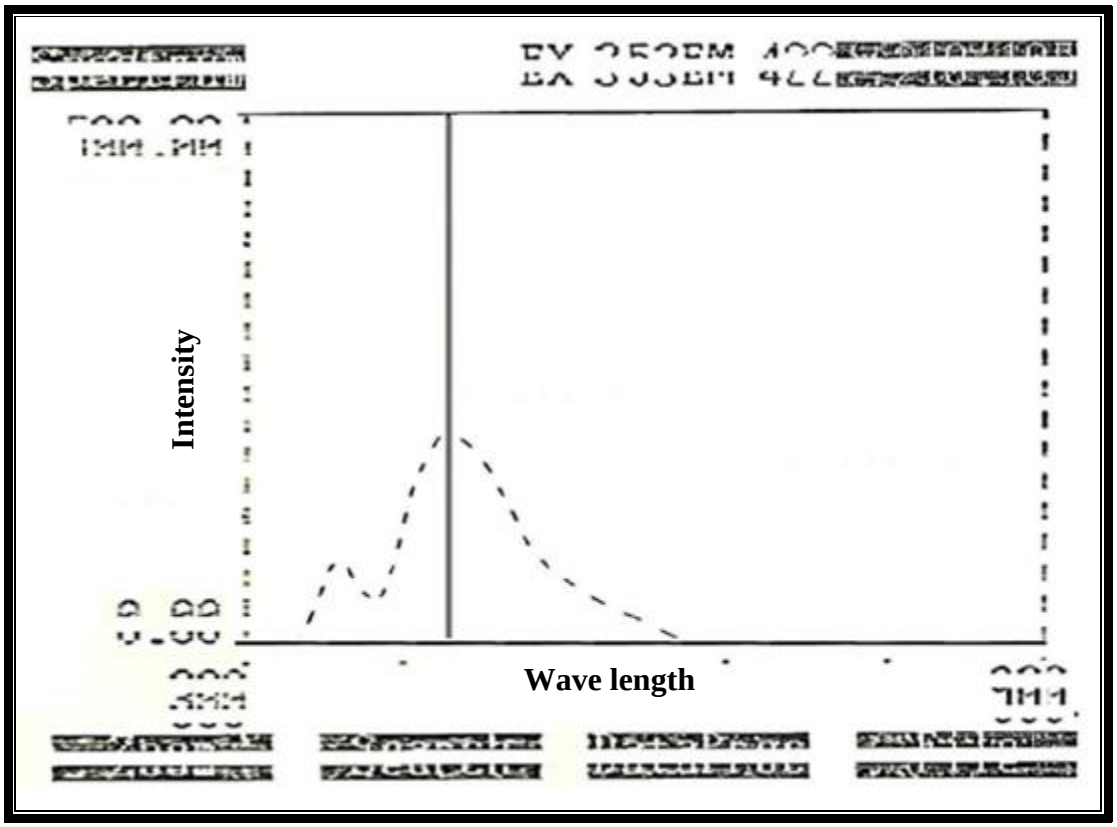


Figure (3.28): Fluorescence spectrum of lumichrome solution.

A calibration curve was then constructed using 353 nm and 422 nm as excitation and emission wavelengths respectively. A straight line was obtained from (3.00×10^{-6} to 2×10^{-5}) M as shown in Figure (3.29), with correlation coefficient of 0.9998 and linear equation:

$$I_{lumi}= 1\times10^{7}\ x + 13.047 \qquad (3.19)$$

Where I_{lumi} is the intensity of fluorescent light, x is concentration of lumichrome at pH4.

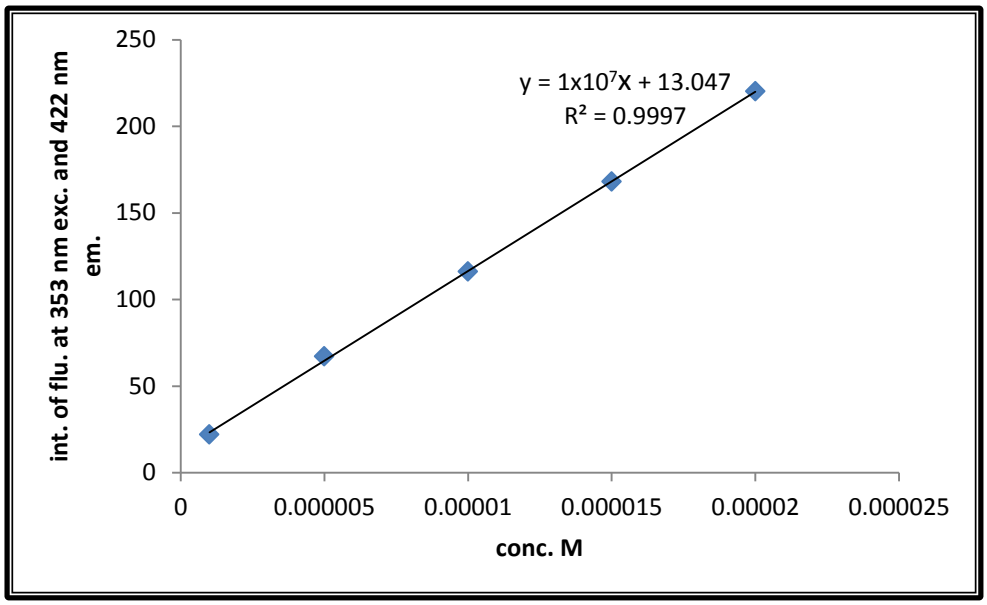


Figure (3.29) calibration curve of lumichrome using spectrofluorometric method

Similar conditions, were employed to prepare new standard solutions. The concentrations of these samples were measured using equation (3.19). The concentration of riboflavin was calculated using equation (3.17). These concentrations were compared with the concentration of riboflavin standard solution. The recovery of this method was 95.75% with relative errors of 4.25%.

***Table (3.16):* Concentration of riboflavin from its photodegradation product lumichrome by spectrofluorometric method.**

Sample	Concentration of vitamin B2		% Recovery	% Error	RSD%
	prepared	Calculated*			
Stand.1	8×10^{-5}	7.68×10^{-5}	96	4	0.78
Stand.2	8×10^{-6}	7.64×10^{-5}	95. 5	4.5	0.66

*Each concentration represents an average of at least three measurements.

Conclusion remarks

- The reaction follows first order reaction.
- The specific rate constant (k_d) at room temperature increase as the pH increase from (2 to7) then begins decreasing from pH (8 to10).
- The quantum yield of photodecomposition process is generally low and is greatly affected by the pH. The value of the quantum yield of photodecomposition increases as the pH increase from (2 to7) then begins to decrease as pH increases from (8 to10).

Future work

1. Optimizing the conditions for the photodegradation conversion of riboflavin.
2. Use another analytical method to determine riboflavin, such as HPLC and compare between them.
3. Study the effect of the interference of other media and pharmaceutical in the determination of the riboflavin concentration.
4. Using derivative spectroscopy as an alternative method to determine riboflavin in the presence of its derivatives.
5. Study the effect of the polar and non- polar solvents on the rate of photodecomposition of the riboflavin.

Summary

This work involved the use of UV-VIS spectrophotometric and spectrofluorometer to study the photoproperties and the determination of riboflavin in the vitamin synthetic standards either directly from the riboflavin present in standard solution samples or indirectly by conversion of riboflavin to its photoderivatives.

Standard solutions of riboflavin were prepared at different pH ranged from (2 -10) to get the pH at which maximum absorbance occurs. Riboflavin showed a maximum absorbance at 445 nm at pH7 after scanning in visible region. Thus this wavelength and pH were used to determine the riboflavin concentration in different standard solution samples by spectrophotometric method. The recovery of this method was found to be 98.68% with relative error of 1.31%.

Scanning of the emission spectra of riboflavin by spectrofluorometer showed (445 nm as excitation wavelength) a maximum emission at 520 nm, this wavelength was chosen for the determination of riboflavin at different standard solution, the recovery of this method was found to be 99.68% with relative error of 0.63%.

Indirect determination of riboflavin was carried out after the conversion of riboflavin to its photoderivatives by irradiating the vitamin solution with UV light. Different standard solutions of riboflavin with the same concentration were prepared at different pH range from (2-10) to get the suitable pH at which lumiflvine and lumichrome formed, pH9 showed the disappearance of the 445 nm peak and the shift of 373 nm to 359 nm, which is indicative for the formation of lumiflavine. pH4 showed the

disappearance of the 445 nm peak and the shift of 373 nm to 353 nm, which is indicative for the formation of lumichrome. Both lumiflavine and lumichrome were the main photoderivatives in basic and in acidic media respectively. The percentage of the degradation of each photoderivative was determined. The produced lumiflavine showed a maximum absorbance at 359 nm and a maximum emission at 465 nm. pH9 and a wavelength 359 nm and 465 nm were used to determine the concentration of riboflavin spectrophotometricaly and spectrofluorometricaly. The recovery of the spectrophotometric method was 95.99% with a relative error of 4%, the recovery of spectrofluorometric method was 96.42% with a relative error of 3.57%. pH4 and both wavelengths (353 nm,422 nm) were used to determine the concentration of riboflavin spectrophotometricaly and spectrofluorometricaly. The recovery of the spectrophotometric method was 95.18 % with relative errors of 4.826%, and the recovery of spectrofluorometric method was 95.75% with relative a error of 4.25%. Spectrofluorometric method showed a better recovery with a less error than the spectrophotometric method.

During the study of the photochemical properties of riboflavin, it was found that the degradation is first order reaction. The specific rate constant (k_d) at room temperature, was evaluated by monitoring the spectral changes during irradiation process.

The quantum yield of the photodecomposition process is generally low and is greatly affected by the pH. The quantum yield of photodecomposition increases as the pH increase from 2-7 then begin to decrease as the pH increase from 8-10.

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تاريخ المناقشه: 24-10-2012

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College of Science
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SPECTROSCOPIC AND PHOTOCHEMICAL STUDY OF RIBOFLAVIN

A Thesis
Submitted to the College of Science Al-Nahrain University as a Partial Fulfillment of the Requirements for the Degree of M. Sc. in Chemistry

By:

Sura Majid Attwan

(B. Sc. 2009)

Supervised by:

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2012 A.D. **1433 A. H.**

References

1. Shari Lieberman, Nancy Bruning, (**1990**), "*The Real Vitamin and Mineral Book*", NY: Avery Group, 2nd edition. USA.

2. Maton, A. Anthea, H. Jean Hopkins, Charles William McLaughlin, Susan Johnson, Maryanna Quon Warner, David LaHart, Jill D. Wright (**1993**), "*Human Biology and Health"*, Englewood Cliffs, New Jersey, and USA: Prentice Hall.

3. Bender, T. David A. (**2003**), *Nutritional biochemistry of the vitamins*, Cambridge, U.K.: Cambridge University Press. 2nd edition.

4. Bolander FF **(2006),** "Vitamins: not just for enzymes". *Curr Opin Investig Drugs* **7** (10): 912–5.

5. Iłowiecki, Maciej **(1981)**, *Dzieje nauki polskiej*. Warszawa: Wydawnictwo Interpress. p. 177. ISBN 83-223-1876-6.

6. Jack Challem **(1997)**, *Subcell Biochem*, 35, 11–26.

7. Jacob, R. (**1996**), *Subcell Biochem*, 25, 1–16.

8. Tsutomu Fukuwatari and Katsumi Shibata. (**2008**), *J. Nutr. Sci. Vitaminol*, 54 (3), 223-229.

9. Said, HM., Mohammed, ZM. (**2006**), Curr. Opin. Gastroenterol. 22 (2): 140–6.

10. Maqbool A, Stallings VA (**2008**), Curr Opin Pulm Med 14 (6): 574–81.

11. British Pharmacopoeia **(2009),** Her Majesty's Stationary Office, London, Electronic version .

12.Robert B. Bucker, **(2001)**, The Handbook of Vitamins, 3rd ed. Marcel Dekker,Inc.257.

13. Spallholz J. (**1989),** The nutrients. In: Spallholz J, ed. 1. Nutrition: Chemistry and Biology. Englewood Cliffs, NJ: Prentice Hill, 65-68.

14. Jane Higdon, Drake Victoria J. (**2007**), "Riboflavin". Micronutrient Information Center. Linus Pauling Institute at Oregon State University.

15. Watson, D.G. **(2005)**, Pharmaceutical Analysis, 2nd ed., Elsevier Churchill Livingstone,153

16. Gropper SS, Smith JL, and Groff JL **(2009),** Advanced Nutrition and Human Metabolism. 5th ed. Wadsworth: CENGAG Learning;. Riboflavin, Chapter 9. p. 329–333),

17. Mccromick,D.B.,and Wright.L.D**.(1980)**, New York, Academic Press.60.

18. Ana Luiza P. Nery, Rodrigo M. Liegel and Carmen Fernandez**.(2009)**, *CEJ*, (13):2:13-22.

19. Ahmad, I., Fasihullah, Q., Vaid, F. H. M. **(2006**), *Photochem. Photobiol. Sci*. 5, 680-685.

20. Ahmad, I., Fasihullah, Q., Noor, A., Ansari, I.A., Ali, Q.N.M. **(2004)**, *Int. J. Pharm*. 280, 199-208.

21. Ahmad, I., Ahmed, S., Sheraz, M.A., Vaid, F.H.M. **(2008)**, *Photobiol. B: Biol.* 93, 82-87.

22. Sato, Y., Chakis, H., Suzuki, Y. **(1984)**, *Chem. Pharm. Bull. (Jpn)*, 32, 1232-1235.

23. Ahmad, I., Fasihullah, Q., Noor, A., Ansari, I.A., Ali, Q.N.M. **(2004)**, *Int. J. Pharm.* 280, 199-208.

24. Ahmad, I., Fasihullah, Q., Vaid, F.H.M. **(2006),** *J.Photochem.Photobiol. B: Biol.* 82, 21-27.

25. Cairns, W.L., Metzler, D.E. **(1971)**, *J. Am. Chem. Soc*. 93, 2772-2777.

26. Ahmad, I., Fasihullah, Q., Vaid, F. H. M. **(2006)**, *Photochem. Photobiol. Sci*. 5, 680-685.

27. Ahmad, I., Ahmed, S., Sheraz, M.A., Vaid, F.H.M. **(2008)**, *J. Photochem. Photobiol. B: Biol*. 93, 82-87.

28. Treadwell, G.E., Cairns, W.L., Metzler, D.E. **(1968)**, *Chromatogr*. 35, 376-388.

29. Heelis, P.F. and Muller, F. (**1991**), *CRC Press*, *Boca Raton*, FL, Vol. 1, pp. 171-193.

30. Ahmad, I., Fasihullah, Q., Vaid, F.H.M. **(2005)**, *J. Photochem. Photobiol*. B: Biol. 78, 229-234.

31. Traber, R., Kramer, H.E.A., Hemmerich, P. **(1982)**, One and two electron transfer pathways in the photoreduction of flavin, Pure Appl. Chem. 54, 1651-1665.

32. Schuman Jorns, M., Schollnhammer, G., Hemmerich, P. **(1975)**, *Eur. J. Biochem.*, 57, 35-48.

33. Traber, R., Kramer, H.E.A., Knappe, W.R., Hemmerich, P. **(1981),** *Photochem. Photobiol.* 33, 807-814.

34. Hemmerich, P. (**1976**), *Fortschr. Chem. Org. Naturst.*, 33, 451-527.

35. Rivlin, R.S. **(2007),**. Riboflavin (vitamin B2), In: Zempleni, J., Rucker, R.B., McCormick,D.B., Suttie, J.W., Eds., Handbook of Vitamins, 4th ed., Taylor & Francis, CRC Press, Boca Raton, FL, Chap. 7.

36. Silva, E., Godoy, J. **(1994),** *Int. J. Vit*.64, 253-256.

37. Quintero, B., Miranda, M.A. **(2000)**, Mechanisms of photosensitization induced by drugs:A general survey, *Ars Pharmaceutica* 41, 27-46.

38. Garcia, N.A., Criado, S.N., Massad, W.A. **(2006)**, Riboflavin as a visible-light-sensitizer in the aerobic photodegradation of ophthalmic and sympathomimetic drugs, In: Silva, E., Edwards, A.M., Eds., Flavin

Photochemistry and Photobiology, The Royal Society of Chemistry, Cambridge, Chap.4.

39. Viteri, G., Edwards, A.M., De la Fuente, J., Silva, E. **(2003)**, Study of the interaction between triplet riboflavin and the α-, βH- and βL-crystallins of the eye lens, Photochem. Photobiol. 77, 535-540.

40. Novak, M., Miller, A., Bruce, T.C., Tollin, G. **(1980)**, *J. Am. Chem. Soc*. 102, 1465-1467.

41. Knobloch, E., Mandys, F., Hodr, R., Hujer, R., Mader, R. **(1991)**, *J. Chromatogr*. 566, 89-99.

42. Miyake, K., Masaki, Y., Miyamoto, I., Yanagida, S., Ohno, T., Yoshimura, A., Pac, C. **(1993),** *Photochem. Photobiol*. 58, 631-636.

43. Encinas, M.V., Previtali, C.M. **(2006)**, Excited state interaction of flavins with amines: application to the initiation of vinyl polymerization, In: Silva, E., Edwards, A.M., Eds., Flavins The Photochemistry and Photobiology, The Royal Society of Chemistry, Cambridge, Chap.3.

44. Montana, P., Pappano, N., Debattista, N., Avila, V., Posadaz, A., Bertolotti, S.G., Garcia, N.A. **(2003)**, *Can. J. Chem*. 81, 909-914.

45. Diaz, M., Luiz, M., Bertolotti, S.G., Miskoshi, S., Garcia, N.A. **(2004)**, *Can. J. Chem.* 82, 1752-1759.

46. Encinas, M.V., Bertolotti, S.G., Previtali, C.M. **(2002)**, *Helv. Chim. Acta* 85, 1427-1438.

47. Fu, P.P., Cheng, S.-H., Coop, L., Xia, Q., Culp, S.J., Tollesen, W.H., Wamer, W.G., Howard, P.C. **(2003*)***, *J. Environ. Sci. Health Part C: Environ.* 21, 165-197.

48. Encinas, M.V., Rufs, A.M., Bertolotti, S.G., Previtali, C.M. **(2001)**, *Macromolecules* 34, 2845-2847.

49. Martin, C.B., Wilforg, E., Ruane, P., Goodrich, R., Platz, M. **(2005)**, *Photochem. Photobiol*. 81, 474-480.

50. Corbin, F. III. **(2002)**, *Int. J. Hematol*. 76, 253-257.

51. Mahns, A., Melchheier, I., Suschek, C.V., Sies, H., Klotz, L.O. **(2003)**, *Free Radical Res*. 37, 391-397.

52. Edwards, A.M., Barredo, F., Silva, E., De Ioannes, A.E., Becker, M.I. **(1999)**, *Photochem. Photobiol*. 70, 645-649.

53. Ugarte, R., Edwards, A.M., Diez, M.S., Valenzuela, A., Silva, E. **(1992*)***, *J. Photochem. Photobiol*. B Biol. 13, 161-168.

54. Sinko, P.J. **(2006)**, Chemical Kinetics and Stability, Martin's Physical Pharmacy and Pharmaceutical Sciences, 5th ed., Lippincott Williams & Wilkins, Baltimore, Chap.15.

55. Casini, G., De Laurentis, N., Maggi, N., Ottolino, S. **(1981),** Protective effect of hydroxybenzoic acid esters in the photodegradation of riboflavin, Farm. Ed. Prat. 36,553-558.

56. Loukas, Y.L. Vraka, V., Gregoriadis, G. **(1996),** *Int. J. Pharm*. 144, 225–231.

57. Margherita Grotzkyj Giorgi, Kevin Howland, Colin Martin, and Adrian B. Bonner. **(2012)**, *The Scientific World Journal*. Article ID 359721, 8.

58. Ashoor Erich g. J, Monte W. C., and Welty J. (**1983)**, *Journal of Food Scienc*e, 48 (1): 92–94.

59. Shahnaz Perveen, Arfa Yasmin and Khalid Mohammed Khan **(2009)**, *The Open Analytical Chemistry Journal*, 3, 1-5.

60. Diaz, M., Luiz, M., Bertolotti, S.G., Miskoshi, S., Garcia, N.A. **(2004)**, *Can. J. Chem.*, 82, 1752-1759.

61. Tomás Perez-Ruiz , Carmen Martínez-Lozano , Virginia Tomás and Otilia Val. **(1994)**, *Analyst*, 119, 1199-1203.

62. Himanshi Shah, Shraddha Patel, Bhavik Patel, Nitin Solanki, Nurudin P. Jivani, Digbijay B. Kumar. **(2012),** *IJPSR*, 3(9): 3462-3466

63. Marc D. Friedman, Radha Pertaub, David Usher, Evan Sherr, Pavel Kamaev, and David Muller. **(2012)**, *Journal of OphthalmologyVolume* 2012, Article ID 303459.

64. Zhang, H. Jinsheng, Z. Houting,L. Huaisheng, W. Renmin, Liu, Jifeng Liu., **(2010),** Int. J. Electrochem. Sci., 5, 295 – 301.

65. C.G. Hatchard and C.A.Parker, Proc.Roy.Soc.A,235 (1956) 518.

66. Ganqlitz G. and Hubiq S. **(1986)**, *pure and appl. Chem*. 61-187.

67. Maki, S. Yousif, E. Salman, E. **(2011**),"Photolysis of Tris(ethylxanthato)chromium(III)complexes in DMSO", Germany:LAP Lambert Academic publishing.

68. Al-Lamee K.G., M.Sc. **(1976)**, Thesis,Baghdad univ., Baghdad university. 70. Ahmad, I., Fasihullah, Q., Vaid, F.H.M. **(2006)**, *J. Photochem.Photobiol*. B: Biol. 82, 21-27.

69. Wu S, Jiang Z, Li H, Li Y, Zeng D. **(2001)**, *Molecules*, 6, 52-60.

70. Cui H, Hwang HM, Cook S, Zeng K. **(2001)**, *Chemosphere*, 44, 621-5.

71. Pan YL, Pinnick RG, Hill SC, Niles S, Holler S, Bottiger JR, Wolf JP, Chang RK.**(2001)**, *Appl. Phys. B*, 72, 449-54.

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Standard solutions of riboflavin were prepared at different pH ranged from (2 -10) to get the pH at which maximum absorbance occurs. Riboflavin showed a maximum absorbance at 445 nm at pH7 after scanning in visible region. Thus this wavelength and pH were used to determine the riboflavin concentration in different standard solution samples by spectrophotometric method. The recovery of this method was found to be 98.68% with relative error of 1.31%.

Scanning of the emission spectra of riboflavin by spectrofluorometer showed (445 nm as excitation wavelength) a maximum emission at 520 nm, this wavelength was chosen for the determination of riboflavin at different standard solution, the recovery of this method was found to be 99.68% with relative error of 0.63%.

Indirect determination of riboflavin was carried out after the conversion of riboflavin to its photoderivatives by irradiating the vitamin solution with UV light. Different standard solutions of riboflavin with the same concentration were prepared at different pH range from (2-10) to get the suitable pH at which lumiflvine and lumichrome formed, pH9 showed the disappearance of the 445 nm peak and the shift of 373 nm to 359 nm, which is indicative for the formation of lumiflavine. pH4 showed the

disappearance of the 445 nm peak and the shift of 373 nm to 353 nm, which is indicative for the formation of lumichrome. Both lumiflavine and lumichrome were the main photoderivatives in basic and in acidic media respectively. The percentage of the degradation of each photoderivative was determined. The produced lumiflavine showed a maximum absorbance at 359 nm and a maximum emission at 465 nm. pH9 and a wavelength 359 nm and 465 nm were used to determine the concentration of riboflavin spectrophotometricaly and spectrofluorometricaly. The recovery of the spectrophotometric method was 95.99% with a relative error of 4%, the recovery of spectrofluorometric method was 96.42% with a relative error of 3.57%. pH4 and both wavelengths (353 nm,422 nm) were used to determine the concentration of riboflavin spectrophotometricaly and spectrofluorometricaly. The recovery of the spectrophotometric method was 95.18 % with relative errors of 4.826%, and the recovery of spectrofluorometric method was 95.75% with relative a error of 4.25%. Spectrofluorometric method showed a better recovery with a less error than the spectrophotometric method.

During the study of the photochemical properties of riboflavin, it was found that the degradation is first order reaction. The specific rate constant (k_d) at room temperature, was evaluated by monitoring the spectral changes during irradiation process.

The quantum yield of the photodecomposition process is generally low and is greatly affected by the pH. The quantum yield of photodecomposition increases as the pH increase from 2-7 then begin to decrease as the pH increase from 8-10.

الخلاصة

يتضمن هذا العمل استخدام الطرق الطيفية_المرئيه_ الفوق البنفسجيه والطرق التوهجيه وتعيين تركيز الرايبوفلافين في عينات الفيتامين بطريقه مباشره من تحضير الرايبوفلافين في محاليل قياسيه, وبطريقه غير مباشره عن طريق تحويل الرايبوفلافين الى مشتقاته بعد التشعيع وفي دراسة الخصائص الضوئيه له.

تم تحضير محاليل قياسيه عند مديات مختلفه من الاس الهيدروجيني (2-10) للحصول على الاس الهيدروجيني الذي يحدث فيه اعلى امتصاصيه, اظهر الرايبوفلافين اعلى امتصاصيه عند الاس الهيدروجيني 7 وعند طول موجي 445 نانومتر بعد قياسها بالمنطقه المرئيه. لذلك تم اختيار هذا الطول الموجي والاس الهيدروجيني لتعيين تراكيز الرايبوفلافين في محاليل قياسيه مختلفه بالطرق الطيفيه المرئيه الفوق- البنفسجيه, وكانت نسبة الاسترجاع 98.68% مع نسبة خطأ1.31.

تم قياس كذلك تم قياس انبعاث الرايبوفلافين بالطرق التوهجيه وأظهر اعلى انبعاث عند 520نانومتر. لذلك تم اختيار هذا الطول الموجي لتعيين تركيز الرايبوفلافين في مختلف المحاليل القياسيه مع نسبة استرجاع 99.68 %ونسبة خطأ0.63%.

تم تعيين نسبة تكسر كل مشتق (الليموفلافين في الوسط القاعدي) و(الليموكروم في الوسط الحامضي). أظهر الليموفلافين الناتج اعلى امتصاص عند طول موجي 359 نانومتر وأعلى انبعاث عند 465 نانومتر. لذا تم استخدام الاس الهيدروجيني 9 والاطوال الموجيه 359 نانومتر,365 نانومتر لقياس تركيز الرايبوفلافين بالطرق الطيفيه _المرئيه الفوق_البنفسجيه والطرق التوهجيه بالتسلسل, كانت نسبة الاسترجاع بالنسبه للطرق الطيفيه 95.99% مع نسبة خطأ 4%

نسبة استرجاع الطرق التوهجيه 96.42% ونسبة خطأ3.57% . كذلك تم استخدام الاس الهيدروجيني 4 وكل من الاطوال الموجيه 353 نانومتر, 422 نانومتر. لقياس تركيز الرايبوفلافين بالطرق الطيفيه_ المرئيه الفوق_البنفسجيه والطرق التوهجيه بالتسلسل. كانت نسبة الاسترجاع بالنسبه للطرق الطيفيه % 95.18 مع نسبة خطأ4.826%. استرجاع الطرق التوهجيه 95.75% مع نسبة خطأ .4.25%.

وبعد ذلك تمت دراسة الخصائص الضوئيه للرايبوفلافين. وكان تكسر الرايبوفلافين تفاعل من الدرجه الاولى, وتم تعيين ثابت السرعه النوعيه بدرجة حرارة الغرفة بملاحظة التغيرات الطيفيه خلال عملية التشعيع. كان الناتج الكمي لعملية التكسر قليله بصوره عامه وتتأثر بالاس الحامضي, يزداد الانتاج الكمي لعملية التكسر بزيادة الاس الهيدروجيني من 2-7 وبعدها بدأ بالنقصان عند 8-10.