



Improving the Photostability of Vitamin K₁ (Phylloquinone) in organic solvent

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ABSTRACT

The photostability of vitamin K₁ (phylloquinone) in bulk powder and in its dosage form was studied according to the conditions suggested by ICH Guideline (option 2). The results showed that the rate of Photodegradation of vitamin K₁ follows first order kinetics and it is higher in its dosage form than in bulk powder. Attempts have been made to improve the photostability of vitamin K₁ by combination with different photoprotective agents such as p-aminobenzoic acid (PABA), tartaric acid, boric acid, citric acid, sodium benzoate, titanium dioxide (TiO₂), zinc oxide (ZnO), propyl 4-hydroxybenzoate and methyl 4-hydroxybenzoate. The photodecompositions products were monitored by UV-Vis spectrophotometric method and HPLC. It was found that 0.01% PABA was the best photoprotective agent, which improved the photostability of vitamin K₁ by 46 fold. The method was validated according to ICH guideline. The calibration graph was linear over the concentration range of 1.1×10^{-5} - 30.0×10^{-5} mol L⁻¹. The limit of detection and the limit of quantification were 2.42×10^{-6} and 7.35×10^{-6} mol L⁻¹, respectively.

Keywords: Phylloquinone, Photostability, 4-aminobenzoic acid; HPLC, UV-Vis spectrophotometry

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INTRODUCTION

Large number of pharmaceutical compounds undergoes degradation on exposure to light. A wide variety of drugs can undergo changes in color and nutrient composition when exposed to light. The photostability of a drug substance may be defined as the response of the drug or drug product to the exposure to solar, UV and visible light in the solid, semisolid, or liquid state that leads to a physical or chemical change.¹

Vitamin K₁ is found in leafy green vegetables, broccoli and Brussels sprouts. Vitamin K₁ (phyloquinone PY) is fat soluble vitamin (2-methyl-3-(3, 7, 11, 15-tetramethylhexadec-2-enyl) naphthalene-1, 4-dione Figure 1. Vitamin K₁ is a highly photosensitive compound, Vitamin K exists naturally in two forms; as phyloquinone (PY) and menaquinones (vitamin K₂), they have similar metabolic pathways during reactions. Overall, vitamin K contains a functional naphthoquinone ring and an aliphatic side chain. PY has a phytyl side chain attached to the homolog three synthetic types of vitamin K are known: vitamins K₃ (Menadione), K₄, and K₅. Poor anticoagulant stability in patients using vitamin K antagonists is a risk factor for both bleeding and thrombosis and low dose vitamin K₁ was shown to improve the stability of anticoagulant control².

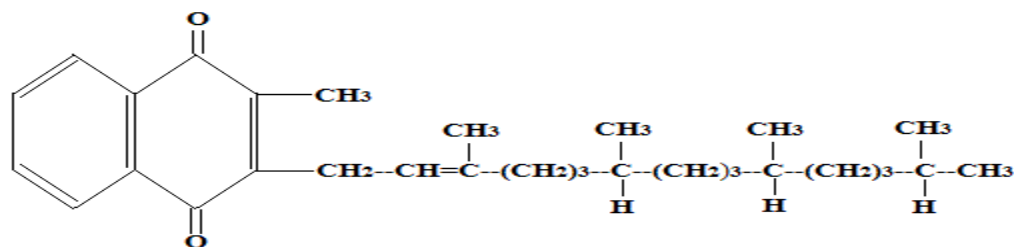


Figure 1: Chemical structures of Vitamin K₁ (phyloquinone)

PY is insoluble in water, slightly soluble in alcohol and readily soluble in non-polar organic solvents, for example in n-hexane, ether, and chloroform

The photostability of PY was studied under a combination of a near UV fluorescent lamp and a cool white fluorescent lamp. Moreover, in the present work demonstrate to improve the photostability of PY by addition of some photoprotective agents. Their photodecompositions products were monitored by UV spectrophotometric method, HPLC and LC-MS/MS.

MATERIALS AND METHOD

Chemicals and reagents

Phylloquinone (99.9%) was kindly supplied from European Egypt Pharmaceutical Company, Egypt. Acetonitrile, formic acid and methanol HPLC grade were purchased from E-Merck

(Darmstadt, Germany). Tartaric acid was purchased from (ADWIC, Egypt). Methyl 4-hydroxybenzoate and propyl 4-hydroxybenzoate were purchased from (Sigma Company, Germany). 4-aminobenzoic acid was purchased from (Win Lab Company, UK). Sodium benzoate was purchased from (Alexandria Company, Egypt). Boric acid was purchased from (El Gomhouria Company, Egypt). Citric acid was purchased from (Nasr Company, Egypt). Zinc oxide was purchased from (Merck, Germany). cyclohexane was purchased from (SdfiNe- Chem Limited Company, India.), Titanium dioxide was purchased from (Qualikems, New Delhi, India). K chewable tablets were kindly supplied by (European Egypt Pharmaceutical IND Company).

Preparation of standard and sample solutions:

Standard stock solution

The standard stock solutions of concentration 4.43×10^{-3} mol L⁻¹ of PY was prepared in cyclohexane. Working standard solution of PY 4.4×10^{-5} mol L⁻¹ was freshly prepared by appropriate dilution using cyclohexane. Care was taken to protect the solutions from light at all the time. The solution of PY was prepared freshly for each experiment to avoid any bacterial action or chemical effects.

Preparation of the working solution of vitamin K₁ in combination with different photoprotective agents

The photostability of PY 4.4×10^{-5} mol L⁻¹ solution was also investigated by typical combination with different photoprotective agents at certain concentration of (0.01%, 0.01%, 0.01%, 0.1%, 0.02%, 0.01%, 2%, 0.05% and 0.015%) tartaric acid, boric acid, citric acid, sodium benzoate, titanium dioxide, zinc oxide, propyl 4-hydroxybenzoate and methyl 4-hydroxybenzoate respectively^{3,4}.

Preparation of tablet sample solution

PY market tablet (label K chewable 10 mg phytomenadione per tablet) was extracted purified and prepared in cyclohexane to obtain the same concentration as corresponding standard stock by weight 20 tablets and grained them, weight an amount of the powder equivalent to 4.43×10^{-3} mol L⁻¹ of PY was transferred to a 100 ml volumetric flask, complete the volume to the mark with cyclohexane, the solution was filtered and put 0.1 ml of filtered solution into 100 mL volumetric flask complete the volume with cyclohexane to obtain a solution of concentration 4.4×10^{-5} mol L⁻¹.

Method validation for the stabilization of vitamin K₁ in presence of (0.01%) PABA.

The validity of the method was tested regarding to linearity, accuracy, precision, and standard addition technique, limit of detection (LOD) and limit of quantitation (LOQ) according to the International Conference on Harmonization (ICH) guideline. Also, this method was applied to pharmaceutical preparations.

Instrumentation

Irradiation conditions

CLIMACELL chamber (CLC111, MMM group, Germany) was used as a photostability testing device). It is equipped with a microprocessor-controlled system of humidification and dehumidification with a powerful lighting system that guarantees excellent homogenous parameters for testing (near UV light fluorescent lamp and cool white fluorescent lamp). Working temperature used ranged from 0.0°C up to 99.9°C (without humidity) and from 10°C to 90.0°C (with humidity). It has Inner glass door and the inner chamber is made of stainless steel DIN 1.4301 (AISI 304). It contains a shelf for putting samples and a sensor for irradiance measurements

Solution of PY at concentration 4.4×10^{-5} mol L⁻¹ was exposed to near UV light fluorescent lamp and cool white fluorescent lamp and the UV spectra of the samples were recorded just after preparation ($t = 0$) and the sample of PY solution was stirred at dark for 30min after the addition of the photoprotective agent, the samples withdrawn at different time interval (0.5,1,3,5,7,10h), 5ml sample was withdrawn, and centrifuged immediately at 3000 rpm for 10 min. The supernatant was filtered through 0.45µm pore size syringe.

UV spectrophotometry

UV spectrophotometric method was performed on double beam UV-visible spectrophotometer (Shimadzu, model 1700) having two matched quartz cells with 1 cm light path. Connected to an IBM compatible personal computer (PC) and a HP-600 inkjet printer.

Chromatographic conditions and HPLC instrumentation

High performance liquid chromatographic system (Waters, Milford, USA) equipped with Waters 486 variable wavelength UV detector, Waters 717 auto sampler injector and pump controlled by Waters 610 controller, The output signal was monitored and processed using Empower software. The output signal was monitored operated by Pentium III (450MHz) processor (Lenovo, UK) was used. The Analysis for PY solution before and after illumination was performed by HPLC using Xterre C18; 250 × 4.6 mm, 5 µm column, mobile phase composed of (Acetonitrile and methanol in isocratic elution (5:95v/v) by isocratic program. Flow rate was set to 2mL min⁻¹ with

maintained at ambient temperature, Detection wavelength was carried out at 327 nm, Injection volume was 20 μ L and the retention times of PY was at 3.99 min Figure 3A.

LC-MS/MS instrumentation and its condition

Separation and detection for Photodegradation product was performed on Agilent Triple quadrupole mass spectrometer (USA). Where Agilent Triple quadrupole mass spectrometer with API source coupled with Agilent pump controlled by Agilent 1200 controller and equipped with Agilent1200 auto sampler injector. The analysis was performed in both positive and negative electro spray ionization modes. The capillary voltage was 6000V. The source temperature was 350°C and the gas flow rate was 13 L min⁻¹. For the data acquisition and integration, Agilent Mass Hunter software operated by Pentium III (450 MHz) processor (HP, USA) .was used Agilent SB-C18 (50 x 4.6 mm), a 1.8 μ m particle size column was used for separation of Photodegradation peaks using acetonitrile: 0.1% formic acid (84:16 v/v) as mobile phase pumping at a room temperature using a flow rate of 0.6 mLmin⁻¹.

RESULTS AND DISCUSSION

Photodegradation of vitamin K₁ (PY) in cyclohexane

Spectrophotometric analysis

A literature survey reveals no photostability studies for PY was determined according ICH guidelines, thus the goal of this work was to study the photostability of PY in bulk powder and its dosage form. In addition, this work clarifies the effect of photoprotective agents on the photostability of PY in cyclohexane.

Photostability of PY in bulk powder was examined in cyclohexane. Sample was irradiated with two light doses for ten hours, Dose (I) 66 w.m⁻².h⁻¹ for UV and Dose (II) 80 kilolux for visible. The effect of radiation on drug solution was monitored by recording their absorption spectra at different time intervals

The maximum absorption of PY was observed at wavelength 327nm, when solution of PY was irradiated, spectral change was observed, the absorption spectrum of PY was remarkably decreased by increasing irradiation time Figure 2. Therefore, organic solution of PY in bulk powder is photochemically unstable and its rate of photodegradation obeys first order of kinetic law, the rate constant of photodegradation was 0.185 h⁻¹.

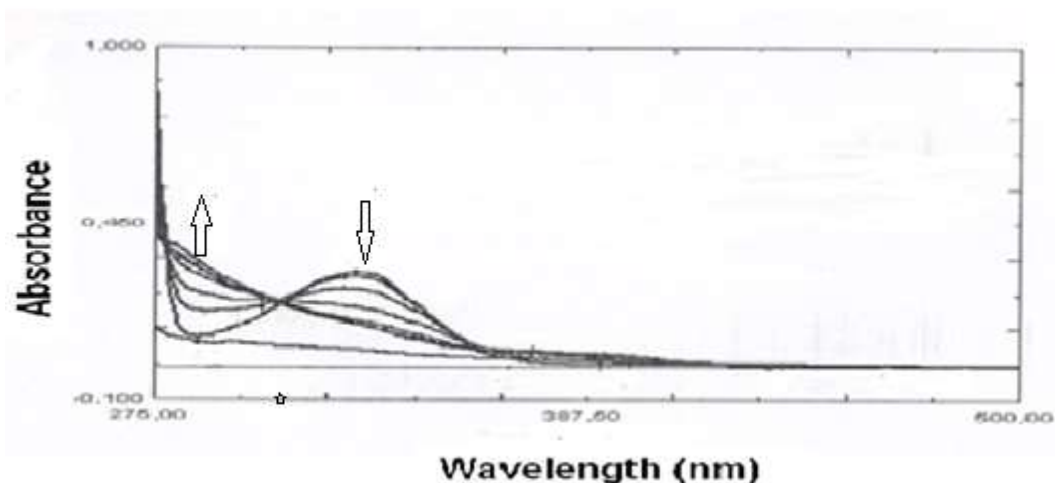


Figure 2: Change in absorption spectra of PY in cyclohexane

HPLC analysis

On the analysis of non-irradiated and irradiated solutions of PY by HPLC, the chromatogram corresponding to non-irradiated solution showed characteristic peak of the PY at retention time 3.99 min. In the case of irradiated solution the characteristic peak of the PY completely disappeared and no degradation products appeared in the chromatogram Figure 3A, 3B

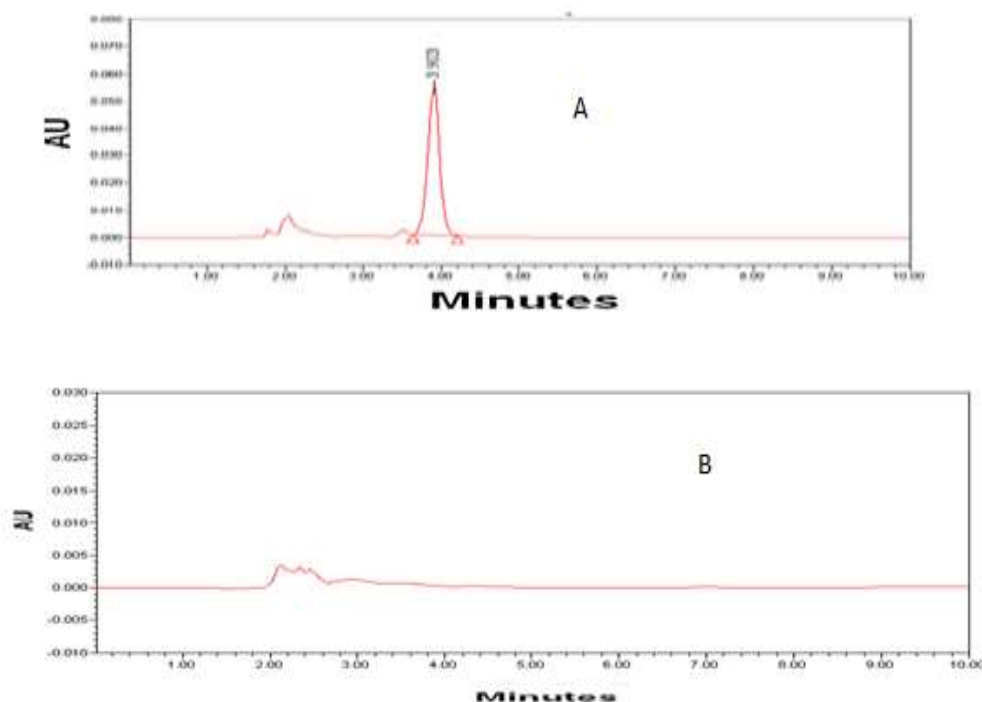


Figure 3: HPLC chromatogram of PY in cyclohexane (A) pre-exposure and (B) post-exposure to the illumination

LC-MS/MS analysis

The LC-MS/MS was used to illustrate the photodegradation pathway of PY. The mass spectra for PY solution before and after illumination were recorded. The molecular ion of PY before illumination was detected at m/z 449.6 (M - H) of PY Figure 4A. The mass spectra of PY solution after illumination exhibited a new product was formed in LC/MS chromatogram 171.3 (M- C₂₀H₃₈) Figure 4B which indicated that complete degradation for the vitamin K₁ and formation of new product. The representative extracted ion chromatograms prove the presence of new product Figure 5. Photodegradation pathway of PY was proposed and illustrated in Scheme (I)

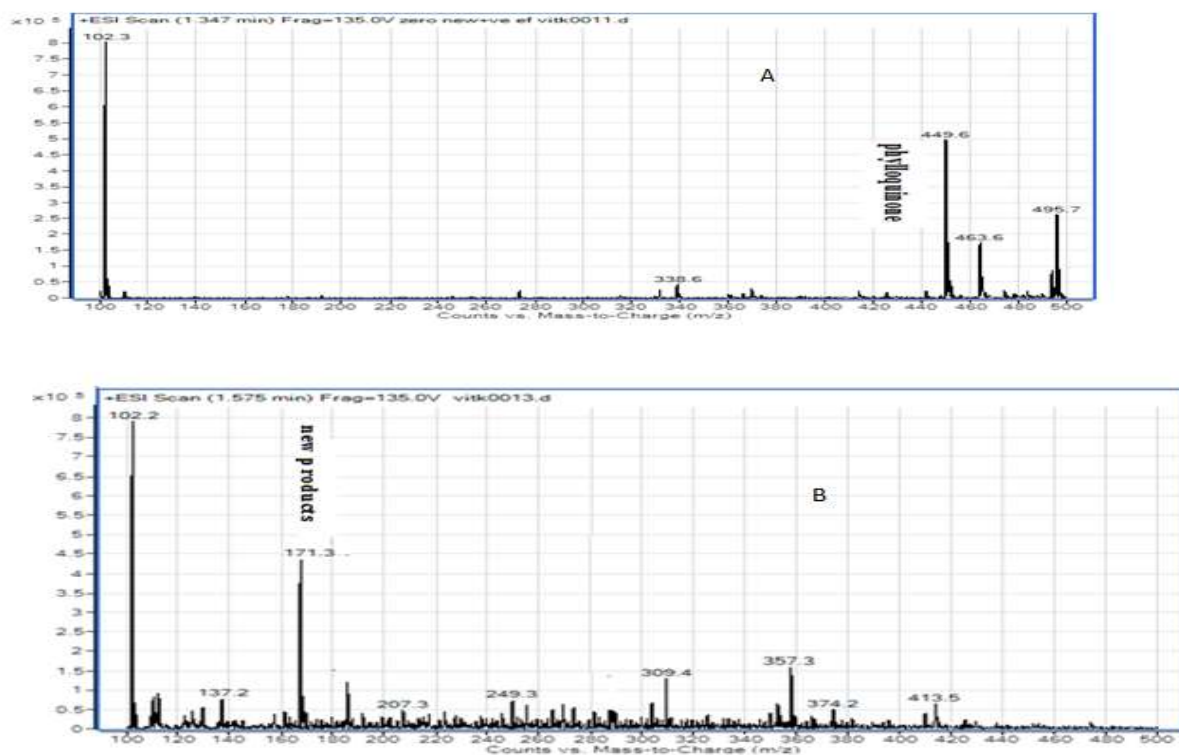


Figure 4: MS/MS spectra of PY (A) pre exposure and (B) post exposure to the illumination.



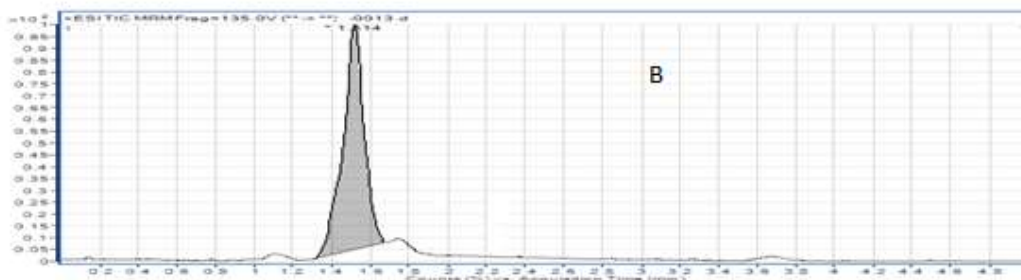
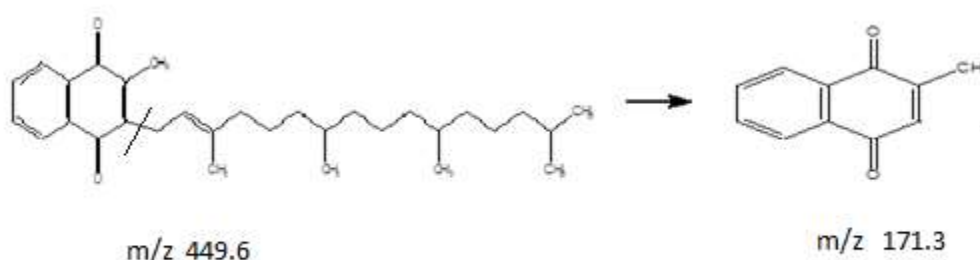


Figure 5: A representative total ion chromatogram in MRM mode of irradiated solution of PY (A) and the extracted ion chromatogram of new products (B).



Scheme (I) Photodegradation pathway of PY

Effect of concentration of vitamin K₁ on its rate of photodegradation .

Stock solutions of concentration $4.43 \times 10^{-3} \text{ mol L}^{-1}$ of PY was prepared, then appropriate dilution in cyclohexane to prepare different concentration ranging from $(30.0 \times 10^{-5} - 0.25 \times 10^{-5} \text{ mol L}^{-1})$. The rate of Photodegradation was calculated by plotting \ln concentration against time. It was found that the rate of Photodegradation increase with increase of concentration of PY, half life time and the percent remaining were also calculated (Table 1) .

Table 1: Rate of Photodegradation of PY solution at different concentration of VitaminK₁

Concentration of PY $\times 10^{-5} \text{ mol L}^{-1}$	Rate of Photodegradation ($\times 10^{-3} \text{ h}^{-1}$)	Half-life times $t_{1/2}(\text{h})$	Percentage remaining (%)
30.0	250.74	3.98	8.72
25.0	243.57	4.10	9.03
20.0	243.87	4.10	9.38
15.0	229.28	4.36	10.14
12.0	228.71	4.37	10.81
10.0	224.83	4.44	10.87
8.8	216.47	4.61	11.44
4.4	187.93	5.32	16.27
2.2	136.45	7.32	24.70
1.1	90.01	11.1	41.37
0.50	20.67	48.36	80.48

Effect of the addition of photoprotective agents on the photostability of vitamin K₁ .

UV spectrum and HPLC analysis of vitamin K₁ in the presence of photoprotective agents

The rates of Photodegradation of PY were changed in the presence of PABA, sodium benzoate, methyl 4-hydroxybenzoate, citric acid, boric acid, tartaric acid, propyl 4-hydroxybenzoate, zinc oxide and titanium dioxide respectively (Table 2). It was found that PABA was the best photoprotective agent which improves the photostability of vitamin K₁ by 46 fold Figure 6.

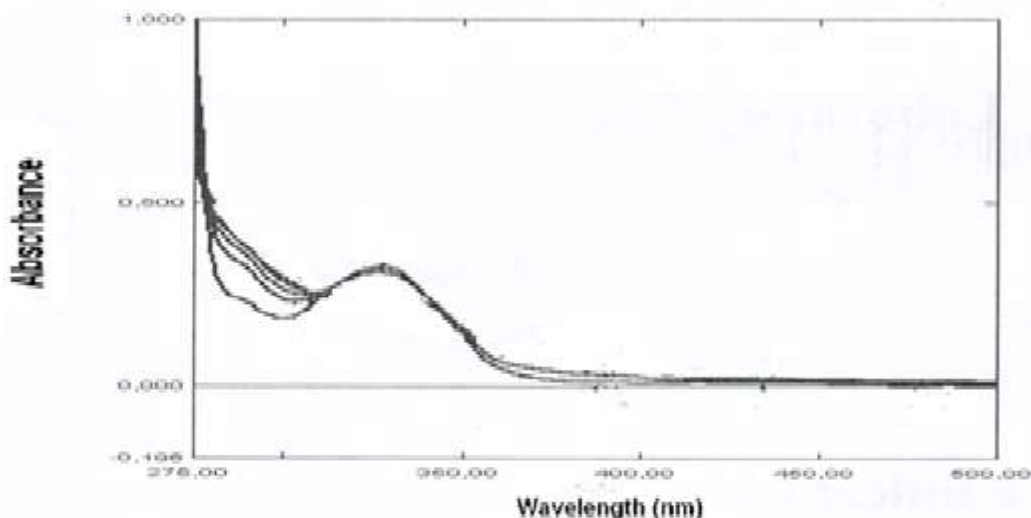


Figure 6: Absorption spectra of PY in presence of PABA

Table 2: Rates of Photodegradation and Half-life times of PY in cyclohexane with photoprotective agents

PY with photoprotective agents	Rate of Photodegradation PY with Photoprotective agents ($\times 10^{-3}$)h ⁻¹	Half-life times t _{1/2} (h)	Percentage remaining (%)
PY without any additives	185.84	5.39	14.64
PABA	4.01	249.37	95.91
Sodium benzoate	61.07	16.37	54.43
Methyl -4-hydroxybenzoate	57.46	17.40	56.25
Citric acid	74.90	13.35	47.50
Boric acid	58.43	17.11	55.77
Tartaric acid	65.69	15.22	52.12
Propyl 4-hydroxybenzoate	49.46	20.21	60.80
ZnO	72.11	13.86	48.48
TiO ₂	80.59	12.40	44.51

PABA contains a benzene ring in which electrons can resonate, between different locations within the six-sided structure. Electrons can also bounce around in an adjacent carboxyl group, made up of carbon, two oxygen and hydrogen (COOH). The mechanism of action of 4-

aminobenzoic acid involves the absorption of UV radiation followed by the excitement of the π HOMO orbital (occupied molecular orbital of higher energy) to the π^* LUMO orbital (unoccupied molecular orbital of lowest energy). These molecules upon returning to their ground states, release excess energy absorbed in the form of heat⁵. The addition of other photoprotective agents slightly improve the photostability of PY about 2-5 fold compared to the photostability of PY without any addition. The half-life and the percent remaining of PY after addition of photoprotective agents (Table 2). The analysis of non-irradiated and irradiated PY solution in the presence of PABA by HPLC was illustrated in Figure 7A,7B.

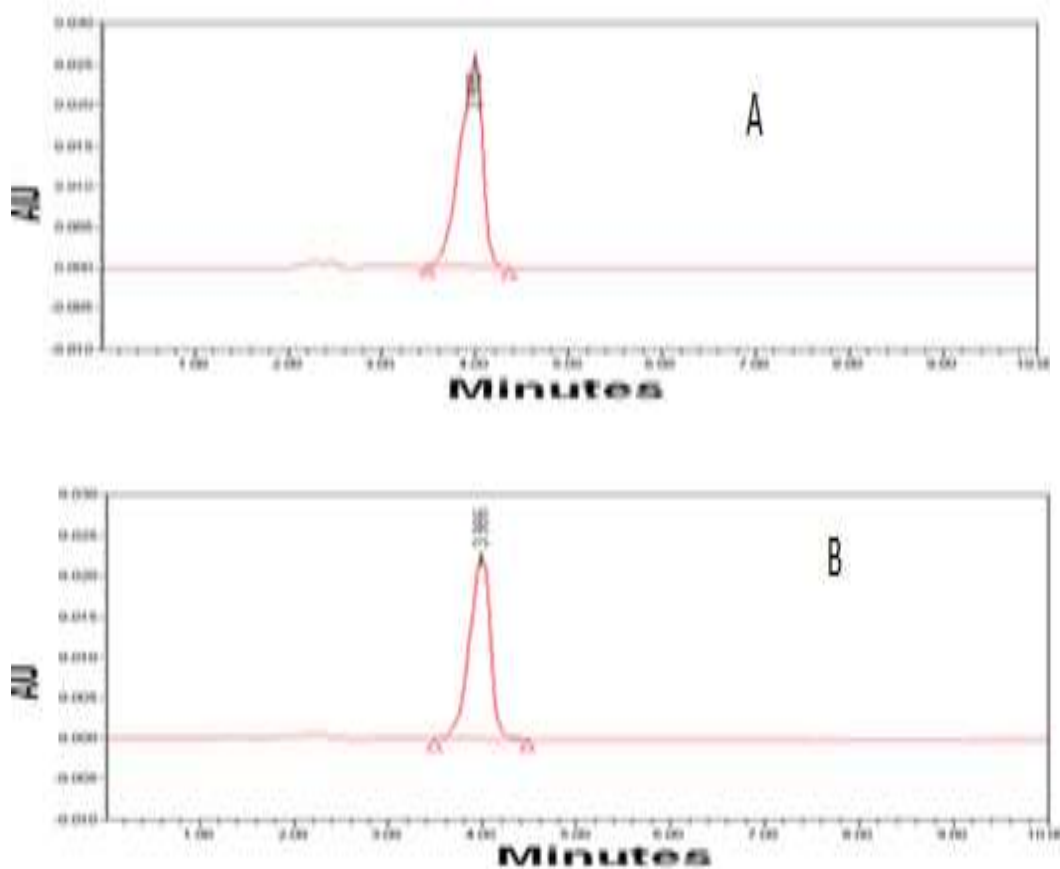


Figure 7: HPLC chromatograms of PY with in presence of PABA pre-exposure(A) and post-exposure (B)

Effect of concentration of PABA and propyl 4-hydroxybenzoate on the rate of photodegradation of vitamin K₁

Different concentrations of PABA (0.4,0.25,0.1,0.05,0.01%) and also different concentrations of propyl 4-hydroxybenzoate (0.4,0.25,0.15,0.1,0.05%) were added to the working solution of PY $4.4 \times 10^{-5} \text{ mol L}^{-1}$. The rate of Photodegradation was plotted against the concentration Figure

8. It is observed that the rate of Photodegradation increases as concentration of PABA and propyl 4-hydroxybenzoate increase.

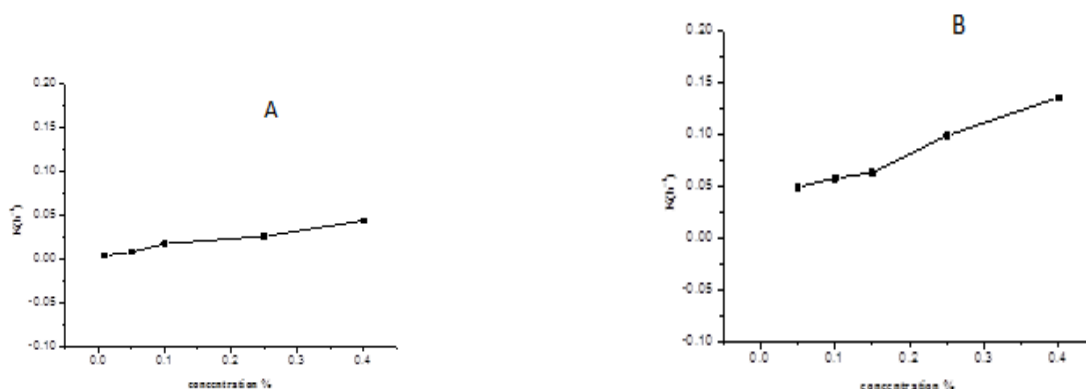


Figure 8: Rate of Photodegradation of PY versus different concentration of A) PABA, B) propyl 4-hydroxybenzoate

Photostability of vitamin K₁ in its dosage form

The photostability of PY in its dosage form was also studied. Remarkable decreases in absorbance was observed as illumination time increased and analysis of the sample in its dosage form before and after illumination by HPLC was shown in Figure 9A, 9B .

The rate of Photodegradation of PY in its dosage form is 0.222 h⁻¹ which is greater than in bulk powder .This is may be attributed to the interference of one or more of the excipients contributing to the increase of its degradation ⁶.Excipients can initiate, propagate or participate in photochemical reactions ^{7,8}. So excipients used in PY preparation strongly influenced the Photodegradation kinetics so the photostability testing for the final pharmaceutical products is very important.

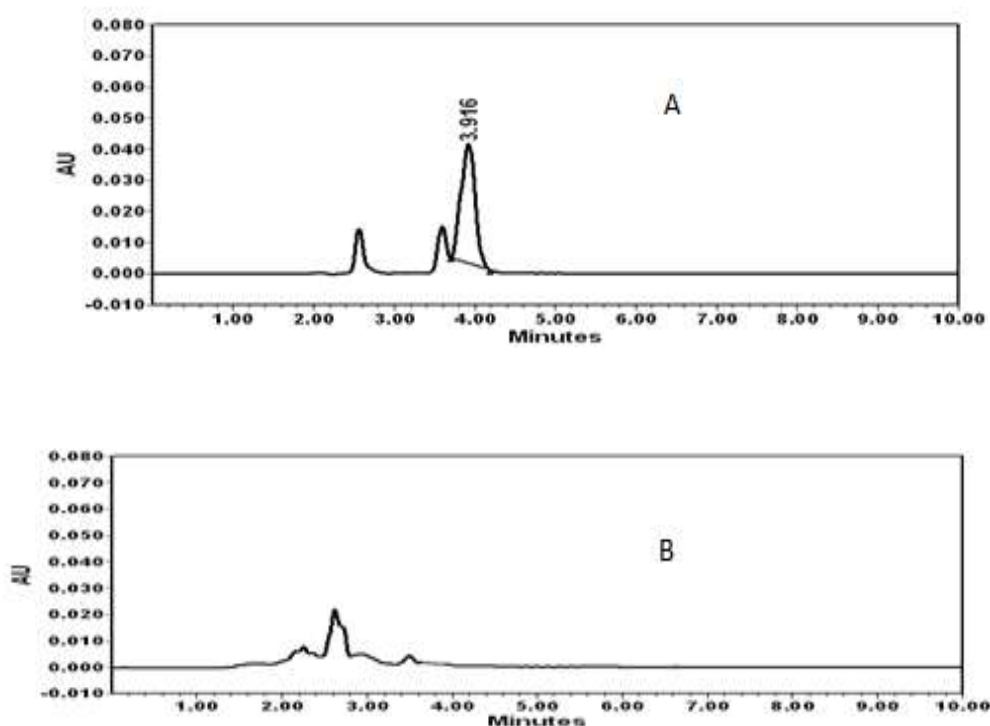


Figure 9: HPLC chromatogram of PY in its dosage form (A) Pre-exposure and (B) post-exposure to the illumination

Method validation for the stabilization of vitamin K₁ in presence of (0.01%) PABA

The method was validated for parameters like linearity range, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy.

The limit of detection (LOD) and limit of quantification (LOQ) were estimated from the standard calibration curve

LOD (the smallest concentration of the analyte that gives the measurable response was calculated using the following equation (1) :

$$\text{LOD} = 3.3 (\sigma / S) \dots\dots\dots(1)$$

LOQ (the smallest concentration of the analyte, which gives a response that can be accurately quantified) was calculated using the following equation (2):

$$\text{LOQ} = 10 (\sigma / S) \dots\dots\dots(2)$$

Where σ = standard deviation of the response, and S = slope of calibration curve^{9,10}.

Linearity study

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range^{11,12}.

Linearity was evaluated by preparing standard concentrations in the range of $(1.1 \times 10^{-5} - 30.0 \times 10^{-5})$ mol L⁻¹ of PY at constant concentration of PABA 0.01%. Three independent solutions for each concentration were prepared the solutions were scanned on a spectrophotometer in the UV range 200–400 nm. The spectrum was recorded at 325 nm after 10h. The calibration curve was constructed by plotting the absorbance versus concentration Figure 10. The regression equation was computed. The linearity parameters for the determination of PY in presence of PABA (Table 3).

Table 3: Linearity parameters for the stability of PY in presence of (0.01%) PABA

Parameters	Results observed PY
Slope	6904.5
Intercept	0.0036
Correlation coefficient (R ²)	0.9999
LOD	2.42×10^{-6}
LOQ	7.35×10^{-6}

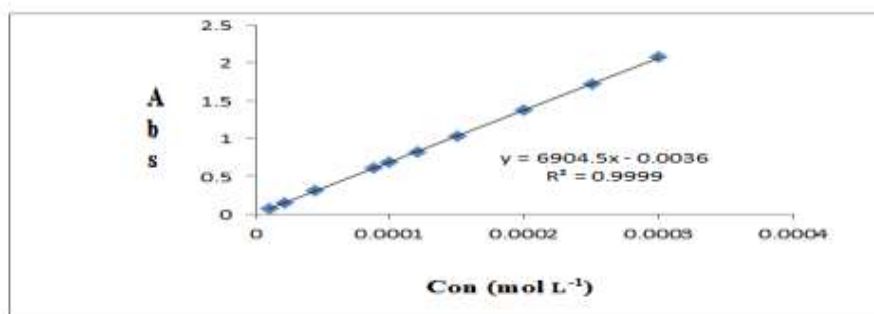


Figure 10: Calibration curve for PY in the concentration range $(30.0 \times 10^{-5} - 1.1 \times 10^{-5})$ mol L⁻¹

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. To confirm the accuracy of this method, the recovery test was performed following the ICH guideline recommendations. This was determined by adding known amounts 0.68, 0.56, 0.45, 0.34, 0.22, 0.2, 0.1, 0.05 and 0.025 mL of PY (4.43×10^{-3} mol L⁻¹) to series of 100 mL volumetric flask with 0.01 g PABA in each volumetric flask and completed to the mark with the cyclohexane to get final concentrations $(1.1, 2.2, 4.4, 8.8, 10, 15, 20, 25, 30) \times 10^{-5}$ mol L⁻¹ for PY and 0.01% PABA. The resulting solutions were analyzed and the recovered percentages were calculated (Table 4). Moreover, the accuracy of the method was also determined by the standard addition method. Known amounts of PY were added to a pre-quantified sample solution, and the

amount of PY was estimated by measuring the absorbance and by fitting these values to the straight-line equation of calibration curve (Table 5).

Table 4: Data of standard calibration curve for the stability of PY in presence of (0.01%) PABA

No	Theoretical Concentration of PY $\times 10^{-5}$ mol L ⁻¹	Absorbance means \pm SD (n=3)	Concentration found of PY $\times 10^{-5}$ mol L ⁻¹	Recovery%
1	30.0	2.08 \pm 0.01	28.18	93.95
2	25.0	1.72 \pm 0.002	23.29	93.14
3	20.0	1.37 \pm 0.003	18.58	92.94
4	15.0	1.03 \pm 0.003	14.02	93.46
5	12.0	0.82 \pm 0.004	11.22	93.55
6	10.0	0.68 \pm 0.004	9.33	93.30
7	8.8	0.61 \pm 0.003	8.39	95.40
8	4.4	0.30 \pm 0.001	4.22	95.99
9	2.2	0.14 \pm 0.001	2.06	93.95
10	1.1	0.06 \pm 0.002	1.05	95.84
Means \pm SD				94.15 \pm 1.15

Table 5: Application of standard addition method on K chewable tablets for the determination of PY in present of (0.01%) PABA

Drug	PY			
	Label claim ($\times 10^{-5}$ mol L ⁻¹)	Amount of standard added ($\times 10^{-5}$ mol L ⁻¹)	Amount Found ($\times 10^{-5}$ mol L ⁻¹)	Recovery (%) ^a
K chewable tablets	4.4	30	29.80	99.35
	4.4	20	19.91	99.58
	4.4	10	9.78	97.82
	4.4	4.4	4.35	98.90
	4.4	2.2	2.15	97.89
	Mean \pm SD			98.71 \pm 0.81
RSD (%)			0.81	

Precision

Precision is the measure of the degree of repeatability of a method under normal operation, and is normally expressed as the percent relative standard deviation (RSD) for a statistically significant number of samples. Precision may be performed at different levels: repeatability and intermediate precision¹³.

Precision was assessed as RSD % at different levels. Repeatability (intraday) was evaluated by the analysis of three different concentrations of pure drugs 6.6, 2.2 and 4.4 mol L⁻¹ of PY with 0.01%PABA each in triplicates in the same day, while intermediate precision interday was evaluated by repeating the analysis of the same concentrations for three times on three consecutive days. The results of intraday and interday precision are indicated in (Table 6). From

the data obtained, the stability of PY in presence of 0.01% PABA given precise and results are revealed high precision (RSD% <2) for this method.

Table 6: Intraday and interday precision for the stability of PY in present of (0.01%) PABA

Chemical	Concentration ($\times 10^{-5}$ mol L ⁻¹)	Intra-day precision		Mean recovery% \pm SD	Mean RSD%
		Amount Found ($\times 10^{-5}$ mol L ⁻¹)	Recovery %		
PY	2.2	2.07	94.23	95.16 \pm 1.08	1.08
	4.4	4.23	96.36		
	6.6	6.26	94.90		
PY	2.2	2.07	94.10	95.00 \pm 0.98	0.98
	4.4	4.22	96.05		
	6.6	6.26	94.85		

Effect of 0.01% PABA and 0.05 % Of propyl 4-hydroxybenzoate in the rate of Photodegradation of vitamin K₁ in its dosage form.

It was found that the addition of 0.01% PABA improves the photostability of PY in its dosage form by 25 fold while with the addition of 0.05% propyl 4-hydroxybenzoate improves in its photostability by 5 fold its dosage form **Figure 11**. (Table 7).

Table 7: Rates of Photodegradation of PY and its Half-life times in its dosage form in the presence of PABA, propyl 4-hydroxybenzoate

Vitamin K ₁ (PY)	Rate of Photodegradation ($\times 10^{-3}$ h ⁻¹)	Half-life times t _{1/2} (h)	Percentage remaining (%)
In Bulk form	185.84	5.39	14.64
In dosage form	222.13	4.50	10.03
In dosage form in presence of PAPA	8.81	113.50	89.35
PY in dosage form with propyl 4- hydroxybenzoate	40.72	24.55	64.13

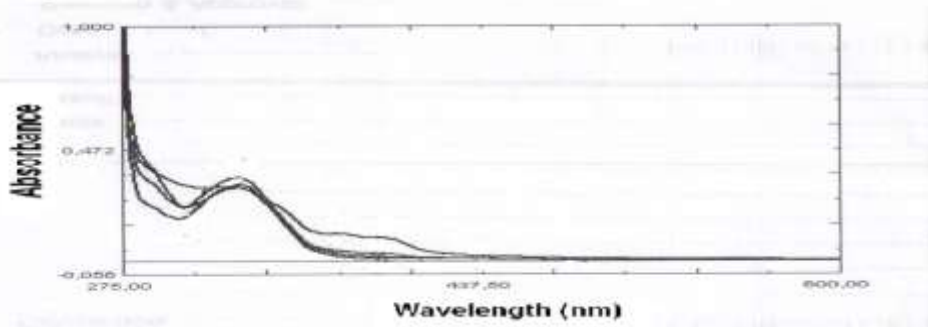


Figure 11: Change in absorption spectra of PY in doses form in present of (0.01%) PABA

CONCLUSION

Vitamin K₁ is photolabile, its rate of Photodegradation obeys first order kinetic law. Its rate of Photodegradation in its dosage form is higher than in bulk powder. Attempts were done to increase its photostability by addition of different photoprotective agents. It was found that 0.01% PABA was the best photoprotective agent which improves the photostability of vitamin K₁ by 46 fold in bulk powder and by 25 fold in its dosage form.

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