Current Research in Pharmaceutical Sciences
Available online at www.crpsonline.com

ISSN: 2250 – 2688

Received: 16/02/2023
Revised: 25/02/2023
Accepted: 27/02/2023
Published: 08/04/2023

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DOI: 10.24092/CRPS.2023.130104

Website: www.crpsonline.com

Quick Response Code:

Simultaneous Determination of Phytonadione and its Degradants by HPLC in Injectable Emulsion Formulation Used to Treat VKDB in Newborns

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ABSTRACT

Phytonadione, also known as Phytomenadione or Vitamin K_1 is used as an Injectable emulsion is used to Newborns for the precautionary treatment for Vitamin K Deficiency Bleeding (VKDB) during birth. In the current study, A Gradient, Sensitive & Cost effective HPLC method has been developed to determine and quantify the degradants of Phytonadione and Benzyl Alcohol into Phytonadione Injectable Emulsion. The chromatographic separation was performed by GL Sciences Inert sustain HP™ C_{18} (250 × 4.6 mm, 3.0 µ) column. The degradants were well separated by a gradient program started with the mixture of 25 mM Ammonium Acetate in water as buffer, pH 3.5 and Methanol in the ratio of 40: 60 V/V at the flow rate of 1.0 mL min^{-1} and UV detection was performed at 254 nm. The degradation products from Phytonadione and Benzyl Alcohol were well resolved from the main peaks and its other impurities by the developed method. The method was validated by complying specificity/selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision following ICH Q2 (R1). The developed method in this study could be applied for the analysis of routine quality control related substances of Phytonadione Injectable Emulsion, since there is no official monograph.

Key words: Phytomenadione, Vitamin K, Method validation, Impurities, Degradants.

1. INTRODUCTION

Vitamin K is responsible as an essential cofactor for the synthesis of active blood-clotting factors such as II, VII, IX, X, protein C and protein S as well as non-coagulation proteins such as osteocalcin and matrix Gla protein.[1] Vitamin K has a common structure named as 2-methyl 1-4-naphthoquinone ring. Vitamin K (VK) naturally forms phylloquinone (PH) or vitamin K1 (VK1), which is originated from a vegetable natural source and menaquinones MK-n (or vitamin K2), which is originated form an animal source or bacterial fermentation.[2] Where, Vitamin K1 acts as a blood clotting factor.

Phytomenadione is chemically named as 1,4-Naphthalenedione,2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-, [R- [R*, R*-(E)]]-. Phylloquinone [CAS No.: 84-80-0]. Phytonadione (Figure 1) is basically a naphthoquinone derivative, works on newborn as a co-factor to maintain normal blood clotting mechanism and preventing hemorrhagic disease.[3] For the infants, Vitamin K deficiency bleeding (VKDB) is a rare and life-threatening bleeding disorder. Storage of Vitamin K during birth and lactation is very low, thus possibilities of this disorder is in higher rate in this stage. By a small dose of vitamin K1 might save a life when, classical VKDB occurs in the first week of life or delayed or inadequate feeding have seen at birth.[4]

Vitamin K1 is basically a yellow, sterile, nonpyrogenic aqueous dispersion available as injection in the market which can be administered through intravenous (IV), intramuscular (IM) or subcutaneous (SC) routes. Each milliliter OF Vitamin K1 injectable emulsion contains phytonadione 2 or 10 mg where benzyl alcohol, 9 mg used as preservative.[5] Vitamin K_1 is oxygen sensitive and decomposes gradually on exposure to light,[6,11,17] which literally introduce Photodegradation products by degrading Vitamin K1. Whereas, Benzyl Alcohol is used as preservative to formulate this injectable emulsion, which itself undergo oxidative degradation to benzaldehyde. Scientific literature reveals that benzaldehyde may cause many toxicities to newborns.[5,13]

Thus, determination of Photodegradation products of vitamin K1 and Benzaldehyde in Phytonadione injectable emulsion is very much necessary to control the impurities into the product at minimum level. Phytonadione Injectable Emulsion is official in British Pharmacopoeia (B.P) & United States Pharmacopoeia (U.S.P) and European Pharmacopoeia (E.P), represents a liquid chromatographic method for its active drug estimation. But there is no method available in these monographs against related substances or degradation products estimation. A deep literature survey reveals that there are some articles regarding determination of Active Vitamin K1 in biological matrices, [3,8,9,12,14,15,18,22] or the HPLC method for determination of Vitamin K1 in pharmaceutical formulations.[2,7,16,19,20,21] These articles are applied to determine the Active (Phytonadione) only.

There is an article on the Vitamin k1 and its impurities which does not represent the photodegradation products of Vitamin K1 and degradant of Benzyl Alcohol.[23] There was no specific literature for estimation of degradants of vitamin K1 injectable emulsion and it's preservative. So, considering all these, the present research article describes a HPLC method for the estimation of all degradant's of Phytonadione and Benzyl Alcohol in Phytonadione Injectable Emulsion in a single run.

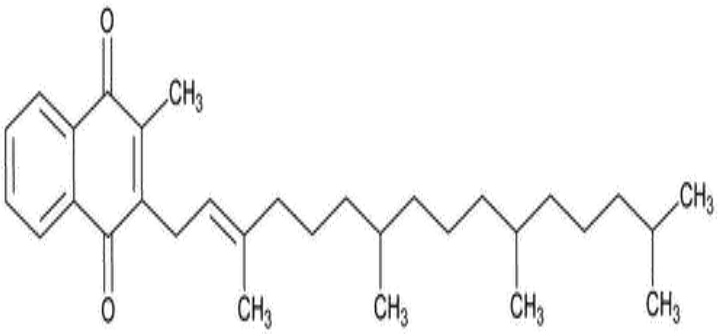

Figure 1: Phytonadione (Vitamin K1)

2. EXPERIMENTAL SECTION

2.1 Instruments

The Waters HPLC with PDA detector is used with Empower 2 software, Mettler Toledo Electronic analytical balance, ultra-sonic bath, Metrohm pH meter and Inertsustain HP, L1Column, 3 µm, 4.6 x 250 mm were used.

2.2 Chemicals and Reagents

Photodegradation Product I (potency: 97.83%) & Photodegradation Product II (potency: 83.09%) obtained from EMCURE Pharmaceutical Ltd., India. Menadione USP (potency: 99.8%), Trans-Epoxyphytomenadione EP CRS (potency: 100.0%) obtained from USP & BP. Benzaldehyde (potency: 98.0%), Benzyl alcohol (potency: 100.08%) obtained from Merck Laboratories, Germany and Phytonadione Working standard (potency: 99.19%) and Phytonadione Inectable Emulsion achieved from the LVP unit of SQUARE Pharmaceuticals Ltd., Kaliakoir, Bangladesh. Ammonium acetate, HPLC grade methanol and glacial acetic acid were used from Merck, Germany, HPLC grade Ethanol was used from Applichem GmbH, Germany.

2.3 Preparation Mobile Phase-A

3.85 g of Ammonium acetate was taken as buffer salt to prepare 2000 mL buffer. Dissolved it with purified water. The pH was adjusted to 3.5 with glacial acetic acid. 0.45 µm sartolon polyamide membrane filter was used to filter the buffer solution before use.

2.4 Preparation Mobile Phase-B

Methanol was used as Mobile Phase-B for the gradient program.

2.5 Preparation of Diluent

Ethanol was used as diluent.

3. METHOD

3.1 Wavelength Detection

20.0 mg of Phytonadione working standard was taken and transferred into a 100 mL clean and dried volumetric flask. Dissolved it with diluent and transfer 1.0 mL of this Phytonadione stock solution to a 100-mL volumetric flask and dilute to volume with diluent and mix. Concentration of Phytonadione was 2.0 µg/ml. Filtered this solution through PTFE syringe filter, 0.45 µm and collected the solution in a clean and dry HPLC vial and scanned between 200 and 400 nm with 2998 PDA detector of Waters 2695 HPLC system. The maximum absorbance of

Phytonadione was around 254 nm, thus the wavelength detection had set at 254 nm.

3.2 Chromatographic Conditions

Different chromatographic conditions were applied and finalized as 55 °C Column Temperature, 5°C Auto sampler (Cooling Temperature) with a UV detection at 254 nm. The flow rate was fixed at 1.0 ml per min at gradient elution (Table 1) and run time was finalized for 85 minutes. The column was equilibrated for 60 min with the mobile phase before injection of drug solution and the injection volume was set to 10 µl. The diluent was injected as blank to check the solvent interference (Figure 2).

Table 1: Gradient program of the Mobile phase

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)
0	40	60
5	40	60
20	10	90
45	5	95
55	3	97
75	3	97
77	40	60
85	40	60

3.3 System Suitability Solution Preparation

25 mg of Phytonadione (Vitamin K1) working standard was taken into a 100 mL clean and dried volumetric flask and diluted to volume up to 100 mL with diluent and mixed well. A 2 µg/ml system suitability solution was prepared through further dilution of this solution.

3.4 Evaluation of System Suitability

The %RSD of 06 replicate system suitability solutions was below 5.0, Theatrical plate count as column efficiency was found more than 2000 USP and USP Tailing factor was also more than 2.0 in the chromatogram as shown in Table 2 & Figure 3.

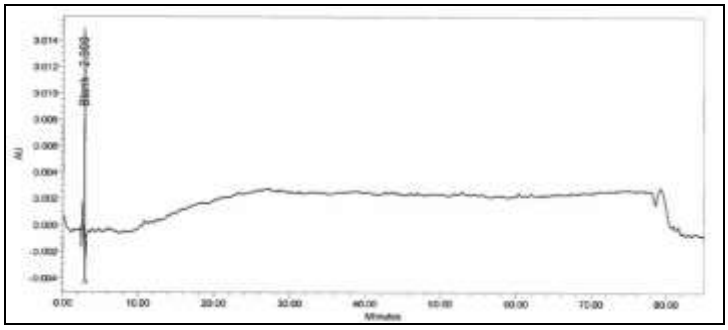

Figure 2: Chromatogram of Diluent/Bank

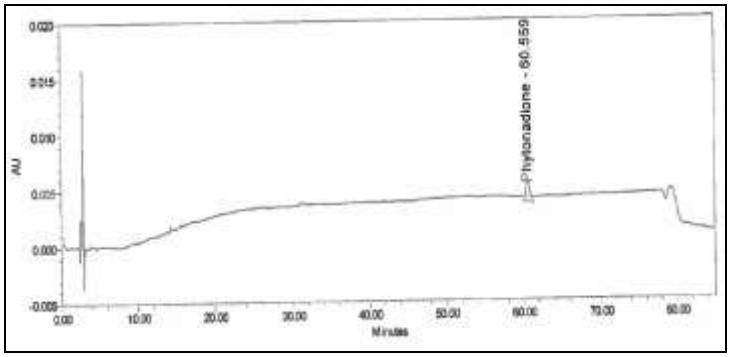

Figure 3: Standrad Chromatogram

3.5 Test Solution Preparation

There are 02 (Two) different drug formulation for Phytonadione Injectable Emulsion. For the sample preparation of Phytonadione injectable emulsion, 2 mg/mL, Took all the contents of 5 vials in a clean, dry & Amber colored test tube. Transferred accurately 2.0 mL of this Phytonadione Injectable emulsion solution into a 4 mL volumetric flask and diluted to volume with diluent and mixed well. For the sample preparation of Phytonadione injectable emulsion, 10 mg/mL, took the contents of 3 vials in a clean, dry & Amber colored test tube. Transferred accurately 1.0 mL of this Phytonadione Injectable emulsion solution into a 10 mL volumetric flask and diluted to volume with diluent and mixed well. Final concentration of both samples was 1.0 mg/ml. Placebo for each injectable emulsion should be prepared by the same way.

Table 2: System suitability Data

Sr. No.	Area of Phytonadione
1	35169
2	33463
3	33149
4	32523
5	33472
6	34691
Mean	33745
Standard Deviation	992.50
(%) RSD	2.94
Theoretical plates of last injection of standard solution	162082
Tailing factor for last injection of standard solution	1.34

4. ANALYTICAL METHOD VALIDATION[10]

4.1 Specificity

Blank was prepared by HPLC grade Ethanol and Placebo was prepared according to test solution preparation. The responses of the Blank and placebo peaks were noted to observe any interference of the Blank and Placebo at the retention time of Phytonadione and its degradants (Figure 4), The data demonstrated in specificity below in Table 3.

Table 3: Selectivity data for spiked test solution

Spiked test solution (10mg/mL)				
Peak Name	**RT (min.)**	**RRT**	**Purity Angle**	**Purity Threshold**
Benzaldehyde	5.286	0.09	0.42	0.65
Photodegradation Product I	39.415	0.65	0.79	0.65
Photodegradation Product II	45.111	0.74	1.08	4.18
Menadione	8.721	0.14	5.55	2.05
Trans-epoxymenadione	53.902	0.89	4.09	18.12
Spiked test solution (1mg/0.5mL)				
Benzaldehyde	5.293	0.09	0.21	0.34
Photodegradation Product I	39.427	0.65	1.08	0.73
Photodegradation Product II	45.116	0.74	7.41	7.99
Menadione	8.727	0.14	5.47	2.14
Trans-epoxymenadione	53.909	0.89	7.63	21.64

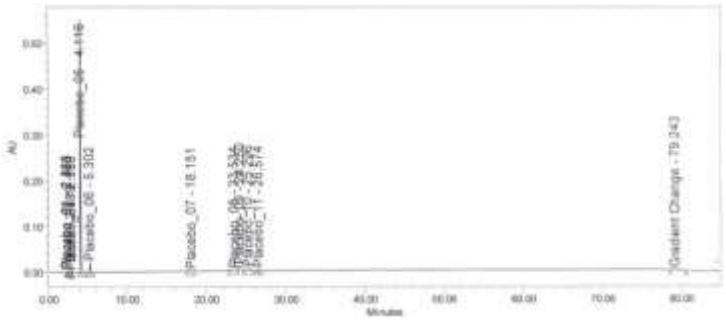

Figure 4.1: Placebo (10 mg/ml)

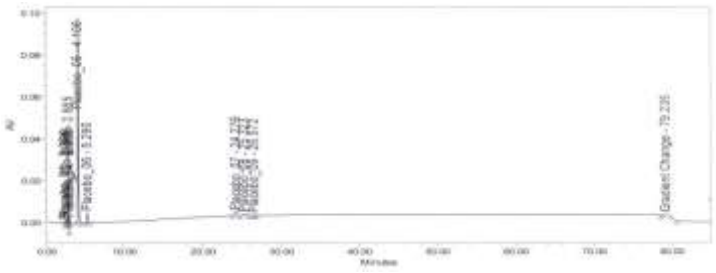

Figure 4.2: Placebo (2 mg/ml)

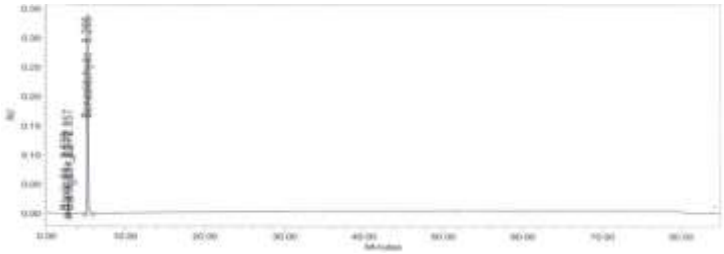

Figure 4.3: Identification of Benzaldehyde

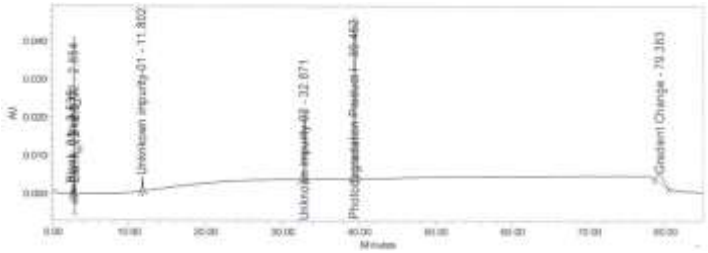

Figure 4.4: Identification of Photodegradation Product I

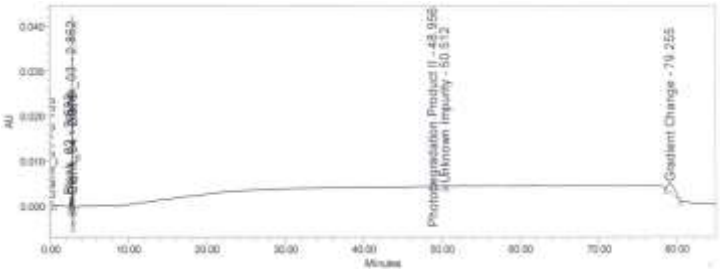

Figure 4.5: Identification of Photodegradation Product II

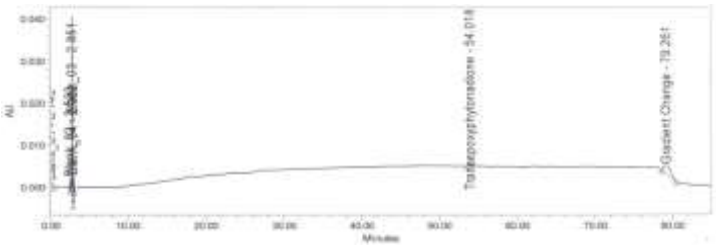

Figure 4.6: Identification chromatogram of Trans-epoxy

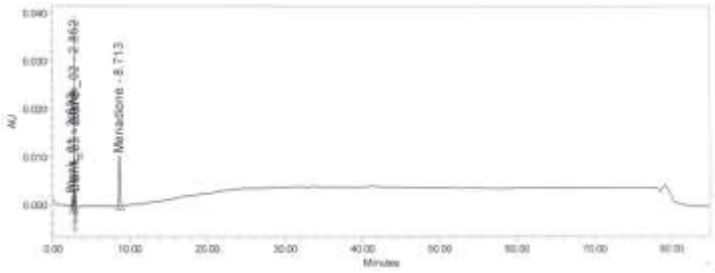

Figure 4.7: Identification chromatogram of Menadione

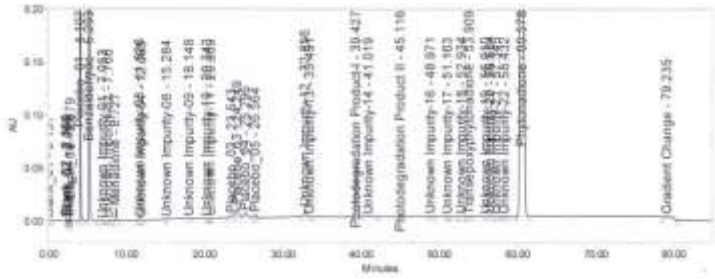

Figure 4.8: Sample spiked with known impurities

4.2 Precision

Precision was performed by spiking the Test solution with known degradants (Photodegradation product I & II, Benzaldehyde) of Phytonadione Injectable emulsion at limit concentration as follows in Table 4.

Table 4: Acceptance Criteria of impurities of Phytonadione Injectable Emulsion

Sr. No.	Name	Relative Retention time	Acceptance Limit
1	Benzaldehyde	~ 0.09	Not more than 0.5%
2	Photo degradation Product I	~ 0.65	Not more than 1.0%
3	Photo degradation Product II	~ 0.74	Not more than 2.0%
4	Any Unspecified impurity		Not more than 0.2%

Two different HPLC system were used to perform the Method precision and Intermediate precision. The peak area of these degradants of six spiked test solutions were calculated and reported. The data reveals that the method and intermediate precision is satisfactory as %RSD is not more than the pre-determined acceptance criteria as shown in Table 5.

Table 5: Comparison of Method Precision and Intermediate Precision

Analyst Number # 01						**Acceptance criteria for impurities of six spiked test solutions**	
HPLC 1							
Column Serial No.: 5FR55064							
Method precision							
Spiked test solution	Benzaldehyde	Photo degradation Product I	Photo degradation Product II	Any unspecified impurity	Total impurities (Excluding Benzaldehyde)		
1	1.033	2.640	0.544	0.154	3.470		
2	1.029	2.658	0.585	0.164	3.515		
3	1.025	2.639	0.574	0.155	3.475		
4	1.042	2.645	0.569	0.159	3.487		
5	1.038	2.635	0.603	0.151	3.514		
6	1.034	2.649	0.596	0.159	3.515		
Intermediate precision						**% Impurity (ies) found**	**% RSD limit**
Analyst Number # 02						<0.05%	Not to be reported
HPLC 2						>0.05% to <0.20%	NMT 20.0
Column Serial No.: 5FR55065						>0.20% to <1.0%	NMT 10.0
1	1.054	2.886	0.581	0.150	3.742	>1.0% to <10.0%	NMT 5.0
2	1.057	2.867	0.509	0.151	3.635	>10.0%	NMT 2.0
3	1.061	2.886	0.567	0.154	3.721		
4	1.054	2.855	0.485	0.151	3.603		
5	1.062	2.872	0.512	0.151	3.645		
6	1.059	2.875	0.520	0.149	3.661		
Mean	1.046	2.759	0.554	0.154	3.582		
SD	0.014	0.120	0.039	0.005	0.098		
%RSD	1.34	4.35	7.04	3.25	2.74		

4.3 Linearity

The peak responses of each degradant like Photodegradation Product I, Photodegradation Product II and Benzaldehyde were linear over the concentration range about 1.0 – 12 µg mL^{-1}, 2.0 – 24.0 µg mL^{-1}, 0.5 – 6.0 µg mL^{-1} respectively, which was with a correlation coefficient (r) ≥0.999.

4.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) was determined from the linearity curve previously determined as per following LOD = 3.3 (SD/S) and LOQ = 10 (SD/S), whereas, SD means standard deviation and S means slope. The LOD and LOQ values were shown in Table 6.

Table 6: LOD and LOQ determination

Name	LOD	LOQ	LOQ precision@ 0.50 ppm	
	ppm	ppm	Average Area	S/N of last Injection
Benzaldehyde	0.15	0.50	13483	94.8
Photodegradation Product I	0.15	0.50	13668	56.0
Phytonadione	0.15	0.50	9870	24.6

4.5 Stability of Sample Solution

The Test solution was prepared as per analytical procedure and the spiked test solution was prepared by spiking the known degradants/impurities with limit concentration and analyzed initially and at different time intervals by keeping the solution at room temperature and refrigerator (2-8^{0}C). The stability studies of solutions show that the test solutions were stable for at least 24 hours at refrigerator (2-8^{0}C) condition only.

4.6 Accuracy

The Accuracy (%Recovery) was performed at LOQ, 50%, 100% & 150% level. The test solution (un-spiked) and spiked test solution with known impurities were prepared at LOQ level to 50%, 100% and 150% level of nominal test concentration. The mean percentage recovery was found v for Benzaldehyde and Photodegradation product I in the range between 101.41 and 112.52 which were within the acceptance limits as shown in Table 7.

5. RESULT AND DISCUSSION

The developed and validated method with the chromatographic conditions achieved was found to be suitable for all combinations to separate and quantify these degradants in quantification level for Phytonadione Injectable Emulsion. The acquired chromatographic conditions gave a selective retention time for all impurities and Phytonadione itself. The specificity of this method was performed by injecting diluents, Placebo and individual specific impurities for phytonadione injectable formulations and no peaks were found at the retention time of Benzaldehyde, Phytonadione, Photodegradation Product I & Photodegradation Product II. The stability of the Test solution and spiked test solution was evaluated up to 24 hours and found that the solutions are stable at refrigerator conditions (2-8°C) for 24 hours. A good linearity was observed for each degradant like Photodegradation Product I, Photodegradation Product II and Benzaldehyde over the concentration range about 1.0 – 12 µg mL^{-1}, 2.0 – 24.0 µg mL^{-1}, 0.5 – 6.0 µg mL^{-1} respectively and correlation coefficient (r) was ≥0.999. Limit of quantitation (LOQ) value was determined as 0.5 ppm for each degradant. Intermediate Precision was done comparing with method precision results by changing the analyst, column, System etc. with the same chromatographic conditions and obtained good results within limit. The accuracy of the method was also performed and calculated the percentage recovery which was also within limits.

6. CONCLUSION

Phytonadione Injectable Emulsion is used for the Neonates during birth for the treatment of VKDB which can cause bleeding in nearly to every organ of the body for the neonates. Vitamin K deficient bleeding diseases involve with bleeding in the brain and brain damage (https://kidshealth.org/en/parents/vitamin-k-shot.html). A simple vitamin K1 injection can protect the babies from a serious, even deadly bleeding disorder. Vitamin K formulation itself carries Benzyl Alcohol as a preservative which literally degraded to toxic Benzaldehyde which must be monitored during analysis. Photodegradation product I & II also introduced from Vitamin K molecule by decomposition. All these impurities must be monitored and control during drug product formation for Vitamin K. There should have a specific method which can determine and quantify the degradants in specific level. In this method, all the possible impurities/degradants of Phytonadione Injectable Emulsion have been separated, identified and quantified to analysis the impurities for Quality control of drug formulations.

The method validation results indicated that this HPLC method for the determination all possible degradants/impurities from Phytonadione Injectable Emulsion including its preservative in a single run is precise, accurate, linear over the test concentration range, rugged and specific and low cost. Consequently, this method is suitable for using of routine impurity analysis of production sample and research work on Vitamin K1 Injectable Emulsion.

ACKNOWLEDGEMENT

The authors are very much thankful to the management of Square Pharmaceuticals Ltd., Dhaka, Bangladesh for providing Laboratory support to carry out this study.

Table 7: Accuracy (% Recovery) summary of Impurities for Phytonadione Injectable Emulsion

Accuracy (% Recovery) of Benzaldehyde For 10mg/mL							
Levels	Preparations	Amount found (%)	Amount added (%)	% Recovery	Mean % Recovery	% RSD	Acceptance Criteria
First Level	LOQ /1	0.104	0.101	103.24	102.40	3.94	For LOQ Level and impurities >0.05 % to < 0.20%: % recovery should be between 80.0 & 120.0 and % RSD of % recovery should be not more than 20.0. For impurities >0.20 % to < 1.0%: % recovery should be between 80.0 & 120.0 and % RSD of % recovery should be not more than 10.0. For impurities >1.0% to <10.0%. % recovery should be between 90.0 & 110.0 and % RSD of % recovery should be not more than 5.0. For impurities >10.0% % recovery should be between 90.0 & 110.0 and % RSD of % recovery should be not more than 2.0.
	LOQ /2	0.099	0.101	98.02			
	LOQ/3	0.107	0.101	105.94			
Second level	Rec-50%/1	0.596	0.527	113.09	112.52	0.51	
	Rec-50%/2	0.590	0.527	111.95			
	Rec-50%/3	0.593	0.527	112.52			
Third level	Rec-100%/1	1.155	1.054	109.58	109.33	0.27	
	Rec-100%/2	1.149	1.054	109.01			
	Rec-100%/3	1.153	1.054	109.39			
Fourth level	Rec-150%/1	1.702	1.582	107.59	107.00	0.86	
	Rec-150%/2	1.676	1.582	105.94			
	Rec-150%/3	1.700	1.582	107.46			
Accuracy (% Recovery) of Photodegradation Product I for 10mg/mL							
First Level	LOQ/1	0.053	0.045	117.78	107.41	8.36	
	LOQ/2	0.046	0.045	102.22			
	LOQ/3	0.046	0.045	102.22			
Second level	Rec-50%/1	1.637	1.504	108.84	109.13	0.31	
	Rec-50%/2	1.647	1.504	109.51			
	Rec-50%/3	1.640	1.504	109.04			
Third level	Rec-100%/1	3.185	3.008	105.88	106.43	0.47	
	Rec-100%/2	3.205	3.008	106.55			
	Rec-100%/3	3.214	3.008	106.85			
Fourth level	Rec-150%/1	4.593	4.512	101.80	101.41	0.34	
	Rec-150%/2	4.566	4.512	101.20			
	Rec-150%/3	4.567	4.512	101.22			

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