

# Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytonadione Injectable Emulsion Dosage Form

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## ABSTRACT

The intention of the study is to identify impurities in Phytonadione injectable emulsion formulation by using NMR, LC-MS, preparative, and HPLC system. To identify and quantify unspecified impurities, a novel HPLC method has been validated. A gradient programme has been used to separate the process-related and degradation impurities that produced under stress, on the Phenomenex Gemini-C18 column. The mobile phase having two pump A and B. pump-A is the 55%:45% v/v of phosphate buffer with pH 2.50: acetonitrile and pump-B is the 90%:10% v/v of acetonitrile and phosphate buffer with pH 2.50. The 1.2 ml/min is used flow rate. 40°C is kept the column temperature at and sample temperature 25°C maintained, 20µL is the injection volume and 268 nm wavelength fixed. During stress conditions, acid, humid, base, ultraviolet light, thermal, and oxidative processes are carried out. The separation is achieved between the peaks and degradation products. The method is validated with respect to current ICH guidelines for solution stability, specificity, linearity, precision, accuracy, and robustness. Preparative HPLC is used to isolate three unknown impurities, which were then identified using LC-MS, NMR, based on literature, This is the first research study to propose five impurities in parenteral dosage forms of Phytonadione with quantitative method.

**Keywords:** Vitamin K, Phytonadione, Forced degradation, Liquid chromatography, Determination of related substances.

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## INTRODUCTION

The Phytonadione formula indicates the 2'-Trans-7R, 11R-stereoisomer has two geometrical isomers (cis-trans or (Z)-(E) isomers). there are eight diastereoisomers (four in the trans- and four in the cis-configuration) as having plus two asymmetric carbon atoms (C7 and C11), each generating two enantiomers (R or S). The others are not vitamins, The Phytonadione name is appropriate only for the 2'-Trans-7R, 11R-stereoisomer. Phytonadione is 2-methyl-3-phytyl-1, 4-naphthoquinone. Its empirical formula is C<sub>31</sub>H<sub>46</sub>O<sub>2</sub>. Phytonadione Injectable Emulsion is a sterile, nonpyrogenic aqueous dispersion yellow color available for injection by the intravenous, intramuscular and subcutaneous routes. Phytonadione Injectable Emulsion shows the following coagulation disorders which are due to faulty formation of factors II, VII, IX and X when caused by interference with Vitamin activity or vitamin K deficiency.

Phytonadione injectable emulsion (Vitamin-K) VTK is indicated in: anticoagulant-induced prothrombin deficiency caused by indanedione derivatives or coumarin; hypoprothrombinemia due to antibacterial therapy; prophylaxis and therapy of hemorrhagic disease of the newborn; hypoprothrombinemia secondary to factors limiting absorption or synthesis of Phytonadione, e.g., biliary fistula, obstructive jaundice, sprue, intestinal resection, ulcerative colitis, celiac disease, cystic fibrosis of the pancreas, and regional enteritis; other drug-induced hypoprothrombinemia

where it is definitely shown that the result is due to interference with Phytonadione metabolism, e.g., salicylates[1].

The proposed research target to develop a single LC method for estimation of impurities in drug substances and drug products. An wide-ranging literature survey found many impurity testing methods in monograph of USP for Phytonadione drug substances and formulated drug products, including tablets and injections [2-3].

The column chemistry and mobile phases defined in the monograph procedure are different; therefore, the methods are not the same for the drug substances and drug formulations. Rare quality control methods are published for quantification of impurities [4-7], but published procedure did not cover for estimation of all the impurities listed in Table-1. Further, several assay procedures are reported for measuring the Phytonadione from drug substance and drug formulations using HPLC [8-10]. Bio-analytical methods and quality control methods for Phytonadione determination have been reported [11-14]. The published assay and bio-analytical procedure are intended to estimate the active drug and its metabolite however not the degradants. Thus, the objective of the present work is to develop a stability indicating HPLC procedure and validation as per ICH14 and Q2(R1) validation guidelines [15-16]. Artificial intelligence is increasingly transforming agricultural practices by improving resource efficiency, crop management, forecasting, and decision-making. However, effective AI adoption also depends on

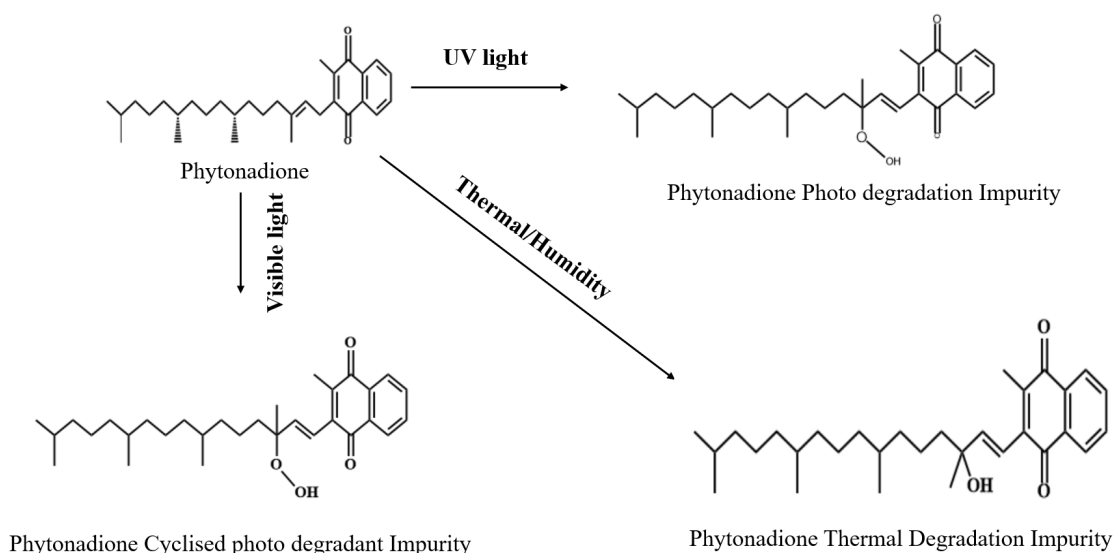
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## Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytonadione Injectable Emulsion Dosage Form

managerial readiness, technological accessibility, organizational support, and appropriate institutional policies [17]. Artificial intelligence and machine learning can facilitate early animal disease detection, reduce the workload associated with conventional disease-monitoring practices, and raise important ethical considerations regarding their application in veterinary healthcare [18].

None of the literature shows the photo and thermal degradants impurities in Phytonadione injectable emulsion dosage form; none of the analytical methods were reported. Based on literature survey, we conclude

that the method is not reported for all impurities of Phytonadione Parenteral dosage forms (i.e., emulsion). The goal of this work was to create a single, efficient HPLC method for separating Phytonadione and their potential impurities, and degradation impurities during acid hydrolysis, oxidative degradation, alkali hydrolysis, photolytic and thermal, stress conditions studies. There are five potential impurities that are possible in Phytonadione (Figure-1). A total of five compounds, including all potential impurities, Phytonadione, are quantified parallelly using these developed impurities method.



**Figure-1-** Phytonadione and its Impurities structure

### EXPERIMENTAL SECTION

#### Reagents or Chemicals

High pure, HPLC grade. Tetra butyl ammonium dihydrogen phosphate, Hydrogen peroxide, potassium hydroxide, Trifluoro acetic acid, Hydrochloric acid, Methanol, Sodium hydroxide Acetonitrile and Milli-Q water HPLC grade procured from Merck, India.

#### Preparation of buffer

Weigh accurately and transfer about 0.68g of Tetra butyl ammonium dihydrogen phosphate in 1Lt of water, sonicate to dissolve, Set the pH with orthophosphoric acid to 2.50. Filter the solution with 0.45µm membrane filter.

#### Preparation of Impurity Identification solution:

Transfer equivalent to 10mg of Phytonadione injectable emulsion sample into a transparent 10 mL volumetric flask, expose the sample to UV light (Short 254nm) for about 60 minutes and after to visible light for about 60 minutes in UV chamber, dilute to volume with diluent and well mixed.

#### Preparation of standard solution

Weigh exactly 50 mg of Phytonadione working/Reference material in a 50 mL volumetric

flask, add about 30mL of methanol, mix well to dissolve completely, make up to the volume with methanol and mix. Dilute 1 mL of the above solution to 200 mL with same diluent and well mixed.

#### Preparation of placebo solution

Transferred 10.0 mL of product placebo to a 20.0 mL with diluent and well mixed.

#### Preparation of sample solution

Pool 15 PFS into a suitable and cleaned glass container. transfer 5.0 mL of the sample to 10 mL with diluent and well mixed.

#### Menadione impurity (MD) stock solution:

Weigh accurately 7.7 mg of Menadione impurity standard into a 50 mL flask, add about 30 mL methanol or suitable solvent, mix well to dissolve completely, dilute up to the volume with diluent or suitable solvent and well mixed.

#### Photolytic degradant impurity (PD) stock solution:

Weigh accurately 31.2 mg of Photolytic degradant impurity into a 10 mL flask, add about 4 mL methanol or suitable solvent, well mix to dissolve completely, dilute up to the mark with diluent or suitable solvent and well mixed.

## Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytonadione Injectable Emulsion Dosage Form

### Trans epoxy Phytonadione (TP) impurity stock solution:

Weigh accurately 5 mg of Trans epoxy Phytonadione impurity into a 10 mL flask, add about 4 mL methanol or suitable solvent, well mixed to dissolve completely, dilute up to the mark with diluent or suitable solvent and well mixed.

### Cyclised photo degradant (CPD) impurity stock solution:

Weigh accurately 26 mg of Cyclised photo degradant impurity into a 10 mL flask, add about 4 mL of acetonitrile or suitable solvent, well mixed to dissolve completely, dilute up to the volume with diluent or suitable solvent and mix.

### Phytonadione Hydroxy impurity (PHI) stock solution:

Weigh accurately 31.2 mg of hydroxy impurity into a 10 mL flask, add about 4 mL methanol or suitable solvent, mix well to dissolve completely, dilute up to the mark with diluent or suitable solvent and well mixed.

### Impurity Mixture stock solution:

Transferred 2.5 mL of Menadione impurity standard stock, 5.0 mL of Photolytic degradant impurity standard stock, 5.0 mL of Trans epoxy Phytonadione impurity standard stock, 5.0 mL of Cyclised photo degradant impurity standard stock and 5.0 mL of hydroxy impurity standard stock into 25 mL flask and dilute to volume with methanol and mixed well.

### Preparation of impurities spiked on sample solution:

Pool 15 PFS into a suitable and cleaned glass container. Dilute 5 mL sample solution to 10 mL flask and add 1.0 mL of impurity mixture stock solution and dilute to volume with diluent and mix well.

## EXPERIMENT DESIGN

### Chromatographic conditions optimization

The knowledge of the nature of API (acidity, functionality or basicity), the steps of process, related impurities, the known degradation products and degradation pathways are needed for stability indicating method development by chromatographic technique. In addition, developed method should result a simple, robust and stability indicating in nature.

The wavelength which gives linear response for related substances and meets the sensitivity of the methodology. Based on the peak response and maximum absorption of VTK, wavelength in proposed method is 268 nm.

Appropriate finding of the column chemistry means stationary phase depends on the chemical nature of the

sample. The VTK was polar compound and could be separated either by NP-Liquid chromatography or RP-Liquid chromatography. From literature search, it was observed that different C<sub>18</sub> stationary phase could be suitably used for the quantification of related substances for VTK.

Different composition of mobile phase and different stationary phases like C<sub>8</sub>, C-18 were tried to develop a stability indicating method for the estimation and separation of degradants in VTK. Different pH of mobile phase was applied to get symmetric peak shapes and better selectivity for the degradants existing in VTK. Poor resolution and asymmetric peak shape were observed when Inertsil ODS C<sub>18</sub> (250mm x 4.6mm, 3μ) and mobile phase run with gradient programmed, the mobile phase in pump- A pH 3.80 buffer (phosphate) and mobile phase in pump- B mixture of water and Acetonitrile. The separation of impurities from VTK was not proper. The efficiency of the peak and Peak shape of VTK is also not attained and interferences are present with trans isomer from the sample matrix.

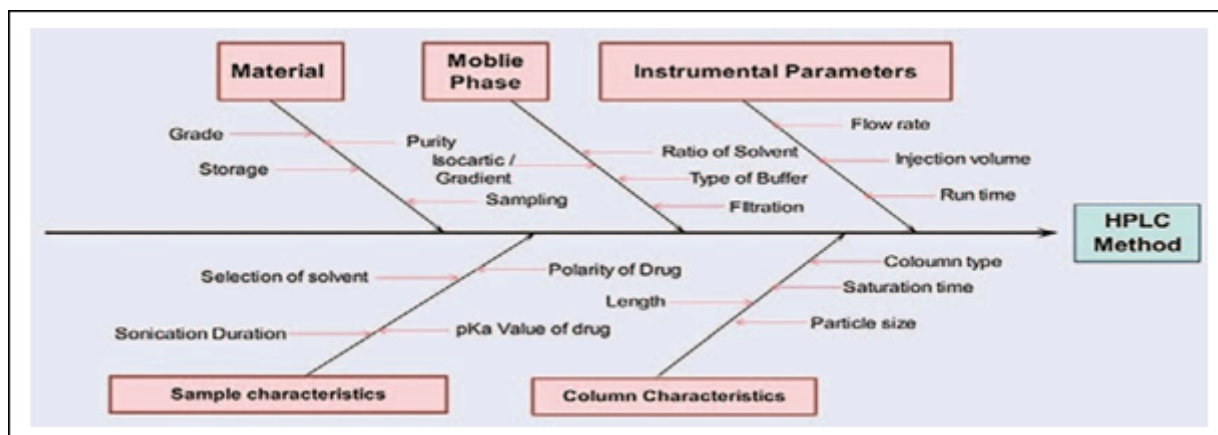
In other effort made using Zorbax SB, C-18, 150 x 4.6 mm, 3.0μm column, and mobile phase gradient programmed modified with mobile phase in pump A pH 2.50 phosphate solution: Acetonitrile and mobile phase in pump B water: Acetonitrile. The separation of VTK and impurities were improved however peak interferences are present with impurities.

Further to improve the peak interferences attempt made by using Phenomenex -C<sub>18</sub>, 4.6 mm X 150 mm, 3.0μm column, mobile phase gradient programmed of mobile phase in pump A pH 2.50 phosphate Buffer: Acetonitrile and mobile phase in pump B pH 2.50 phosphate buffer: Acetonitrile.

### Risk assessment studies

In the development by the RP-HPLC method, risk assessment is needed to evaluate the parameters that affect the target method quality profile (TMQP). First, critical analytical attributes (CAAs) are identified to study the correlation between critical method parameters and TMQP<sup>17</sup>. It is used to decide and control areas of variation or potential sources of failure. The factors are ranked based on their (high, medium, or low) risk level and prioritized according to seven key parameters. Three critical analytical factors (CAFs) are selected for systematic optimization of CAAs using experimental designs. Key CAAs, such as peak area, retention time, and tailing factor, are essential for method efficiency. Figure 1 highlights the identified CAFs and their associated risks.

## Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytonadione Injectable Emulsion Dosage Form

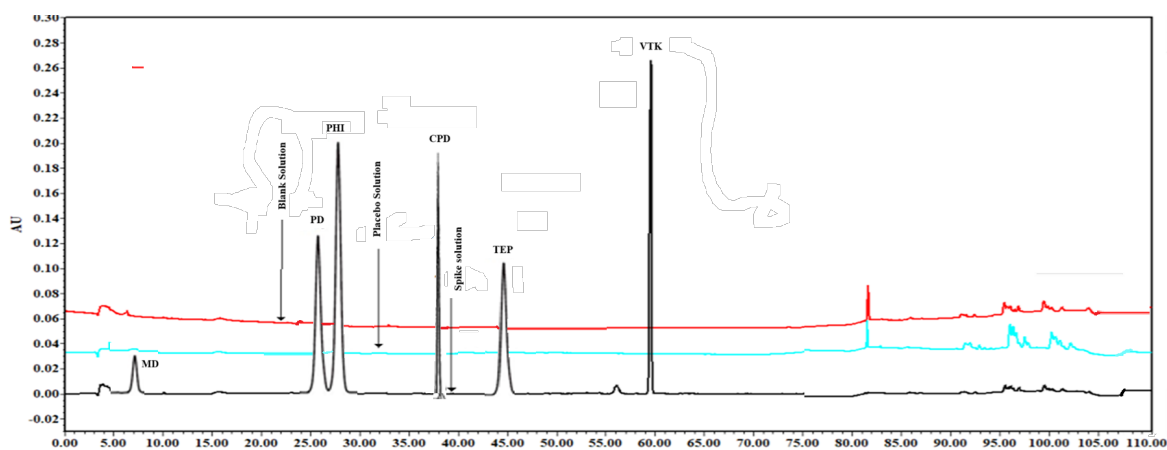


**Figure-2** Risk assessment parameters applied in the method development study.

### Equipment and Liquid Chromatographic settings

A LC method was developed for the analysis of the unknown impurities of VTK injection. Chromatographic analysis was performed on Waters HPLC Alliance System (Waters, Milford, MA, USA). Data acquisition and processing were performed using Waters Unifi software (Version 1.9.4.053). Chromatographic separation was achieved on Phenomenex -C18, (150 mm × 4.6 mm and 3 μm particle size). The mobile phase A consisted of a mixture of 0.68g of Tetra butyl ammonium dihydrogen phosphate in 1Lt of water (pH 3.00±0.05 with dilute ortho phosphoric acid acid) and acetonitrile in the ratio of 550 and 450. Mobile phase B consists of a mixture of 100 ml of buffer (pH 3.00±0.05), 900 ml of acetonitrile.

A stock solution of sample (5 ml/10 ml v/v) was prepared by dissolving an appropriate amount in diluent. The linear gradient programme was optimized by the percentage of mobile phases as follows: T time/mobile phase-A: B (%): T0/100:00, T5/100:00, T10/00:100, T80/00:100, T85/100:00, T105/100:00. The flow rate was set at 1.2 ml min<sup>-1</sup>. Injection volume was 25 μl and the column temperature was maintained at 40 °C. The analytes were detected in photo diode array detector at 268 nm with the sample temperature maintained at 5±1 °C. Individual impurity and drug substance standards were injected to identify the peaks. VTK and their impurities were spiked at specification level (Figure 3).



**Figure 3:** Typical overlay chromatogram of blank, placebo and sample with impurity spike solution.

### Preparative liquid chromatography conditions for isolation of unknown impurities

To isolate the impurity, a Knauer Preparative HPLC (made in Germany) equipped with a PUMP 1800 module and a Detector (S-2500) UV detector was used. Clarity chrome Software was used to analyse the data. To achieve chromatographic separation, a YMC ODS AQ C18 (250 x 50 mm, 10 μm) column (Japan) was used. In diluent, a sample concentration of 500 mg/mL was produced. As mobile phases A and B, aqueous trifluoroacetic acid (0.1%) and acetonitrile were used. Mobile phases A and B were used as the diluent in a

90:10, %v/v. At 268 nm, the column eluent was measured. The fractions were gathered, combined, and lyophilized using a lyophilizer for further characterization of isolated impurities, LCMS and NMR instrument was used.

### High resolution mass spectrometry

The aforementioned Thermo Vanquish UHPLC system was paired with a Thermo QExactive Orbitrap mass spectrometer and used for HRMS analyses. Electrospray Ionization (ESI) was employed in positive ionization mode. Mass spectrometric data were recorded within m/z range of 100-1500 using full scan

## Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytonadione Injectable Emulsion Dosage Form

acquisition at a resolution of 70,000. The analysis was conducted with an Automatic Gain Control (AGC) target of 3e6 and a maximum Injection Time (IT) of 200 ms. A spray voltage of -3.30 kV was used, along with capillary temperature of 350°C, and S-lens RF level of 50 and auxiliary gas heater temperature of 200°C. Sheath, aux, and sweep gas flow rates were set at 40, 12, and 0 (arbitrary units) respectively, with an aux gas heater maintained at 350°C. These parameters were specific to Q Exactive HRMS and were optimized before analysis.

### Nuclear magnetic resonance spectroscopy

The impurity's structure was elucidated using NMR spectroscopy. One-Dimensional (1D) NMR experiments including <sup>1</sup>H, <sup>13</sup>C, <sup>13</sup>C DEPT as well as Two-Dimensional (2D) NMR techniques such as <sup>1</sup>H-

<sup>1</sup>H COSY, <sup>1</sup>H-<sup>1</sup>H ROESY, <sup>1</sup>H-<sup>13</sup>C HSQC and <sup>1</sup>H-<sup>13</sup>C HMBC were conducted. The NMR analysis of was conducted in deuterium oxide (D<sub>2</sub>O, NMR analysis was conducted in deuterium oxidanalyzed in deuterated Dimethyl Sulfoxide (DMSO-d<sub>6</sub> ). The probe temperature was regulated at 25°C. The <sup>1</sup>H NMR chemical shifts were referenced to the singlet signal of Tetramethylsilane (TMS) at 0 ppm, while the <sup>13</sup>C NMR spectra referenced to the septet signal of DMSO-d<sub>6</sub> at 28.5 ppm.

NMR spectroscopic analysis was anticipated to confirm structure of unknown impurities. We did not detect any appreciable variation in the chemical shift values in the impurities in NMR spectra. The suggested structure of unknown impurities was reported in Figure-4-6.

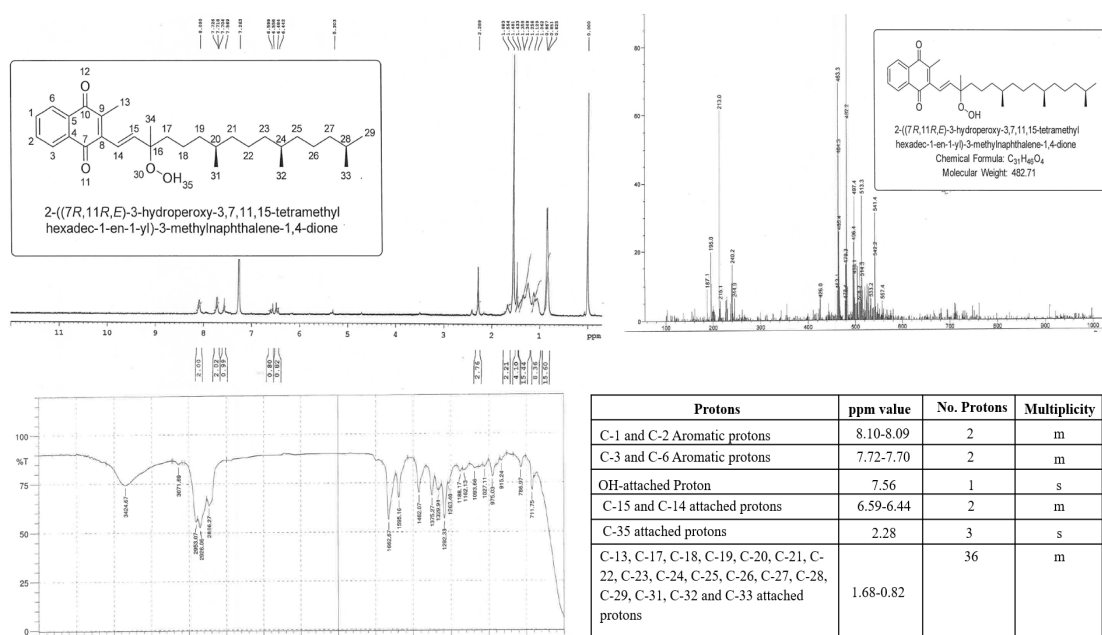


Figure-5- NMR, Mass, IR and structure elucidation of Phytonadione Photo degradation Impurity

## Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytonadione Injectable Emulsion Dosage Form

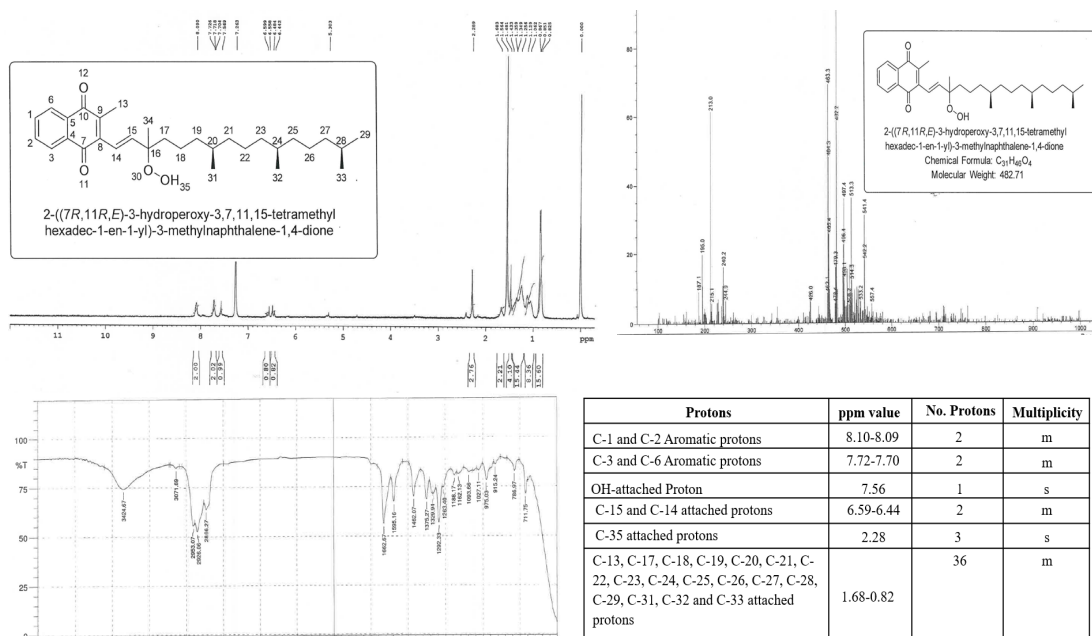


Figure-5: NMR, Mass, IR and structure elucidation of Phytonadione Photo degradation Impurity

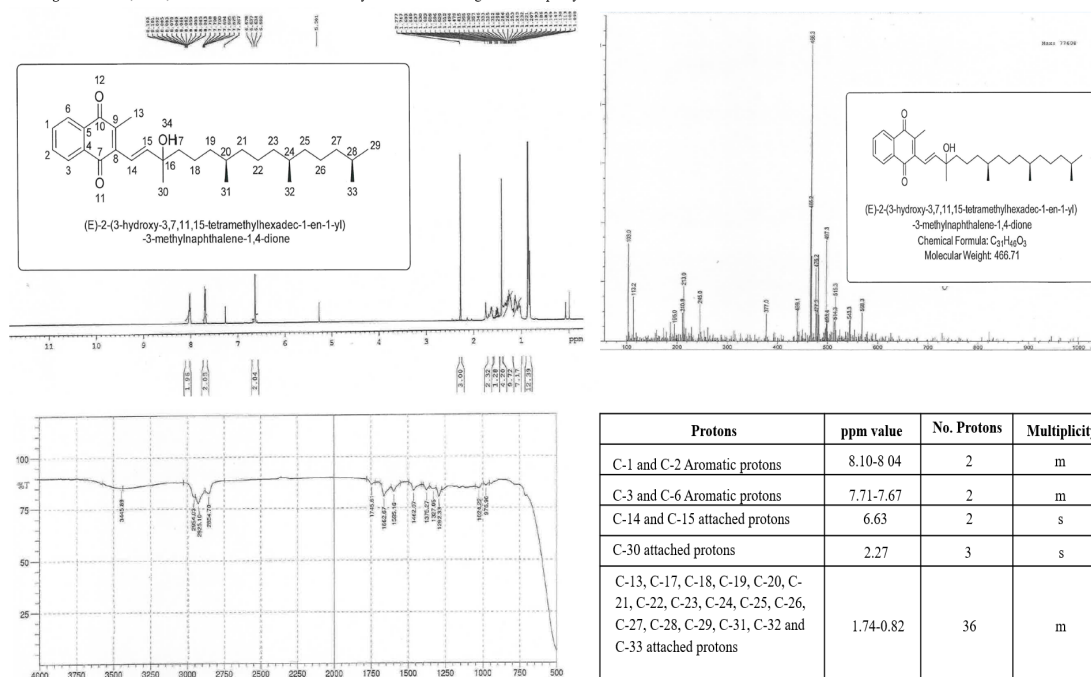


Figure-6: NMR, Mass, IR and structure elucidation of Phytonadione Thermal Degradation Impurity

### METHOD VALIDATION

The established analytical technique was verified in accordance with the most recent ICH Q2(R1) guidelines. We evaluated all required validation parameters, including specificity, linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), precision, solution stability, and robustness.

#### System precision

To demonstrate the system suitability or system precision, prepared an Impurity Identification solution, standard solution as per the test procedure, injected into the chromatographic system and evaluated the system suitability parameter.

#### Specificity (Selectivity)

Specificity of method was established by introducing both placebo and blank solution into the system by injected blank solution, placebo solution, standard solution, sample solution, spiked sample and individual impurities and analyzed as per the test method.

#### Force degradation (Interference from degradation products)

Force degradation study of drug products and drug substances can guide us to know degradation products, which can facilitate to set up the degradation route and inherent stability of the compound. It works well as a

## Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytonadione Injectable Emulsion Dosage Form

tool for figuring out to establish the stability of the product, an analysis of force degradation was carried out employing acid hydrolysis, peroxide oxidation, alkali degradation, ultraviolet radiation, thermal and humidity, which might help prove the method's specificity in terms of drug degradation.

Sample solutions and placebo solutions were exposed to the following stress conditions to degradation. Stressed and unstressed samples were injected into the HPLC system with photo diode array detector by following test method conditions.

### LOD and LOQ

The limit of detection (LOD) should be determined by injecting the smallest amount of substance that can be reliably sensed. The limit of quantification (LOQ) is the smallest concentration of an analyte that can be measured in a sample. LOQ, LOD for Phytonadione and all their impurities were established. Matching signal-to-noise ratios of 3:1 and 10:1. Precision study and accuracy at the LOQ level were also performed by injecting separate preparations of Phytonadione and their impurities, and figuring out the impurity peak area's %RSD.

### Method precision

The degree of agreement between replicate measurements of an analyte is known as precision. Six samples were prepared in accordance with the test procedure, and impurities of Phytonadione were spiked at the specified quantity in each sample to find out the method's precision. Fresh six sample solutions were made for the daily precision investigation and injected by another column, another instrument, another day, and different analysts.

### LOQ precision:

Determined the Precision at limit of quantitation level. Prepared six sample solutions having Phytonadione and its specified impurities at about limit of quantitation level on placebo and injected into HPLC. Calculated the %RSD for peak area of Phytonadione and its specified impurities from six preparations and the results were found within the acceptance criteria. The results are summarized in Table 7.

### Linearity

The linearity of detector response for analytes and for impurities was demonstrated by preparing solutions

over the range of LOQ to 150% of specification limit with respect to sample concentration. These solutions were injected into the HPLC system and the responses of the same were recorded.

### Accuracy (Recovery)

Recovery of VTK impurities in VTK was performed. The sample was taken and the amounts of Phytonadione impurities representing LOQ to 150 % of specification level were prepared. The spiked samples were prepared as per the method.

### Solution stability of analytical solutions

Standard and sample and spiked sample solutions were kept for 48 hr at room temperature in amber bottles in auto sampler and in refrigerator 2-8°C. The stability of standard and sample and spiked sample solutions was determined by comparison of "old" prepared standard solutions with freshly prepared standard

### Robustness

Robustness was assessed by deliberately varying intrinsic method parameters such as pH, column temperature, and flow rate. Ruggedness was evaluated by testing the method with different analysts.

The robustness of the analytical method was assessed by making slight changes to the HPLC parameters, including buffer pH, column oven temperature, flow rate, and mobile phase ratio. In every one of the adjusted conditions, the selectivity as well as the separation between each closely spaced peak was examined.

## RESULTS AND DISCUSSION

### Specificity

No peak interference was observed from blank and placebo at the retention time of all specified impurities. The observations are tabulated below Table 1 and Figures 7, demonstrating the suggested approach's specificity. PDA detector was employed to check and ensure the homogeneity and purity of each analyte peak and peak purity found passing.

## Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytionadione Injectable Emulsion Dosage Form

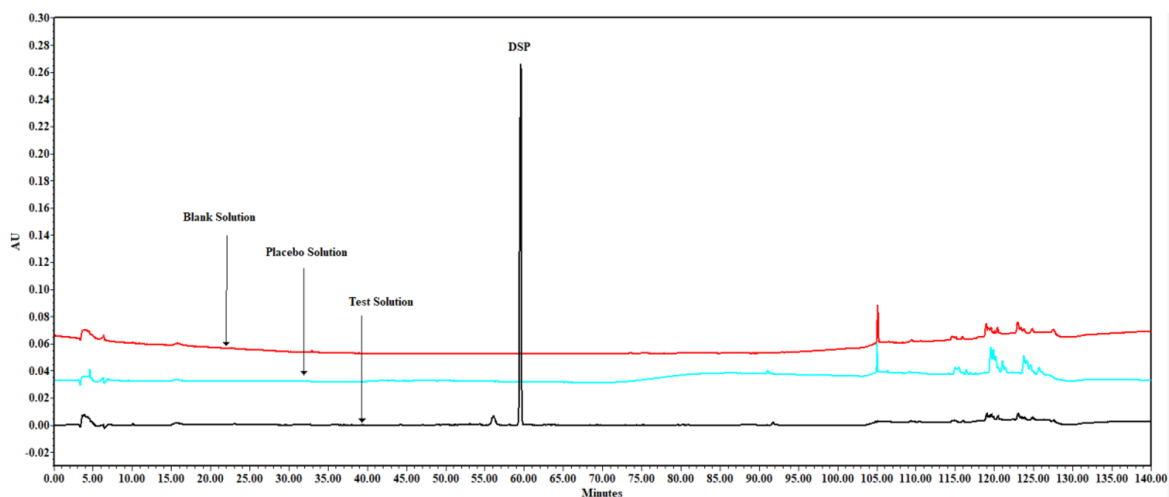


Figure-7: Overlay chromatograms of Blank, Placebo and test solution during specificity test

### Interference from degradation products

All degrading peaks were resolved from VTK peak in the chromatograms of all samples and placebo did not show any interference at the retention time of VTK and impurities under the above conditions. VTK was sensitive to stress condition like alkali and photo. The

results proved that the developed method has good selectivity and specificity, and is suitable for quantification of impurities in VTK emulsion formulation. The observations are tabulated below Table 2.

| Name | Retention Time (min) | Blank interference | Placebo interference | Purity Angle | Purity Threshold |
|------|----------------------|--------------------|----------------------|--------------|------------------|
| MD   | 5.020                | NO                 | NO                   | 0.080        | 0.377            |
| PD   | 24.974               | NO                 | NO                   | 0.078        | 0.247            |
| PHI  | 26.092               | NO                 | NO                   | 0.080        | 0.224            |
| CPD  | 38.556               | NO                 | NO                   | 0.143        | 0.232            |
| TEP  | 44.205               | NO                 | NO                   | 0.125        | 0.348            |
| VTK  | 65.035               | NO                 | NO                   | 0.180        | 0.307            |

Table 1: Peak Purity data and impurity interference data (Specificity results).

| Sample Name                                                             | MD    | PD    | PHI   | CPD   | TEP   | Total |
|-------------------------------------------------------------------------|-------|-------|-------|-------|-------|-------|
| As such Sample                                                          | 0.002 | 0.240 | 0.018 | 0.096 | 0.060 | 0.649 |
| Thermal Degradation (80°C days)                                         | 0.003 | 0.307 | 0.040 | 0.030 | 0.215 | 0.790 |
| Peroxide Degradation Sample (30% H <sub>2</sub> O <sub>2</sub> /2 days) | 0.002 | 0.292 | 0.017 | 0.036 | 0.257 | 0.916 |
| Neutral (Water 2 Days)                                                  | 0.003 | 0.250 | 0.019 | 0.074 | 0.199 | 0.748 |
| Acidic (0.5N HCl, 2 Days)                                               | 0.002 | 0.330 | 0.129 | 0.040 | 0.217 | 0.961 |
| Basic (0.5N NaOH 2 Days)                                                | 0.002 | 0.239 | 0.155 | 0.077 | 0.535 | 1.429 |
| Photolytic (130 K lux Hours)                                            | 0.003 | 0.568 | 0.057 | 4.699 | 0.219 | 5.888 |
| UV (21 watt Hours/square meter)                                         | 0.002 | 2.007 | 0.072 | 4.841 | 0.223 | 7.509 |

Table 2: Force degradation data in different stress conditions

### Linearity

For known impurities, linearity was performed at different concentration levels, ranging from 200% to

LOQ. Each impurity in the linear regression equation's y-intercept, correlation coefficient (r), slope and %bias was examined and observed to be greater than 0.995, as exposed in Table 3.

| Parameter name                 | VTK       | MD        | PD       | PHI       | CPD      | TEP      |
|--------------------------------|-----------|-----------|----------|-----------|----------|----------|
| Linearity                      |           |           |          |           |          |          |
| Range (µg/mL)                  | 0.05-0.70 | 0.02-0.25 | 0.05-9.0 | 0.05-9.0  | 0.05-8.0 | 0.10-1.5 |
| Slope                          | 468719.3  | 1039284.0 | 234988.2 | 372882.8  | 388942.3 | 140122.5 |
| Intercept                      | 449.951   | 1041.532  | 2624.754 | 11012.895 | 9928.088 | 291.494  |
| Correlation coefficient >0.999 | 1.000     | 1.000     | 1.000    | 1.000     | 0.999    | 1.000    |

Table 3: Linearity data of VTK and its impurities

### LOD and LOQ

All five of their impurities, as well as the LOQ and LOD for phytodione, were investigated at 10:1 and 3:1

signal-to noise ratios, respectively. There was also accuracy and precision performed at the LOQ level computing the impurity peak areas' % RSD presented in Table 4.

## Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytonadione Injectable Emulsion Dosage Form

| Name | Concentration |        | S/N Ratio |      |
|------|---------------|--------|-----------|------|
|      | LOQ           | LOD    | LOQ       | LOD  |
| VTK  | 0.0505        | 0.0167 | 12.9      | 7.9  |
| MD   | 0.0154        | 0.0051 | 127.6     | 42.6 |
| PD   | 0.0489        | 0.0161 | 28.7      | 10.0 |
| PHI  | 0.0483        | 0.0160 | 34.8      | 11.0 |
| CPD  | 0.0550        | 0.0182 | 23.0      | 4.4  |
| TEP  | 0.1200        | 0.0396 | 15.5      | 4.8  |

**Table 4:** LOD and LOQ concentrations and S/N values for VTK and impurities.

### Precision

The associated method's precision and intraday precision study was calculated. Table 5 provides a summary of the % RSD for each impurity. Within 5% of the %RSD of area, the difference between intraday precision and intermediate precision was identified.

The Intermediate precision analysis was carried out with different columns, analysts, and HPLC systems on different days to be able to demonstrate the method's reliability. Based on the data, the %RSD was computed and found to be within the tolerances (5%). Table 5 provides the precision results.

| Parameter name                            | VTK   | MD  | PD  | PHI | CPD | TEP |
|-------------------------------------------|-------|-----|-----|-----|-----|-----|
| <b>Pre validation System suitability</b>  |       |     |     |     |     |     |
| USP Tailing (Less than 2.0)               | 1.2   | NA  | NA  | NA  | NA  | NA  |
| USP Plate (more than 5000)                | 22882 | NA  | NA  | NA  | NA  | NA  |
| RSD percent (n = 6, less than 2.0)        | 2.1   | NA  | NA  | NA  | NA  | NA  |
| <b>LOQ Precision</b>                      |       |     |     |     |     |     |
| Intraday, n = 6 %RSD less than 5.0        | 2.1   | 3.2 | 0.9 | 1.4 | 2.6 | 3.1 |
| <b>Precision</b>                          |       |     |     |     |     |     |
| Intraday, n = 6 %RSD less than 2.0        | 1     | 0.3 | 0.2 | 0.3 | 0.2 | 0.7 |
| <b>Intermediate precision</b>             |       |     |     |     |     |     |
| Intraday, n = 6 %RSD less than 2.0        | 0.4   | 0.8 | 0.8 | 0.2 | 0.1 | 0.3 |
| Ruggedness, n = 12 %RSD less than 2.0     | 7.6   | 0.6 | 1.5 | 5.6 | 6.1 | 4.2 |
| <b>Post validation system suitability</b> |       |     |     |     |     |     |
| USP Tailing (Less than 2.0)               | 1.1   | NA  | NA  | NA  | NA  | NA  |
| USP Plate (More than 5000)                | 25721 | NA  | NA  | NA  | NA  | NA  |
| RSD percent (n = 6, less than 2.0)        | 1.3   | NA  | NA  | NA  | NA  | NA  |

**Table 5:** System Precision, method precision, Precision at LOQ level and intermediate precision data from method validation experiments

### Accuracy

The outcome were confirmed by adding known quantities of phytodione impurities in triplicate at five distinct concentration levels of the prescribed limit. the average recovery result was found to be between 90 to

110% for each contaminant. The RSD values were less than 5%, which shows that the measured value and true value agree well (Table 6).

| Parameter name                               | VTK       | MD        | PD        | PHI       | CPD       | TEP       |
|----------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| <b>Accuracy Study (n = 6 avg percentage)</b> |           |           |           |           |           |           |
| LOQ avg ± SD                                 | 105.8±4.5 | 102.8±3.3 | 106.8±4.3 | 105.1±3.9 | 103.7±5.4 | 105.9±4.6 |
| 50% avg ± SD                                 | 99.8±2.5  | 98.8±0.5  | 103.9±0.1 | 102.1±0.1 | 103.1±0.2 | 101.1±0.6 |
| 100% avg ± SD                                | 101.8±0.5 | 99.6±0.8  | 102.8±0.3 | 99.8±0.3  | 105.1±0.1 | 104.8±0.8 |
| 150% avg ± SD                                | 103.8±1.5 | 97.8±0.4  | 95.1±1.5  | 91.8±1.3  | 96.1±0.8  | 97.8±3.2  |

**Table 6:** Accuracy data of VTK and its impurities from LOQ concentration

### Robustness

The robustness of the analytical method was assessed by making slight changes to the HPLC parameters, including buffer pH, column oven temperature, flow rate, and mobile phase ratio. The system suitability parameter were verified and remained well within predefined limits. These results confirm that the proposed method is optimized and robust for the estimation of the analytes.

### Solution stability

Standard and sample and spiked sample solutions were kept for 48 hr at room temperature in amber bottles in auto sampler and in refrigerator 2-8°C. The stability of standard and sample and spiked sample solutions was determined by comparison of “old” prepared standard solutions with freshly prepared standard solutions. The observations are tabulated below Tables-7.

## Characterization and Quantification Approach for Process Impurities and Major Photo Degradants in Phytonadione Injectable Emulsion Dosage Form

| Name | Concentration |        | S/N Ratio |      |
|------|---------------|--------|-----------|------|
|      | LOQ           | LOD    | LOQ       | LOD  |
| VTK  | 0.0505        | 0.0167 | 12.9      | 7.9  |
| MD   | 0.0154        | 0.0051 | 127.6     | 42.6 |
| PD   | 0.0489        | 0.0161 | 28.7      | 10.0 |
| PHI  | 0.0483        | 0.0160 | 34.8      | 11.0 |
| CPD  | 0.0550        | 0.0182 | 23.0      | 4.4  |
| TEP  | 0.1200        | 0.0396 | 15.5      | 4.8  |

Table 7: LOD and LOQ concentrations and S/N values for VTK and impurities.

### Photo Degradants proposed structural elucidation by NMR and ESI-MS

All Five impurities were identified through analytical HPLC. Through preparative HPLC, the remaining three unidentified degradants were separated and subsequently characterised. and identification were carried out using UPLC-PDA-MS and NMR analysis. The suggested structure of unknown impurities was reported in Figure-4-6.

### CONCLUSION

For the parental dose form of Phytonadione, a related substance method was created and validated in accordance with the most recent ICH recommendation. The study's findings shows that all of the degradation products are extremely clearly separated, proving the technique's suitability as a stability indicator. Impurity analysis was performed on same batch of drug material and combination dosage forms using the established method. This work presents a simple, robust, rugged, and validated methodology for quantifying Phytonadione and its impurities. The method adheres to stability indicating in nature and making it suitable for routine and quality control stability sample analysis.

### Conflict of Interests

The authors declare that there is no conflict of interest.

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### Ethical Approvals

This study does not involve experiments on animals or human subjects.

### Data Availability

All data generated and analyzed are included in this research article.

### Use of Artificial Intelligence (Ai)-Assisted Technology

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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