Research Paper

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Method Validation of High Performance Liquid Chromatography Mass Spectrometric Method for the Estimation of Cis And Trans Isomers of Phytonadione In Human Plasma Using D-Labeledtrans Phytonadione As An Internal Standard

Chemistry

KEYWORDS : -Cis and Trans Phytonadione; LCMS-MS; Method validation; Clinical study

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ABSTRACT

A novel, simple, specific, sensitive and reproducible liquid chromatography mass Spectrometric (LCMS-MS) assay method has been developed and validated for estimation of Cis and Trans isomers of Phytonadione. The LCMS-MS method includes use of D-labeledtransphytonadione as an internal standard (IS). The chromatographic analysis was performed using API 4000, Applied biosystems Liquid Chromatography Mass Spectrometer system equipped with MS detector and PC based data system with Analyst 1.6.1 supplied by Lab India. Chromatographic separation was achieved on C30 column, 250×4.6mm, 200A, 5µ column maintained at 40ºC temperature using isocratic mobile phase composition with (0.1% Formic acid in Methanol V/V) at a flow rate of 0.8 ml/min, without splitter with a total run time of 18 min. Method validation was performed as per USFDA guidelines and the results met the acceptance criteria. The calibration curve was linear over a concentration range of 0.650 to 40.596 ng/ml for Cis Phytonadione and 0.652 to 81.461 ng/ml for Trans Phytonadione using (D-labeledTrans-Phytonadione) as internal standard (r2 ≥ 0.99). Precicion& accuracy batches analysed on 3 different day's revealed accuracy of Inter-batch calibration standard accuracy for ranged from 93.69% to 103.11% for Cis Phytonadione and 98.19% to 101.60% for Trans Phytonadione with inter-batch precision values of 1.41% to 3.96% for Cis Phytonadione and 1.24% to 3.00% for Trans Phytonadione during the course of validation, demonstrating acceptable assay linearity. The validated LCMS-MS method was successfully applied for assay and stability determination of clinical study samples.

INTRODUCTION

Vitamin K is an essential cofactor for the gamma-carboxylase enzymes which catalyze the posttranslational gamma-carboxylation of glutamic acid residues in inactive hepatic precursors of coagulation factors II (prothrombin), VII, IX and X. Gamma-carboxylation converts these inactive precursors into active coagulation factors which are secreted by hepatocytes into the blood. Supplementing with Phytonadione results in a relief of vitamin K deficiency symptoms which include easy bruisability, epistaxis, gastrointestinal bleeding, menorrhagia and hematuria.

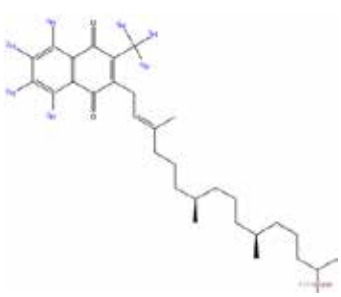

Figure 1: Molecular structure of phytomenadione

It's a mixture of 2-methyl-3-[(2E)-(7R,11R)-3,7,11,15-tetramethylhexadec-2-enyl]naphthalene-1,4-dione (trans-phytomenadione), 2-methyl-3-[(2Z)-(7R,11R)-3,7,11,15-tetramethylhexadec-2-enyl] naphthalene-1,4-dione (cis-phytomenadione). Empirical formula: C31H46O2 with molecular weight 450.69.

It is a fat-soluble vitamin that is stable to air and moisture but decomposes in sunlight. It is found naturally in a wide variety of green plants. Phylloquinone is also an antidote for coumatetralyl. Vitamin K is needed for the posttranslational modification of certain proteins, mostly required for blood coagulation. Phytonadione is a vitamin, indicated in the treatment of coagulation disorders which are due to faulty formation of factors II, VII, IX and X when caused by vitamin K deficiency or interference with vitamin K activity. Phytonadione aqueous colloidal solution of vitamin K1 for parenteral injection, possesses the same type and degree of activity as does naturally-occurring vitamin K, which is necessary for the production via the liver of active prothrombin (factor II), proconvertin (factor VII), plasma thromboplastin component (factor IX), and stuart factor (factor X). Oral phytonadione is adequately absorbed from the gastrointestinal tract only if bile salts are present. After absorption, phytonadione is initially concentrated in the liver, but the concentration declines rapidly. Very little vitamin K accumulates in tissues. The intravenous LD 50 of phytonadione in the mouse is 41.5 and 52 mL/kg for the 0.2% and 1% concentrations, respectively.

The available literature with respect to quantification of Vitamin K were, opinion on Vitamin K1 (Phytonadione), Scientific Committee on Consumer Safety, Directorate general for Health and consumers, Euoropean Commission, SCCS/1313/10 24 March 2010. And simultaneous and accurate determination of water- and fat-soluble vitamins in multivitamin tablets by using an rp-hplc method. And A Validated HPLC Method for the Determination of Vitamin K in Human Serum – First Application in a Pharmacological Study" The Open Clinical Chemistry Journal, 2011, 4, 17-27.

Literature survey shows that there is no LC-MS/MS method available for quantitative analysis of cis and trans phytonidone in biological samples. Hence in present research, we demonstrate an analytical strategy for quantisation of chiral Phytonadione in biological samples. Further the method was validated as per regulatory guidelines recommendations and employed for evaluation of experiments related to stability and clinical studies.

EXPERIMENTAL

Materials and Methods

Chemicals and reagents: Cis Phytonadione, Trans Phytonadione and D-labeled Trans-Phytonadione (IS) was procured from Vivan Life Sciences Pvt. Limited, Mumbai. LCMS grade acetonitrile were obtained from JT Baker Formic Acid - AR Grade and Ethyl acetate, Iso propanol was procured from Merck India. All other chemicals/reagents were of analytical grade and used without further purification.

LCMS operating conditions

Mass spectrometric detection was carried out on an API 4000 triple quadrupole instrument equipped with a heated nebulizer (APCI) source operated in the positive ion mode. Cis Phytonadione and Trans Phytonadione was selectively isolated from 500µl plasma by solid phase extraction using Agilent Bond Elute cartridge C18. Estimation was done by mass spectrometric method and chromatographed using a C30, 250×4.6mm, 200A 5 micron column. Sample processing was done under yellow monochromatic light. An isocratic mobile phase was used consisting of 0.1% Formic acid in Methanol V/V. The flow rate was 0.8 ml/min, without splitter under ambient temperature. The autosampler temperature was maintained at 10°C ± 4°C and the injection volume was 15 µl. The run time was 18 min. During the optimization of the mass spectrometric parameters, strong and stable signals of analytes and internal standards were noted and the ion transitions m/z 451.400/187.000→ 451.400/187.001→ 458.400/194.200 were selected for the MRM of Cis Phytonadione, Trans Phytonadione and D-labeled Trans-Phytonadione (IS) respectively. The source/gas parameters were optimized as follows: curtain gas: 25, collision Activated Dissociation (CAD): 2, ion source gas-1: 25, ion source gas-2: 30, ion spray voltage: 5500 V and temperature: 300°C.

Sample preparation

Transfer accurately weighed (about 5 mg) Cis Phytonadione into a 5 ml volumetric flask and dissolve in 0.25% Ethyl acetate in Acetonitrile. Make up the volume with the same and vortex. Concentration of the resultant solution will be about 1000 µg/ml. Calibration curves were prepared by spiking drug free plasma samples (980 µL) with standard solutions of 20 µL (0.0325 to 59.700 µg/mL, 0.650 ng– 40.956 ng) of Cis Phytonadione and the internal standard (500 ng/mL; 50 µL) to give concentrations in the range of 0.650 ng– 40.956 ng.

Transfer accurately weighed (about 5 mg) Trans Phytonadione into a 5 ml volumetric flask and dissolve in 0.25% Ethyl acetate in Acetonitrile. Make up the volume with the same and vortex. Concentration of the resultant solution will be about 1000 µg/ml. Calibration curves were prepared by spiking drug free plasma samples (980 µL) with standard solutions of 20 µL (0.652 ng– 81.461 ng) of Trans Phytonadione and the internal standard (500 ng/mL; 50 µL) to give concentrations in the range of 0.652 ng– 81.461ng.

RESULTS AND DISCUSSION

Method development and optimization

Optimization of chromatographic conditions was performed, particularly the composition of mobile phase, through several trials to achieve the separation of the two chiral analytes. Resolution positive mode phytoniodone was achieved by methanol in the mobile phase.

Method Validation

Specificity and selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Plasma selectivity was evaluated by analyzing six lots of blank K_2EDTA human plasma obtained from independent sources. Two separate aliquots of each of the blank samples collected from a minimum of six different sources were taken. One aliquot of each blank sample is spiked with the analyte at LLOQ level and with internal standard. Analysed all spiked and blank samples using the method being validated. There is no interference at the analyte RT, so Method is selective for Phytoniodine analysis.

There was no interferences were observed at the retention times of Cis Phytonadione and Trans Phytonadione and IS (D-labeled Trans-Phytonadione) in all the six lots evaluated and % CV of selectivity was 5.46% for Cis Phytonadione and 8.31% for Trans Phytonadione, demonstrating acceptance criteria were met. The results met the acceptance criteria (i.e. response of interfering peak(s) at the retention time of the Cis and Trans Phytonadione and internal standard peak should be ≤ 20% & ≤ 5 % respectively, to the corresponding LLOQ standard). These results suggest the competence of the method to differentiate and measure Cis and Trans Phytonadione and internal standard D-labeled Trans-Phytonadione.

Ruggedness

To assess ruggedness one batch with injection of standard blank, zero standard blank and 8 non zero calibration standard with duplication of LLOQ and ULOQ injection was performed by a different analyst using a different column (LCMS-COL-118). The variation observed in the results were negligible, hence the method meets ruggedness criteria.

Determination of LOQ and LOD

The measure of LOD and LOQ, as a measure of method sensitivity, were calculated by signal to noise ratio (S/N). The limit of detection has been established by analyzing the processed biological matrix by decreasing the concentration of the analyte by 2, 4, 8 & 16 times at LLOQ and inject. Limit of detection of concentration for Cis Phytonadione were observed by 2, 4 and 8 times at LLOQ and Trans Phytonadione were observed by 2 and 4 times at LLOQ.

Linearity of calibration standards

Linearity of calibration standards were plotted out on three different days (three times along with P&A batches on three different days). A standard curve was comprising of 8 non-zero standards including lowest and highest concentration in duplicate.

Linearity of calibration standards analysed along with three different precision & accuracy batches revealed accuracy of all the standard curve points were in the range of 96.2 to 103 %, 96.4 to 108 % and 96.3 to 103 % respectively of three different batches which met acceptance criteria (i.e.75% of standards must have accuracy within or equal to 85 to 115% of theoretical and 80 to 120% of theoretical for the LLOQ). The correlation coefficient (r2) of calibration plots was greater than 0.997 for all batches, which met the acceptance criteria (i.e. correlation coefficient (r2) value for standard curve should be ≥ 0.98).

Recovery

Recovery of Cis Phytonadione and Trans Phytonadione from K_2EDTA human plasma was determined by comparing peak areas of extracted QCL, QCM and QCH samples with peak areas determined from freshly prepared unextracted (aqueous) samples prepared at similar concentrations in mobile phase. Mean overall % recovery was 72.21% and Overall %CV was 5.84% for Cis Phytonadione and % recovery was 76.76% and %CV was 14.43% for Trans Phytonadione and % recovery was 78.49% and %CV was 11.57% for IS (D-labeled Trans-Phytonadione).

CONCLUSIONS

A sensitive and selective LC-MS/MS method to quantitate Cis Phytonadione and Trans Phytonadione in K_2EDTA Human plasma over the concentration range 0.650 to 40.596 ng/ml for Cis Phytonadione and 0.652 to 81.461 ng/ml for Trans Phytonadione was successfully validated. This method is suitable for incurred sample analyses to support bioequivalence / bioavailability and /or pharmacokinetic studies involving formulations of Cis Phytonadione and Trans Phytonadione.

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TABLES

Table 1: Summary of the Experimental Parameters of Cis Phytonadione in Human Plasma

Experimental Parameters	Results
Analyte	Cis Phytonadione
Biological Matrix	Human Plasma
Specificity and Selectivity % CV	5.46%
Analytical range	0.650 to 40.596 ng/ml
Sensitivity : Precision, Accuracy	 5.41% , 96.97%
Recovery Cis Phytonadione % CV , % Recovery D-labeled Trans-Phytonadione % CV, % Recovery	 5.84%, 72.21% 11.57%, 78.49%

Table 2: Summary of the Experimental Parameters of Trans Phytonadione in Human Plasma

Experimental Parameters	Results
Analyte	Trans Phytonadione
Biological Matrix	Human Plasma
Specificity and Selectivity % CV	8.31%
Analytical range	0.652 to 81.461ng/ml
Sensitivity : Precision , Accuracy	 3.94%, 113.11%
Recovery Cis Phytonadione % CV, % Recovery D-labeled Trans-Phytonadione % CV, % Recovery	 14.43%, 76.76% 11.57% , 78.49%

FIGURES

Fig. 2: Representative Calibration Curve for Cis Phytonadione in Human Plasma (K_2EDTA)

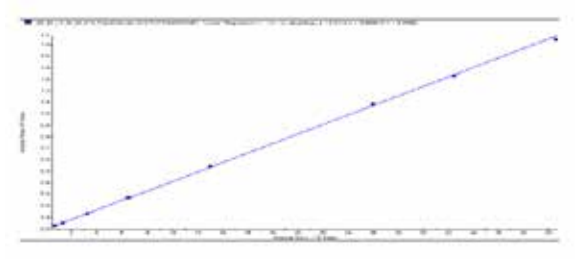


Fig. 3: Representative Calibration Curve for Trans Phytonadione in Human Plasma (K_2EDTA)

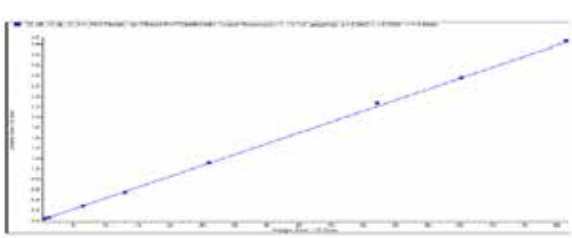


Fig. 4: Chromatogram of the LLOQ Calibration Curve Standard for Cis Phytonadione with Internal Standard (D-labeled Trans-Phytonadione)

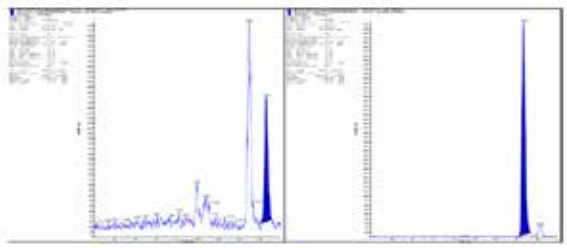


Fig. 5: Chromatogram of the LLOQ Calibration Curve Standard for Trans Phytonadione with Internal Standard (D-labeled Trans-Phytonadione)

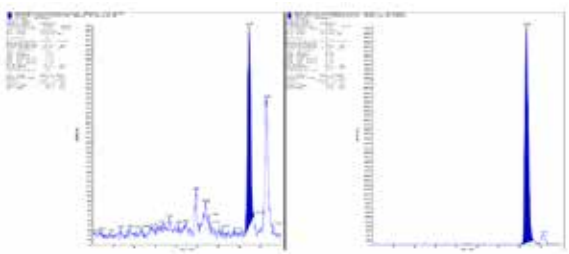
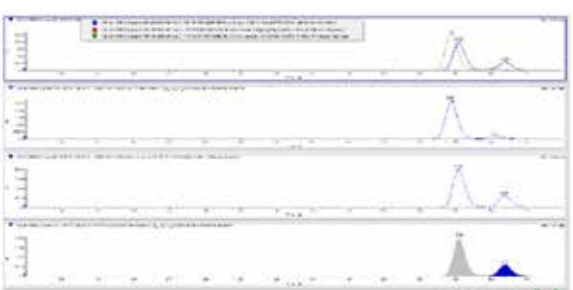


Fig. 6: Chromatogrphic separation of Cis Phytonadione, Trans Phytonadione and D-labeled Trans-Phytonadione (Internal Standard)



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