

# THE IDENTIFICATION OF PHYLLOQUINONE IN CATTLE SAMPLES USING T.L.C. METHOD

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## *Summary*

*A case of hemorrhage of unknown origin was observed in cattle and liver samples were submitted for assay of Vitamin K or possible cuomarol. After evaluating normal and reverse phase Thin Layer Chromatography (TLC) plates and different solvents, reverse phase TLC plate with indicator and methylene chloride:methanol 70:30 were chosen for direct method of detection and indication of Vitamin K. Gass Chromatography and Densitometry were used to quantitate the Vitamin K present in liver samples and confirmation of Vitamin K was done by Mass Spectroscopy.*

## **INTRODUCTION**

Microorganisms in the rumen synthesize large amounts of Vitamin K and deficiency is seen when metabolic antagonists such as dicumarol from moldy sweet clover, hemorrhagic sweet clover disease, are present. Animals may consume sweet clover for months before signs of disease appear or death may occur suddenly with little preliminary evidence of disease. A number of studies have shown that phylloquinone is especially concentrated and retained in the liver. Almost 50% of a physiological dose of phylloquinone is localized in the liver about 3 hours after administration. Carboxylic derivatives appear to be the major excretion product.

There have been reports of assays of Vitamin K in human serum by reverse phase high performance liquid chromatography (HPLC). Concentrations as low as 500 pg/ml have been detected and identification was confirmed by UV spectroscopy. Gas chromatography of phylloquinone and menaquinone has been reported also. Determination of Vitamin K is usually done by prothrombin levels and also by immunologic method, as an indicator of Vitamin K status. Disease such as cirrhosis can also result in hypoprothrombinemia which would reflect Vitamin K deficits.

Reverse phase high performance liquid chromatography was used to determine fasting plasma phylloquinone concentrations in a population of young human subjects. The normal range derived from log normal distribution was 0.29 – 2.64 nmol/L. Serum phylloquinone concentration assessed in normal healthy red cross volunteer donors was 1.1 ng/ml with mean value of 1.3 +/- 0.64 (SD) ng/ml. Administration of Vitamin K1 subcutaneously to anticoagulant poisoned (Diphendione) dogs provided diagnostic information when canine sera was measured using gas chromatography for Vitamin K1 epoxide.

High performance liquid chromatography was used to study dietary intake of Vitamin K concentration in surgical patients. Plasma phylloquinone concentration decreased rapidly in patients on low phylloquinone diet. Phylloquinone concentration was 28.0 +/- 4.3 pmol/g liver (wet weight) on standard diet, whereas it was 6.8 +/- 1.1 pmol/g on low phylloquinone diet after 3 days.

Reverse phase thin layer chromatography appears to be an easy and rapid method for the detection of Vitamin K present in liver. We found that concentrations as low as 0.2 µg can be seen easily. Vitamin K fluoresces readily on reverse phase TLC plate with indicator when viewed under long-wave ultraviolet (UV) light. Our aim was to develop a method that is selective and sensitive to Vitamin K in tissues. The separation and retention of Vitamin K was studied. Eluents were selected and optimized for separation and detection. Liver samples were homogenized and extracted then cleaned up. Peaks were resolved from interfering co-extracts in liver samples. Normal liver sample along with case sample were used.

## **MATERIAL AND METHODS**

Vitamin K working standard (2.43 µg / µl) was prepared from a stock dissolved in hexane: chloroform (95:5). Extraction solvents used were hexane: chloroform (95:5) and 95% ethanol. Development solvents were methylene chloride:methanol (MECL<sub>2</sub>: MEOH 90:30). Methanol was used to elute Vitamin K and chloroform for phase separation during centrifugation. Celite was used for preparation of sample before

extraction. Homogenation was done by vortex mixer and rotary mixer: Rotor Rack with sample in 50 ml test tubes with Teflon screw caps. Extract was removed from each test tube with Pasteur pipets. Centrifuge was used for separation of extraction solvents phase separation of Vitamin K after clean-up. Samples were transferred to 2 dram vials covered with aluminium foil for drying. Regulated prepurified nitrogen supply, nitrogen effusion apparatus and steambath used for drying samples. Thin Layer Chromatography Reverse Phase plates were used for detection and identification of Vitamin K. Long and short wave UV light were used to examine plates. Concentrate Tubes (18.25 x 114 ml 0.90 wall 3 TPB) were used to concentrate samples.

#### **DETERMINATION STEPS**

- weigh out two 5 g of homogenized test samples and one control sample into separate 50 ml tubes with Teflon-lined screw caps.
- add vitamin K at 1 ppm to one of the test samples for spike.
- add 1 g celite to each tube; mix so that the liver balls up.
- add 10 ml 95% ethanol and 20 ml 95:5 hexane: chloroform to each tube. Mix contents of tube on vortex mixer.
- place tubes on a rotary mixer. Place in the dark or cover with box to protect from light. Mix for 30 minutes.
- after 30 minutes, remove tubes from mixer and centrifuge up to 2000 rpm for 2 minutes.
- transfer the upper solvent extract layer of each sample to a 125 ml Erlenmeyer flask covered with foil and concentrate to dryness under a stream of nitrogen gas and low heat steam bath.
- transfer quantitatively to 2 dram vials with MeCl<sub>2</sub>, and reconcentrate to dryness under stream of nitrogen gas and low heat steam bath.
- clean up sample. Dissolve sample.

#### **Column Cleanup**

1. prepare a chromatographic column for each extract with 10 g of deactivated Florisil topped with 20 g anhydrous sodium sulfate
2. wet each column with 25 ml petroleum ether
3. add sample extract to each column
4. elute each column with following fractions:
5. 100 ml petroleum ether
6. 100 ml 8:92 diethylether:petroleum ether
7. 100 ml 40:60 diethylether : petroleum ether. Collect this portion for Vitamin K.
8. concentrate each extract portion eluted in 40:60 diethylether:petroleum ether to dryness using a stream of nitrogen gas and low heat steam bath
9. transfer each sample quantitatively to 2 dram vials using MeCl<sub>2</sub> covered with aluminium foil and reconcentrate to dryness under nitrogen and low heat steam bath.
10. dissolve in 200 µl HPLC-MEOH for Sep Pak Cleanup

#### **Sep Pak Cartridge Cleanup**

1. obtain a 15 ml test tube for each extract. Prepare a cartridge for each extract with 2 ml MEOH, then 2 ml water. Push some air through
2. add sample to cartridge in 200 µl volume HPLC-MEOH.
3. wash cartridge with 4 ml water, discard, wash with 4 ml 50:50 methanol:water, discard, elute Vitamin K with 5 ml MEOH into each test tube.
4. add 5 ml water to each test tube MEOH fraction
5. add 2 ml of chloroform. Mix and centrifuge up to 2,000 rpm for 2 minutes
6. remove the bottom layer (chloroform layer) from each test tube using Pasteur pipette and transfer to 2 dram vials covered with aluminum foil.
7. repeat steps 5 and 6.
8. concentrate the chloroform layer in vials to dryness under nitrogen gas and low heat steam bath.

#### **Thin Layer Chromatography**

Each extract was dissolved in 100 µl HPLC-MEOH. Fifty µl of each extract was placed in a 18.25 x 114 ml 0.90 3 TPB tube and concentrated down to 10 µl. Each 10 µl sample was spotted on reverse phase thin layer chromatography plate along with 0.1 µl, 0.5 µl and 1 µl of the 2.43 µg/ µl of Vitamin K standard. The

plate was developed in 70:30 MECL<sub>2</sub>: MEOH. Detection and identification of Vitamin K in samples was done by examination of plate under long and short wave ultraviolet (UV) light at Rf value corresponding to Vitamin K standard.

#### **Gas chromatography**

The Vitamin K standard was checked by a 6 ft 3 mm ID 1% OV 101 column at 255 C with sensitivity of 10. Two 0.5 µl of standard (1.22..) and two 2.5 µl of each sample taken from the remaining 50 µl of each sample were injected into GC. Quantitation of Vitamin K was done by comparing the ratio of peak height of each sample to peak height of the Vitamin K standard at the corresponding retention time. Confirmation of Vitamin K was done by Mass spectroscopy.

#### **Mass Spectroscopy**

Spots of sample and Vitamin K standard spotted on TLC reverse phase plates were scraped at Rf value corresponding to Vitamin K standard and placed in 2-dram vials. Three ml of HPLC-MEOH was added to each vial and allowed to sit overnight in order to elute Vitamin K. The HPLC-MEOH was removed from each vial and dried under nitrogen and low heat steam bath. Each sample was redissolved in 100 µl of HPLC-MEOH, vortexed, added to concentrate tubes and concentrated down to approximately 10 µl which was used for confirmation of Vitamin K.

#### **Densitometry**

The densitometer was set on B-A scan mode with a scan rate of 1 cm/min and attenuation of 32. Visible light was used with the TLC fluorescent plate. The chart recorder was set at a rate of 2.5 cm/min. Quantitation of Vitamin K present in samples was done from a standard curve constructed from peak height of intensity corresponding to the amount of Vitamin K standard spotted on TLC plate.

#### **Results and Discussion**

Thin Layer Chromatography results showed Vitamin K fluorescent spots for sample and standard were observed at Rf value of 0.75 under long-wave UV light. Under short-wave UV light, Vitamin K showed an intense purple color.

Gas chromatography results showed that Vitamin K retention time was 1.2 minutes. Two injections of each sample and standard were averaged for calculation of Vitamin K concentration. The results showed that the concentration of Vitamin K present in the case sample was lower than that present in the normal liver. The normal liver sample contained 1.70 +/- 0.3 (SD) µg of Vitamin K/g, whereas the case sample contained 0.38 µg of Vitamin K/g.

Densitometry results from standard curve showed that the concentration of Vitamin K in the normal liver sample was higher than that which was present in the case sample. The normal liver contained 0.47 µg of Vitamin K/g of liver, whereas the case sample contained 0.20 µg of Vitamin K/g liver. The difference in Vitamin K concentrations from GC contained other compounds which co-eluted with Vitamin K resulting in reduced interference and more accurate reading for Vitamin K present. Standard addition or additional cleanup of samples could have been done to achieve more accurate reading by GC.

The results suggest that the case sample showed possible Vitamin K deficit. Determination of Vitamin K status is usually done by prothrombin levels and no direct method for determination of Vitamin K status or normal Vitamin K values were found in literature. Data for sample-to-sample and day-to-day reproducibility were not available because this case was received for evaluation by the laboratory and no other samples were submitted.

Mass spectroscopy results showed that in Vitamin K standard the major ions were observed with base peak at 225 and molecular ion at 450 mass units respectively. The most abundant ions were at 225 which were about 7,000 compared to 2,000 at 450 with retention time of approximately 12.5 minutes. The results from samples showed only ions at 225 for Vitamin K but none at 450. This indicates that the quantity of ions at 225 was greater and showed up in the samples, while no 450 ions were detected.

#### **CONCLUSIONS**

The assay of Vitamin K by reverse phase thin layer chromatography showed that Vitamin K was resolved from other co-extracts at Rf 0.75 and detection and identification can be done under long-wave and short-wave UV light. Levels as low as 0.2 µg can be easily seen.

Gas chromatography, and densitometry can be used to quantitate Vitamin K present in bovine liver. Mass spectroscopy can be used to confirm Vitamin K present in the extracts. Further studies should be conducted to establish the normal Vitamin K levels expected in animals. This method may be important to clinicians in the diagnosis and treatment of animals with hypoprothrombinemia and hemorrhage diseases.

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