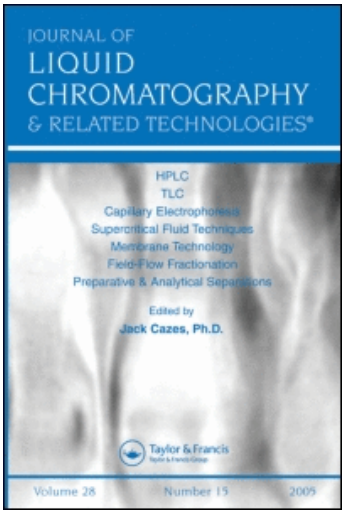


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# HPLC Determination of Vitamin $K_1$ in Neonatal Plasma Following Oral or Parenteral Supplementation with Konakion

M. Lucock[a]; R. Hartley[a]; N. J. Wild[a]
[a] University of Leeds Paediatric Research Unit D Floor, Clarendon Wing

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# HPLC DETERMINATION OF VITAMIN $K_1$ IN NEONATAL PLASMA FOLLOWING ORAL OR PARENTERAL SUPPLEMENTATION WITH KONAKION

M. Lucock, R. Hartley, and N. J. Wild

*University of Leeds*
*Paediatric Research Unit*
*D Floor, Clarendon Wing*
*The General Infirmary at Leeds*



## ABSTRACT

A rapid high-performance liquid chromatographic method for the determination of Vitamin $K_{1(20)}$ in plasma after a single hexane extraction is described. A ternary mobile phase consisting of acetonitrile/propan-2-ol/dichloromethane (68.5/22.2/9.3, v/v) with a flow rate of 1.8 ml/min was used in combination with a Waters Associates Z Module RCSS containing a Nova-Pak $C_{18}$ Radial-Pak cartridge to provide separation from co-extracted UV absorbing contaminants. The analytical column was protected by a Waters Associates Guard-Pak precolumn module with a Guard-Pak μBondapak $C_{18}$ cartridge. Using only 250μl of sample, plasma levels in the region of 15-20 ng/ml for Vitamin $K_{1(20)}$ can be determined using UV detection at 270 nm. Vitamin $K_{1(25)}$, a synthetic homologue of $K_{1(20)}$, was used as internal standard. The method has been developed for measuring plasma levels in neonates after supplementation with Vitamin $K_{1(20)}$.

## INTRODUCTION

Vitamin $K_{1(20)}$ (2-methyl-3-phytyl-1,4-naphthoquinone) is essential in the synthesis of clotting factors II, VII, IX and

X(1). Deficiency of these clotting factors in neonates can lead to a bleeding disorder known as haemorrhagic disease of the newborn (HDN) which occurs in the first 8 weeks of life(2). For many years routine prophylactic use of parenteral vitamin $K_{1(20)}$ ($K_1$) was successful in preventing HDN. However, as a result of diminished incidence, cost and trauma this form of treatment has been discontinued in many areas(3), except in high risk cases. Recently, there has been an increase in the occurrence of HDN(4) and this may be related to the dietary intake of $K_1$; the current trend towards breast-feeding resulting in fewer babies receiving $K_1$ supplemented infant formula milk. Consequently, a case may exist for the re-introduction of $K_1$ prophylaxis. It has been suggested that oral administration of the vitamin offers safer and equally effective protection against HDN(5). In order to evaluate the efficacy of oral versus parenteral administration, a rapid and reliable method for monitoring the elevation of $K_1$ levels in plasma following supplementation is required.



Existing HPLC methods for the quantitation of K vitamins in plasma can be divided into two general categories. The first group includes those assays which attempt to measure endogenous levels by employing off-line multidimensional chromatography(6-12). Using this approach, preliminary fractionation of the lipid components by liquid-liquid extraction is followed by further separation of the lipids using adsorption chromatography. The fraction of the eluant containing the lipoid material of interest is then collected, evaporated and, following reconstitution in an

appropriate solvent, subjected to a second chromatographic stage using reversed-phase conditions. The mode of detection used in conjunction with this multidimensional chromatographic approach has included ultraviolet(6-10), photochemical reaction(11) and electrochemical(12). Although not strictly a member of this category, a variant single column reversed-phase method exists which employs fluorimetric detection after post-column electrochemical reduction(13). The second category includes analytical procedures which are adequate for therapeutic monitoring of $K_1$ concentrations in plasma(14) and determination of vitamin levels in extracts of microsomal protein(15), but lack sufficient sensitivity to be of value in measuring endogenous levels. The methods in this group tend towards a rapid, single column approach employing either reversed or normal-phase chromatography followed by ultraviolet (UV) detection.



The sensitive and specific reversed-phase method described falls into the latter category and provides a simple and rapid procedure for therapeutic monitoring of $K_1$ plasma concentrations following oral or parenteral administration of the vitamin.

## EXPERIMENTAL

### Reagents

Vitamins $K_{1(20)}$ (Konakion), $K_{1(25)}$, $K_1$2,3-epoxide, $K_{2(20)}$, $K_{2(30)}$ and $K_{2(35)}$ were donated by Hoffman-La Roche, Basle, Switzerland. Acetonitrile (HPLC S grade) and dichloromethane (HPLC grade) were obtained from Rathburn

Chemicals, Walkerburn, UK. Propan-2-ol (Analar quality) and n-hexane (Laboratory Reagent grade) were purchased from BDH Chemicals, Poole, UK. Ethanol (Absolute Alcohol, AR quality) was supplied by James Burrough, London, UK.

Standard Solutions

Solutions of $K_1$ were prepared by diluting Konakion (containing $K_1$ 10 mg/ml) with ethanol to provide working concentrations of 0.1, 1 and 10 µg/ml. The internal standard solution was prepared by dissolving Vitamin $K_{1(25)}$ in ethanol to give a working concentration of 0.624 µg/ml. All solutions were protected from fluorescent light by storage in low actinic glassware and long term storage in the dark at 0-4$^0$C.



Apparatus

The high-performance liquid chromatograph (Waters Associates, Hartford, UK.) consisted of a Model 510 pump, U6K injector and a Lambda-Max Model 481 variable wavelength L.C. spectrophotometer operating at 270 nm which was connected to a Model 730 Data Module. Chromatography was performed using a Waters Associates Z-Module RCS system containing a NOVA-PAK $C_{18}$ Radial-Pak cartridge (10 cm x 8 mm ID). The analytical column (fully end capped 4 µm spherical, octadecylsilane bonded-silica) was protected by a Waters Associates Guard-PAK precolumn module containing a Guard-Pak µBondapak $C_{18}$ cartridge.

Chromatographic Conditions

The mobile phase, consisting of acetonitrile/propan-2-ol/dichloromethane (68.5/22.2/9.3, v/v), was filtered through a

0.5 µm Millipore filter (type FH) prior to use. Chromatography was effected at ambient temperature using a flow-rate of 1.8 ml/min which produced a back-pressure of 6.8 MPa. Column eluant was monitored with a UV detector operating at 270 nm and a maximum sensitivity of 0.002 aufs.

Blood Sampling

Blood was collected from newborn babies 24 hours post-partum and diluted with 50 µl of 3.13% trisodium citrate dihydrate to a final volume of 500 µl. Anticoagulated blood was centrifuged at 1400 "g" for 15 min and plasma stored at -20°C until required.

Procedure

Into a light-shielded 15 ml stoppered glass centrifuge tube, pipette 250 µl of plasma, add 500 µl of internal standard solution and Vortex mix for 10 sec. Add 2 ml of n-hexane and shake for 30 min on a mechanical shaker. Following centrifugation for 5 min at 1000 rpm (approx 300 "g") the hexane layer was removed, evaporated under nitrogen at 50°C and re-dissolved in 200 µl of ethanol. 25 µl aliquots of this ethanolic extract were injected onto the chromatograph.

Calibration

Aliquots of the $K_1$ standard solutions in ethanol referred to earlier were added to cord plasma obtained from patients who were not receiving Konakion, in order to produce a series of calibration standards. The concentration increments chosen for these calibration standards were 0.025, 0.05, 0.1, 0.25, 0.5 and 1µg/ml. These were extracted using the procedure described and,

following analysis, calibration graphs comparing peak height ratio with actual concentration of $K_1$ were constructed.

## RESULTS

Fig. 1A illustrates the chromatogram obtained following injection of an ethanolic solution of authentic components. Peaks corresponding to $K_1$2,3-epoxide, $K_1$ and $K_{1(25)}$ (internal standard) have retention times of 4.28, 5.96 and 10.55 min, respectively. The retention times of some additional $K_2$ analogs which were evaluated for use as internal standard are 3.49, 6.06 and 8.54 min for $K_{2(20)}$, $K_{2(30)}$ and $K_{2(35)}$, respectively. The chromatogram represented in Fig 1B is that of extracted blank cord plasma obtained from a patient receiving no $K_1$ supplementation. Fig. 1C is a typical chromatogram of extracted plasma obtained from a patient receiving Konakion. Comparison of Fig. 1B and 1C clearly shows the absence of endogenous components which might interfere with the quantitation of $K_1$ using this technique.

The recovery of vitamins $K_1$ and $K_{1(25)}$ was calculated after extracting two series of replicate samples. These were prepared by spiking blank plasma with 50 ng/ml and 500 ng/ml vitamin $K_1$. The extraction efficiency was determined by comparing the peak heights obtained when these samples were subjected to the standard extraction procedure and subsequently chromatographed, with those from injections of standard solutions. The mean recovery of vitamin $K_1$ was 84.3%±0.60 S.D. and

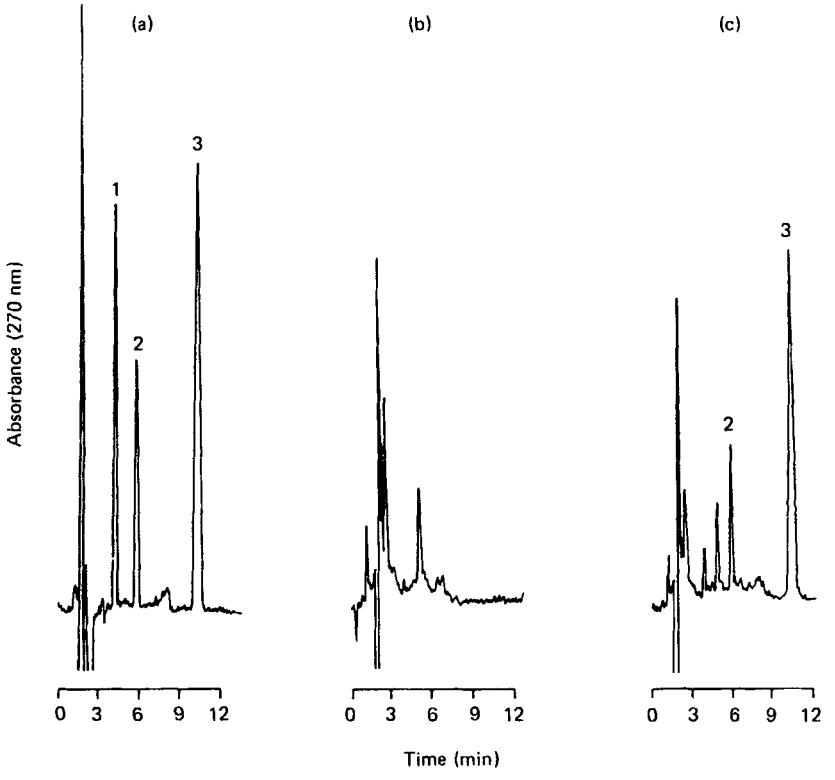


Figure 1: Chromatograms of (A) an ethanolic solution of authentic components (peaks 1, 2 and 3 represent $K_1$ 2,3 -epoxide, $K_{1(20)}$ and $K_{1(25)}$, respectively); (B) an unsupplemented plasma extracted without internal standard; (C) a plasma from a patient supplemented with Konakion (Vitamin $K_{1(20)}$).

86.3%±6.40 S.D. at low and high concentrations, respectively. The mean recovery of vitamin $K_{1(25)}$ at the internal standard working concentration was 86.6%±4.97 S.D. (in all cases n = 5).

The calibration curve was obtained by comparing the peak height ratio (vitamin $K_1$/internal standard) with the actual concentration of vitamin $K_1$ in spiked aliquots of plasma. The relationship was linear over the concentration range 0-1µg/ml with

correlation coefficient (r) and regression slope values of 0.997 and 0.002, respectively.

The effect of sample storage on reproducibility of results was examined by analysing replicate samples containing 50 and 500 ng/ml of vitamin $K_1$ in plasma. Following initial analysis (zero time), samples were stored in the dark at -20°C and assayed weekly for four weeks followed by monthly assays. The final measurements were taken after three months storage. Inter-batch coefficients of variation calculated from peak height ratios are 9.85% (n = 10) and 6.17% (n = 11) for 50 and 500 ng/ml vitamin $K_1$, respectively. Clearly storage at -20°C in the dark for up to three months has had no serious effect.



## DISCUSSION

Interference from co-extracted UV absorbing contaminants poses a major problem in the determination of $K_1$ in plasma, particularly in those methods designed for measuring endogenous levels of the vitamin. Consequently, multidimensional chromatography (MDC) has been widely used in order to overcome this difficulty(6-12). Shearer's group used MDC with UV detection at 270 nm(8-10) and electrochemical detection(12) to measure endogenous levels of $K_1$ as low as 0.08 ng/ml in human plasma. However, this high level of sensitivity was achieved using very large sample volumes (10-20 ml). Lefevere also used MDC with UV detection at 248 nm which permitted endogenous $K_1$ plasma concentrations in the region of 500 pg/ml to be quantitated(6).

Using the same approach, but with a Photochemical Reaction Detector, sensitivity was increased to provide a detection limit of 150 pg(11). In common with the previous MDC methods(8-10,12) this high level of sensitivity was also achieved by using large sample volumes (10 ml). Furthermore, Lefevere's methods(6,7,11) incorporate tritiated $K_{1(20)}$ as the internal standard so that collection of fractions for analysis by Liquid Scintillation Counting is an additional requirement subsequent to the chromatographic separation. The complexity of these MDC techniques(6-12) and their large sample requirement make them unsuitable for routine therapeutic monitoring of $K_1$ plasma levels in children.



An alternative approach devised by Langenberg(13) uses a single column reversed-phase chromatographic separation and post-column electrochemical reduction followed by fluorimetric detection. Although far less complicated than those previously described, this method does require two expensive detector systems to provide the detection limit of 25 pg of $K_1$ using 1 ml of plasma.

In another study Wilson's group(14) evaluated both normal and reversed-phase single column methods for monitoring the elevation of $K_1$ in rabbit(14) and human plasma(16,17) following pharmacological doses of the vitamin. Wilson opted for the improved selectivity and sensitivity of the normal-phase system, which enabled levels in the region of 20 ng/ml to be quantitated using 1 ml of plasma.

The present method employs a ternary non-aqueous mobile phase in conjunction with a Nova-pak $C_{18}$ column to provide sufficient selectivity to overcome problems from co-extracted UV absorbing contaminants. Levels in the region of 15-20 ng/ml can be measured using UV detection at 270 nm with only 250µl of plasma. Clearly, for therapeutic drug monitoring applications in neonates the small sample requirement using this method is preferable to that of the alternative techniques cited(6-14).

Mean 24 hr $K_1$ plasma levels determined using this method after oral (n = 12) or intramuscular (n = 14) administration of the vitamin are 73.4 ng/ml (range 19.2-210.9 ng/ml) and 347.7 ng/ml (range 174.0-608.9 ng/ml), respectively. These figures are of the same order as the median values obtained by McNinch et al(18) 24 hrs following an equivalent dose of Konakion given orally (23 ng/ml) or intramuscularly (444 ng/ml).



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