The Enzymatic Conversion of Phylloquinone to Menaquinone-4

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Abstract

All forms of vitamin K share the naphthoquinone ring, but differ in the side chain. The naphthoquinone ring is the active site for the enzyme cofactor activity of vitamin K. Mammals have the ability to convert dietary phylloquinone into MK-4 and store the latter in specific tissues. This suggests that MK-4 plays a role beyond that of an enzyme cofactor. Several functions unique to MK-4 have been proposed. Recently, UBIAD1 was identified as the enzyme catalyzing prenylation of menadione with geranylgeranyl side chain forming MK-4. However, the exact mechanism by which phylloquinone is converted to MK-4 and the tissue localization(s) for this conversion are not known.

We hypothesized that the phylloquinone's phytyl side chain is removed and a preformed geranylgeranyl side chain is added to the resultant menadione to form MK-4. The overall goal of this thesis was to provide direct evidence that menadione is the intermediate in this conversion. Menadione detection would provide insight into the location and mechanism by which phylloquinone is converted to MK-4.

A highly reproducible HPLC method was developed and validated to measure menadione in urine. Archived urine samples of participants in a human phylloquinone supplementation study were analyzed (n=367). Phylloquinone supplementation resulted in a significant increase in urinary menadione secretion (P<0.0001). These results lend support to the hypothesis that menadione is an intermediate in the conversion of phylloquinone to MK-4. Furthermore, changes in urinary menadione excretion were significantly correlated with changes in biomarkers of vitamin K status, including serum phylloquinone and percent serum undercarboxylated osteocalcin (P=0.0008 and 0.02, respectively). In a separate metabolic study of 42 healthy men and women, we used deuterium-labeled (-d) collard greens to confirm that urinary menadione-d (MW 173-174) was formed from phylloquinone-d. For an intake of 255 nmol phylloquinone-d, up to 14.9%± 10.0% (mean± SD) was recovered as menadione-d in urine.

The urinary menadione extraction method developed as part of this thesis, was subsequently modified to extract menadione from different matrices including rat urine, serum, tissues, cells and cell culture media. New menadione detection methods were developed using both LC/MS and LC-APCI-MS/MS. Male Fischer 344 rats (8 months; n=15) were fed 1.6 mg of phylloquinone-d (MW 459-463) per kg diet for 0 (control), 1 and 7 days. Using LC/MS, phylloquinone-d and MK-4-d (MW 446-449) were detected in tissues after 1 day. MK-4-d carried the d-label on the naphthoquinone ring, but not on the side chain, confirming the need for side chain removal for its formation. Using LC/MS, unconjugated menadione-d (MW 173-174) was measured in urine. Using LC-MS/MS, unconjugated menadione-d was detected in serum of rats (n=4/5 at day 1; n= 3/5 at day 7).

A Caco-2 cell culture model was used to study the role of the enterocytes in the conversion process. Neither MK-4 nor menadione was detected in cells treated with phylloquinone. However, when treated with menadione, MK-4 was formed. These data suggest that enterocytes are not the location for the phytyl side chain removal step of this conversion.

In conclusion, menadione can be measured in both urine and serum. This is the first time unconjugated menadione has been detected in biological materials. We demonstrated that newly formed MK-4 is synthesized from the dietary phylloquinone by the means of replacing the phylloquinone's phytyl side chain with the geranylgeranyl side chain. This conversion does not appear to occur in the enterocytes. Menadione is produced from dietary phylloquinone. Our findings support that menadione is an intermediate in phylloquinone to MK-4 conversion. Future studies are required to determine the exact location and function of this conversion**.**

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Abbreviations and Acronyms

7-EC	7-ethoxycoumarin
9-cis-RA	9-cis-retinoic acid
AI	adequate intake
ALP	alkaline phosphate
APOE	apolipoprotein E
APTT	activated partial thromboplastin time
DMAPP	dimethylallyl pyrophosphate
BMD	bone mineral density
CID	collision induced dissociation
-d	deuterium-labeled
DBD	DNA-binding domain
DMEM	Dulbecco's modified Eagle's medium
D2O	deuterium oxide
ECM	extracellular matrix
ER	endoplasmic reticulum
FBS	fetal bovine serum
FPP	farnesyl pyrophosphate
FPP synthase	farnesyl pyrophosphate synthase
Gas 6	growth arrest-specific 6
GFP	green fluorescent protein
GGCX	Gamma glutamyl carboxylase
GGPP	geranylgeranyl pyrophosphate
GGPP synthase	geranylgeranyl pyrophosphate synthase
Gla	gamma carboxyglutamate

GLM	general linear model
Glu	Glutamate
GPP	geranyl pyrophosphate
GPP synthase	geranyl pyrophosphate synthase
GRP	Gla-rich protein
HDL	high density lipoproteins
HMG-CoA	hydroxy-3-methylglutaryl coenzyme A
2H-NMR	deuterium nuclear magnetic resonance
HPLC	high performance liquid chromatography
INR	international normalized ratio
IPP	isopentenyl pyrophosphate
IPP isomerase	isopentenyl pyrophosphate isomerase
KH2	hydronaphthoquinone form of vitamin K
LBD	ligand-binding domain
LC-APCI-MS/MS	liquid-chromatography/ atmospheric-pressure chemical ionization tandem mass spectrometry
LDL	low-density lipoprotein
L-MD	deuterium-labeled menadione
L-MK-4	deuterium-labeled MK-4
L-PK	deuterium-labeled phylloquinone
MD	Menadione
menadione-6, 7-3H	tritium-labeled menadione
menadione-d8	deuterium-labeled menadione
MGP	matrix Gla protein
MK-n	Menaquinone

MK-2	menaquinone-2
MK-4	menaquinone-4
MK-4-d7	deuterium-labeled MK-4 on naphthoquinone ring but not side chain
MK-4-d9	deuterium-labeled MK-4 on both naphthoquinone ring and side chain
MK-4-d12	deuterium-labeled MK-4 on both naphthoquinone ring and side chain
MKO-4	menaquinone-4 epoxide
MRM	multiple reaction monitoring
MRU	metabolic research unit
MS	mass spectrometry
NADH	nicotinamide adenine dinucleotide
NADHP	nicotinamide adenine dinucleotide phosphate
OA	oleic acid
OPG	Osteoprotegerin
PGGT-I	protein-geranylgeranyl transferase I
PGGT-II	protein-geranylgeranyl transferase II
phylloquinone-d7	deuterium-labeled phylloquinone on naphthoquinone ring but not side chain
phylloquinone-d9	deuterium-labeled phylloquinone on both naphthoquinone ring and side chain
PIVKA-II	proteins induced in the vitamin K absence or antagonism-factor II
PK	Phylloquinone
pre-OLs	oligodendrocyte precursors

PRGP-1	proline rich Gla protein 1
PRGP-2	proline rich Gla protein 2
PT	prothrombin time
PXR	pregnane X receptor
REP	Rab escort protein
RT-PCR	real time polymerase chain reaction
RXR	retinoid X receptor
SFM	serum-free media
Sf9	Spodoptera frugiperda
siRNA	short interfering RNA
SPE	solid phase extraction
SXR	steroid xenobiotic receptor
TC	taurocholic acid sodium salt hydrate
TCA	Tri-Chloro-Acetic acid
TMG-3	transmembrane Gla protein 3
TMG-4	transmembrane Gla protein 4
TRL	triglyceride rich lipoproteins
Tween-40	Tween
UBIAD1	UbiA prenyltransferase containing 1
ucOC	percent serum undercarboxylated osteocalcin
UV	Ultraviolet
VCOR	vitamin K epoxide reductase
VKDB	vitamin K deficiency bleeding
VLDL	very low density lipoproteins

CHAPTER ONE

INTRODUCTION

1.1 Significance of proposed research

Vitamin K is a fat soluble vitamin. The only well-established biological role of vitamin K is a cofactor for the enzyme vitamin K dependent carboxylase. All forms of vitamin K share the naphthoquinone ring but differ in the position 3' side chain. Mammals have the ability to convert dietary phylloquinone into menaquinone-4 (MK-4) and store the latter in certain extrahepatic tissues. It seems unlikely that a metabolic pathway leading to MK-4 would have evolved unless MK-4 has unique biological roles. These roles are unlikely to involve the vitamin K–dependent carboxylase, because phylloquinone and MK-4 have similar activity as a substrate for this enzymatic activity [1]. Emerging roles unique to MK-4 beyond coagulation have been identified, including: inhibition of oxidative cell death in primary cultures of oligodendrocyte precursors (pre-OLs) and immature neurons [2], apoptosis induction in leukemia and other malignant cell lines [3-4], and being a ligand for the steroid xenobiotic receptor (SXR) in bone cells [5].

The mechanism by which MK-4 is formed is not well understood. The early attempts to characterize the mechanism of phylloquinone to MK-4 conversion go back to the early 1950s [6]. A recent breakthrough in these efforts has been reported by identifying UbiA prenyltransferase containing 1 (UBIAD1) as the enzyme catalyzing prenylation of menadione with geranylgeranyl side chain forming MK-4 [7]. Still, despite all of the efforts made to characterize phylloquinone to MK-4 conversion, *the exact mechanism and tissue localization of this conversion process is not fully understood. In addition, direct evidence identifying the intermediate in the conversion process was lacking.*

The identification of the mechanisms by which phylloquinone is converted to MK-4 will provide insight in these potential unique roles of MK- 4, and will provide the basis for future studies on the dietary requirements for vitamin K.

1.2 Specific aims and hypotheses

The overall goal of this thesis research is to characterize the conversion mechanism of dietary phylloquinone into tissue MK-4. This characterization includes identifying the intermediate in the conversion process and tissue localization where the conversion takes place.

Central hypothesis: Phylloquinone is converted to MK-4 in target tissues by an initial cleavage of the phytyl side chain to menadione, followed by addition of a preformed geranylgeranyl side chain to form MK-4.

Specific aim # 1

To develop and validate a sensitive and reproducible high performance liquid chromatography (HPLC) assay to measure menadione in human urine. The lack of an existing sensitive and reproducible method for measurement of menadione constituted the major limitation for data interpretation in previous work. This assay was used to measure changes in urinary menadione in archived urine samples from a human phylloquinone supplementation study [8].

Hypothesis: It is hypothesized that menadione concentrations in human urine will increase in response to phylloquinone supplementation. The first specific aim is intended to show that menadione is a byproduct of phylloquinone metabolism. Our central hypothesis states that phylloquinone loses its phytyl side chain and forms menadione as the first step in the conversion pathway to MK-4. In order to do so, menadione has to be produced from phylloquinone.

Specific aim # 2

To ascertain the role of the enterocytes in the removal of the phylloquinone's phytyl side chain forming menadione. We examined the ability of colon cancer cell lines, Caco-2

cells, to cleave off the phylloquinone's phytyl side chain during phylloquinone's absorption. Caco-2 cells exhibit small intestinal characteristics upon differentiation and routinely used as a model for intestinal absorption.

Hypothesis: It is hypothesized that the small intestinal cells are candidates for the central compartment where the phylloquinone's phytyl side chain is removed producing menadione. We tested the ability of differentiated Caco-2 cells to cleave off the phylloquinone's phytyl side chain forming menadione.

Specific Aim # 3

To confirm in a rat model that the phytyl side chain of dietary phylloquinone is removed and the resultant menadione is alkylated with a preformed geranylgeranyl side chain, converting dietary phylloquinone into tissue MK-4. Eight month-old male Fischer 344 rats were fed deuterium-enriched collard greens as a source of deuterium-labeled phylloquinone. Phylloquinone and MK-4 were measured in serum and tissues. Total and unconjugated menadione was measured in urine. Unconjugated menadione was measured in serum and tissues.

Hypothesis: It is hypothesized that in Fischer 344 rats the phytyl side chain in the deuterium-labeled phylloquinone is cleaved off forming deuterium-labeled menadione. A preformed unlabeled geranylgeranyl side chain is added to the newly formed menadione forming MK-4 that carries the deuterium label on the ring, but not on the side chain. We expected the newly formed MK-4 to carry the deuterium label on the naphthoquinone ring, but not on the side chain, showing that it was produced from dietary phylloquinone by means of side chain removal-addition. We also expected to measure deuterium-labeled menadione, showing that it is a product of dietary phylloquinone and supporting that menadione is an intermediate in the phylloquinone to MK-4 conversion.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Forms of vitamin K

The term “vitamin K” is used as a generic descriptor for 2-methyl-1, 4-naphthoquinone (menadione) and all its derivatives that exhibit an anti-hemorrhagic activity in animals fed a vitamin K–deficient diet [1]. By definition, all forms of vitamin K share the naphthoquinone ring but differ in the position-3 side chain.

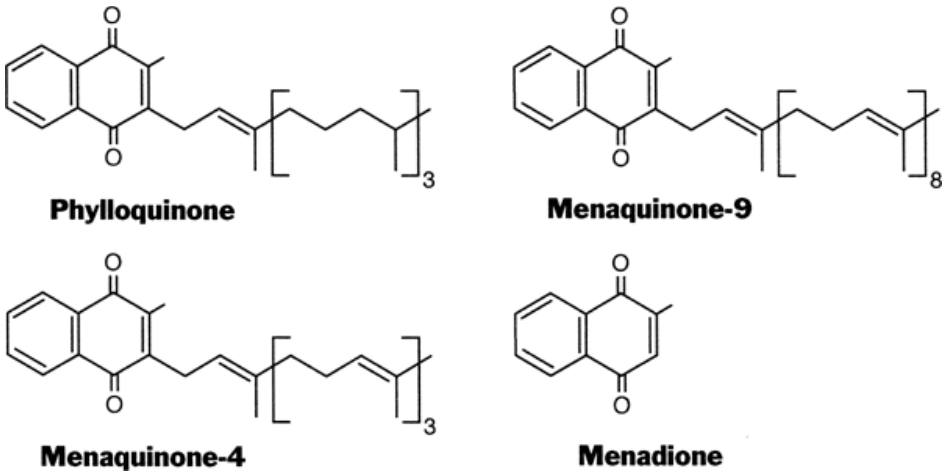


Figure-1 Forms of vitamin K.

Phylloquinone (2-methyl-3-phytyl-1, 4-naphthoquinone) contains the phytyl group as its side chain. It is synthesized in plants where it plays a role in the photosynthetic process, and is the main dietary source of vitamin K [1-2]. Menadione, which lacks the side chain, is best known as a synthetic form of vitamin K that is added to animal feed. Menaquinones (MKs) are a group of vitamin K-related compounds that contain poly-isoprenoid side chain of varying length at position-3 of the naphthoquinone ring. The menaquinones are designated by the number of isoprenoid units, i.e. MK-n. As a general

rule, gut bacteria play an important role in synthesis of longer chain menaquinones (MK-7-11) [1-2]. The human gut has long been known to contain substantial amounts of vitamin K in the form of long-chain menaquinones [1]. Menaquinones with up to 13 isoprenoid units have been identified [1].

On contrast to its low concentration in the U.S food supply, menaquinone-4 (MK-4, 2-methyl-3-geranylgeranyl-1, 4- naphthoquinone) accumulates in high concentrations in animals' tissues, specifically extrahepatic tissues.

2.2 Vitamin K cycle

The only established function of vitamin K is as a cofactor for the enzyme vitamin K-dependent glutamate carboxylase. The active form needed by the carboxylase is the reduced hydronaphthoquinone form of vitamin K (KH2) [3]. In a post-translational process, vitamin K-dependent glutamate carboxylase adds a second carboxyl group to specific glutamate residues in a limited number of proteins, converting glutamate (Glu) into gamma carboxyglutamate (Gla) [1, 4]. During this process, the hydronaphthoquinone form of vitamin K is oxidized to 2, 3-epoxide vitamin K. Vitamin K epoxide is then reduced to vitamin K quinone (K) by the action of vitamin K epoxide reductase (VCOR), a warfarin-sensitive dithiol-driven enzyme. Vitamin K quinone is the form of vitamin K in our diet. Vitamin K quinone in turn undergoes further reduction back to the hydronaphthoquinone form completing the vitamin K cycle. This final reduction of the quinone form of vitamin K to its hydronaphthoquinone form can be catalyzed by either the warfarin-sensitive VCOR or by one or more the hepatic reduced nicotinamide adenine dinucleotide (NADH) or NADH phosphate (NADHP)-linked quinone reductases, which are less sensitive to warfarin [1, 5].

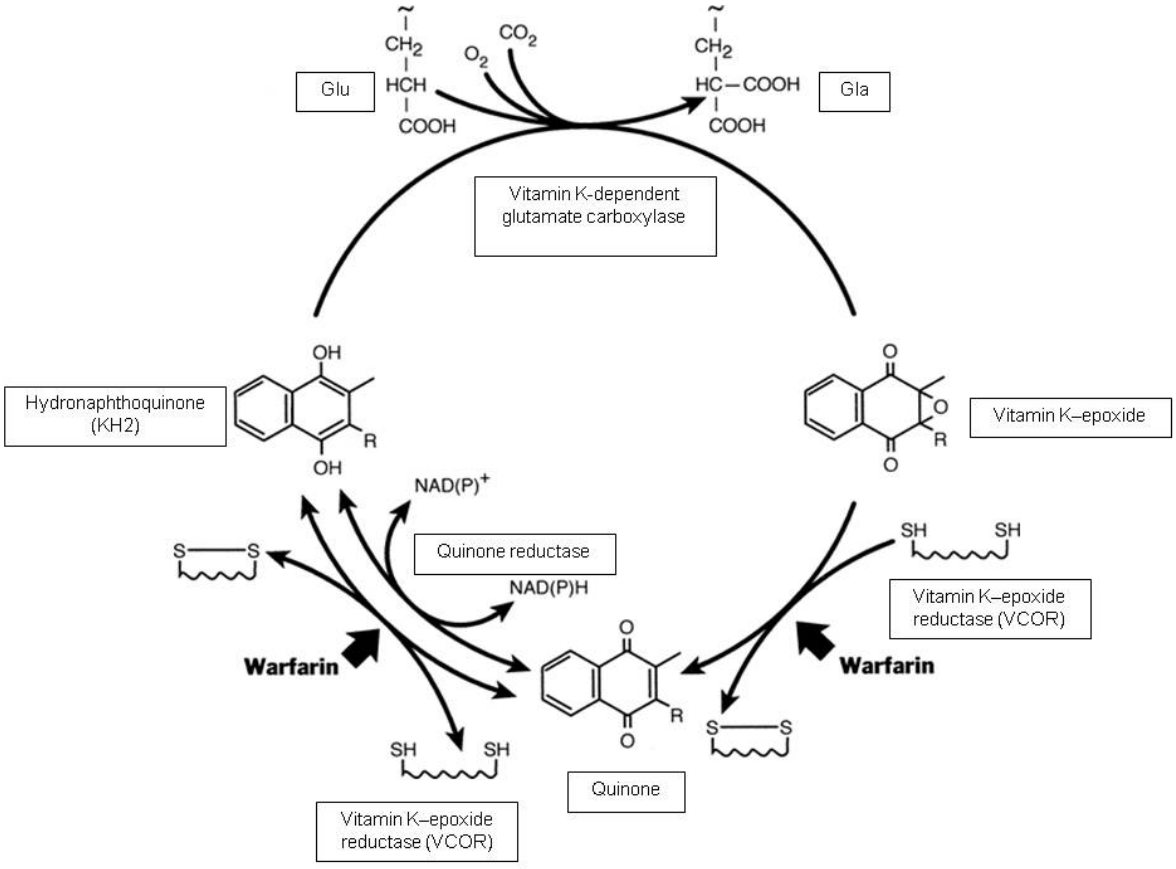
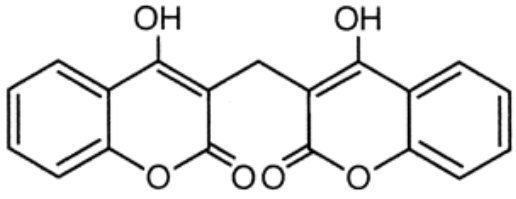


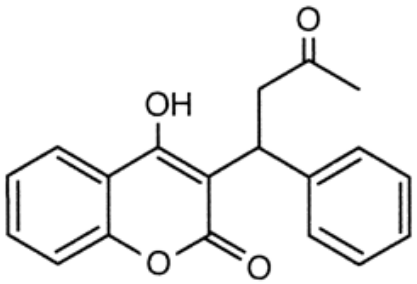
Figure-2 Vitamin K cycle.

2.3 Vitamin K antagonists

Coumarin-based oral anticoagulants (e.g. warfarin and the naturally occurring dicumarol) (Figure-3) are antagonists of vitamin K that can induce vitamin K clinical deficiency. *Matschiner et al.* [6] discovered that warfarin administration induced the accumulation of vitamin K 2,3-epoxide in rat tissues. This discovery came before the discovery of the gamma carboxylation function of vitamin K. The characterization of vitamin K 2, 3-epoxide led to the discovery of VCOR as a target of coumarin action and eventually to the discovery of the vitamin K cycle. Coumarin-based oral anticoagulants action as vitamin K-antagonists is mediated by their ability to inhibit VCOR, the enzyme responsible for the reduction of vitamin K epoxide to its quinone form, and thus interrupting the vitamin K cycle. As a result, the concentrations of vitamin K epoxide in tissues and plasma increase while the concentrations of the hydronaphthoquinone needed for the carboxylation reaction drop. Consequently, more vitamin K-dependent proteins lacking all or portion of Gla residues are secreted into the circulation [1, 3].



Dicumarol



Warfarin

Figure-3 Structure of dicumarol and warfarin.

2.4 Vitamin K-dependent proteins

Vitamin K dependents proteins (also referred to as Gla-proteins) are specific proteins that contain the Gla residues in their primary structure. These proteins undergo a post-translational modification in which the vitamin K-dependent glutamate carboxylase adds a second carboxyl group to specific glutamate residues in the amino acid sequence in these proteins converting Glu into Gla [1, 4]. Since Gla residues are stronger calcium chelators than Glu residues, this vitamin K-dependent modification significantly increases the calcium-binding capacity of those proteins [4]. The number of well characterized vitamin K-dependent proteins is limited and can be classified into three categories:

a) Vitamin K dependent clotting proteins

   There are a total of seven vitamin K-dependent proteins involved in coagulation and synthesized in liver. Among those are four proteins that are pro-coagulants: prothrombin (factor II), and factors VII, IX and X. Prothrombin was the first protein shown to be dependent on vitamin K for its synthesis, and also the first protein shown to contain Gla residues [7-9]. The four proteins circulate as a zymogen of serine protease until they are converted to their active form [1]. The amino terminal, which is the Gla domain, of these four proteins is homologous [1]. Two anticoagulant proteins (proteins C and S) are also vitamin K-dependent proteins. Their role in blood coagulation regulation includes inactivation of the activated coagulation factors V and VII [10-12]. Protein Z, which is also vitamin K-dependent, has been shown recently to have an anticoagulant function under some conditions [13].

b) Vitamin K-dependent proteins involved in regulation of calcification

Two well-characterized vitamin K-dependent proteins are found in bones: Osteocalcin and matrix Gla protein (MGP). Osteocalcin was the first Gla protein discovered that originated outside the liver [14-16]. Although osteocalcin is a very abundant protein in bone, its function is not clearly defined [1]. Osteocalcin is exclusively synthesized in osteoblasts and odontoblasts [17-18]. After synthesis most of the protein is bound to the hydroxyapatite in bones but a small fraction is secreted to the circulation. Unlike osteocalcin, MGP is present in other tissues in addition to bone. Both MGP and its mRNA have been detected in cartilage and many other soft tissues [19]. MGP appears to be a regulator of calcification, and the phenotype of the MGP knockout mice was spontaneous calcification of arteries and cartilage [20].

c) Other proteins

Few other mammalians proteins have been found to contain the Gla residues. The list includes: Gla-rich protein (GRP), which is expressed in many tissues and thought to be associated with calcification pathologies [21], Growth arrest-specific 6 (Gas 6), a ligand for tyrosine kinase Axl [22], periostin, which is synthesized by bone marrow-derived mesenchymal stromal cells and thought to play a role in extracellular matrix (ECM) mineralization [23], transmembrane proteins: proline rich Gla proteins 1 and 2 (PRGP-1, PRGP-2) [24], and transmembrane Gla protein 3 and 4 (TMG-3 and TMG-4) [25]. The specific functions of those cell-surface receptors are not clearly known.

Name of protein	Primary action	Location
prothrombin (factor II)	pro-coagulant	circulation
factors VII	pro-coagulant	circulation
factors IX	pro-coagulant	circulation
Factors X	pro-coagulant	circulation
proteins C	Anticoagulant	circulation
proteins S	Anticoagulant	circulation
Protein Z	conditional anticoagulant	circulation
Osteocalcin	bone mineralization	bones
Periostin	ECM mineralization	bones
matrix Gla protein	calcification regulator	bones, cartilage and other soft tissues
Gla-rich protein	calcification regulator	universal
Gas 6	ligand for tyrosine kinase Axl	universal

Table-1 Major vitamin K-dependent proteins.

2.5 Dietary intakes and requirements of vitamin K

The mean daily phylloquinone intake in the United States is estimated to range between 60–160 μg/day [26]. The adequate intakes (AI) of vitamin K for adult men and women are 120 and 90 μg/day, respectively [27]. AI is determined by the reported median intake among adults in the United States [27]. In infants, the AI is set at 2-2.5 μg/day based on the estimated phylloquinone intake from breast milk. Infants exclusively breast-fed have an estimated daily intake of 0.5-2.6 μg [27], and are at greater risk of vitamin K deficiency because breast milk is a poor dietary source of vitamin K. In contrast, infant formulas contain appreciable amounts of phylloquinone so the average daily intake of infants in the United States 2-6 months old is 63 μg [27].

MK-4, rather than phylloquinone, is the form of vitamin K routinely used in Japan and other Asian countries to prevent hemorrhagic disease of the newborn [1]. MK-4 is also used in Asian countries in doses of 45 mg/day Japan to treat osteoporosis.

2.6 Tissue distribution

Whereas phylloquinone is most abundant in plants, MK-4 is exclusively of animal origin. *Okano et al.* [28] reported that concentrations of MK-4 and its epoxide (MKO-4) were much higher than those of phylloquinone and its epoxide in all tissues examined among rats fed normal chow diet, despite the high phylloquinone concentration in the diet. The pancreas had the highest MK-4 concentration, and relatively high MK-4 concentrations were found in brain (cerebrum, cerebellum and medulla oblongata), thymus, thyroid and adrenal glands. The lowest MK-4 concentrations were detected in liver, skeletal muscles, lung and heart. (Table-1) Dietary phylloquinone may have been the dietary source of the tissue MK-4, but interpretation of the data were confounded by the high

concentrations of menadione in the diet, which is readily converted to MK-4 in animal tissues [5, 29-31].

A study conducted in postmortem human tissue samples showed a similar distribution of MK-4 concentrations, with the highest concentrations noted in pancreas, kidney and brain, whereas phylloquinone was concentrated mainly in heart and liver [32]. Collectively, these results suggest a tissue-specific distribution phenomenon for different forms of K that cannot be entirely explained by selective uptake and/or lipophilicity of MK-4. This selective distribution phenomenon suggests a specific function for MK-4 in extrahepatic tissues that is different from the well-established function of vitamin K as a cofactor for glutamate carboxylase enzyme.

	Phylloquinone		Phylloquinone epoxide		MK-4		MK-4 epoxide	
	Male	Female	Male	Female	Male	Female	Male	Female
Cerebrum	1.4±0.7	1.1±0.4	0.1±0.0	0.1±0.1	106.0±8.2	252.5±10.3	13.4±1.0	28.9±1.7
Cerebellum	4.8±1.8	26.2±21.5	0.1±0.1	0.4±.02	200.5±17.5	487.7±28.2	36.3±3.0	81±4.1
Medulla oblongata	7.6±2.8	22.4±15.5	0.1±0.1	0.4±0.1	116±8.3	253.3±5.5	22.4±1.6	39.4±1.9
Liver	1.2±0.1	1.5±2.9	0.1±0.0	0.1±0.0	18.2±2.7	35.0±2.2	1.8±0.4	4.4±0.4
Pancreas	3.0±0.6	3.2±0.4	0.1±0.0	0.2±0.0	520.1±47.4	829.4±56.7	184.5±15.5	355.3±35.0
Kidney	1.1±0.1	1.7±0.2	0.3±0.2	0.2±0.1	66.1±4.9	212.7±20.8	7.6±1.0	30.6±3.2
Adrenal gland	50.7±10.9	50.7±10.6	1.2±1.2	1.0±0.5	148.6±14.7	417.5±139.6	15.1±7.2	77.6±11.1
Thymus	7.5±1.2	9.0±3.9	0.1±0.1	3.9±3.5	131.2±5.5	232.5±12.0	56.2±3.9	104.4±11.9
Thyroid gland	134.9±45.5	274.8±140.3	13.9±6.2	60.8±34.6	247.3±30.4	370.3±64.0	188.2±53.7	256.0±26.1
Skeletal muscles	2.2±0.8	1.4±0.2	N.D.	N.D.	19.1±1.5	56.3±7.5	4.1±0.6	12.6±1.2
Lung	2.1±0.4	5.5±2.9	0.1±0.0	0.1±0.0	38.1±4.2	67.1±5.4	13.3±1.9	34.8±3.4
Heart	4.9±2.3	80.4±76.3	0.5±0.3	7.7±4.6	46.4±4.2	107.7±10.0	5.6±0.8	11.7±1.9
Plasma	0.6±0.0	0.6±0.0	N.D.	N.D.	0.7±0.1	1.2±0.1	N.D.	N.D.

Table-2 Tissue distribution of vitamin K in mice fed a standard chow diet [28].

2.7 Conversion of dietary phylloquinone and menadione to MK-4

Overall evidence from literature supports the side chain removal-addition model of dietary phylloquinone to tissue MK-4 conversion. According to this model, the phytyl side chain of phylloquinone is removed, and a preformed geranylgeranyl side chain is donated by geranylgeranyl pyrophosphate (GGPP) [33] to form MK-4. In this model, menadione, or its epoxide, serves as an intermediate in the conversion process.

When labeled menadione was fed to vitamin K-deficient chicks, labeled MK-4 was extracted from animals tissues [29]. Likewise, ^{3}H- labeled MK-4 was detected in all tissues examined (liver, kidney, heart, viscera, and skeletal muscles) 18 hours post intracardial injection of tritium-labeled menadione (Menadione-6, 7-^{3}H) in vitamin K-deficient rats [5]. At 3 hours post I.M. injection of labeled menadione in vitamin K-deficient chicks, MK-4 was detected in the liver [34]. In a study conducted in male Wistar rats [35], animals were placed on a vitamin K-deficient diet for nine days. The diet was then supplemented with 31 nmole/g menadione and fed to animals for five days. Menadione supplementation resulted in significantly higher MK-4 and MKO-4 concentrations in all organs examined compared to controls. The greatest accumulations were in non-hepatic organs, particularly the pancreas and salivary gland, followed by the brain and the sternum. In contrast, liver and plasma had low MK-4 concentrations. No other menaquinones such as MK-3, MK-5, MK-6 or higher were detected in any of the examined organs.

To test the hypothesis that phylloquinone's phytyl side chain is replaced by a geranylgeranyl side chain forming MK-4, phylloquinone was labeled with tritium on the methyl group of the naphthoquinone ring and with ^{14}C in positions 1` and 2` in the phytyl side chain [29]. Following intake, extrahepatic organs contained unlabeled

phylloquinone, presumably obtained from the diet, plus labeled MK-4 on naphthoquinone ring with unlabeled geranylgeranyl side chain [29]. These data confirmed that phylloquinone is converted to MK-4.

In rat studies using phylloquinone-supplemented diet with no menadione [35-36], MK-4 was detected in relatively high concentrations in pancreas and the submaxillary gland, sternum and brain contained lower concentrations, while MK-4 concentrations in liver and heart were the lowest. Liver and heart were the main organs to accumulate phylloquinone in phylloquinone supplemented groups [35-36]. MKO-4 was detected in lower concentrations than MK-4 in pancreas, salivary gland and sternum, and was not detected in liver or kidney [35]. In a different study, MK-4 was found to accumulate in relatively higher concentrations than phylloquinone in the brains and testis of Wistar rats that were orally fed phylloquinone at 4mg/kg body weight [37].

Whereas phylloquinone is converted to MK-4, it does not appear to be a reversible reaction. In the study conducted by *Sakamoto et al*. [38], when MK-4 was the exclusive form of vitamin K administered to animals, phylloquinone was not detected in liver or plasma, regardless of the route of administration (oral, intravenous or intra-peritoneal). Oral administration of phylloquinone to animals resulted in an increase in MK-4 concentration in both liver and plasma, whereas intravenous and intra-peritoneal routes did not [38]. In a different study, phylloquinone concentrations, in organs of Wistar rats fed MK-4 or menadione as the sole source of vitamin K, were not different from concentrations in organs of the vitamin K deficient rats [35]. In a rat study conducted by *Davidson et al.* [30], the presence of menadione in phylloquinone supplemented diet did not influence phylloquinone tissue concentrations. In a human study, the concentrations of both phylloquinone and MK-4 in breast milk increased with phylloquinone

supplementation in a dose-dependent manner [39], indicating that dietary phylloquinone is the source of MK-4 in milk.

Okano et al. [28] used a deuterium labeled phylloquinone carrying the label on the naphthoquinone ring but not the phytyl side chain. The resulting compound (phylloquinone-d7) was orally given to mice, and the naphthoquinone ring-labeled MK-4 (MK-4-d7) was detected in cerebra. Furthermore, when mice were orally fed deuterium-labeled phylloquinone on both the ring and the side chain (phylloquinone-d9), MK-4-d7, the parent compound (phylloquinone-d9) and both their epoxides were measured in cerebra. In contrast, MK-4 labeled on both the ring and the geranylgeranyl side chain (MK-4-d9) was not detected, which indicates that the phytyl side chain of phylloquinone is replaced by geranylgeranyl side chain to produce MK-4. Similar finding were noted for the MKO-4. In the same study, MK-4-d7 and its epoxide were detected in cerebral slice culture, and at lower concentration, in cerebral primary culture incubated for 24 hours at 10^{-5} M of either phylloquinone-d7 or deuterium labeled menadione. More MK-4 was formed per mole of menadione than per mole of phylloquinone. Primary culture cells of astrocytes or neurons were able to convert menadione, but not phylloquinone, to MK-4 [28]. The tissues that are capable of converting menadione, but not phylloquinone, to MK-4 presumably lack the enzymatic activity that cleaves the side chain of phylloquinone to form menadione.

In conclusion, these findings show that animals are capable of converting both menadione and phylloquinone into MK-4, which is stored in certain tissues. These findings also support the hypothesis that in order to convert phylloquinone to MK-4, the organisms have to remove the phytyl side chain in phylloquinone and add a geranyl-geranyl side chain to the menadione nucleus producing MK-4. In addition these findings

show that the conversion to, or at least the accumulation of MK-4 occurs mainly in extrahepatic tissues.

2.8 The role of gut bacteria in the conversion

Unlike longer chain menaquinones, e.g. MK-7-11 [2], gut bacteria is not needed for the synthesis of tissue MK-4. This statement is based on studies in which phylloquinone is converted to MK-4 in both germ-free rats [30, 40] and in aseptic mammalian cell cultures [28, 30, 41].

Davidson et al. [30] showed that both controls and gnotobiotics (germ-free) male rats were able to accumulate MK-4 in tissues when fed vitamin K-deficient diet supplemented with intraperitoneal phylloquinone (1500 μg/Kg diet consumed) as the only source of vitamin K. MKO-4 was detected in the same tissues MK-4 was found, albeit at 15-30% of the MK-4 concentrations. The highest concentrations of MK-4 in tissues examined were in the mandibular salivary gland, followed by the kidney. Phylloquinone supplementation increased the concentrations of phylloquinone in all tissues assayed, and tissue concentrations did not differ in both control and germ-free rats. In the same study, phylloquinone and menadione supplementation resulted in similar MK-4 concentrations. In a different study [40], vitamin K-deficient germ-free rats given menadione or phylloquinone in drinking water were able to accumulate MK-4 mainly in extrahepatic tissues, such as pancreas and brain. This establishes that the tissue-specific formation of MK-4 from phylloquinone is a metabolic transformation that does not require action of intestinal bacteria.

Direct tissue conversion that was shown in several cell and slice culture studies lends further evidence that gut bacteria have no role in phylloquinone or menadione to MK-4

conversion. A transformed kidney cell line (HEK- 293) was able to synthesize MK-4 from phylloquinone [30] and menadione [41]. Other cell lines, including the pancreatic cell line (Panc-I), synthesized MK-4 from menadione [41]. MK-4-d7 and its epoxide were detected in cerebral slice culture, and at lower concentration, in cerebral primary culture incubated for 24 hours at 10^{-5} M of either deuterium-labeled phylloquinone-d7 or deuterium labeled menadione. The conversion was more evident when menadione was added. Primary culture cells with high concentration of astrocytes or neurons were able to convert menadione, but not phylloquinone, to MK-4 [28]. One possibility is that tissues that are capable of converting MK-4 from menadione, but not phylloquinone, lack the enzymatic activity that releases menadione from phylloquinone.

2.9 Tissue location of the conversion

There are two possible pathways regarding tissue localization of the specific catalytic activities. In the first pathway, phytyl side chain removal, as well as the subsequent geranylgeranyl side chain prenylation, is a metabolic activity specific to certain tissues. Evidence for this pathway is the detection of MK-4 in cultured cells and slice culture incubated with phylloquinone. MKO-4 and MK-4 were detected in kidney-derived cell line HEK- 293 incubated with 2.2 μM phylloquinone [30], cerebral slice culture, and to a lesser extent, in a cerebral primary culture incubated with phylloquinone [28]. In the absence of a detection method, in neither of these studies were menadione, its epoxide or the side chain detected. *Detection of menadione and the side chain in cells of interest is a critical gap in knowledge.*

The alternative pathway requires the phytyl side chain to be removed in a central body compartment (the enterocytes and the liver are the main candidates), and menadione, or its epoxide, to be released into the circulation where it is prenylated in target tissues with

the preformed geranylgeranyl, forming MK-4. In support of the alternative pathway, urinary menadione excretion rapidly increased in response to increased oral phylloquinone intake, but not subcutaneous administration [41], suggesting that the oral route of administration is needed for phytyl side chain removal. Urinary menadione was detected 1-2 hours after oral phylloquinone ingestion, and peaked at 3 hours. The rapid appearance in urine after an oral, but not subcutaneous administration, suggests that side chain removal occurs during the first-pass metabolism. *Okano et al.* [28] administered phylloquinone-d7 to animals via four different routes: oral, enteral (bypasses mouth), intravenous (bypasses intestine), and intracerebroventricular (bypasses blood-brain barrier). Only oral and enteral routes resulted in MK-4-d7 accumulation in the cerebra, suggesting a role for the oral route in the conversion process. In the absence of a detection method, menadione was not measured in circulation, which constitutes a gap in knowledge. It is not known if the phytyl side chain is removed as a whole or if there are different sites of cleavage on the side chain. After oral administration, absorbed phylloquinone is integrated in chylomicrons and probably enters the liver as part of chylomicron remnants [42-43], while the majority of phylloquinone administered via I.M. route is transported as part of LDL and HDL fractions [44]. This difference in lipoprotein transport may partially explain the different results from different routes of administration.

A third possible pathway would be that MK-4 is formed in a central compartment and released into circulation to reach target tissues. In animal studies, MK-4 was detected in specific tissues, but not in circulation, which argues against this proposed pathway [30]. Also, very low concentrations of MK-4 were detected in the livers of rats fed normal chow diet [28]. In humans, MK-4 is usually undetectable or present in very low

concentrations in plasma and liver [32, 41, 45], which also argues against the hypothesis of forming MK-4 in a central compartment, specifically the liver.

Collectively, the data suggest that menadione release from phylloquinone may take place in limited number of tissues, as well as a central compartment (liver or enterocytes). The subsequent prenylation with the preformed geranylgeranyl side chain occurs in target tissues. The gap in knowledge is direct measurement of menadione in target tissues and in circulation to confirm this hypothesis.

2.10 Candidate enzymes for the alkylation reaction

Based on evidence from literature, it is unlikely that the enzyme responsible for phylloquinone to MK-4 conversion is a desaturase. In the presence of a desaturase, phylloquinone with a labeled phytyl side chain would form MK-4 with a labeled geranylgeranyl side chain. Instead, phylloquinone with a labeled phytyl side chain (phylloquinone-d9) resulted in MK-4 with an unlabelled geranylgeranyl side chain (MK-4-d7) [28].

GGPP donates a preformed geranylgeranyl side chain in a well-established protein prenylation reaction in the body. Protein prenylation is a lipid modification [46]. In this enzymatic-catalyzed reaction, the geranylgeranyl is covalently attached via thioester linkage (R-S-R) to specific cysteine residues at or near the carboxyl terminus of a limited number of proteins; the thiolate in cysteine forms the thioester bond (R-S-R) by a nucleophillic attack (S becomes negative) on C1 in the geranylgeranyl chain releasing pyrophosphate [46-48]. Protein substrates of the prenyltransferases include: Ras, Rho, Rab and other Ras-related small GTP-binding proteins [48].

There are two enzymes capable of catalyzing this prenylation reaction: protein-geranylgeranyl transferase I and II (PGGT-I and II). Both PGGT-I and II are cytosolic [49-50]. Zn^{+2} metaloenzymes [51]. Those enzymes are classified as prenyltransferases. PGGT-I (ec: 2.5.1.59) adds geranylgeranyl side chain to proteins with a Ca1a2X (C=Cysteine, X= Lucien, a1 and a2= aliphatic amino acids) sequence on the carboxyl end of the polypeptide chain [47]. Mammalian PGGT-I is a heterodimer that consists of 48kDa alpha subunit and 43kDa beta subunit [46-47]. PGGT-II, which is also called Rab geranylgeranyl transferase, is also a heterodimer [46-47, 49]. PGGT-II (ec: 2.5.1.60) [47] attaches geranylgeranyl groups to two C-terminal cysteine residues in the Rab family of Ras-related GTPases (CC or CXC) [48]. PGGT-II requires Rab escort protein (REP) in order to recognize its substrate, the unprenylated Rab [49].

There are many characterized commercially available specific inhibitors of PGGT-I and II [47, 52]. Inhibitors can act as competitive inhibitors for one or both of the two substrates [47], or by depleting the cells from the precursor molecules [52].

In the broad sense, prenyltransferase is any enzyme that catalyzes the transfer of prenyl groups from dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), GGPP, etc…) to acceptors that include isopentenyl pyrophosphate (IPP), aromatic compounds and proteins, etc [53]. By definition, our alkylation enzyme of interest is a prenyltransferase since it must add geranylgeranyl, a prenyl group, to menadione. *Nakagawa et al.* [54] screened the human genome database for prenylation enzyme homologues of critical prenyltransferase enzymes in E.coli. Two prenyltransferase-encoding human genes were identified as the candidates for encoding the prenylation enzyme that catalyses MK-4 biosynthesis in human: UbiA prenyltransferase containing 1 (UBIAD1), a human homologue of the E.coli menA gene,

and COQ2. In E.coli, menA gene encodes a prenyltransferase enzyme that is involved in the vitamin K biosynthetic pathway [55].

Short interfering RNA (siRNA) against the UBIAD1 gene, but not against the COQ2 gene, inhibited the conversion of deuterium-labeled menadione (menadione-d8), and deuterium-labeled MK-4 that carried the deuterium label on both the ring and the side chain (MK-4-d12), into MK-4-d7, in human osteoblast-like MG-63 cells. Furthermore, MG-63 cells trasfected with UBIAD1 expression vector contained increased levels of MK-4-d7 compared to non transfected cells. This increase was correlated with UBIAD1 mRNA and protein expression levels [54]. Thus UBIAD1 was singled out as the gene of interest.

UBIAD1 expression was not detected in Spodoptera frugiperda (Sf9) insect cells. In addition, Sf9 cells showed no measurable conversion activity to MK-4. On the other hand, when transfected with UBIAD1 baculovirus expression vector, UBIAD1 protein was expressed in those cells and Sf9 cells were able to convert menadione-d8, MK-4-d12, and to a lesser extent phylloquinone-d7, into MK-4-d7 [54]. These data showed that the presence of UBIAD1 gene and consequently UBIAD1 protein is sufficient for MK-4-d7 synthesis.

Prenylation activity (menadione-d8 to MK-4-d7) of UBIAD1 in microsomes prepared from UBIAD1 baculovirus-infected Sf9 cells increased in a dose-dependent manner with addition of GGPP, showing that GGPP is a substrate for UBIAD1 in the MK-4 synthesis prenylation reaction.

The chemical structure of MK-4-d7 was confirmed using liquid-chromatography/ atmospheric-pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS) and deuterium nuclear magnetic resonance (^{2}H-NMR).

UBIAD1 was identified as a human prenyltransferase enzyme responsible for the geranylgeranyl side chain addition step of the conversion process from phylloquinone to MK-4 [54]. Evidence regarding UBIAD1 role as the enzyme responsible for the phytyl side chain removal is rather weak. More experiments need to be done to elucidate that role. The inability to measure menadione, the proposed intermediate in the conversion, represents a major limitation in data interpretation regarding UBIAD1 role in the phytyl side chain removal step of the conversion process.

2.11 Cellular enzymatic location

After identifying the UBIAD1 to be the prenyl transferase enzyme responsible for MK-4 formation, *Nakagawa et al.* [54] studied UBIAD1 subcellular localization in MG-63 cells. Constructs expressing green fluorescent protein (GFP)-tagged UBIAD1 fusion proteins (UBIAD1–GFP) were developed. MG-63 cells were transfected with UBIAD1–GFP expression vector. Cells were stained with either an endoplasmic reticulum (ER) marker or a Golgi marker.

The green fluorescence of UBIAD1–GFP co-localized exactly with the ER marker label but did not co-localize with the Golgi marker. Based on these results, the authors concluded that UBIAD1 is located in the ER.

Previous work prior to the identifying of UBIAD1 lends support to this conclusion that the enzyme is located in the ER. In the search for the menadione to MK-4 alkylation cellular

localization, *Dialameh at al.* [34] showed that in vitamin K-deficient chicks injected intramuscularly with tritium-labeled menadione, microsomes were found to contain 66% of total MK-4 measured in the cells. In addition, *in vitro* deactivation of chicks' liver microsomes prevented alkylation of menadione to MK-3 in the presence of FPP as a side chain donor, suggesting that the ER is the location of geranylgeranyl side chain addition.

However, other studies suggest that the mitochondria, not the ER, is the cellular enzymatic location, albeit the evidence is weaker. *Martius* [29] reported that the side chain can be introduced *in vitro* in the presence of an enzyme system that is found in the mitochondria. In a different study, a relatively higher mitochondrial accumulation of phylloquinone and MK-4 was measured in pancreas, kidney and salivary gland; phylloquinone and MK-4 were also measured in the microsomes of those organs [36]. There are no published data regarding the cellular localization of the phylloquinone phytyl side chain removal.

2.12 Effect of warfarin administration on phylloquinone to MK-4 conversion

Several studies tested the effect of coumarin-based vitamin K-antagonists anticoagulants (e.g. warfarin and dicumarol) on phylloquinone to MK-4 conversion.

In animals injected intracardially with tritium-labeled menadione, dicumarol lowered MK-4 concentrations in all examined tissues (liver, kidney, heart and viscera), with the exception of the skeletal muscle [5]. It is unclear whether the decrease in MK-4 concentrations was due to an inhibition of the conversion reaction or due to other mechanisms, such as MK-4 being used by vitamin K-dependent glutamate carboxylase and accumulated as MKO-4. Unfortunately, MKO-4 was not measured.

In the presence of warfarin, less phylloquinone accumulated in kidney-derived cell line HEK-293 incubated with 2.2 μM phylloquinone (with warfarin: 1.23±0.32, without warfarin: 5.20±1.09, means ± SD, pmol/mg cellular protein) [30]. In contrast, phylloquinone epoxide concentrations remained the same (with warfarin: 0.62±0.11, without warfarin: 0.65±0.11 means ± SD, pmol/mg cellular protein). Warfarin inhibits VCOR and thus accumulation of vitamin K epoxide combined with a decrease in the quinone form of vitamin K is expected. Low levels of MK-4 and MKO-4 were detected with no warfarin added. In the presence of warfarin, both MK-4 and MKO-4 were below detection limits [30]. It is possible that there was too little substrate to form detectable amounts of MK-4 and MKO-4.

In Wistar rats fed phylloquinone as the exclusive source of vitamin K in the diet [35], warfarin administration resulted in significantly lower phylloquinone concentrations in lung, kidney, liver, sternum, heart and plasma, with a concomitant increase in phylloquinone epoxide concentrations in plasma, liver and sternum. No phylloquinone epoxide was detected in other organs. MK-4 concentrations were >65% lower while MKO-4 concentrations were relatively higher. In animals fed menadione as the exclusive source of vitamin K in the diet [35], warfarin administration resulted in a greater decrease in MK-4 concentration in most organs (-90%). MKO-4 accumulation was seen in the liver and sternum only whereas other organs contained lower or comparable epoxide concentrations.

MK-4-d7 concentrations in UBIAD1-expressing Sf-9 cells were similar when treated with menadione-d8 and with 0, 6.25, 12.5 or 25.0 μM warfarin, indicating that warfarin did not affect the conversion of menadione-d8 to MK-4-d7 [54]. No MK-4-d7 epoxide was

detected in any of the cells. Sf9 cells don't express the enzymes of vitamin K cycle, and thus the inability to measure MK-4-d7 epoxide was not an unexpected outcome. In UBIAD1-expressing MG-63 cells treated with either menadione-d8 or phylloquinone-d7, warfarin decreased the concentration of MK-4-d7, although MK-4-d7 epoxide did accumulate [54]. MG-63 cells are a human osteoblast-like cell line in which the enzymes of the vitamin K cycle are expressed. The observation that warfarin induced the accumulation of the epoxide while the quinone form decreased is in accordance with warfarin's mechanism of action as a VCOR inhibitor (Figure-2. Vitamin K cycle). Collectively, these results indicate that warfarin does not affect MK-4 biosynthesis by UBIAD1, but can result in the accumulation of MKO-4.

Although some have proposed that warfarin blocks the conversion of phylloquinone to MK-4, it appears more likely that warfarin has no direct inhibitory effect on the conversion mechanism. Instead, consistent with warfarin's well-characterized function as a VCOR inhibitor, warfarin treatment causes a decrease in MK-4 once it is formed from phylloquinone, while MKO-4 accumulates.

2.13 Proposed novel functions of MK-4

Mammals are capable of converting menadione and phylloquinone to MK-4, and store the latter in specific tissues. It seems unlikely that a metabolic pathway leading to MK-4 has evolved unless MK-4 has a unique role that cannot be fulfilled by other vitamin K forms. This role is unlikely to involve the vitamin K–dependent carboxylase, because phylloquinone and MK-4 have similar activity as a substrate for this enzyme [1]. Several non-carboxylase related roles for MK-4 have been proposed.

a) Ligand for SXR

MK-4 was shown to act as a ligand for the nuclear receptor steroid xenobiotic receptor (SXR) in bone cells [56]. SXR, which is known as pregnane X receptor (PXR) in mice [57], is a transcriptional regulator activated by various biological and xenobiotic substances (ligands). SXR contains both an N-terminal DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). SXR and retinoid X receptor (RXR), a 9-cis-retinoic acid (9-cis-RA) receptor, form a heterodimer that binds to specific SXR response elements in the genome regulating the transcription of SXR target genes. Known ligands of SXR include many pharmaceutical agents, such as the macrolide antibiotic rifampicin, the antidiabetic drug troglitazone, the cholesterol-lowering drug SR12813, the antimycotic clotrimazole, the barbiturate phenobarbital, hyperforin [58-59], and the herbal antidepressant St. John's wort (Hypericum perforatum) [60-61]. When activated, SXR performs an important role in the transcriptional regulation of xenobiotic detoxifying enzymes and transporters, such as the hepatic cytochrome P450 CYP3A4 and the multidrug resistance gene MDR1 that encodes ABCB1, a member the ATP-binding cassette transporters [62-66]. Therefore, SXR functions as a xenobiotic sensor to coordinately regulate drug clearance in the liver and intestine [64-65].

Tabb et al. [56] conducted a series of experiments to establish that MK-4 is a ligand for SXR in bone cells. Using real time polymerase chain reaction (RT-PCR), SXR was shown to be expressed in three human osteosarcoma cell lines: HOS, MG-63 and Saos-2. These cell lines are osteoblastic in nature and are commonly used as models for osteoblast formation [67-69]. To demonstrate that MK-4 activates SXR, COS-7 cells transiently transfected with GAL-SXR or control were treated with MK-4 or rifampicin, a known SXR ligand. Both MK-4 and rifampicin activated SXR in a dose-dependent manner. This activation was not observed in the controls, indicative of the need for MK-

4's interaction with the SXR's LBD. In another experiment, prototypical SXR target gene CYP3A4 was induced by both MK-4 and rifampicin in a dose-dependent manner in HOS, MG-63 and Saos-2 cell lines, where SXR mRNA was expressed. MK-4 has also been shown to bind directly to SXR *in vivo* and in *vitro. In vivo*, both MK-4 and rifampicin were able to induce SXR to interact with its coactivator proteins (PBP, SRC-1, TIF2 and ACTR) in COS-7 cells. *In vitro*, both MK-4 and rifampicin were able to bind to purified SXR protein. Collectively, these data show that MK-4 acts a ligand for SXR in bone cells.

b) Effect on bones

MK-4 is clinically used in Japan and other Asian countries to treat osteoporosis [70]. The underlying mechanism of action could include stimulating deposition of bone mineralization [69, 71-72], and decreasing bone resorption [72-73].

Several human studies have reported a positive role played by MK-4 in bone mass. Daily administration of MK-4 for one year suppressed the decrease in lumbar spine bone mineral density (BMD) in postmenopausal women as compared to controls [70]. A two-year study conducted on postmenopausal women with osteoporosis concluded that MK-4 significantly increased lumbar spine BMD in patients compared to controls [74]. The Yamaguchi Osteoporosis Prevention Study reported that at the end of two years study period, MK-4 administration reduced vertebral fractures by 56% compared with placebo [75]. In those studies [70, 74-75] a high dose of 45mg MK-4/day was used. In contrast, a number of other studies have reported no effect of MK-4 on bone health [76-77].

A similar positive effect of MK-4 administration on bone health has been reported in animal models. Bone loss in rats, assessed mainly by measuring bone density and bone

strength, and induced by either prednisolone administration [78] or ovariectomy [79], was shown to be suppressed by MK-4 administration.

There are few reports of the action of vitamin K in human osteoblasts. The findings of these studies suggest that MK-4 plays an anabolic role in bone cells that is independent of γ-carboxylation. In a cell culture study conducted on human osteosarcoma cell lines (HOS, MG-63 and Saos-2) [56], MK-4 (as well as rifampicin) induced the expression of osteoblastic marker genes, alkaline phosphate (ALP), MGP and osteoprotegerin (OPG). Both protein and mRNA of these genes were measured. Bone ALP is located on the surface of the osteoblasts and is thought to play a major role in bone formation and mineralization [80-81]. OPN is a major non-collagenous bone matrix protein produced by osteoblasts as an early marker of osteoblasts differentiation [82-83]. MGP is a vitamin K-dependent protein with a wide tissue distribution. The induction effect of MK-4 on osteoblast marker genes was not noticed in PXR knockout cells, indicating that this effect requires the expression of PXR/SXR is bone cells [56]. In a different study, *Chikawa et al.* [84] used microarray to show that MK-4 regulated the transcription of genes encoding ECM proteins in an SXR-dependent manner and increased the accumulation of collagen in osteoblastic cells. This MK-4-SXR interaction might explain some of MK-4's reported positive effects on bone.

c) Inhibition of oxidative cell death

Although vitamin K is not known as a classical antioxidant, both phylloquinone and MK-4 effectively inhibited oxidative cell death mediated by glutathione depletion in primary cultures of oligodendrocyte precursors (pre-OLs) and immature neurons [85]. Warfarin treatment had no effect on this protective function of vitamin K on oxidative injury, suggesting that it is not a carboxylase-dependent function [85]. In a subsequent study

[86], it was shown that phylloquinone and MK-4 protect pre-OLs from arachidonic acid-induced oxidative injury through blocking 12-lipoxygenase activation and thus preventing ROS accumulation. Phylloquinone and MK-4 didn't directly inhibit 12-lipoxygenase suggesting that they work upstream of it. Unfortunately, the ability of pre-OLs to convert phylloquinone into MK-4, as assessed by measuring MK-4 in cells treated with phylloquinone, was not tested. Thus, data interpretation could not exclude the possibility that phylloquinone serves as a precursor for MK-4 in those cells, and MK-4 is the form of vitamin K responsible for oxidative cell death inhibition.

d) Induction of apoptosis

Multiple *in vitro* studies have reported an apoptosis induction effect exerted by MK-4 on several tumor cell lines. Both menadione and MK-4, but not phylloquinone, inhibited the growth of the hepatocellular carcinoma cell lines [87]. Apoptosis induction was shown to play a role in this inhibition [88]. MK-4, but not phylloquinone, also induced apoptosis in leukemia cell lines [89-91]. MK-4 also induced apoptosis in lung carcinoma [90], and gastric cancer cell lines [92]. Finally, MK-4 pro-apoptotic effect was shown in rheumatoid fibroblast-like synovial cells [93].

These unique functions attributed to MK-4, and not to other forms of vitamin K, may explain why mammals need MK-4. However, more research needs to be done.

2.14 Previous attempts for menadione measurement

In the enzymatic conversion of phylloquinone to MK-4, the phytyl side chain of dietary phylloquinone is thought to be removed forming menadione, and a preformed geranylgeranyl side chain is donated by GGPP [29] to form tissue MK-4. According to this mechanism, menadione is an intermediate in the conversion. Sensitive and

reproducible assays for measurement of menadione are required for the study of this conversion of phylloquinone to MK-4. Such an assay can be used to help elucidate the role played by menadione in MK-4 formation.

Previous attempts to measure menadione lack the sensitivity to detect this compound in response to a variety of dietary and physiological conditions [41, 94-96]. Some assays were not designed to measure menadione in biological fluids [97-99]. Those assays designed to measure menadione in biological fluids failed to measure endogenous menadione [94-96], except for the one described by *Thijssen et al.* [41]. Most assays did not use an internal standard [95, 97-99], and of those that did, they used internal standards with chemical properties that are dissimilar to those of menadione [41, 94, 96], which limits their utility.

In the method described by *Thijssen et al.* to measure menadione in urine [41], 7-ethoxycoumarin (7-EC) was used as an internal standard. Vitamin K-related compounds are not endogenously fluorescent, thus they require a postcolumn zinc reduction to their corresponding fluorescent hydroquinones for fluorescent detection [100]. In the same reaction, the zinc metal is oxidized, depleted and its effect diminishes over time, thus, the zinc column needs to be repacked periodically [101]. In contrast to menadione and other vitamin K related compounds; 7-EC is endogenously fluorescent and does not require zinc reduction for fluorescent detection. As a consequence, changes in 7-EC's response do not reflect the zinc column conditions that could influence calculation of menadione concentrations, whereas an internal standard that is not endogenously fluorescent, does. The assay sensitivity was not explicitly stated.

Complete drying of extraction solvent prior to high performance liquid chromatography (HPLC) injection resulted in high menadione losses, as previously reported [96]. The exact cause for this phenomenon is not known. *Thijssen et al.* [41] used 50 μL 50% ethylene glycol in water to prevent menadione loss.

HPLC with ultraviolet (UV) detection has previously been used to measure menadione in plasma [95-96]. However, fluorescence detection is more sensitive and specific for vitamin K compounds compared to UV detection.

This thesis research will address the gaps in the field by developing a sensitive and reproducible method for detection of menadione in biological samples. Once developed, this assay will be applied to multiple study designs to confirm that menadione in an intermediate in the conversion of phylloquinone to MK-4.

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CHAPTER THREE

MANUSCRIPTS

3.1 Measurement of Menadione in Urine by HPLC

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Abstract

Menadione is a metabolite of vitamin K that is excreted in urine. A high performance liquid chromatography (HPLC) method using a C_{30} column, post-column zinc reduction and fluorescence detection was developed to measure urinary menadione. The mobile phase was composed of 95% methanol with 0.55% aqueous solution and 5% DI H_2O. Menaquinone-2 (MK-2) was used as an internal standard. The standard calibration curve was linear with a correlation coefficient (R^2) of 0.999 for both menadione and MK-2. The lower limit of quantification (LLOQ) was 0.3 pmole menadione/mL urine. Sample preparation involved hydrolysis of menadiol conjugates and oxidizing the released menadiol to menadione. Using this method, urinary menadione was shown to increase in response to 3 years of phylloquinone supplementation. This HPLC method is a sensitive and reproducible way to detect menadione in urine.

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Key words: menadione, HPLC, vitamin K, phylloquinone, menaquinone-4.

Abbreviations

MD: Menadione, MK-4: Menaquinone-4, MK-2: Menaquinone-2, GGPP: geranylgeranyl pyrophosphate, PK: Phylloquinone, 7-EC: 7- ethoxycoumarin, LLOQ: lower limit of quantification.

Introduction

Vitamin K is a fat soluble vitamin known for its role in coagulation [1]. New physiological functions have been attributed to vitamin K, including regulation of calcification in vessel walls and protection against oxidative stress [2-3].

All forms of vitamin K share a common naphthoquinone ring, but differ in the position-3 side chain. Phylloquinone, which contains the phytyl group as its side chain, is the main dietary source of vitamin K [1]. Menadione, which lacks the side chain, is best known as a synthetic form of vitamin K added to animal feed. Menaquinones are a group of vitamin K-related compounds that contain a poly-isoprenoid side chain of varying lengths at position-3 of the naphthoquinone ring. Even though MK-4 is not abundant in the food supply, it is found in high concentrations in certain tissues. MK-4 may have unique roles in novel functions of vitamin K.

The phytyl side chain of dietary phylloquinone is thought to be removed forming menadione, and the preformed geranylgeranyl side chain is donated by geranylgeranyl pyrophosphate (GGPP) [4] to form tissue MK-4. The removal of the side chain may take place in a central compartment. Menadione is then released to the circulation and prenylated in target tissues forming MK-4. Circulating menadione is most likely conjugated and excreted in the urine.

Sensitive assays for measurement of menadione are required for the study of this conversion of phylloquinone to MK-4. Previous attempts to measure menadione lack the sensitivity to detect this compound in response to a variety of dietary and physiological conditions [5-7].

We have developed and validated a high performance liquid chromatography (HPLC) method using a C_{30} column, post-column zinc reduction and fluorescence detection for the purpose of measuring urinary menadione. This HPLC method presents a sensitive and reproducible way to detect menadione in urine, and can be used to help elucidate the role played by menadione in MK-4 formation.

Materials and Methods

Reagents and Standards

Solvents used for extraction and chromatography were HPLC grade (Fisher Scientific, Springfield, NJ). Menadione standard (Sigma-Aldrich, St Louis, MO. Product number M 5625) and internal standard, Menaquinone-2 (MK-2, 2-methyl-3-geranyl-1, 4-Naphthoquinone, a gift from Hoffman La Roche®) were prepared in iso-octane, and characterized spectrophotometrically [8] and chromatographically before use. All operations were performed under yellow light to avoid any losses due to light sensitivity of vitamin K.

Hydrolysis and Extraction

Menadione is excreted in urine in the form of glucuronide and sulfate conjugates of menadiol [9]. An acid treatment procedure for hydrolysis and oxidation of menadione conjugates in urine was used as previously described [5]. The internal standard (MK-2) was added and the samples were vortexed. Three mL of iso-octane was added to each sample in 16x100 borosilicate culture tubes, and the tubes were capped with Teflon-lined screw caps. Samples were vortexed for 3 minutes and centrifuged for 5 minutes at 1800xg to separate the aqueous and organic layers. The top, iso-octane, layer was aspirated and transferred to a clean set of tubes. The iso-octane was evaporated using a

gentle stream of nitrogen until approximately 200 μL of iso-octane was left in each tube. The remaining iso-octane residue was transferred to an amber vial, and a suitable aliquot was injected into the HPLC.

HPLC

The HPLC method is adapted from that used for measurement of vitamin K compounds [10]. The method uses online post-column reduction with zinc metal and fluorescence detection. The HPLC consists of a Waters 2695 Separations Module equipped with a vacuum degasser and auto injector. Excitation and emission wavelengths using a Shimadzu Fluorescence Detector were 244 and 430 nm, respectively. We used a ProntoSil C_{30} column (5μm, 250 x 4.6 mm) (MAC-MOD Analytical Inc., Chadds Ford, PA), which was cooled to 5 ^{o}C using a Cool Pocket ^{TM} (Thermo Electron Corporation, Waltham, MA). To achieve post-column reduction of vitamin K compounds that are not otherwise fluorescent, 100-mesh zinc particles (Alfa Aesar; Ward Hill, MA) were dry-packed into a 50 mm X 3.9 mm (i.d) stainless-steel column equipped with 2 μm frits. The mobile phase consisted of 95% methanol and 5% DI H_2O. The methanol has 0.55% aqueous solution added (2.0 M zinc chloride, 1.0 M acetic acid and 1.0 M sodium acetate). The flow rate was 0.6 mL/minute.

Assay Validation

Recoveries and Lower Limit of Quantification (LLOQ)

For spiking recovery experiments, 3 aliquots of a urine pool (6.1 pmol menadione/mL) were spiked with 4, 10 or 20 pmole menadione/mL, respectively. Four replicates of each aliquot were assayed for menadione.

For dilution recovery experiments, a urine pool containing 25.4 pmol menadione/mL was used to prepare three dilutions (urine: total volumes; 5:7, 1:2 and 1:4) using DI H_2O as the diluent. Five replicates of each dilution were analyzed for menadione.

For LLOQ determination, a urine pool was serially diluted with DI H_2O. Four replicates of each dilution were assayed. The LLOQ was defined as the lowest level of menadione detected which met the following criteria: 1) a coefficient of variation ≤20%; 2) a percentage of measured/expected menadione of 80-120%; and 3) statistically different from adjacent concentrations. Group means were compared using general linear model (GLM) with Tukey-Kramer adjustment for multiple comparisons. Results were considered statistically significant if $P < 0.05$.

Intra- and Inter-Assay Variation

Two pooled urine samples with different menadione concentrations [17.3 pmole/mL in control pool one (C-1) and 4.6 pmole/mL in control pool two (C-2)] were used to determine intra- and inter- assay variations. Six replicates of each control were tested per assay; four assays were run on four different days. All individual results were pooled to calculate the inter-assay variability, as expressed by CV%. To obtain the intra assay variability, also expressed by CV%, the individual coefficient of variation of each of the four assays was calculated, and their mean was defined as the intra assay CV%.

Stability

Four aliquots of fresh urine were spiked with different menadione concentrations (final concentrations: 10.4, 15.4, 18.9, and 23.4 pmole menadione/mL urine). Four freeze and thaw cycles were done. For each aliquot, six replicates of fresh urine and each freeze thaw cycle were tested. Results were expressed as % of menadione in fresh urine. GLM

with Dunnett adjustment for multiple comparisons was used. Results were considered statistically significant if $P < 0.05$.

To assess long-term stability, low and high urine controls were tested 48 times over a 5 month period. All aliquots were frozen and thawed once prior to analysis.

Clinical Applications

Archived urine samples from a human phylloquinone supplementation study [11] were analyzed for menadione. Urine samples collected at baseline (month 0) and at the end of the study (month 36) were used for each participant from either the control group (n=181) or the phylloquinone-supplemented group (500 μg phylloquinone/day, n=186). We hypothesized that changes in 24 hour urinary menadione excretion over the 36 month study period would be significantly higher in the phylloquinone-supplemented group compared to the control group.

Statistical Analysis

Student's *t* test with Bonferroni adjustment for multiple comparisons was used for comparing 24 hour urinary menadione excretion at baseline, end of study, and change in urinary menadione excretion in phylloquinone-supplemented group versus control group. Paired student's *t* test with Bonferroni adjustment for multiple comparisons was used for comparing menadione excretion at baseline versus menadione excretion at the end of the study for both phylloquinone-supplemented and control groups. Results were considered statistically significant if the observed significance value was less than 0.01 due to multiple testing ($P < 0.01$). All statistical analyses were performed using SAS 9.1 software.

Results and Discussion

Choice of Internal Standard

MK-2 was selected as the internal standard for multiple reasons. First, MK-2 shares a naphthoquinone ring with menadione. Second, MK-2 is not a proposed by-product of phylloquinone metabolism, and has not been detected in biological material. Third, under the specified conditions, MK-2 elutes in less than 20 minutes after injection. In contrast, $K_{1\ (25)}$, an internal standard used in vitamin K HPLC methods [12], did not elute under these conditions. Although 7- ethoxycoumarin (7-EC) has been used as an internal standard by others [5], it is endogenously fluorescent and does not require zinc reduction for fluorescent detection. As a consequence, changes in 7-EC's response do not reflect the zinc column conditions that could influence calculation of menadione concentrations, whereas MK-2 does.

Urine Sample Preparation

Acid treatment prior to analysis was an essential step in detecting menadione in urine because urinary menadione is conjugated. After acid treatment, menadione was detected in urine samples (Figure. 1a), whereas in its absence, no menadione was detected (Figure. 1b).

Iso-octane was found to be the optimal solvent for liquid-liquid extraction. Using Iso-octane resulted in equivalent recoveries of both menadione and MK-2 (paired *t* test *P* value = 0.382) (Figure. 2), which is an essential requirement for a valid assay. Multiple extractions of the urine samples did not confer any improvement in total recoveries of either menadione or MK-2 (data not shown).

Complete drying of extraction solvent prior to HPLC injection resulted in high menadione losses, as previously reported [13]. We found that the combination of using iso-octane and avoiding complete evaporation was most effective in attaining consistent recoveries of both menadione and MK-2. Therefore, approximately 200 μL residue was left in each tube prior to HPLC injection. More iso-octane was added to reach 200μL if needed. The use of internal standard compensated for any changes in volume of the final residue.

HPLC

The C_{30} column was cooled to 5^{o} C to allow better separation of peaks of interest. Increasing the percentage of water in the mobile phase resulted in better separation but loss of sensitivity (Figure. 3). The optimum percentage of H_2O in the mobile phase was 5%, which allowed baseline separation of peaks of interest while providing the highest sensitivity. HPLC with UV detection has also been used to measure menadione in plasma [7, 13]. However, fluorescence detection is more sensitive and specific for vitamin K compounds compared to UV detection.

Assay Validation

Recoveries and Lower Limit of Quantification (LLOQ)

For spiking recovery experiments, the expected amount of menadione was the sum of endogenous and spiked menadione. Three aliquots of a urine pool (6.1 pmol menadione/mL) were spiked with 4, 10 or 20 pmole menadione/mL, respectively. Recoveries of menadione, defined as measured/expected, from the three aliquots were (mean % ± SD): 104.4±6.0, 100.6±5.8 and 111.7±9.0, respectively.

For dilution recovery experiments, three dilutions of a urine pool containing 25.4 pmol menadione/mL were prepared using DI H_2O as the diluent (urine: total volumes; 5:7, 1:2 and 1:4). The mean %± SD recoveries of menadione were 112.0±6.9, 103.9±2.9 and 85.1±2.5, respectively.

As additional strategies to validate the assay, removal of the zinc column eliminated the menadione and MK-2 peaks. Vitamin K is not endogenously fluorescent, thus it requires a post-column zinc reduction for fluorescent detection. Removing the zinc column is a simple technique for eliminating the possibility that we were measuring a fluorescent non-vitamin K compound that co-elutes with menadione. In addition, menadione peak disappearance when acid treatment of the urine was eliminated is a further validation of the assay. Finally, the molecular weight of the peak corresponding to menadione in representative samples and in standard solution was also confirmed by mass spectrometry (MW= 172 g/mole).

The standard calibration curve was linear from 0 to 3.64 pmole of menadione and 5.83 pmole of MK-2 injected, with a correlation coefficient (R^2) of 0.999 between them. Exceeding the amount injected of both menadione and MK-2 caused the peaks to go off-scale. We routinely performed single point calibration forcing the slope of the lines through zero. The LLOQ was determined to be 0.3 pmole menadione/mL urine (Table 1). This level of sensitivity in measuring menadione is an improvement over previous reports [5], and allows us the capacity to measure menadione in response to manipulation of physiological doses of vitamin K in a heterogenous population in terms of vitamin K status.

Intra- and Inter-Assay Variation

Intra assay CV% was 3.3% and 4.1% for C-1 and C-2, respectively. Inter assay CV% was 4.8% and 4.9% for C-1 and C-2, respectively.

Stability

Menadione concentrations in the first two freeze thaw cycles were not statistically different from fresh urine (*P* value=0.224 and 0.737, respectively), whereas the third and fourth cycles were (*P* value<0.0001). These results suggest that urinary menadione is stable up to two freeze-thaw cycles.

In a long-term stability study, mean and CV% for low and high controls were 5.6 and12.3%, and 21.5 and 11.6%, respectively, showing that menadione in urine is stable for at least 5 months when stored at -80^{o}C.

Clinical Applications

As predicted, at the end of the 3 years study period, daily urinary menadione excretion for the phylloquinone-supplemented group was significantly higher than the baseline excretion (paired *t* test *P* value <0.0001) (Table 2). In contrast, for the control group there were no statistically significant differences between baseline and 3 years menadione excretion (paired *t* test *P* value =0.2951). There were no statistically significant differences in urinary menadione excretion between the phylloquinone-supplemented group and control group at baseline prior to supplementation. The change in urinary menadione excretion, as well as urinary menadione excretion at 3 years, were significantly higher in the phylloquinone-supplemented group compared to control group.

Menadione excretion increases in response to phylloquinone supplementation, which supports the hypothesis that menadione is an intermediate metabolite in the conversion of phylloquinone to MK-4. This assay will be useful for future studies on the role of menadione in this conversion process.

Conclusions

In conclusion, this HPLC method presents a sensitive and reproducible way to detect menadione in urine, and can be used in future studies to elucidate the role played by menadione in MK-4 formation.

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Figure Legend

1. Chromatograms of menadione (MD) in a urine sample collected from a healthy volunteer:

    1.a. Sample subjected to acid treatment prior to measurement.

    1.b. Sample not subjected to acid treatment.

2. Bland-Altman Plot: Comparison of average % recoveries of each simultaneous injection (n=19) of menadione (MD) and menaquinone-2 (MK-2). Average % recovery= [(MK-2 + MD)/2] X 100.

    Difference in % Recovery= (MK-2 – MD) X 100.

    Paired student's *t* test *P* value=0.382.

    ○: MD concentration = 13.3 pmol/mL, MK-2 concentration = 13.0 pmol/mL

    Δ: MD concentration = 8.7 pmol/mL, MK-2 concentration = 11.1 pmol/mL

    I: MD concentration = 7.0 pmol/mL, MK-2 concentration = 8.9 pmol/mL

3. Effect of H_2O content in mobile phase on sensitivity. Exponential trend line was fitted to the data.

Figure. 1.a.

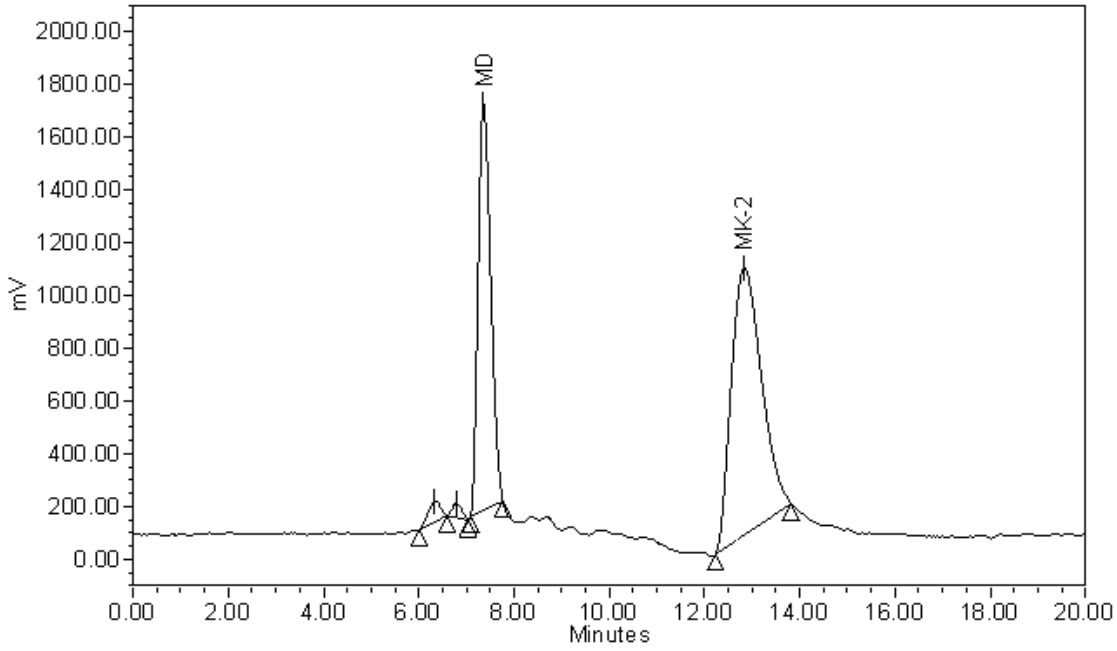

Figure. 1.b.

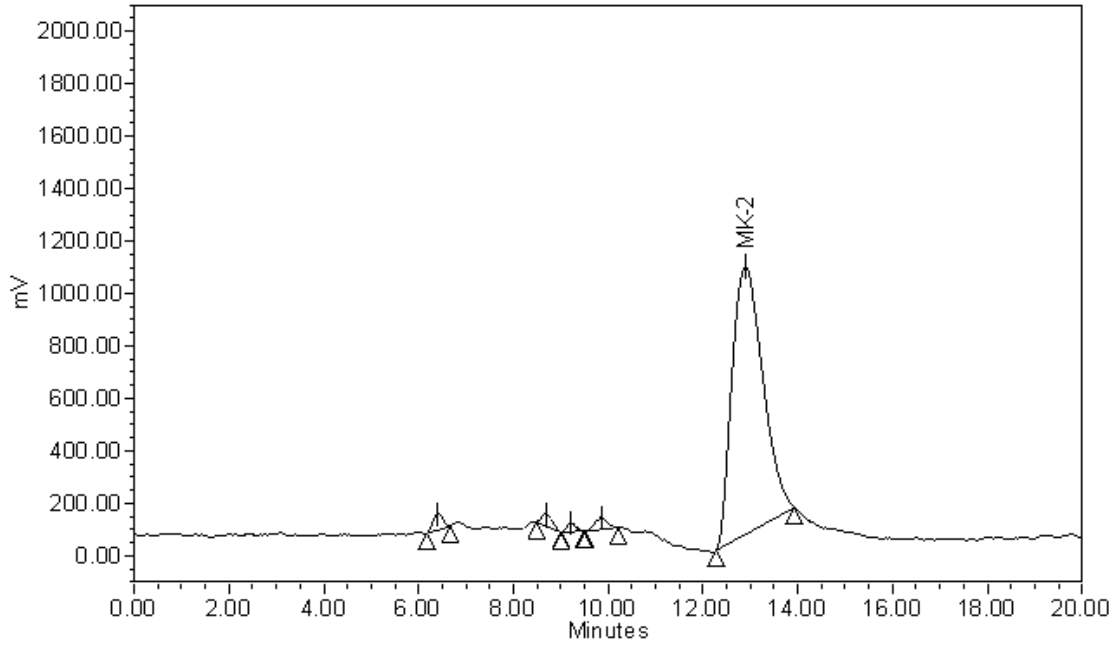

Figure. 2.

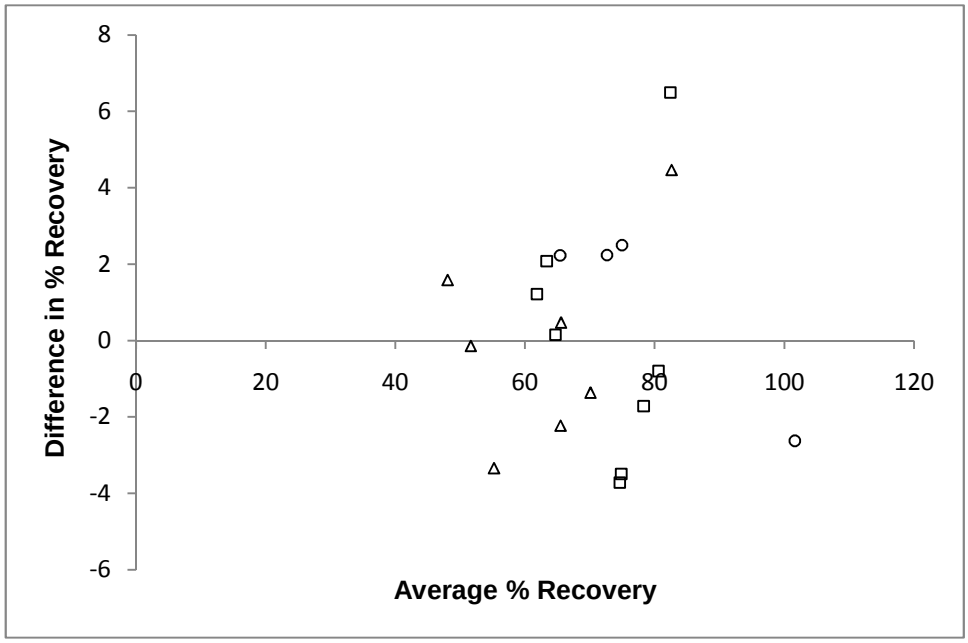

Figure. 3.

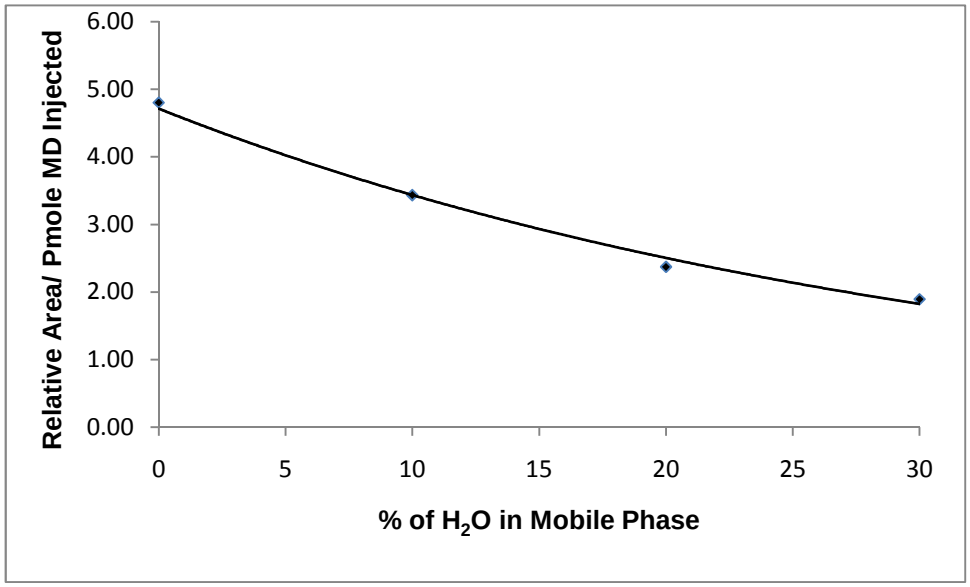

Table. 1. Menadione (MD) lower limit of quantification (LLOQ)

Lower Limit of Quantification					
Dilution Factor	Expected Concentration (pmole MD/mL urine)	Measured Concentration [B] (pmole MD/mL urine)	Measured/ Expected (%)	SD (%)	CV (%)
Initial					
1	N/A	2.37	N/A	0.16	6.8
Dilutions					
2	1.19	1.14 [w]	96.2%	0.11	9.39
4	0.59	0.60 [x]	101.3%	0.06	9.36
8	0.30 [A]	0.30 [y]	101.3%	0.02	6.84
16	0.15	0.16 [z]	108.0%	0.04	31.84
32	0.07	0.09 [z]	121.5%	0.07	71.72

[A] LLOQ of menadione

[B] Concentrations with different superscripts are significantly different using Tukey's adjusted multiple comparisons of groups's means (*P* values <0.05).

Table. 2. Changes in urinary menadione excretion in response to 3 years of 500 μg/day (1109 nmole/day) of phylloquinone (PK) supplementation. Values are presented as mean± SD**.**

MD (nmole/24 hours urine excretion)			
	PK –Supplemented Group (n=186)	Control Group (n=181)	*P* Value [A]
Baseline	12.55± 9.53 [m]	13.26± 10.11 [m]	0.4893
3 Years [B]	31.59± 29.73 [n]	12.27± 10.67 [m]	<0.0001
Δ (3 Years- Baseline)	19.05± 28.29	-0.98± 12.60	<0.0001

A. *P* value based on student's *t* test adjusted for multiple comparisons.

B. Paired student's *t* test: PK group at 3 years is significantly different from baseline.

3.2 Menadione is an Intermediate in Conversion of Phylloquinone to Menaquinone-4 among Fischer 344 Male Rats

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Abbreviations

APCI-LC/MS, atmospheric pressure chemical ionization LC/MS; BM, basolateral medium; deuterium-labeled menaquinone-4, L-MK-4; deuterium-labeled phylloquinone, L-PK; deuterium-labeled unconjugated menadione, L-MD; GGPP, geranylgeranyl pyrophosphate; MD, menadione; MK-4, menaquinone-4; PK, phylloquinone; SFM, serum-free medium; SXR, steroid xenobiotic receptor.

Abstract

Phylloquinone (PK) is converted into menaquinone-4 (MK-4) via side chain removal-addition. Direct evidence that menadione (MD) is the conversion intermediate is lacking. Following a 14 day PK-deficient diet, male Fischer 344 rats (8-mo; n=15) were fed deuterium-labeled collard greens as source of 1.6 mg L-PK per kg diet for 0 (Group 1), 1 (Group 2) and 7 days (Group 3). Using LC/MS, L-PK and labeled MK-4 (L-MK-4) were detected in tissues in Groups 2 and 3. Both tissue PK and urinary MD concentrations significantly increased ($p<0.05$) with collard greens administration compared to controls. L-MK-4 carried the deuterium-label on the naphthoquinone ring, but not on the side chain, confirming the need for side chain removal for its formation. Labeled unconjugated MD (L-MD) was detected in urine and serum of rats following deuterium label intake (n=4/5 and n= 3/5 in Groups 2 and 3, respectively) using MS. The presence of protein was confirmed in the urine of all animals, which suggests that unconjugated urinary MD reflects what is in circulation. A Caco-2 cell monolayer model was used to study the role of the enterocytes in the conversion process. Neither MK-4 nor MD was detected in Caco-2 cell treated with PK. However, when treated with MD, MK-4 was formed. This is the first direct evidence that MD is an intermediate in the dietary PK to tissue MK-4 conversion. However, this conversion does not appear to occur in the enterocytes. Future studies are required to determine the location and function of this conversion.

Introduction

Vitamin K is a generic descriptor for 2-methyl-1, 4-naphthoquinone (menadione; MD) and all its derivatives that exhibit an anti-hemorrhagic activity in animals fed a vitamin K–deficient diet. By definition, all forms of vitamin K share the naphthoquinone ring but differ in the position-3 side chain. The naphthoquinone ring is the active site for vitamin K's established role as a cofactor for the vitamin K–dependent carboxylase.

Mammals have the ability to convert dietary phylloquinone (PK), and MD, into menaquinone-4 (MK-4) and store the latter in specific tissues [1]. It is unlikely that a metabolic pathway leading to MK-4 would have evolved unless MK-4 has unique biological roles. These roles are unlikely to involve the vitamin K-dependent carboxylase, because PK and MK-4 have similar activity as a substrate for this enzymatic activity [2]. This suggests that MK-4 plays a role beyond the classical enzyme cofactor role of vitamin K. Several functions unique for MK-4 have been proposed, including: inhibition of oxidative cell death in primary cultures of oligodendrocyte precursors (pre-OLs) and immature neurons [3]; apoptosis induction in leukemia and other malignant cell lines [4-5]; and a ligand for the steroid xenobiotic receptor (SXR) in bone cells [6].

Recently, UBIAD1 was identified as the enzyme catalyzing prenylation of MD with a geranylgeranyl side chain forming MK-4 [7]. However, the exact mechanism by which PK is converted to MK-4, and the location of where this conversion occurs are not known. Furthermore, direct evidence identifying the MD as the intermediate in the conversion process has been lacking.

We fed deuterium-labeled phylloquinone (L-PK) to Fischer 344 rats to test the hypothesis that the phytyl side chain in the L-PK is cleaved off forming deuterium-

labeled menadione (L-MD). A preformed unlabeled geranylgeranyl side chain that is added to the labeled MD to form MK-4 would demonstrate that MK-4 was produced from dietary PK by means of side chain removal-addition. We also proposed to measure L-MD, supporting the observation that MD is an intermediate in the PK to MK-4 conversion. In a second study designed to test the hypothesis that cells of the small intestine the central compartment where the phylloquinone's phytyl side chain is removed producing MD ascertain the role of the enterocytes in this conversion, we examined the ability of colon cancer cell lines, Caco-2 cells, to convert PK to MK-4 via the intermediate, MD. The identification of the location and mechanisms by which PK is converted to MK-4 will provide insight into the potential unique roles of MK- 4.

Materials and Methods

Animals and Diets

Male Fischer 344 rats (8 months old, n=15) obtained from National Institute of Aging were acclimated for 2 weeks on a vitamin K-deficient diet (TD.09686, Harlan Teklad) in suspended wire caging to minimize coprophagy [8]. The rats were weight-matched and placed in individual metabolic cages to enable monitoring of food consumption (including spillage), and urine collections, while minimizing coprophagy. The rats were then randomly assigned to one of three experimental groups of 5 animals each: **Group 1** (control) which was killed at 0 day; and **Groups 2** and **3,** which were fed the vitamin K-deficient diet ad libitum with deuterium-labeled collard greens added as a source of 1.6 mg L-PK per kg diet for 1 and 7 days, respectively. Urine was collected daily. Animals were killed by terminal exsanguination and serum was collected. Tissues of interest (duodenum, jejunum, ileum, liver, kidney, pancreas, salivary glands, testes, visceral fat

and brain) were harvested and frozen immediately in liquid nitrogen. Urine, serum and tissues were stored at -80 ℃ until analysis.

Deuterium-labeled collard greens were grown hydroponically in an environmental growth chamber at USDA/ARS Children's Nutrition Research Center in Houston Texas, as previously described [9].This protocol was approved by the USDA Human Nutrition Research Center on Aging at Tufts University Institutional Animal Care and User Committee.

Cell Culture

Caco-2 cells (passages 20-40) were obtained from the American Type Culture Collection (Rockville, MD) and grown as described previously [10]. For experiments, cells (5×10^4) were plated on 12-well Transwells (12 mm diameter, 0.4μm pore size; Corning Costar Corp. Cambridge, MA) in the presence of Dulbecco's modified Eagle's medium (DMEM) high glucose with L-glutamine plus 20 % heat-inactivated FBS, 1% nonessential amino acids, and 1% penicillin-streptomycin solution. The medium was changed every 48 hrs for 21 days.

Before the transport experiments on Transwells, the integrity of the cell monolayer was tested by determining the diffusion of phenol red from the apical side to the basolateral side as previously described [11]. The 10μM PK, MK-4 and MD solubilzed by 29 μg /mL Tween-40 were evaporated under nitrogen and solubilized in serum-free medium (SFM) by vortexing [12]. Differentiated Caco-2 cells were supplemented on the apical side with 0.5mL SFM with Tween-40-solubilzed PK, MK-4 or MD for 24 hrs, and 1.5mL HBSS was added to basolateral side. At 24hrs, cells were washed by cold PBS three times, and them digested by 0.5 mL Trpysin for 10min at 37 ^{o}C. The cells were sonicated for 1min

on ice. The homogenized cells were stored at -20 ^{o}C for further analysis. The protein concentration of the cell lysate was determined with a Coomassie Plus Assay Kit (Thermo). Three independent experiments were carried out.

Vitamin K Analysis

Rat tissues (100 -200 mg wet weight) from each animal were homogenized using a Powergen homogenizer (Fisher Scientific) in PBS. The internal standard, $K_{1(25)}$, was used for all homogenate analysis. Concentrations of unlabeled PK, labeled phylloquinone (L-PK), unlabeled MK-4, and labeled menaquinone-4 (L-MK-4) were measured in tissue homogenates, serum and cell lysate by APCI-LC/MS, as described elsewhere [13]. Data were collected using Agilent Chemstation software (Version B.03.01).

Total MD (including both conjugated and unconjugated forms of MD) in urine and cell lysate were measured by HPLC, as described elsewhere [14]. The assay was adapted for measurement of unconjugated MD using either APCI-LC/MS in rat urine or APCI-LC-MS/MS in rat serum. Specifically, urine or serum samples were precipitated with 10% trichloroacetic acid (TCA) (w/v, final concentration) in 16×100 mm borosilicate culture tubes with Teflon lined screw caps [15]. Protein precipitation treatment releases unconjugated, fat-soluble MD from its binding proteins and makes it readily accessible for liquid-liquid extraction. MD was extracted using the method we previously described [14] with some modifications. No internal standard was used for serum sample, and extraction was done 3 times using 2 mL isooctane each time. The LC/MS consisted of an Agilent HP series 1100 G1946D MSD with an APCI source connected to an Agilent series 1200 HPLC instrument. The HPLC condition was described elsewhere [14]. The

MS ion source was negative APCI. Data were collected using Agilent Chemstation software (Version B.03.01).

The APCI-LC/MS/ MS consisted of AB SCIEX QTRAP® 5500 MS/MS with an APCI source connected to an Agilent series 1200 HPLC instrument. A ProntoSil C_{30} column was used (5 μm, 250 mm × 4.6 mm) (MAC-MOD Analytical Inc., Chadds Ford, PA). The mobile phase consisted of 100% methanol. Flow rate was set to be 0.60 mL/minute. One cycle was 24 minutes. The MS/MS ion source was negative APCI with temperature set at 400 ℃. Multiple Reaction Monitoring (MRM) was used to detect isotopomers of parent compounds and fragments of MD [m/z (parent: fragment) 172:146] and L-MD [m/z (parent: fragment) 173:147, 174:148, 175: 149]. Data were collected using Analyst 1.5.1.The detection methods for the various forms of vitamin K measured in this study are summarized in **Table 1**.

Protein Determination

The presence of protein in urine was determined by visual reading of colorimetric reactions on reagent strip (Bayer Multistix-10 SG, Item #2161)

Statistical Methods

Data are reported as means ± SD. The main effect of the number of days consuming labeled collard greens on body weights, the tissue concentrations of total and labeled PK and MK-4, as well as for the individual forms of MD (total and unconjugated MD in urine, including L-MD and unlabeled MD in urine), was analyzed using a one way ANOVA with Tukey's honestly significant difference for multiple comparisons. All analyses were carried out using SAS v.9. Significance was set at $P< 0.05$.

Results

The mean of body weights did not differ among groups in response to collard greens intake. We measured PK, MK-4 and MD in a variety of matrices in response to intake of deuterium-labeled phylloquinone in collard greens, as summarized in **Table 1**. The scans of all labeled isotopomers in diet, tissue, urine and serum samples were also analyzed for isotopomer profiles. The most abundant isotopomers of L-PK in serum (*m/z* 459-463) corresponded to 63% of the total deuterium label in the collard greens. The most abundant isotopomers for unlabeled and labeled MK-4 corresponded to *m/z* 445 and *m/z* 446-449, respectively. As expected, there were no labeled forms of vitamin K in tissues obtained from animals in Group 1 (**Table 2**). With the exception of liver and serum, all tissues measured had labeled and unlabeled MK-4 even though the diet only contained PK. Testes and brain only had detectable amounts of labeled and unlabeled MK-4, and no PK. Duodenum, pancreas, testes, brain and visceral fat all had higher amounts of total MK-4, but not PK, compared to the L-MK-4.

Total urinary MD excretion peaked at day 1, and did not further change in response to daily intake of L-PK (**Figure 1A**). Excretion of the labeled unconjugated form of MD that originated from the labeled collard greens, increased at day 1, with a general further increase thereafter (**Figure 1B**). Unlabeled conjugated MD excretion did not change over the course of the study. We were also able to confirm L-MD in serum using APCI-LC-MS/MS. L-MD was detected in serum of rats who received collards (n=4/5 Group 2; n= 3/5 Group 3) but not in those who did not (n=0/5 Group 1). However we were unable to calculate the actual amounts present in each due to the lack of an appropriate internal standard.

The Caco-2 cells were harvested and tested for the formation of MK-4 and MD (**Table 3**). Neither MK-4 nor MD was detected in Caco-2 cell culture model treated with PK. However, when treated with MD, MK-4 was formed.

Discussion

In this study of male Fischer 344 rats fed deuterium-labeled collard greens as source of L-PK. L-PK and L-MK-4 were detected in tissues within 7 days of intake. Based on the principle of random distribution of deuterium labels, L-MK-4 MW indicated that L-MK-4 carried the deuterium-label on the naphthoquinone ring, but not on the side chain, confirming the need for side chain removal for its formation. Labeled MD that had its origin from the labeled collard greens, was detected in serum and urine, thereby providing direct evidence that MD is an intermediate in the dietary PK to tissue MK-4 conversion.

Unlike longer chain menaquinones [16], gut bacteria is not needed for the synthesis of tissue MK-4 [1, 17]. Overall evidence from literature supports the side chain removal-addition model of dietary PK to tissue MK-4 conversion. *Okano et al*. [18] used a deuterium labeled phylloquinone carrying the label on the naphthoquinone ring but not the phytyl side chain. The resulting compound (PK-d7) was orally given to mice, and the naphthoquinone ring-labeled MK-4 (MK-4-d7) was detected in cerebra. In contrast, MK-4 labeled on both the ring and the geranylgeranyl side chain (MK-4-d9) was not detected, which indicates that the phytyl side chain of PK is replaced by geranylgeranyl side chain to produce MK-4. The current study now provides direct evidence that MD is the intermediate.

When labeled MD was fed to vitamin K-deficient chicks [19] and Wistar rats [20], labeled MK-4 was extracted from animal tissues. Animals supplemented with MD resulted in significantly higher MK-4 concentrations in all organs examined compared to controls [20]. The greatest accumulations were in non-hepatic organs. In contrast, liver and plasma had low MK-4 concentrations, similar to our findings presented here using labeled PK. Of interest in our study is the observation that the total MK-4, which contained both labeled and unlabeled forms, was higher than the L-MK-4 in the duodenum, pancreas, testes, brain and visceral fat. Although the animals were on a vitamin K-free diet for 14 days prior to intake of the deuterium label, it is plausible that the MD in the normal chow fed to rats prior to shipment were converted to MK4 and stored in these tissues during the 14-day period of vitamin K restriction. With the exception of the duodenum, the total MK-4 concentrations did not change in response to the L-PK, whereas the L-MK-4 concentrations did. This suggests that MK-4 is regulated in certain tissues.

There are two possible pathways regarding tissue localization of the specific catalytic activities. In the first pathway, phytyl side chain removal, as well as the subsequent geranylgeranyl side chain prenylation, is a metabolic activity specific to certain tissues [1, 18]. The alternative pathway requires the phytyl side chain to be removed in a central body compartment (the enterocytes and the liver are the main candidates), and MD, or its epoxide, to be released into the circulation where it is prenylated in target tissues with the preformed geranylgeranyl, forming MK-4 [20]. Collectively, the data suggest that MD release from PK may take place in limited number of tissues, as well as a central compartment (liver or enterocytes). The subsequent prenylation with the preformed geranylgeranyl side chain occurs in target tissues. The detection of L-MD in serum suggests that the phytyl side chain removal step may partially take place in a

central compartment. Although we measured L-PK and L-MK-4 in the intestine, with the highest concentrations in the jejunum, neither MK-4 nor MD was detected in Caco-2 cell culture model treated with PK. We concluded that the enterocytes where not the central compartment where MD is formed.

UBIAD1 was recently identified as a human prenyltransferase enzyme responsible for the geranylgeranyl side chain addition step of the conversion process from phylloquinone to MK-4 [7]. Evidence regarding UBIAD1 role as the enzyme responsible for the phytyl side chain removal, as also suggested by these authors, was weak. Indeed our data suggest that Caco-2 cells are capable of converting MD, but not PK, to MK-4 because they presumably lack the enzymatic activity that cleaves the side chain of PK to form MD. More research is required to confirm if UBIAD1 is indeed the enzyme responsible for both steps.

Conclusions

In conclusion, these findings provide direct evidence that in order to convert PK to MK-4, the organisms have to remove the phytyl side chain in PK and add a geranyl-geranyl side chain to the MD nucleus producing MK-4. Although there is suggestion that MD may be produced in a central compartment, our data does not support that enterocytes are involved.

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Statement of Authors' Contributions to Manuscript

AA and XF analyzed data and wrote the paper, JWP and DS analyzed data and contributed to the study design, SLB designed the research and had primary responsibility for final content, SWC and JS contributed to the study design, the interpretation of the data and preparation of the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Changes in A) total [1] and B) unconjugated [2] urinary MD concentrations in response to 1.6 mg L-PK /Kg diet for 7 days. [3]

[1] Total urinary MD concentrations were measured using HPLC; total includes both conjugated and unconjugated forms of MD.[2] Unconjugated urinary MD concentrations were measured using HPLC; percent enrichment of unconjugated MD was measured using LC-APCI/MS, and corresponding concentrations of labeled and unlabeled unconjugated MD were calculated retrospectively based on percent enrichment and unconjugated MD concentrations.[3] Values are means ± SD, n= 5; male Fischer 344 rats were fed deuterium-labeled collard greens as source of 1.6 mg L-PK per kg diet for 7 days; concentrations with different superscripts are significantly different within each measure using Tukey's adjusted multiple comparisons of means (*P* values <0.05).

Table 1 Confirmation of deuterium labeling in each form of vitamin K.[1]

Vitamin K	Sources	MW	Major isotopomers *(m/z)*		Detection methods
			Unlabeled	Labeled	
PK	Diet, tissues & serum	451	451	459-463[2]	LC-APCI/MS
MK-4	Tissues	445	445	446-449	LC-APCI/MS
MD	Urine	172	172	173-174	LC-APCI/MS
	Serum	172	172	173-175	LC-APCI/ MS/MS

[1] No deuterium was detected in control diet or in rats in control group; tissues and serum PK isotopomers distribution matched that of PK in collard greens.[2] m/z 459-463 corresponds to 63% of total deuterium label.

Table 2 Vitamin K concentrations in Fischer 344 rat serum and selected tissues, with and without supplementation of 1.6 mg L-PK /Kg diet[1]

		Vitamin K concentrations (pmol/g wet tissue or mL) [2]					
		Labeled vitamin K			Total [3]		
		Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
Duodenum	PK	ND [a]	34.8±22.6 [b]	18.1±3.1 [ab]	1.3±1.2 [a]	36.9±23.9 [b]	19.9±2.7 [ab]
	MK-4	ND [a]	28.2±15.7 [b]	51.4±7.6 [c]	45.1±14.1 [a]	62.3±13.1 [a]	97.4±26.7 [b]
Jejunum	PK	ND [a]	171.5±49.4 [b]	135.7±69.8 [b]	5.1±2.6 [a]	178.7±50.3 [b]	139.4±71.4 [b]
	MK-4	ND [a]	37.7±4.3 [b]	57.3±9.2 [c]	64.8±47.7	94.0±14.1	106.6±21.7
Ileum	PK	ND [a]	35.6±2.0 [b]	88.2±9.0 [c]	1.7±1.7 [a]	38.2±2.4 [b]	90.0±9.8 [c]
	MK-4	ND [a]	37.8±5.4 [b]	55.3±13.8 [c]	24.3±4.8 [a]	71.4±10.9 [b]	55.3±13.8 [b]
Liver	PK	ND [a]	194.9±51.1 [b]	209.4±111.8 [b]	3.6±0.8 [a]	198.7±52.0 [b]	212.8±113.5 [b]
	MK-4	ND	ND	ND	ND	ND	ND
Serum	PK	ND [a]	28.7±18.1 [b]	7.7±2.7 [a]	ND [a]	28.7±18.1 [b]	7.7±2.7 [a]
	MK-4	ND	ND	ND	ND	ND	ND
Kidney	PK	ND [a]	8.3±3.0 [b]	15.2±3.2 [c]	2.6±5.9 [a]	8.6±3.4 [a]	16.2±3.6 [b]
	MK-4	ND [a]	27.3±5.0 [b]	43.2±9.2 [c]	9.2±20.7 [a]	27.3±5.0 [ab]	43.2±9.3 [b]
Pancreas	PK	ND [a]	1.8±4.0 [a]	38.5±8.0 [b]	22.8±9.6 [a]	15.5±4.4 [a]	54.8±6.4 [b]
	MK-4	ND [a]	100.7±10.8 [b]	363.6±43.0 [c]	979.3±164.8	1234.7±38.5	1059.3±201.1
Salivary	PK	ND	ND	1.7±3.7	3.4±3.2	2.7±2.7	3.6±5.0
glands	MK-4	ND [a]	311.4±46.8 [b]	757.2±111.8 [c]	134.8±38.1 [a]	466.9±47.6 [b]	903.5±139.9 [c]
Testes	PK	ND	ND	ND	ND	ND	ND
	MK-4	ND [a]	ND [a]	55.9±12.6 [b]	428.0±93.9	338.7±72.2	317.5±43.5
Brain	PK	ND	ND	ND	ND	ND	ND
	MK-4	ND [a]	15.4±6.0 [b]	38.6±8.3 [c]	67.5±20.4	82.6±18.2	63.9±14.9
Visceral fat	PK	ND	ND	8.5±14.4	25.1±9.2	14.7±2.2	24.0±21.3
	MK-4	ND [a]	73.3±10.3 [b]	68.6±23.9 [b]	120.9±53.1	114.8±13.1	108.9±49.8

[1] Group 1: control, Group 2: 1 day and Group 3: 7 days of 1.6 mgL-PK/Kg diet; values are means ± SD, n= 5 per group; ND, values were below the minimum detectable concentration of 0.05 pmol/g wet tissue.[2] Individual tissue concentrations with different superscripts are significantly different among groups within each measure using Tukey's adjusted multiple comparisons of means (*P* values <0.05).[3] Total is the sum of the labeled and unlabeled measures

Table 3 Vitamin K concentrations in Caco-2 cells after supplement of 10μM PK, MK-4 and MD for 24 h.

Vitamin K supplement	Vitamin K in cells (pmol/mg)		
	PK	MK-4	MD
PK	2215±570	ND	ND
MK-4	ND	1737±59.7	ND
MD	ND	15.3±0.4	ND

[1] Values are means ± SD of three independent experiments, n= 3 per group. [2] ND, values were below the minimum detectable concentration of 0.005 pmol/g protein.

Figure 1A

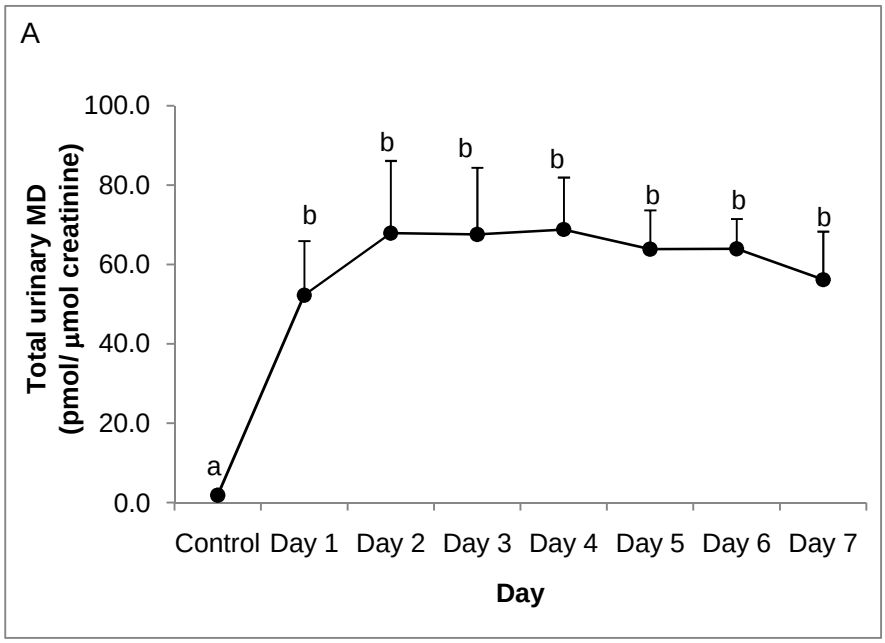
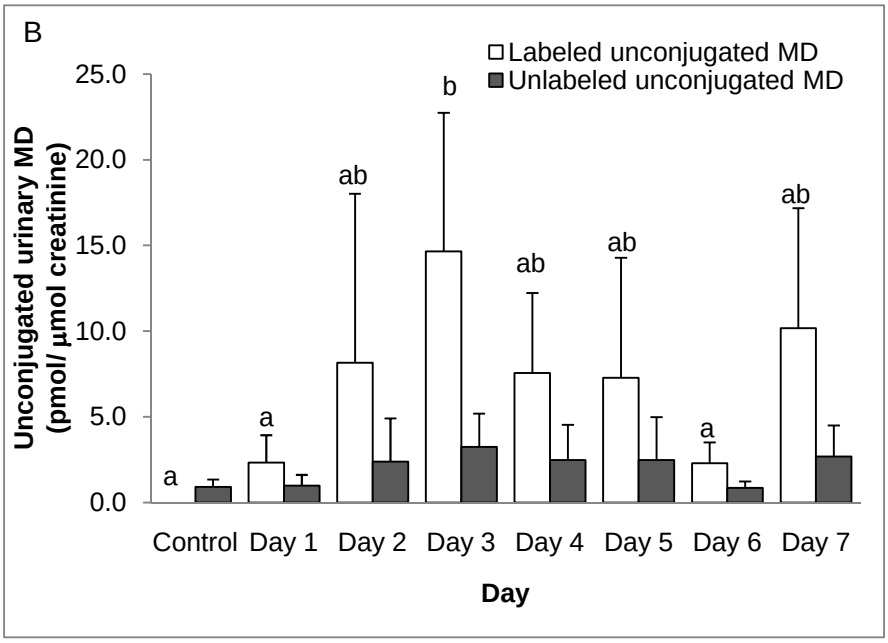


Figure 1B



3.3 Urinary Menadione is a Vitamin K Metabolite in Healthy Adults

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Abbreviations

APCI-LC/MS, atmospheric pressure chemical ionization LC/MS; deuterium-labeled menaquinone-4, L-MK-4; deuterium-labeled phylloquinone, L-PK; deuterium-labeled menadione, L-MD; GGPP, geranylgeranyl pyrophosphate; MD, menadione; MK-4, menaquinone-4; PK, phylloquinone; SXR, steroid xenobiotic receptor.

Abstract

Phylloquinone (PK) is converted into menaquinone-4 (MK-4) via side chain removal-addition. Direct evidence that menadione (MD) is the conversion intermediate is lacking in humans. Archived urine samples of participants in a human PK supplementation study (n=368) were analyzed for MD. PK supplementation resulted in a significant increase in the urinary MD secretion ($P<0.0001$); lending support to the hypothesis that MD is an intermediate in the conversion of PK to MK-4. Furthermore, changes in urinary MD excretion were significantly correlated with changes in biomarkers of vitamin K status, including serum PK and percent serum undercarboxylated osteocalcin (ucOC; $P=0.0008$ and 0.02, respectively). In a separate metabolic study of 42 healthy, younger and older men and women, we used deuterium-labeled collard greens to confirm that urinary deuterium-labeled menadione (L-MD; MW 173-174) was formed from deuterium-labeled phylloquinone (L-PK). For an intake of 255 nmol L-PK, between (mean± SD) 12.4%± 6.2% and 14.9%± 10.0% was recovered as L-MD in urine within 5 days of intake. These estimates do not take into account bioavailability of PK from plant sources. However, existing vitamin K status did not affect amount excreted. Future studies are required to determine the location and function of this conversion.

Introduction

Mammals have the ability to convert the primary dietary form of vitamin K, phylloquinone (vitamin K_1; PK) into menaquinone-4 (MK-4), which is stored in specific tissues [1]. It is unlikely that a metabolic pathway leading to MK-4 would have evolved unless MK-4 has unique biological roles. These roles are unlikely to involve the vitamin K-dependent carboxylase, because PK and MK-4 have similar activity as a substrate for this enzymatic activity [2]. This suggests that MK-4 plays a role beyond the classical enzyme cofactor role of vitamin K. Several functions unique to MK-4 have been proposed, including: inhibition of oxidative cell death in primary cultures of oligodendrocyte precursors (pre-OLs) and immature neurons [3]; apoptosis induction in leukemia and other malignant cell lines [4-5]; and a ligand for the steroid xenobiotic receptor (SXR) in bone cells [6].

Overall evidence from literature supports the side chain removal-addition model of dietary PK to tissue MK-4 conversion. According to this model, the phytyl side chain of PK is removed, and a preformed geranylgeranyl side chain is donated by geranylgeranyl pyrophosphate (GGPP) [7] to form MK-4. In this model, menadione (MD), or its epoxide, serves as an intermediate in the conversion process. Recently, UBIAD1 was identified as the enzyme catalyzing prenylation of MD with a geranylgeranyl side chain forming MK-4 [8]. However, the exact mechanism by which PK is converted to MK-4, and the location of where this conversion occurs are not known. Furthermore, direct evidence identifying the MD as the intermediate in the conversion process has been lacking.

A highly reproducible HPLC method has been developed and validated to measure MD in urine [9]. In a randomized-controlled trial of vitamin K supplementation (Study A), we found that older community-dwelling adults who were randomized to a PK supplement

had higher vitamin K status, as measured by a variety of biomarkers, compared to those who received no PK [10]. The assessment of urinary MD from archived baseline and post-intervention urine samples from this study provided an opportunity to determine the effect of PK supplementation on urinary MD. We hypothesized that increased urinary MD would be associated with higher vitamin K status.

In a separate metabolic study of healthy, younger and older men and women (Study B), we used deuterium-labeled PK (L-PK) obtained from collard greens to test the hypothesis that urinary MD was formed from dietary PK.

Materials and Methods

Study participants

In Study A, 452 community-dwelling men and postmenopausal women, aged 60-80 years, were recruited through direct mailings, newspaper advertisements, and notices in the community. Study inclusion and exclusion criteria, as well as study eligibility, have previously been described [10]. Of the 452 eligible participants, equal numbers were randomly assigned to either the treatment (n=229) or non-treatment (n=223) group.

In Study B, 42 men and women, divided into a younger age group (18-40 years) and older age group (55-80 years), were recruited through direct mailings, newspaper advertisements, and notices in the community. Exclusion criteria included kidney stones within the past two years; smoking within the last six months; therapy with bisphosphonates, hormone replacement therapy, oral contraceptive use, estrogen or progesterone use within the past three months; oral antibiotics, seizure medications, diuretics, Coumadin use within the past 12 months; use of proton pump inhibitors;

diabetes; inflammatory bowel disease or other malabsorption problems; laboratory evidence of kidney or liver disease; atrial fibrillation or flutter; unstable coronary artery disease; pregnancy or planning on becoming pregnant; history of gastritis within the past six months; prior gastric surgery; history of coagulopathy; and International Normalized Ratio (INR) greater than 1.2. Women in the older age group were postmenopausal for at least three years.

Study design

In Study A, 452 participants participated in a three-year randomized controlled trial designed to determine the effect of vitamin K supplementation (500 µg/d) on age-related bone loss, as previously described [10]. Equal numbers of men and women were randomized to receive a daily multi-vitamin with 500 µg of PK or the same multi-vitamin without PK. All participants also received a second supplement that contained 600 mg of elemental calcium and 10 µg (400 IU) of cholecalciferol. The nutrient composition of all supplements has been previously described [10]. Supplements were manufactured specifically for this study by Hermes Arzeneimittel GMBH (Munich Germany). This study was approved by the Institutional Review Board at Tufts University Medical Center and Massachusetts General Hospital and is registered with clinicaltrials.gov (NCT00183001). Of the original 452 participants, 368 had urine samples from baseline and the end of the study, and were included in these analyses.

In Study B, 21 younger (aged 18-40 years) and 21 older (aged 55-80 years) healthy study participants participated in a 65-day un-randomized, un-blinded metabolic study at the Metabolic Research Unit (MRU) of the Jean Mayer USDA Human Nutrition Research Center on Aging (HNRCA) at Tufts University. The study was designed to assess dietary and non-dietary determinants of vitamin K status in healthy younger and older adults and

also to compare the effects of vitamin K depletion and repletion on markers of bone turnover. Prior to enrollment, all participants consented to a medical exam, completed a detailed medical questionnaire, and were instructed to stop taking dietary supplements. Participants were in general good health and free from cardiovascular disease, diabetes, malabsorption disorders, liver or kidney disease, or blood clotting disorders.

During the study period, participants were provided with effervescent supplements to be taken each morning dissolved in a five to six-ounce glass of water. For 30 days prior to the baseline run-in period, participants received supplements containing 600 mg of elemental calcium in the form of calcium carbonate and 10 µg (400 IU) of vitamin D in the form of cholecalciferol. The baseline run-in period consisted of 5 days during which the participants were fed a PK diet of 200 µg/day. On days 6 to 35, participants completed the PK depletion period (Phase I) and were fed a PK-restricted diet of 10 µg/day and continued to take a 600 mg calcium and 10 µg vitamin D supplement. On days 36 to 65, the participants completed the PK repletion period (Phase II) and continued on a PK-restricted diet of 10 µg/day and were given a supplement of 600 mg calcium, 10 µg vitamin D, plus 500 µg PK. The sequence of the dietary PK depletion-repletion was identical for all participants, with all members of the study team un-blinded to the intervention.

Participants received all their meals from the MRU during the 65-day study period. In addition, participants were in residency for nine days of the study period at the MRU (days 1, 2, 3, 26, 27, 28, 56, 57, 58). All other days of the study period (days 4-25, 29-55, 59-65) the participants were free-living and visited the MRU a minimum of two to three days per week to consume their meals as well as pick up meals for the next two days.

On days 26 to 28 (corresponding to PK depletion), and days 56 to 58 (corresponding to PK repletion), participants resided in the MRU for two absorption components of the study. On days 28 and 56, deuterium-labeled collards were introduced at breakfast as part of the participant's study diet. On the absorption days following ingestion of the labeled collards (days 29-30 and 57-58), blood was drawn at hours 0, 4, 5, 6, 7, 9, 12, 16, 24, 48 and 72 in order to measure L- PK in plasma. Twenty-four hour urines were also collected during the two absorption components. This stable isotope tracer technique was used to compare the absorption efficiency, transport, and excretion of PK during a vitamin K-deplete state to that of a vitamin K-replete state.

All participants signed a written informed consent, and this study was approved by the Institutional Review Board at Tufts University. This study was registered with ClinicalTrials.gov (NCT00336532).

Biochemical measurements

All blood samples were drawn after a 12 hours fast, and urine samples were a 24-hour collection. Dedicated serum, plasma and urine aliquots were stored at -80°C until time of analysis.

Urinary MD

Total MD in urine was measured by HPLC, as described elsewhere [9]. The assay was adapted for measurement of labeled MD using high-performance liquid chromatography/mass spectrometry with atmospheric pressure chemical ionization (APCI-LC/MS) in urine. The LC/MS consisted of an Agilent HP series 1100 G1946D MSD with an APCI source connected to an Agilent series 1200 HPLC instrument. The

HPLC condition was described elsewhere [9]. The MS ion source was negative APCI. Data were collected using Agilent Chemstation software (Version B.03.01).

Vitamin K Status

Plasma PK was measured using reverse-phase HPLC [11]. L-PK was measured APCI-LC/MS), as described elsewhere [12]. The serum total and uncarboxylated osteocalcin (ucOC) were measured using the radioimmunoassay method of Gundberg [13-14]. The uncarboxylated prothrombin, known as PIVKA-II , was measured at baseline only in citrated plasma using ELISA (American Bioproducts Company, Parsippany, NJ) [15].

Statistical Analyses

To determine the effect of vitamin K supplementation on urinary MD, we compared the 3-year change in urinary MD between the vitamin K supplementation group and the control group in Study A using an independent samples t-test, in an intent-to-treat analysis. Pearson correlations were computed to explore the association of urinary MD with baseline measures of vitamin K status and potential determinants (SAS version 9.1, Cary NC). A two-sided $P<0.05$ was considered statistically significant.

Results

Study A. Baseline characteristics of participants are summarized in **Table 1**. As previously reported [9], at the end of the 3 year study period, urinary MD excretion for the PK-supplemented group was significantly higher than the baseline excretion (paired *t* test *P* value <0.0001) (**Table 2**). In contrast, for the control group there were no statistically significant differences between baseline and 3 years MD excretion (paired *t* test *P* value =0.30). There were no statistically significant differences in urinary MD

excretion between the PK-supplemented group and control group at baseline prior to supplementation. The change in urinary MD excretion, as well as urinary MD excretion at 3 years, were significantly higher in the PK-supplemented group compared to control group.

Urinary MD was significantly correlated with plasma PK and serum % ucOC both cross-sectionally (at baseline and at the end of the study), and longitudinally (as measured by 3-year change) (**Table 3**). Similar to plasma PK and serum %ucOC, urinary MD was not correlated with PIVKA-II, as measure of vitamin K status in liver.

Study B. We measured plasma PK and urinary MD in response to intake of L-PK in collard greens. The scans of all labeled isotopomers in diet, urine and plasma samples were also analyzed for isotopomer profiles. The most abundant isotopomers of L-PK in plasma (*m/z* 459-463) corresponded to 63% of the total deuterium label in the collard greens. The most abundant isotopomers of L-MD in urine corresponded to *m/z* 173-174 compared to *m/z* 172 for unlabeled menadione. L-MD was measured in urine within 1 day of intake of L-PK (**Figure 1**). During Phase I, which corresponded to the depletion period, we were still able to measure L-MD, albeit at lower concentrations compared to the first day of intake. We collected urine in 9 of the 15 days followed the intake of the L-PK. An average of 18.6% was recovered as L-MD in urine; 12.4% was accounted for in 4 of the first 6 days post intake (**Table 4**). During Phase II, which corresponded to the repletion period, we only had 4 days of follow-up urine collection in the 5 days period followed L-PK intake. An average of 15.0% was recovered. There were no differences in absolute L-MD excretion between the two phases. However, the percent enrichment was lower for Phase II, suggesting that total MD excretion was higher due to the intake of the PK supplement during the time of sample collection.

Discussion

Urinary MD responded to PK supplementation, consistent with other biomarkers of vitamin K status. Specifically, urinary MD excretion was inversely associated with %ucOC, and positively associated with circulating vitamin K concentrations in older adults. These observations were strengthened by the post-intervention analysis which found a significant increase in the urinary MD among older adults who received 500 µg/day of PK for 3 years compared to those who did not receive PK. In a separate metabolic study of younger and older men and women, we used deuterium-labeled collard greens to provide the first direct evidence in humans that that urinary MD was formed from dietary PK.

Previous attempts to measure MD lacked the sensitivity to detect this compound in response to a variety of dietary and physiological conditions [16-19]. Some assays were not designed to measure MD in biological fluids [20-22]. Using a highly reproducible HPLC method that was developed and validated by our laboratory to measure MD in urine [9], we were able to demonstrate that MD is a valid measure of vitamin K status, based on its associations with other measures of vitamin K status, such as plasma PK and %ucOC. Although urinary MD was not associated with PIVKA-II, neither were the other biomarkers. PIVKA-II has poor sensitivity as a marker of vitamin K sufficiency [23], and therefore it is not surprisingly that we were unable to detect a correlation with urinary MD or other biomarkers.

Previous studies have shown that PK is rapidly metabolized and excreted via urine and bile within a few days [24-27]. This rapid metabolic turnover of PK explains the appearance of labeled MD within 24-h in Study B. Because we used L-PK, we were able to rule out that the sources of MD included endogenously-produced MD and/or

mobilized tissue stores of PK. We currently lack the data from which to calculate vitamin K bioavailability from the labeled collards. Recent studies estimate the relative bioavailability of PK from plants to be 4-7% [26]. It is assumed that delivery of the L-PK in a supplement form, which has a higher bioavailability, would yield much higher MD excretion.

Surprisingly, the existing vitamin K status did not influence the absolute amount of the L-MD excreted. Similar findings were obtained with the corresponding plasma PK concentrations (data not shown). In contrast, the percent enrichment of MD during the PK repletion phase was lower, suggestive of higher total MD concentrations during periods of higher vitamin K intake. Although the data are currently not available to confirm this, data from Study A supports a correlation between MD excretion and VK intake.

One of the limitations of this study is the inability to measure MK-4. Menaquinone-4 is not routinely measured in serum in the absence of intake of pharmacological MK-4 doses. No tissue samples were obtained from which to confirm that the L-MD measured in urine was the intermediate in the conversion of phylloquinone to MK-4. This would be an important step in future human studies because use of a urinary metabolite such as MD, would be a non-invasive, albeit indirect measure of the formation of MK-4 from dietary sources of vitamin K. Similarly, future studies would require longer periods of complete urine collections for accurate estimates of menadione produced from a known quantity of phylloquinone because we were able to still detect the deuterium label 14 days after intake.

In conclusion, urinary MD is correlated with changes in biomarkers of vitamin K status in response to phylloquinone supplementation. The data presented here provide the first direct evidence in humans that menadione is produced from dietary phylloquinone. Future studies are required to determine the location and function of this conversion.

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Table 1 Subject Baseline Characteristics (mean ± SEM).

Characteristics	Study A	Study B
N	368	42
Age, years	68.9 ± 0.4	67.6 ± 0.3
Plasma PK, nmol/L	1.2 ± 0.1	1.1 ± 0.1
Serum ucOC, %	37 ± 1.2	41 ± 1.1
BMI, kg/m^2	28.0 ± 0.3	27.9 ± 0.3
PIVKA-II, ng/mL	2.43± 0.06	2.44± 0.06
Urinary MD, nmole/24hr	13.1±0.53	12.5±1.2

Table 2. Changes in 24-h urinary menadione excretion (nmole) in response to 3 years of 500 µg/day of PK supplementation.

	PK –Supplemented (n=186)	Control (n=181)	*P* Value [A]
Baseline	12.55± 9.53	13.26± 10.11	0.4893
3 Years [B]	31.59± 29.73	12.27± 10.67	<0.0001
Δ (3 Years- Baseline)	19.05± 28.29	-0.98± 12.60	<0.0001

Values are presented as mean± SD**.** (permission will be requested to reproduce from Al Rajabi A, *et al* 2010[9]). [A]*P* value based on student's *t* test adjusted for multiple comparisons. [B] Paired student's *t* test: PK group at 3 years is significantly different from baseline.

Table 3 Correlations between different measures of vitamin K status at A) baseline B) 3 Years and Δ (3 Years – baseline) (n=368). Participants in the study group (n= 187) received 500μg/day (1109 nmol/day) of phylloquinone supplementation. *r* : Pearson correlation coefficient.

A. Baseline

	PK-Baseline		%ucOc-Baseline		PIVKA-Baseline	
	R	*P*	*r*	*P*	*r*	*P*
MD- Baseline	0.19	0.0002	-0.27	<0.0001	-0.04	0.46
PK- Baseline	1		-0.21	<0.0001	-0.03	0.55

B. 3 Years and Δ (3 Years – baseline)

	PK-3 Years		%ucOc-3 Years	
	R	*P*	*r*	*P*
MD-3 Years	0.15	0.0035	-0.19	0.0004
PK-3 Years	1		-0.29	<0.0001
	ΔPK		Δ%ucOc	
	R	*P*	*r*	*P*
ΔMD	0.18	0.0008	-0.12	0.0184
ΔPK	1		-0.26	<0.0001

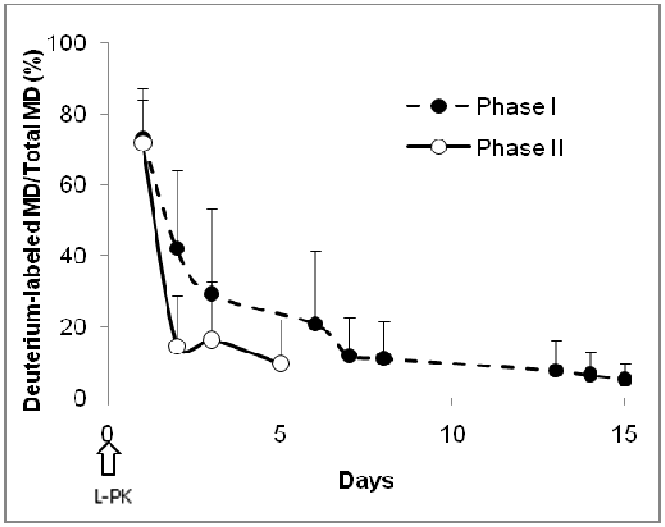
Table 4. Percentage L-PK excreted as L-MD in urine (Proportional to 64% of PK in collard greens) (Study B; means ±SD, n=42).

Phase I [a]		Phase II [b]	
Day	% L-MD	Day	% L-MD
28	10.08±5.83%	56	9.10±6.18%
29	1.09±1.13%	57	2.07±2.85%
30	0.70±0.71%	58	2.24±1.96%
33	0.48±0.57%	60	1.48±1.98%
Subtotal	12.35±6.22%		
34	1.46±1.52%		
35	1.46±1.64%		
40	1.14±1.20%		
41	1.07±1.38%		
42	1.04±1.15%		
Total	18.52±8.53%		14.90±9.96%

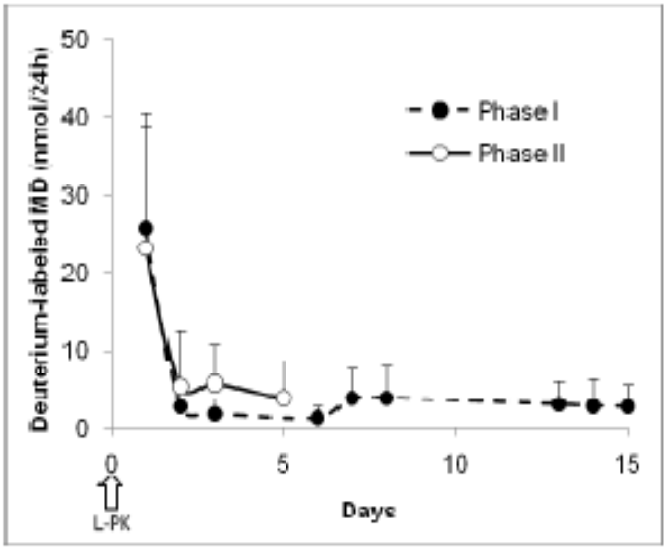
[a] Phase I, PK consumption of 10 µg/d from diet plus L-PK from collard greens; [b] Phase II, PK consumption of 500 µg/d in the form of a supplement and 10 µg/d from diet plus L-PK from collard greens.

Figure 1. Effect of L-PK intake on urinary MD excretion. **A.** The percentage enrichment of deuterium-labeled menadione (MD) in urine after the intake of deuterium-labeled collard greens by APCI-LC/MS (Study B; means ±SD, n=42). Phase I, PK consumption of 10 µg/d from diet plus L-PK from collard greens; Phase II, PK consumption of 500 µg/d in the form of a supplement and 10 µg/d from diet plus L-PK from collard greens. **B.** The means ±SD concentrations of deuterium-labeled menadione (MD) after intake of deuterium-labeled collard greens (421 nmol L-PK).

A



B



3.4 Determinants of Vitamin K Status in Humans

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Abstract

To understand the role of vitamin K in human health, it is important to identify determinants of vitamin K status throughout the life cycle. Our current understanding of vitamin K physiology and metabolism only partially explains why there is wide inter-individual variation in vitamin K status, as measured by various biochemical measures. Dietary intake of vitamin K is one of the primary determinants of vitamin K status, and intakes vary widely among age groups and population subgroups. How dietary sources of vitamin K are absorbed and transported varies with the form and food source of vitamin K. Likewise, the role of plasma lipids as a determinant of vitamin K status varies with the form of vitamin K ingested. There is also some evidence that other fat-soluble vitamins antagonize vitamin K under certain physiological conditions. Infants are at the greatest risk of vitamin K deficiency because of a poor maternal-fetal transfer across the placenta and low vitamin K concentrations in breast milk. During adulthood, there may be subtle age-related changes in vitamin K status but these are inconsistent and may be primarily related to dietary intake and lifestyle differences among different age groups. However, there is some suggestion that absence of estrogen among postmenopausal women may be a determinant of vitamin K status. Genetics may explain some of the observed inter-individual variability in vitamin K but to date, there are few studies that have systematically explored the associations between individual genetic polymorphisms and biochemical measures of vitamin K status.

I. Introduction

An inadequate diet is an important component in the multifactorial nature of numerous diseases. Infants are born deficient in vitamin K, and when unsupplemented, are at risk of hemorrhage due to vitamin K-deficient bleeding within the first few months of life. In contrast, frank dietary vitamin K deficiency in adults, without accompanying illness or predisposing factors, is rare. However, poor vitamin K nutrition has been recently linked to several chronic diseases associated with abnormal calcification, including osteoporosis [1], vascular calcification [2], and osteoarthritis [3]. To understand the impact of vitamin K nutrition on health, it is necessary to assess the determinants of vitamin K nutritional status throughout the life cycle.

II. Assessment of Vitamin K Status

A. Function

Vitamin K is a cofactor specific to the formation of γ-carboxyglutamyl (Gla) residues from specific glutamate residues in certain proteins. The Gla residues in these vitamin K-dependent proteins confer calcium-binding properties, hence function, as discussed in greater detail in this volume. Coumarin-based oral anticoagulants, such as warfarin, inhibit coagulation by decreasing the γ-carboxylation of the Glu residues in vitamin K-dependent coagulation proteins. The antagonism of vitamin K results in the secretion of undercarboxylated proteins that have reduced calcium-binding properties. As demonstrated in controlled metabolic studies in humans, inadequate amounts of available vitamin K in the diet also result in the secretion of undercarboxylated vitamin K-dependent proteins [4-6].

B. Biochemical Measures

Although dietary intake has historically been considered the primary determinant of vitamin K status [7], other factors are emerging. Biochemical markers may be more preferable than dietary assessment alone because the former capture the role of both dietary and non-dietary determinants of vitamin K status.

1. Circulating Concentrations of Vitamin K

Circulating phylloquinone concentrations are the primary form in circulation. Plasma phylloquinone concentrations reflect dietary intake over the previous 24 hours, and have high intra- and interindividual variation compared to other fat-soluble vitamins [8]. Less is known about the diet-plasma associations for the menaquinones. As discussed in section III.B, there is controversy regarding the interdependence of circulating forms of vitamin K with plasma lipids, and the implications when assessing vitamin K status.

2. Coagulation Times

The prothrombin time (PT), also expressed as an International Normalized Ratio (INR), and activated partial thromboplastin time (APTT) are routine tests of coagulation that can reflect vitamin K deficiency. These tests are nonspecific because prolongation of these measures is also indicative of hepatic dysfunction, hematologic disease unrelated to vitamin K deficiency, and multiple other acute or chronic conditions. PT becomes elongated only when the prothrombin concentrations drops below 50% of normal, demonstrating its low sensitivity for detecting vitamin K deficiency [9]. In otherwise healthy adults, sustained intakes as low as 10 µg/d of phylloquinone for several weeks do not prolong PT. In contrast,

other more sensitive measures do respond to dietary restriction and supplementation and are more appropriate for identifying determinants of vitamin K status.

3. Undercarboxylated Vitamin K-Dependent Proteins

Measurement of circulating undercarboxylated proteins is currently considered to be a more sensitive indicator of vitamin K deficiency. Undercarboxylated prothrombin, also known as PIVKA-II (proteins induced in the vitamin K absence or antagonism-factor II) has advantages in that it detects abnormalities in prothrombin before the prolongation of PT. Most infants with vitamin K deficiency have elevated PIVKA-II concentrations, but an elevated PIVKA-II is not necessarily a predictor of hemorrhagic disease. PIVKA-II has also been reported to increase in response to low-dose (1mg) warfarin [10] and vitamin K dietary restriction [6, 11]. Osteocalcin is one of the most abundant non-collagenous proteins in bone, and is thought to act as a regulator of bone mineral maturation, as described in detail elsewhere in this volume. The mineral-binding capacity of osteocalcin is dependent on the vitamin K-dependent γ-carboxylation of its three glutamate residues, such that partially carboxylated osteocalcin may have reduced binding to the mineral in bone. The proportion of serum osteocalcin (expressed as either %ucOC or ucOC/tOC) that is not carboxylated is used as a sensitive marker of vitamin K status. A high proportion of uncarboxylated osteocalcin is indicative of poor vitamin K status. In contrast, the absolute concentrations of ucOC are not particularly meaningful in terms of vitamin K status because these values are dependent on the total amount of osteocalcin available for carboxylation. For example, absolute levels of ucOC may decrease in response to vitamin K supplementation but if the total osteocalcin also decreases due to other factors, then the proportion of OC that is not carboxylated has remain unchanged, indicative of a null effect of vitamin K. Although there is a weak

negative correlation between circulating concentrations of phylloquinone and %ucOC, it is not strong enough to have predictive value as a measure of individual vitamin K status. Recent studies also suggest that the determinants of %ucOC differ from those of serum phylloquinone[12], which emphasize the limitations of using a single biochemical measure as a measure of individual vitamin K status.

4. Urinary Measures

Metabolites of vitamin K can now be measured in urine and respond to dietary manipulation of vitamin K [13]. It is anticipated that these metabolites will provide a novel marker for assessment of overall vitamin K status. A second urinary measure for which there has been more collective experience, is the measurement of urinary Gla excretion. Gla cannot be recycled and is excreted in urine during the turnover of individual vitamin K-dependent proteins. Urinary Gla excretion is an overall measure of vitamin K-dependent proteins, including those for which there are currently no known functions [14]. Urinary Gla excretion is highly correlated with lean body mass so it is usually corrected for urinary creatinine excretion [15]. In metabolic studies, urinary Gla excretion has been shown to decrease and increase in response to vitamin K dietary restriction and supplementation, respectively [6, 16].

III. Non-Genetic Determinants

A. Dietary Intakes of Vitamin K

1. Dietary Forms and Sources of Vitamin K

The term “vitamin K” represents a family of compounds with a common chemical structure, 2-methyl-1,4-napthoquinone (**Figure 1**). **Phylloquinone**, or vitamin K_1, is

a compound present in all photosynthetic plants [17]. In the human diet, phylloquinone is the predominant dietary form of vitamin K and is present in foods of plant origin. In general, green, leafy vegetables contain the highest known phylloquinone concentrations and contribute approximately 60% of total phylloquinone intake [18-19]. Certain plant oils and margarine, spreads and salad dressings derived from these plant oils, are also important dietary sources of phylloquinone [20-21]. **Menaquinone**s are primarily of bacterial origin, and differ in structure from phylloquinone in their 3-substituted lipophilic side chain. The major menaquinones contain 4-10 repeating isoprenoid units, indicated by MK-4 to MK-10; forms up to 13 isoprenoid groups have been identified. **Menaquinone-4** (MK-4) is not a major constituent of bacterial production; instead it is alkylated from menadione present in animal feeds or is the product of tissue-specific conversion directly from dietary phylloquinone [22-23]. Because poultry feed is a rich source of menadione, which is subsequently converted to MK-4 in certain tissues, poultry products are the primary dietary sources of MK-4 in the U.S. food supply [24-25]. **Menaquinone-7** (MK-7) is primarily found in natto, a soybean product that is fermented using *bacillus natto*. Although certain cheeses contain some menaquinones [26], generally the longer chain menaquinones are not thought to be present in the food supply. Very little is known about the contribution of dietary menaquinones to overall vitamin K nutrition and although it is a generally held belief that approximately 50% of the daily requirement for vitamin K is supplied by the gut flora through the production of endogenous menaquinones, there is insufficient experimental evidence to support this conviction [27]. In one small study in individuals with acute bacterial overgrowth as induced by omeprazole, menaquinones produced by these bacteria had some contribution to vitamin K nutriture during dietary phylloquinone restriction, but no enough to restore normal vitamin K status [28].

2. Dietary Intakes

An Adequate Intake (AI) for vitamin K was set at 120 and 90 µg/d for men and women, respectively (**Table 1**) [29]. The AI is determined by the reported median intake among adults in the United States [29]. In infants, the AI is set at 2 to 2.5 µg/d based on the estimated phylloquinone intake from breast milk. Infants exclusively breast fed have an estimated daily intake of 0.5-2.6 µg (IOM 2001), and are at greater risk for vitamin K deficiency because breast milk is a poor dietary source of vitamin K. In contrast, infant formulas contain appreciable amounts of phylloquinone so the average daily intake of U.S. infants two to six months of age is 63 µg (IOM 2001), which is far in excess of the AI for this age group. The AI for children aged 1 year and older is set on the basis of the median intake for each age group as estimated in U.S. national surveys (**Table 1**). The adequacy of these intakes for health has not yet been determined because there are currently no physiological outcomes available that can be reliably used to assess adequacy. Recent surveys in North America, Europe and Asia indicate that wide ranges in dietary intakes of phylloquinone exist across different geographic regions and among different ages. Subgroups of populations with an average reported phylloquinone intake below current recommendations, include nursing home residents in Hong Kong [30], and adults [18, 31-32] and children [33] in the United Kingdom. Furthermore, phylloquinone intakes have been decreasing over the last two decades in the United Kingdom, consistent with a concomitant decline in leafy green vegetable consumption [34]. In contrast, reported average phylloquinone intakes among adults in Germany [35], the Netherlands [36], Japan [37] and Northern China [31] are within the current dietary recommendations. Of note is the type of dietary assessment used and the source of food composition data, which may result in measurement

errors that contribute to the wide reported ranges of vitamin K intakes within and between subgroups [7].

3. Associations with Biochemical Measures

Dietary restriction of phylloquinone to < 35 µg/d causes rapid decreases in plasma phylloquinone (**Table 2)** and urinary excretion of Gla residues, and increases in the under-γ-carboxylated forms of the vitamin K-dependent proteins, osteocalcin (%ucOC) and prothrombin (PIVKA-II), without affecting classic measures of blood coagulation [4-6, 38]. The rapidity of the observed changes in these studies suggest that the complete carboxylation of the Gla residues in vitamin K-dependent proteins may require adequate intakes of vitamin K on a daily basis. In reviewing the data, it is of importance to note that most metabolic studies have examined the response of biochemical markers of vitamin K status to phylloquinone supplementation in adults who are already vitamin K-replete. However, recent population-based data suggest that ~15% of adults have plasma phylloquinone concentrations equivalent to those concentrations attained with vitamin K dietary restriction (i.e. below 0.5 nmol/L) [18] [39]. In observational studies, dietary phylloquinone intakes are positively, albeit weakly associated with circulating concentrations of phylloquinone [34] and inversely associated with %ucOC [19]. There appears to be a threshold of an intake of ~ 200 µg /day, above which there is no longer a linear association with circulating concentrations of phylloquinone when dietary intakes are assessed by food frequency questionnaires [19]. This observation most likely reflects limitations in the food frequency questionnaire in assessing high intakes of vitamin K because this threshold effect is not supported by the dietary supplementation studies in which plasma phylloquinone concentrations continue to increase with supplementation of up to 1,000 µg/day [40]. More recently, MK-4 and MK-7 plasma or serum

concentrations have been measured in certain populations that are supplemented and/or consume high intakes of fermented soybean products, respectively [41]. Neither PIVKA-II nor urinary Gla excretion measurements have been used to assess associations between vitamin K status and dietary intakes in observational studies.

B. Absorption, Transport and Metabolism of Vitamin K

Most disease states that detrimentally influence vitamin K status are related to malabsorption or other gastrointestinal disorders in origin, including biliary atresia, cystic fibrosis, celiac disease, and short bowel syndrome, as extensively reviewed elsewhere [42]. The absorption of vitamin K has not been well studied in healthy adults, particularly in context of dietary sources, different forms and in regards to different stages of the life cycle. In common with other fat-soluble vitamins, phylloquinone is absorbed from the proximal intestine and the absorption is dependent on bile and pancreatic secretion [43]. In the intestine, phylloquinone is incorporated into nascent chylomicron particles that are secreted directly into the lymph and ultimately into peripheral circulation. Phylloquinone remains associated with these particles during delipidation in circulation and subsequent uptake by the liver. Vitamin K is not known to have a carrier protein; instead, triglyceride rich lipoproteins (TRL), primarily chylomicron remnants and very low density lipoproteins (VLDL), are thought to be the main transporters of phylloquinone [44-46]. Plasma phylloquinone concentrations are consistently correlated with triacylglycerols (Tsugawa, 2006). Age-related differences in the clearance of phylloquinone from circulation may in part explain the documented high inter-individual differences in plasma phylloquinone concentrations [8, 47]. There is current controversy regarding the adjustment of plasma phylloquinone for total plasma lipids to assess the predictive value of fasting plasma phylloquinone concentrations on health outcomes, such as hip fracture risk [48-49]. Absolute plasma phylloquinone concentrations are higher among individuals with

elevated triglyceride concentrations. Fasting plasma phylloquinone and triglyceride concentrations are both higher in older adults compared to younger adults [50-51], but when phylloquinone concentrations are adjusted for triglycerides, the adjusted plasma phylloquinone concentrations are lower in the older adults compared to the younger adults [50]. If adjusted fasting plasma phylloquinone concentrations are a reliable measure of body stores, then these results suggest that older adults have decreased body stores of phylloquinone. However, this has not been consistently substantiated by the metabolic data (**Table 2**). In observational studies, it is difficult to interpret the interrelationship between markers of vitamin K and lipid status and plasma phylloquinone concentrations also have a high intra-individual variability that is thought to be dietary in origin. Less is known about the absorption of menaquinones, but of the limited data available, MK-4 and MK-7 do not appear to be correlated with plasma lipids [41].

Phylloquinone is tightly bound to the membranes of plant chloroplasts, and is less bioavailable compared to phylloquinone obtained from plant oils and/or dietary supplements [7]. MK-7, when administered in the form of natto in equimolar amounts to phylloquinone administered in the form of spinach, has a peak height difference of more than 10-fold compared to phylloquinone, with a half-life of 56 h, compared to 7.5 h for phylloquinone [26]. Whereas all forms of vitamin K appear to be initially associated with TRL, the longer chain menaquinones, including MK-7 and MK-9, are also associated with low-density lipoprotein (LDL). MK-4 has been reported in TRL, LDL, and high density lipoproteins (HDL). These preliminary data suggest that the menaquinones have different transport pathways and distribution, which has implications for transport to extra-hepatic tissue, such as bone. Animal data support this differential transport of the different forms of vitamin K, because MK-7 shortened the prolonged prothrombin time in

vitamin K deficient rats for a longer period compared to phylloquinone or MK-4 [52]. There are no data on potential interactions between different forms of vitamin K when co-administered.

C. Age

Infants are born deficient in vitamin K because of poor maternal-fetal transfer across the placenta [53]. Breast milk contains very low phylloquinone concentrations, which does not appear to be related to the maternal diet, and initially there is limited bacterial colonization of the gut that could produce endogenous menaquinones. In addition, coagulation factors concentrations are low at the time of birth which increases the risk of hemorrhagic bleeding known as vitamin K deficiency bleeding (VKDB). Vitamin K prophylaxis in the form of a single, intramuscular dose of 0.5 to 1 mg of phylloquinone is routinely administered as an effective intervention against VKDB. There are few studies on the safety and bioavailability of oral formulations and optimal dosing regimens of vitamin K to prevent late VKDB. Recent data suggest that intramuscular prophylaxis of 0.2 mg of phylloquinone maintains adequate vitamin K status in preterm infants, a group that is at higher risk for VKDB, without evidence of the hepatic overload seen in premature infants receiving the higher doses that are consistent with current public policy [54]. The incidence of VKDB declines at 12 weeks of age, and spontaneous bleeding beyond that age is rare and limited to lipid malabsorption syndromes, as discussed in section II.B.

As also discussed in section III.B., there is controversy regarding age-related differences in the vitamin K status among adults, particularly in observational studies. These differences may in part be explained by age-related differences in lipid concentrations. In controlled dietary studies, older adults have been reported to be relatively more

resistant to the development of subclinical vitamin K deficiency when fed a low vitamin K diet [6]. It has been proposed that there are age-related differences in absorption and catabolism of phylloquinone, the turnover of vitamin K-dependent proteins is lower in older adults, and/or they have lower vitamin K requirements [6]. Alternatively, these data may simply reflect the age-related differences in phylloquinone intakes, hence hepatic stores, prior to participation in metabolic studies. At least one metabolic study demonstrated that subclinical vitamin K deficiency can be created in elderly women following at least six weeks of restricted vitamin K intake [5]. There was one report of an age-dependent effect, with no change in %ucOC in response to vitamin K supplementation among younger women [55], but this has not been a consistent finding across studies (**Table 2**). In a cross-sectional study of healthy Japanese women who resided in a region with high *natto* consumption, women in the ≥70 year age group had overall poorer vitamin K status as measured by %ucOC, when compared to younger women [41]. In contrast, the older women had similar circulating levels of both phylloquinone and MK-7 when compared to the younger women (30-49 y age group), which suggests that even though the younger women had the lowest levels of phylloquinone or MK-7 in circulation, there was more vitamin K available for carboxylation of the osteocalcin in bone compared to the other age groups. Unfortunately no dietary data were reported to confirm observations in other studies that older women consume higher intakes of vitamin K, but clearly there are factors other than diet that are contributing to this disparity in vitamin K status among different ages. Further, these data confirms previous reports on differences in vitamin K status among women of different estrogen status from the Framingham Offspring Study [39], and suggest that estrogen status may be an important determinant of vitamin K status, independent of diet.

C. Role of Estrogen

In general, there have been so systematic reported differences in determinants of vitamin K status when compared between men and women. However, estrogen withdrawal at menopause may result in impairment in vitamin K metabolism [56]. Among early postmenopausal women not using hormonal replacement therapy, there was a higher phylloquinone level (i.e. indicative of superior vitamin K status) and high %ucOC (i.e. indicative of inferior vitamin K status) compared to premenopausal women. Triglycerides were equal across the groups studied; hence the higher phylloquinone levels in the postmenopausal women were not being influenced by elevated triglycerides [56]. This suggests that the degree of carboxylation of the osteocalcin due to the presence of vitamin K is not linearly related to amount of phylloquinone transported. Further, this relationship between the amount of phylloquinone in circulation and the amount of phylloquinone available in osteoblasts to carboxylate osteocalcin may be modulated by presence of estrogen. Along the same lines of investigation, the change in ucOC was examined in response to two different regimens of hormonal replacement therapy in postmenopausal women [57]. In this study, there was a decrease in ucOC which the authors attributed to the effect of hormonal replacement therapy on increased triglyceride levels, which in turn elevated levels of vitamin K available to the bone for carboxylation. However, the proportion of ucOC to total osteocalcin (i.e. a higher %ucOC) actually increased, suggestive of a decline in vitamin K status in the bone in response to hormone replacement therapy. Unfortunately no measures of circulating concentrations of vitamin K were reported. Collectively, these studies implicate the absence of estrogen as a determinant of vitamin K status in postmenopausal women, although the mechanisms are currently unknown, which limits our ability to interpret the current data.

E. Interactions with other Nutrients

A relatively understudied area of vitamin K nutrition is how interaction with other nutrients, in particular the other fat-soluble vitamins, influence vitamin K status. In a recent study by Thane *et al* [34], ten percent of the variation in plasma phylloquinone concentrations was explained by intake of other fat-soluble vitamin concentrations, including vitamins E and A. In animal studies, relatively small increments in supplemental α-tocopherol appear to affect the tissue content of vitamin K, particularly under conditions of vitamin K deficiency or antagonism [58-60]. Doses of vitamin E at the Tolerable Upper Limit of 1,000 IU RRR-α-tocopherol can result in an increase in PIVKA-II in adults with normal coagulation status [61]. Reports of bleeding episodes have been limited to individuals taking vitamin E supplements concurrent with vitamin K-antagonists, such as warfarin [62]. One proposed mechanism for the interactions between vitamins E and K is the competitive inhibition of vitamin K-dependent carboxylase by the vitamin E metabolite, tocopherol quinone [63]. An alternative hypothesis is that supraphysiologic doses of vitamin E interferes with vitamin K activation of the pregnane X receptor (PXR) [64]. In one small rodent study, intake of a lutein mixture preserved with vitamin E resulted in decreased absorption, uptake and transport of phylloquinone and MK-4 [65]. Similarly a hemorrhagic toxicity was induced in rats following treatment with pharmacological doses of vitamin E and ß-carotene [66], and various retinoids [67]. The mechanisms by which vitamin A may directly antagonize vitamin K have yet to be elucidated, but warrant further exploration. In a study by Szulc et al [68], there was an inverse association between ucOC and serum 25(OH)D concentrations, and a subsequent reduction of ucOC by vitamin D supplementation. These findings cannot be explained by our current understanding of the biochemical role of vitamin K, but suggest that vitamin D may influence the ucOC level. In contrast, serum 25(OH)D and phylloquinone concentrations are not correlated [12, 47].

F. Antibiotics & other Medications

Vitamin K deficiency is often attributed to broad-spectrum antibiotic use, presumably by a reduction in endogenous menaquinone production by intestinal bacteria [69]. The use of certain antibiotics containing a *N*-methylthiotetrazole side chain (e.g. cefamandole, cefoperazone) can result in a vitamin K antagonism, possibly through a weak inhibition of the γ-carboxylase among individuals with poor vitamin K status. However any direct effect of broad-spectrum antibiotic use on menaquinone production, hence vitamin K status, among individuals consuming an adequate dietary intake remains controversial [69-70]. Salicylates and anticonvulsants are also associated with vitamin K deficiency in some individuals. Those who take these medications and have coexisting compromised nutritional status may be of particular risk for vitamin K deficiency.

G. Lifestyle Factors

By nature of their study design, observational studies usually preclude the ability to isolate the effects of a single nutrient from those of the dietary patterns associated with high intakes of food(s) rich in that nutrient. A high phylloquinone intake may simply be a marker for an overall healthy diet that includes high vegetable consumption given that green leafy vegetables are consistently the primary form of vitamin K in the diet [71]. Further, individuals with high phylloquinone intake tend to consume more fruits and vegetables, dietary fiber, dietary supplementation and consume less saturated fat [72]. The consistent inverse associations between smoking and plasma phylloquinone concentrations [47, 72] may also be explained by this association between phylloquinone intake and healthy lifestyle. Likewise, soybean is a rich dietary source of MK-7, such that high plasma MK-7 concentrations may be indicative of an overall healthy diet.

IV. Genetic Determinants

A. Inter-individual Variability

There has been a recent interest in the role of genetics as a determinant of the interindividual variation in nutrient status. Similar to other nutrients, the response to dietary vitamin K supplementation under controlled conditions has been shown to widely vary among healthy individuals [73]. Non-genetic determinants, as reviewed in Section III, account for approximately 20% of the inter-individual variation in vitamin K status when studied in primarily Caucasian adults [12, 34]. In one study of 264 sib-pairs of Caucasian origin, the biochemical measures for vitamin K status were not significantly heritable, hence did not explain much of the observed inter-individual variability [12]. What is not known though is the extent to which genetics explains inter-individual variability in vitamin K status when comparing populations of diverse ethnic and racial origin. Potential genetic determinants of vitamin K status include variation in the genes involved in the transport or uptake of vitamin K into the tissues, and genes involved in the tissue specific availability and recycling of vitamin K. At the time of this writing, there have been very few published studies that had assessed the effect of these polymorphisms on measures of vitamin K status.

B. APOE

The gene encoding apolipoprotein E (APOE) codes for a 34-kDa lipoprotein primarily found on the triglyceride rich lipoproteins (VLDL and chylomicrons) and HDL [74]. APOE is a ligand for the LDL receptor and other triglyceride-rich lipoprotein receptors, and is therefore largely responsible for the cellular uptake of triglyceride-rich lipoproteins. Three common alleles, made up of amino acid substitutions at positions 112 and 158, are found in the population, and are referred to as E2, E3, and E4 [75]. The various

APOE isoforms interact differently with the LDL and other lipoprotein receptors, ultimately altering circulating levels of cholesterol and triglyceride [75]. The ability of APOE to clear vitamin K-rich intestinal lipoproteins from circulation is greatest with E4 and least with E2 [76, 77]. In a study of hemodialysis patients, serum phylloquinone concentrations in patients with the E3/4 or E4/4 genotypes were less than half that of patients with the genotype E3/3. Those with the genotype E2/3 or E2/2 had the highest serum phylloquinone concentrations [76]. It is thought that the liver is the primary organ for vitamin K uptake and carriers of the E4 allele may have increased hepatic vitamin K uptake and decreased vitamin K in circulation, hence decreased vitamin K available for γ-carboxylation of osteocalcin in the bone [78]. This is supported by the observation that E4 homozygotes require, on average, a higher dose of warfarin, a vitamin K antagonist, compared to non-E4 carriers [79]. A higher warfarin dose in E4 homozygotes would be necessary to compensate for the increased hepatic vitamin K uptake. In contrast, in a study of healthy older adults from China and the UK, carriers of the E4 allele had higher plasma phylloquinone concentrations and lower %ucOC [80]. These authors suggested that the carriers of the E4 allele had slower clearance of the triglyceride-rich lipoprotein remnants from circulation, and subsequently more phylloquinone available for uptake in the bone. In another study, ethnic differences in ApoE genotype, with Gambian postmenopausal women having the highest frequency of E4 allele (32.6%) compared to UK and Chinese postmenopausal women (13.8 and 6%, respectively), were associated with differences in %ucOC, but not plasma phylloquinone concentrations [81]. Clearly more research is required to determine the direction and magnitude of this putative effect that ApoE genotype has on vitamin K nutrition status.

C. VKOR

Vitamin K epoxide reductase (VKOR) is a necessary enzyme for the vitamin K cycle, as discussed elsewhere in this volume. Common polymorphisms and haplotypes within the VKORC1 gene have been associated with interindividual variability in warfarin dose [82-84]. Because warfarin is a vitamin K antagonist that works by directly inhibiting VKOR and thus the recycling of vitamin K, polymorphisms affecting vitamin K recycling in the liver may modulate vitamin K status. One recent study has shown that SNPs and haplotypes within the *VKORC1* locus were associated with ucOC and PIVKA-II concentrations in a Chinese cohort [85]. Paradoxically, the genotype that was associated with lower PIVKA-II concentrations, hence better vitamin K status, was also associated with greater risk of vascular disease. The ucOC was expressed as an absolute concentration, and as discussed in section II.B.3, it is the percentage of the osteocalcin that is carboxylated that provides insight in the availability of vitamin K. Unfortunately there were also no corresponding data on plasma phylloquinone concentrations in this cohort to confirm vitamin K status relative to the individual genotypes.

D. GGCX

Gamma glutamyl carboxylase (GGCX) is necessary for the carboxylation of vitamin K-dependent proteins, as discussed in greater detail in this volume. Common variations within the *GGCX* locus have been analyzed for association with transcriptional activity and required warfarin dose [86]. However, little has been done to examine the effect of these polymorphisms on direct measures of vitamin K status or γ-carboxylation of extrahepatic proteins such as osteocalcin.

V. Conclusions and Future Directions

To understand the role of vitamin K in human health, it is necessary to identify determinants of vitamin K status throughout the life cycle. To date, the research in this area has been focused on the roles of diet, age, and to a lesser extent, the role of circulating lipid concentrations in explaining the inter-individual variation in vitamin K status. Dietary intakes vary widely in the amount and forms consumed among age-groups and different subgroups of populations. This variation in turn influences how vitamin K is absorbed and transported. Likewise, the role of plasma lipids as a determinant of vitamin K status varies with the form of vitamin K ingested. Our current understanding of vitamin K physiology and metabolism only partially explains the sources of inter-individual variation in vitamin K status, as measured by various biochemical measures. It is plausible that dietary intakes of vitamin K contribute more than can be currently quantified, but methodological limitations may attenuate diet-plasma correlations. Despite its role as a primary determinant of vitamin K status, there are insufficient dose-response data and a poor understanding of the physiological relevancy of the biological markers from which to establish recommended dietary allowances [87].

It is well recognized that infants are the only age group at risk of frank vitamin K deficiency as characterized by vitamin K-deficient bleeding. There are few published studies in children and adolescents from which to evaluate determinants of vitamin K status that may be potentially unique to these age groups. In contrast, most of our understanding about vitamin K has been focused on the aging process. During adulthood, there may be subtle age-related changes in vitamin K status but the literature is inconsistent. There is some suggestion that absence of estrogen among postmenopausal women may be a determinant of vitamin K status.

Genetics may explain some of the observed inter-individual variability in vitamin K but to date, there are few studies that have systematically explored the associations between individual genetic polymorphisms and biochemical measures of vitamin K status. This appears to be a promising area of research that may also serve to explain some of the inconsistent data on the putative roles of age and plasma lipid concentrations as determinants of vitamin K status.

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Table-1 Adequate Intakes for vitamin K (IOM 2001).

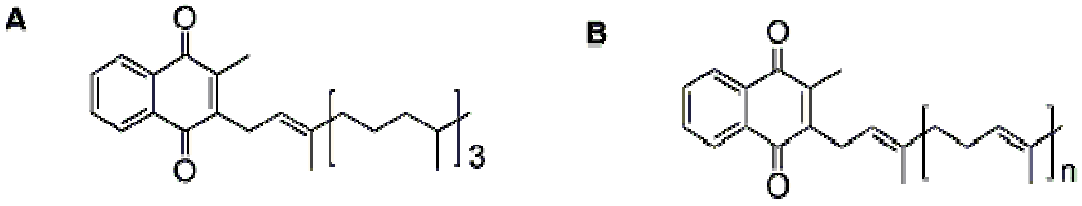
Age Group	Adequate Intake (µg/d)
Infants	
0 - 6 mo	2
7 - 12 mo	2.5
Children	
1 - 3 y	30
4 - 8 y	55
9 -13 y	60
14 -18 y	75
Men	
19 -70 y	120
>70 y	120
Women	
19 - 70 y	90
>70 y	90
Pregnancy and Lactation	
14 -18 y	75
19 - 50 y	90

Table-2 Response of biomarkers of vitamin K status to phylloquinone restriction and supplementation.

Reference	Participants	Intervention with Phylloquinone	Plasma Phylloquinone	Serum % ucOC
[55]	100 women 25-40y; 55-75y	14-d @ 1 mg/d	n/a	↓ (55-75 y) no Δ (24-40y)
[88]	145 women 20-85 y	14-d @ 1mg/d	n/a	↓
[89]	10 women 52-73y	14-d @ 1mg/d	n/a	↓
[90]	8 women mean age: 35 y	30-d @10 mg/d	n/a	↓
[16]	36 men and women 20-40y; 60-80y	5-d @ 400 µg/d	↑	↓
[91]	112 men and women 18-30y; ≥65 y	14-d @1 mg/d	↑	↓
[11]	15 men and women 20-40y	15-d @10 µg/d	↓	↑
		10-d @ 200 µg/d	↑	↓

Reference	Participants	Intervention with Phylloquinone	Plasma Phylloquinone	Serum % ucOC
[40]	10 men and women 19-36y	7-d @ 0.5-2 mg/d	n/a	↓
	100 men and women mean age: 26y	14-d @ 250 µg/d	↑	↓
		14-d @ 375-1000 µg/d	↑	↓
[5]	21 women 60-80y	30-d@18 µg/d	↓	↑
		30-d@86-200 µg/d	no Δ	no Δ
		15-d@450 µg/d	↑	no Δ

Figure-1 Dietary forms of vitamin K: phylloquinone (A) and menaquinones (B).



CHAPTER FOUR

SUMMARY AND DISCUSSION

4.1 Summary

All forms of vitamin K share a common naphthoquinone ring, but differ in the position-3 side chain. The phytyl side chain of dietary phylloquinone (MW 451) is assumed to be removed forming menadione (MW 172), and the preformed geranylgeranyl side chain is donated by geranylgeranyl pyrophosphate (GGPP) to form tissue MK-4 (MW 445). To date, direct evidence that menadione is the conversion intermediate has been lacking. In addition, tissue location of the conversion process is not known. While the geranylgeranyl side chain addition seems to takes place in target tissues, the removal of the phytyl side chain may take place in a central compartment or in the target tissues themselves.

Sensitive assays for measurement of menadione are required for the study of this conversion of phylloquinone to MK-4. Previous attempts to measure menadione lacked the sensitivity to detect this compound in response to a variety of dietary and physiological conditions [1-3]. As part of this thesis research, we developed and validated a sensitive and reproducible HPLC method using a C_{30} column, post-column zinc reduction and fluorescence detection for the purpose of measuring urinary menadione [4]. We then applied this method to archived samples from a 3 year randomized controlled phylloquinone supplementation trial in older men and women [5]. A conjugated, water-soluble form of menadione is present in the urine of healthy humans. Urinary menadione increased in response to 3 years of phylloquinone supplementation whereas it remained unchanged in those participants not receiving vitamin K supplementation. Our findings show that menadione is a metabolite of phylloquinone that is excreted in urine, and support the hypothesis that menadione is an intermediate in the conversion of phylloquinone to MK-4.

We then conducted a rodent study to provide direct evidence that menadione is the intermediate product in the phylloquinone to MK-4 conversion. Following a 14 day phylloquinone-deficient diet, male Fischer 344 rats (8-month; n=15) were fed deuterium-labeled (-d) collard greens as source of 1.6 mg phylloquinone-d [MW 459-463 (64% of total)] per kg diet for 0 (control), 1 and 7 days. Using LC/MS, the majority of phylloquinone-d and MK-4-d (MW 446-449) were detected in tissues after 1 day. The majority of tissues contained both phylloquinone-d and MK-4-d; the salivary gland and brain contained MK-4-d (757.2±111.8 and 38.6±8.3 pmol/g, respectively on day 7), but no phylloquinone-d. Based on the principle of random distribution of deuterium labels, MK-4-d MW indicated that MK-4-d carried the d-label on the naphthoquinone ring, but not on the side chain, confirming the need for side chain removal for its formation. In contrast, but consistent with the existing literature, the liver and serum contained phylloquinone-d (209.7±111.8 pmol/g and 28.7±18.1 pmol/mL on day 7, respectively), but no MK-4-d, supporting the hypothesis that conversion to MK-4 is tissue-specific.

In contrast to human urine that only contains the conjugated form of menadione, rats' urine contains both conjugated and unconjugated forms of menadione. To the best of our knowledge, this is the first time unconjugated menadione has been detected in biological materials. Unconjugated menadione is fat soluble, and is bound to protein in rats' urine. The presence of protein was confirmed in the urine of all animals throughout the study. The presence of protein in urine suggests that unconjugated urinary menadione reflects what is in circulation. To account for the two forms, urinary menadione extraction method was modified to extract both conjugated and unconjugated menadione from different matrices, including urine, serum and tissues. New methods for menadione detection using LC/MS and LC-MS/MS were also developed based on modification to the HPLC menadione detection method.

Unconjugated menadione was measured in rats' urine by HPLC. Using LC/MS, unconjugated menadione-d (MW 173-175) was detected in urine. Using LC-MS/MS, unconjugated menadione-d was detected in serum in the majority of the animals (n=4/5 at day 1; n= 3/5 at day 7). Dietary phylloquinone-d was clearly shown to be the source of both menadione-d (in urine and serum) and tissues MK-4-d, and as expected, there was no detected deuterium label on any form of vitamin K in the control group. This is the first direct evidence that menadione is an intermediate in the dietary phylloquinone to tissue MK-4 conversion.

One of the outstanding questions is the location of the side chain cleavage. One hypothesis is that the side chain is cleaved off in the intestine, releasing menadione into circulation. To test this, a Caco-2 cell culture model was used to study the role of the enterocytes in the conversion process. Upon differentiation, Caco-2 cells exhibit intestinal characteristics and are routinely used in other fat-soluble vitamins absorption studies. The model used allowed the cells to differentiate and form an intact monolayer. Tween-40 was used to deliver different treatment (phylloquinone, MK-4 or menadione) to the apical side of the differentiated Caco-2 cells. HBSS was added to basolateral side. The basolateral media and the cells themselves were harvested. and tested for the formation of MK-4 and menadione. Using LC/MS, neither MK-4 nor menadione was detected in Caco-2 cell culture model treated with phylloquinone, suggesting that the phylloquinone side chain removal does not take place in the enterocytes. However, when treated with menadione, enterocytes formed MK-4.

Archived urine samples of participants in a human PK supplementation study (n=368) were analyzed for MD. Changes in urinary MD excretion were significantly correlated with changes in biomarkers of vitamin K status, including serum phylloquinone and

percent serum undercarboxylated osteocalcin (ucOC; *P*=0.0008 and 0.0184, respectively). In a separate metabolic study of 42 healthy, younger and older men and women, we used deuterium-labeled collard greens to confirm that urinary deuterium-labeled menadione (L-MD; MW 173-174) was formed from deuterium-labeled phylloquinone (L-PK). For an intake of 255 nmol L-PK, between (mean± SD) 12.4%± 6.2% and 14.9%± 10.0% was recovered as L-MD in urine. These estimates do not take into account bioavailability of PK from plant sources. However, existing vitamin K status did not affect amount excreted.

4.2 Future directions

4.2.1 Location of conversion

The data generated as part of this thesis research confirmed that menadione is the intermediate in the conversion of dietary phylloquinone to MK-4 that occurs in the majority of tissues. Menadione was also identified as a urinary metabolite of phylloquinone metabolism, and responds to manipulation of phylloquinone depletion, repletion and supplementation. Our initial attempts to identify the location of this conversion were not successful. Detection of unconjugated menadione in rat serum suggests that the formation of menadione occurs in a central compartment. However using a Caco-2 cell culture model, it does not appear that enterocytes are the location for this initial cleavage. It was suggested that the first pass metabolism is important for phylloquinone to MK-4 conversion, making the liver a top candidate as the central compartment where menadione is formed. Future studies are essential to determine the location of this conversion.

4.2.2 Enzymes responsible for the conversion

During the course of this thesis research, UbiA prenyltransferase containing 1 (UBIAD1) was identified as a human prenyltransferase enzyme responsible for the geranylgeranyl side chain addition step of the conversion process from phylloquinone to MK-4 [6]. Although the authors identified UB1AD1 as the as the enzyme also responsible for the phytyl side chain removal, their evidence was rather weak. The inability to measure menadione, the proposed intermediate in the conversion, represented a major limitation in data interpretation regarding UBIAD1 role in the phytyl side chain removal step of the conversion process. The Caco-2 cell data presented in this thesis suggest that a different enzyme is responsible for the formation of menadione because we were unable to produce MK-4 from phylloquinone, but we were able to produce MK-4 from menadione. Similarly, it is unlikely that our observations that menadione is present in serum whereas MK-4 is not, would be consistent with one enzyme being responsible for both steps in the conversion process. Development of a sensitive and reproducible assay for menadione detection now provides an essential tool that can now be used to study the role of UBIAD1 and/or other enzymes in the phytyl side chain removal.

4.2.3 Exploring the potential antagonists to MK-4 synthesis

Geranylgeranyl pyrophosphate, the purported donor of geranylgeranyl in the alkylation reaction, is synthesized in animal tissues using isopentenyl pyrophosphate (IPP). IPP, a 5 carbon molecule, is the active isoprene unit in isoprenoid synthesis. Isoprenoids are large and diverse class of naturally-occurring organic chemicals derived from the 5 carbon isoprene (2-methylbuta-1, 3-diene). Isopentenyl pyrophosphate isomerase (IPP isomerase) converts IPP to dimethylallyl pyrophosphate (DMAPP) in the first step of isoprenoid synthesis. Then several prenyltransferases catalyze the head to tail condensation of IPP with the acceptor molecule. In this reaction, the head of IPP, which

is the C=C end, is added to the tail in the prenyl molecules, which is the pyrophosphate end. For example, geranyl pyrophosphate synthase (GPP synthase) catalyses the condensation of IPP with DMAPP producing GPP (10 C), farnesyl pyrophosphate synthase (FPP synthase) catalyses the condensation of IPP with GPP producing FPP (15 C), geranylgeranyl pyrophosphate synthase (GGPP synthase) catalyses the condensation of IPP with FPP yielding GGPP (20 C), and so on [7]. The short chain prenyl pyrophosphates (e.g. FPP and GGPP) can either be further elongated or serve as biosynthetic precursors of various isoprenoids, including MK-4. In animals, FPP sits at branch point between sterol and longer-chain non-sterol synthesis [8].

Animals, fungi and archaebacteria synthesize their isoprenoids exclusively through the mevalonate pathway. Briefly, the mevalonate pathway converts acetyl-CoA to mevalonate and then to IPP via a number of enzymatic steps [9]. In this pathway, hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) is converted by HMG-CoA reductase into mevalonate. Depletion of mevalonate can be caused by the HMG-CoA reductase inhibitors (Statins, e.g. Lipitor) [9], thus HMG-CoA reductase inhibitors, through their ability to deplete cells of mevalonate-derived FPP and GGPP have been known to result in the inhibition of isoprenylation [8].

Another widely used drug family that is known for its ability to inhibit isoprenylation is the nitrogenous bisphosphonates. Nitrogen-containing bisphosphonates, (e.g. alendronat and risedronate) are used clinically to reduce bone resorption associated with osteoporosis or metastatic bone disease [10]. Clinical bisphosphonates inhibit FPP synthase, an enzyme of the mevalonate pathway [11], leading to cellular FPP and GGPP depletion. This results in a variety of downstream biological effects that include inhibition of isoprenylation [12-13].

Through similar mechanism to isoprenylation inhibition, both HMG-CoA reductase inhibitors and nitrogenous bisphosphonates could potentially inhibit the MK-4 synthesis through depletion the cells of GGPP, one of the two substrates needed for MK-4 formation. Thus, both widely used drug facilities could potentially interfere with proposed roles unique to MK-4. Future studies could exploit these potential pathways to advance our knowledge of MK-4 function.

4.2.4 Importance of MK-4 formation

As previously stated, it seems unlikely that a metabolic pathway leading to MK-4 would have evolved unless MK-4 has unique biological roles. While emerging roles unique to MK-4 beyond coagulation have been proposed based on *in vitro* studies, this area of research is still relatively unexplored. The ability of different biological systems to convert phylloquinone and menadione into MK-4 should be accounted for. Failure to address the possibility that MK-4 formation could account for functions attributed to other individual forms of vitamin K constituted a major limitation in many previous studies. It is also not known if phylloquinone and menadione are the only precursors for MK-4. It is plausible that an enzyme responsible for cleaving off the side chain of phylloquinone could also perform similar actions for menaquinones. .Future research should avoid such uncertainty by measuring MK-4 formation in biological systems treated with other forms of vitamin K.

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CHAPTER FIVE

APPENDICES

5.1 Methods

5.1.1 HPLC system

Purpose: To measure menadione concentrations in different matrices.

5.1.1.1 HPLC system specifications

1) Instrument: Waters 2695 Separations Module equipped with a vacuum degasser and auto injector.
2) Column: ProntoSil C_{30} column (5 μm, 250 x 4.6 mm) (MAC-MOD Analytical Inc., Chadds Ford, PA USA). The column was maintained at a constant temperature of 5 ℃ using a Cool Pocket ™ (Thermo Electron Corporation, Waltham, MA).
3) Detector: Shimadzu Fluorescence Detector.
    a) Excitation wavelength 244nm, emission wavelength 430nm.
    b) Gain= 1, sensitivity= 1, response= 4.
4) Postcolumn Zinc reactor: Stainless steel column (50 X 2.0 mm i.d.) dry packed with zinc metal (-100 mesh, 99.9% metal basis) with 2 μSS Frits in carbon (Alltech part # 99001) at each end.
5) Mobile phase solvents:
    a) Solvent A: Methanol with 0.55% aqueous solution.
    b) Solvent B: Methylene chloride.
    c) Solvent C: Methanol.
    d) Solvent D: Deionized water (DI H_2O).

5.1.1.2 Preparation of 200 mL of aqueous solution

Aqueous solution =	2.0 M zinc chloride
	1.0 M acetic acid
	1.0 M sodium acetate

1) Tare a 400 mL beaker.
2) Add about 100 mL of DI H_2O and a magnetic stir bar to the beaker.
3) While stirring:
   a) Add 27.22 g of sodium acetate ($C_2H_3O_2Na.3H_2O$. FW = 136.1).

      Calculations: (0.2 L)*(1.0 mole/L)*(136.1 g/mole) = 27.22 g.

   b) Add 54.52 g of zinc chloride ($ZnCl_2$. FW = 136.3).

      Calculations: (0.2 L)*(2.0 mole/L)*(136.3 g/mole) = 54.52 g.

   c) Add 12.08 g of glacial acetic acid ($C_2H_3O_2H$. Molarity= 17.4 M; density= 1.05 g/mL).

      Calculations: (0.2 L)*(1.0 mole/L)/ (17.4 mole/L) = 0.01149 L = 11.5 mL (11.5 mL)*(1.05 g/mL) = 12.08 g.
4) Stir until all solids are dissolved.
5) Remove the stir bar and place the beaker on a balance.
6) Add DI H_2O until the weight of the solution is 245.0 g.
7) Tare weight of the beaker + 245.0 g = final weight of the beaker and solution.
8) Filter the solution through a 0.45 micron filter using an appropriate filtering apparatus.
9) Store in a labeled amber bottle.

5.1.1.3 Preparation of mobile phase solvent A

Mobile phase solvent A consists of methanol with 0.55% aqueous solution. Add 5.5 mL of aqueous solution (see section 5.1.1.2 for preparation instructions) to about 900 mL methanol, qs to 1000 mL with absolute methanol.

5.1.1.4 General menadione HPLC setup and run procedure for a Waters 2690 system

1) Turn on HPLC system and set the vacuum degasser to “on”.
2) Equilibrate the column and detector by pumping a 95% solvent A and 5% solvent D through the column at 0.60 mL/min for about 30 minutes.
3) The excitation wavelength is 244 nm and the emission wavelength is 430 nm.
4) The calibration standard contains both menadione and internal standard (menaquinone-2; MK-2) in iso octane. A calibration standard is injected every six to ten samples in an HPLC run to compensate for possible changes in chromatographic conditions.
5) Standard curves are prepared from each calibration injection. The fluorescence responses for menadione and for MK-2 are linear beyond normal physiological concentrations with the slope of the lines bisecting zero. We therefore routinely perform single-point calibration, forcing the slope of the line through zero. Quantitation is achieved by direct comparison of peak area ratios (menadione to MK-2) generated from the calibration standard to those generated by the sample.
6) At the end of each sample set run, the column is washed with100% methanol at a flow rate of 0.8 mL/minute for about 45 minutes.
7) Peak integration and sample concentration calculations are performed using Empower Pro Empower 2 software from Waters Corporation.

5.1.2 Determination of total menadione in human urine using HPLC [1]

5.1.2.1 Sample preparation

5.1.2.1.1 Aliquot samples

Vortex the urine sample and pipette 0.5 mL of urine into 16X100 MM borosilicate culture tubes with Teflon lined screw caps. Menadione in the urine of healthy human subjects will be in the conjugated form.

5.1.2.1.2 Hydrolysis and extraction of menadione from human urine

1) In order to hydrolyze menadione conjugates and oxidize the released menadiol to menadione, add 35 μL sulfuric acid-dichromate solution (potassium dichromate, 0.1 M, in sulfuric acid, 0.35 M) [2] to each tube. Vortex for five seconds.
2) Cap & heat the solution for 30 minutes at 60 ℃ in a water bath with moderate shaking.
3) Cool at room temperature for approximately 10 minutes. Neutralize with 45 μL sodium carbonate buffer (1 M, pH= 9.0). Vortex for five seconds.
4) To each sample, add approximately 16 pmole of MK-2 internal standard in iso octane. Vortex for five seconds.
5) For liquid-liquid lipid extraction, add 3 mL iso octane to each borosilicate tube. Recap and vortex tubes vigorously for 3 minutes.
6) Centrifuge for 5 minutes at 3,000 rpm (1800xg).
7) Remove the upper (iso octane) layer with a Pasteur pipette and aspirate into previously labeled 12 X 75 mm disposable Borosilicate glass culture tubes.

Alternatively place the samples at -80 ℃ for approxim ately 30 minutes and then decant the organic layer.

8) Evaporate the iso octane with nitrogen until about 200 μL of iso octane is remaining. The remaining 200 μL iso octane contains both menadione and MK-2 internal standard. Do not evaporate to dryness. Complete evaporation results in major losses of menadione. If samples are evaporated to below 200 μL, add iso octane to bring volume back to about 200 μL.
9) Vortex the samples for five seconds and transfer each to an amber sample vial with conical insert.
10) Cap each vial with Teflon lined rubber septum and an aluminum crimp cap. Centrifuge the sample vials at 3,000 rpm (1800xg) for 5 minutes to precipitate any particulate material.

5.1.2.2 Urinary menadione HPLC procedure

1) Set up the HPLC instrument according to the procedure given in section 5.1.1.4.
2) The 95:5 ratio of solvents A and D is maintained throughout the 20 minute injection cycle (isocratic elution).
3) One assay run typically consists of a calibration standard injection every 6 samples, low and high controls, and 22 urine samples.

5.1.3 Determination of menadione in Fischer 344 rat urine using HPLC

In contrast to human urine that contains conjugated form of menadione and is protein-free, Fischer 344 rats' urine was found to contain both conjugated and unconjugated forms of menadione (refer to section 3.2. of this dissertation for details). Unconjugated menadione is fat soluble, and is bound to protein in rat urine. Proteins in rat urine

interfere with menadione extraction. Urinary menadione HPLC procedure was modified to account for the differences between human and Fischer 344 rat urine. Modifications included the addition of protein precipitation steps, and extraction of both conjugated and unconjugated menadione from Fischer 344 rat urine

5.1.3.1 Determination of total menadione in Fischer 344 rat urine using HPLC

5.1.3.1.1 Sample preparation

5.1.3.1.1.1 Aliquot samples

Vortex Fischer 344 rat urine sample and pipette 0.5 mL of urine into 16X100 MM borosilicate culture tubes with Teflon lined screw caps.

5.1.3.1.1.2 Protein precipitation

1) To each tube, add 56 μL 100% Tri-Chloro-Acetic acid (TCA) in order to precipitate the protein. Vortex for five seconds.

   Calculations: Aim for final TCA concentration of 10% following the formula

   $M_1V_1=M_2V_2$

   $(100)*(V_1) = 10*(500+V_1)$

   $90*V_1=5000$

   V_1= 56 μL of 100% TCA.

2) Leave on ice for about 30 minute to allow protein to precipitate.

5.1.3.1.1.3 Hydrolysis and extraction of menadione from Fischer 344 rat urine

Follow the procedure in section 5.1.2.1.2, with the following exceptions:

- Use rat urine instead of human urine
- Extract the solution 3 times with 2.0 mL portions of iso octane.

5.1.3.1.2 Urinary menadione HPLC procedure

To run the samples on the HPLC, follow the steps of given in section 5.1.2.2.

5.1.3.2 Determination of unconjugated, protein-bound menadione in Fischer 344 rat urine using HPLC

Follow the steps for "Determination of total menadione in Fischer 344 rat urine using HPLC" given in section 5.1.3.1, except:

- Delete steps 1-3 from section 5.1.2.1.2 which relate to hydrolysis and neutralization.

5.1.4 Determination of menadione in serum using HPLC

Compared to human urine, serum contains many interfering materials such as proteins and lipid that can interfere with menadione extraction. Thus, protein precipitation is necessary to extract menadione from serum.

5.1.4.1 Determination of total menadione in serum using HPLC

5.1.4.1.1 Sample preparation

Follow the steps for "Sample preparation" given in section 5.1.3.1.1 except:

- Use serum instead of rat urine

5.1.4.1.2 Serum menadione HPLC procedure

1) Set up the HPLC instrument according to the procedure given in section 5.1.1.4.
2) An assay run generally consists of low and high controls and 22 serum samples.
3) The 2695 should be programmed to keep the 95:5 A/D solvents ratios of mobile phase for the first 18 minutes of the injection cycle (Isocratic elution). Both menadione and MK-2 elutes within the 18 minutes.
4) The rest of the run (40 minutes total) is used to wash the column and bring it back to equilibration. Serum contains more interfering materials than urine thus the column washing step is essential in each run.
5) The flow chart for the run is as follows (Table-1):

	Time	Flow rate (mL/min)	Mobile phase composition (Solvents %)			
			A	B	C	D
1	[redacted]	0.6	95.0	0.0	0.0	5.0
2	18.00	0.6	95.0	0.0	0.0	5.0
3	18.50	1.0	0.0	0.0	100.0	0.0
4	21.5	1.0	0.0	0.0	100.0	0.0
5	22.0	1.0	0.0	30.0	70.0	0.0
6	26.0	1.0	0.0	30.0	70.0	0.0
7	26.5	1.0	0.0	0.0	100.0	0.0
8	29.5	1.0	0.0	0.0	100.0	0.0
9	30.0	1.0	95.0	0.0	0.0	5.0
10	33.0	1.0	95.0	0.0	0.0	5.0
11	33.5	0.6	95.0	0.0	0.0	5.0
12	40.0	0.6	95.0	0.0	0.0	5.0

Table-1 Flow chart for serum menadione HPLC procedure.

5.1.4.2 Determination of unconjugated, protein-bound menadione in serum using HPLC

Note: The same method can be used to measure unconjugated menadione in Caco-2 cells and cell culture media.

5.1.4.2.1 Sample preparation

Follow the steps for “Sample preparation” given in section 5.1.3.1.1, except:

- Use Serum instead of rat urine
- Delete steps 1-3 from section 5.1.2.1.2.

5.1.4.2.2 Serum menadione HPLC procedure

To run the samples on the HPLC, follow the steps given in section 5.1.4.1.2.

5.1.5 Determination of unconjugated, protein-bound menadione in animal tissues using HPLC

We believe that conjugated menadione is a metabolite that has been either excreted (in urine) or being transported for the purpose of excretion (in plasma). Thus, we don’t expect to be able to measure any conjugated menadione in tissues. Any menadione in tissues, if found, would be in the unconjugated form, and in our opinion, serve as an intermediated in the conversion process of phylloquinone to MK-4. Homogenizing is an essential step to break down tissue structure and allow menadione to be readily accessed by different treatments. Like serum, tissues contain many interfering materials such as proteins and lipid that can interfere with menadione extraction. Thus, protein precipitation is necessary to extract menadione from serum.

5.1.5.1 Sample preparation

5.1.5.1.1 Sample weighing and homogenizing

1) Allow tissues to thaw at room temperature.
2) In a previously labeled 2.0 mL cryogenic vials weigh approximately 0.2 g of wet tissue. Alternatively, disposable culture tubes (12X75mm) can replace the cryogenic vials. The exact amount of tissue used can be adjusted depending on expected menadione contents. Make sure to get rid of extra and unwanted fat deposits that accumulate in certain tissues before weighing.
3) To each sample add 1mL of Phosphate Buffered Saline, 1X (PBS, pH=7.4). Final volume= 1mL+wet tissue weight (g).
4) Homogenize samples using PowerGen 125 homogenizer. Homogenizing time varies depending on the tissues. Usually 30 seconds will suffice. Clean the homogenizer head after each sample by operating it in a container filled with distilled water. Blot the homogenizer to remove the water. Make sure no samples debris is stuck on the homogenizer head.

5.1.5.1.2 Protein precipitation

1) Vortex and add 0.25ml of homogenate into 16X100 MM borosilicate culture tubes with Teflon lined screw caps.
2) Dilution Factor Calculations:
    a) Tissue concentration= wet weight of tissue (g)/ [1+wet weight of tissue (g)] =g/mL.
    b) g tissue tested= (tissue concentration g/mL)*(mL homogenate used).
    c) Dilution factor= 1/g tissue tested.
3) Final results are expressed as pmole menadione/g wet tissue.

4) To each tube, add 0.25 ml 1X PBS (pH=7.4) to reach a volume of 0.5 mL.

5) Follow steps given in section 5.1.3.1.1.2.

5.1.5.1.3 Extraction of menadione from tissue homogenate

Follow the procedure in section 5.1.2.1.2, with the following exceptions:

- Use tissue homogenate instead of human urine.
- Extract the solution 3 times with 2.0 mL portions of iso octane.
- Do not perform steps 1-3 which relate to hydrolysis and neutralization.

5.1.5.2 Tissue menadione HPLC procedure

To run the samples on the HPLC, follow the steps given in section 5.1.4.1.2.

5.1.6 Solid Phase Extraction (SPE) on silica

Some samples contain more interfering materials than others. Those samples require extra separation steps using SPE techniques.

1) Prepare 50% Methyl-ter-butyl-ether (MTBE) in iso octane, prepare 12 mL for each sample, use within 8 hours.
2) Place 3 mL (500 mg) silica SPE columns (Varian part # 12113036) on the Vac Elute SPS 24 (Varian part # 12234022), one column for each sample.
3) Wash each column with 4 mL of 50% MTBE/iso octane.
4) Wash each column with 4 mL of iso octane.
5) Add the samples in the 6mL iso octane to the column.
6) Wash each column with 4 mL of iso octane.
7) Change to collect. Elute Menadione and MK-2 with 8 mL of 50% MTBE/iso octane.
8) Collect elute and proceed to iso octane evaporation using nitrogen evaporator.

5.1.7 Detection of menadione using HPLC-APC/MS system

HPLC/ MS with atmospheric pressure chemical ionization (LC-APCI/ MS) can be used for menadione detection. LC/MS utilizes both chromatographic and mass spectrometry techniques to detect menadione. This enables LC/MS to differentiate between unlabeled and labeled menadione based on their molecular weights. The method we have developed failed to measure the internal standard used (MK-2). Thus, quantification of menadione was not achieved using LC/MS. We used LC/MS to confirm menadione detection and to measure the percent enrichment of menadione measured using HPLC.

5.1.7.1 LC-APCI/ MS system specifications

1) Instrument: Agilent HP series 1100 G1946D MSD with an APCI source connected to an Agilent series 1200 HPLC instrument.
2) The temperature-controlled HPLC column compartment was set to 20 ℃.
3) Column: ProntoSil C_{30} column (5 μm, 250 mm × 4.6 mm) (MAC-MOD Analytical Inc., Chadds Ford, PA).
4) Mobile phase: Mobile phase consisted of 85% solvent A (methanol) with 15% solvent B (DI H_2O). Flow rate was set to be 1.0 mL/minute. One cycle was 20 minutes.
5) The MS detector conditions: The MS ion source was negative APCI with the spray chamber gas temperature set at 350 ℃, vaporizer temp erature set at 400 ℃, the drying nitrogen 7.0 L/min, capillary voltage was 3800 V, nitrogen nebulizer pressure was set to 45 psi, and corona current set at 5 μA.
6) Selected ion monitoring (SIM) was used to detect isotopomers of menadione (m/z 172) and deuterium-labeled menadione (m/z 173-174). Data were collected using Agilent Chemstation software (version B.03.01).

5.1.7.2 Sample preparation

In order to prepare samples from different matrices, follow sample preparation steps in previous sections as follows:

- Total menadione in human urine: follow sample preparation steps in section 5.1.2.1.
- Unconjugated menadione in Fischer 344 rat urine: follow sample preparation steps in section 5.1.3.1.1, except:
  - Delete steps 1-3 from section 5.1.2.1.2 which relate to hydrolysis and neutralization.
- Unconjugated menadione in serum: follow the steps for "Sample preparation" given in section 5.1.3.1.1, except:
  - Use Serum instead of rat urine.
  - Delete steps 1-3 from section 5.1.2.1.2.
- Unconjugated menadione in tissues: follow sample preparation steps in section 5.1.5.1.

5.1.7.3 Limitations

Total menadione in both Fischer 344 rat urine and in serum could not be detected using LC-APCI/ MS system due to the presence of interfering peak that masks the peak corresponding to the compound of interest (menadione).

5.1.8 Detection of menadione using HPLC-APCI-MS/MS system

Liquid chromatography/tandem mass spectrometry with atmospheric pressure chemical ionization (LC-APCI- MS/MS) is a very specific and sensitive way for compound detection. LC-APCI-MS/MS produces $(M)^-$ parent ions which are activated by collision induced dissociation (CID) breaking menadione into fragment ions which are specific to its chemical structure. Like LC-APCI/MS, LC-APCI-MS/MS utilizes both chromatographic and mass spectrometry techniques to detect menadione and the major fragment created by CID; this allows LC-APCI-MS/MS to be more sensitive and specific than LC/MS. LC-APCI-MS/MS can be used to differentiate between unlabeled and labeled menadione based on molecular weight of parent compounds and fragments. The method we have developed did not measure the internal standard used (MK-2). Thus, quantification of menadione was not achieved using LC-APCI-MS/MS. We used LC-APCI-MS/MS to confirm menadione detection. When unlabeled menadione was subjected to CID, the main fragment formed has m/z=146. Based on that, we hypothesized the following scenario for menadione fragmentation. (Figure-1. Hypothesized pathway for menadione fragmentation in LC-APCI-MS/MS)

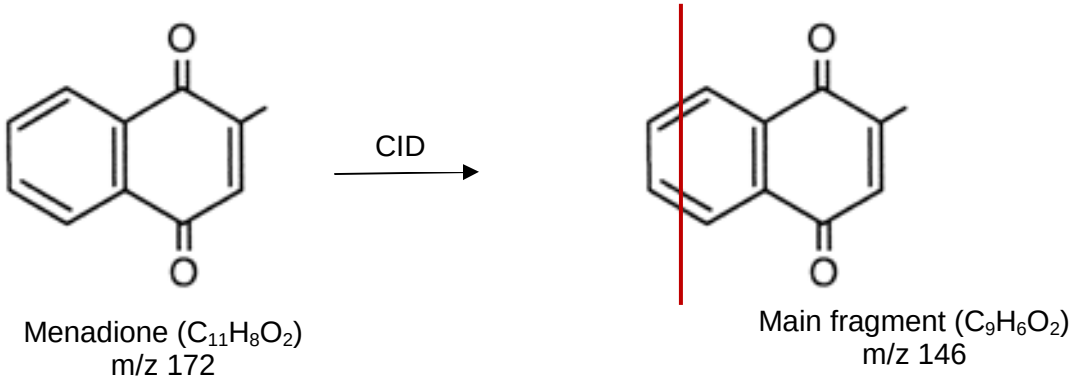

Figure-1 Hypothesized pathway for menadione fragmentation in LC-APCI-MS/MS.

5.1.8.1 LC-APCI-MS/ MS system specifications

1) Instrument: AB SCIEX QTRAP® 5500 MS/MS with an APCI source connected to an Agilent series 1200 HPLC instrument.
2) Column: ProntoSil C_{30} column (5 μm, 250 mm × 4.6 mm) (MAC-MOD Analytical Inc., Chadds Ford, PA).
3) Mobile phase: Mobile phase consisted of 100% solvent A (methanol). Flow rate was set to be 0.60 mL/minute. One cycle was 24 minutes.
4) The MS detector conditions: The MS/MS ion source was negative APCI with temperature set at 400 ℃.
5) Multiple Reaction Monitoring (MRM) was used to detect isotopomers of parent compounds and fragments of menadione [m/z (parent: fragment) 172:146] and deuterium-labeled menadione [m/z (parent: fragment) 173:147, 174:148, 175: 148].
6) Data were collected using Analyst 1.5.1. (**Table-2**. LC-APCI-MS/MS conditions)

5.1.8.2 Sample preparation

In order to prepare samples from different matrices, follow sample preparation steps in previous sections as follows:

1) Total menadione in human urine: follow sample preparation steps in section 5.1.2.1.
2) Unconjugated menadione in Fischer 344 rat urine: follow sample preparation steps in section 5.1.3.1.1, except:
3) Delete steps 1-3 from section 5.1.2.1.2 which relate to hydrolysis and neutralization.
4) Unconjugated menadione in serum: follow the steps for “Sample preparation” given in section 5.1.3.1.1, except:

- Use Serum instead of rat urine.
- Delete steps 1-3 from section 5.1.2.1.2.

5) Unconjugated menadione in tissues: follow sample preparation steps in section 5.1.5.1.

Period 1:		
Scans in Period	713	
Relative Start Time	0.00msec	
Experiments in Period	1	
Period 1 Experiment 1:		
Scan Type	MRM	
Scheduled MRM	No	
Polarity	Negative	
Scan Mode	N/A	
Ion Source	Heated nebulizer	
Resolution Q1	Unit	
Resolution Q3	Unit	
Intensity Thres.	0.00 cps	
Settling Time	0.0000 msec	
MR Pause	5.0070	
MCA	No	
Step Size	0.00 Da	
Q1 Mass (Da) 172	Q3 Mass (Da) 146	Dwell (msec) 500.00
Q1 Mass (Da) 173	Q3 Mass (Da) 147	Dwell (msec) 500.00
Q1 Mass (Da) 174	Q3 Mass (Da) 148	Dwell (msec) 500.00
Q1 Mass (Da) 175	Q3 Mass (Da) 149	Dwell (msec) 500.00
Parameter Table (Period 1 Experiment 1):		
CUR	10	
TEM	400	
GS1	30.00	
GS2	0.00	
NC	-5.00	
CAD	Medium	
DP	-80.00	
EP	-10.00	
CE	-35.00	
CXP	-13.00	

Table-2 LC-APCI-MS/MS conditions.

5.1.9 Medium preparation for Caco-2 cell culture study

All supplies and equipment used for the Caco-2 cell culture study must be sterile. Study is conducted under strict sterile conditions.

5.1.9.1 Supplies

1) Dulbecco's Modification of Eagle's Medium (DMEM) with high glucose (4,500 mg/liter glucose) and L-glutamine, without sodium pyruvate. (Cellgro, cat. no. MT-10-017-CV)
2) Heat inactivated fetal bovine serum (FBS). (BenchMark, cat. no. 100-106)
3) MEM Non-Essential Amino Acids Solution (100X). (HyClone, cat. no. SH30238.01)
4) Penicillin-Streptomycin solution (100X PEST solution) (10,000 unit/ mL penicillin, 10,000 μg /mL streptomycin). (HyClone, cat. no. SV30010)

5.1.9.2 Method

1) Place DMEM and supplements into 37 ℃ water bath. Warm to temperature for approximately 30 min.
2) Two versions of DMEM are required:
    a) 20% FBS in DMEM (DMEM-20), preparation:
        i) 500 mL DMEM.
        ii) 100 mL FBS.
        iii) 5 mL non-essential amino acids (100X).
        iv) 5 mL PEST solution (100X).
    b) 10% FBS in DMEM (DMEM-10), preparation:
        i) 500 mL DMEM.
        ii) 50 mL FBS.
        iii) 5 mL non-essential amino acids (100X).

iv) 5 mL PEST solution (100X).

3) Label bottle: “DMEM-20”, or “DMEM-10”, 1% PEST, date and initials.
4) Store media in refrigerator (4 ºC) until time of use. Media could be stored for at least 3 months.

5.1.10 Resuscitation of frozen Caco-2 cells

It is important to handle frozen ampoules with care; wear a laboratory coat and full protective gloves.

5.1.10.1 Supplies

1) DMEM-20.
2) DMEM-10.
3) 70% ethanol.
4) Trypsin-Versene mixture: 0.25% (w/v) Trypsin-0.03% (w/v) versene (EDTA). (BioWhittaker, cat. no. BW17-161F)
5) Phosphate Buffer Solution without calcium or magnesium (PBS, 1X). (BioWhittaker, cat.no. BW17-517Q)
6) 15mL centrifuge tubes. (Corning, cat.no.430052)
7) 25 cm^2 sterile tissue culture flasks (BD, cat. no. 353808)
8) 75 cm^2 sterile tissue culture flasks (BD, cat. no. 13-680-65)

5.1.10.2 Method

- Day 1:

1) Heat DMEM-20 to 37 ºC in water bath.

2) Quickly transfer the frozen ampoule to a 37 ºC water bath until only one or two small ice crystals, if any, remain (1-2 minutes). It is important to thaw rapidly to minimize any damage to the cell membranes.
3) Wipe ampoule with a tissue soaked in 70% ethanol prior to opening.
4) In laminar flow hood, slowly pipette the whole content of the ampoule into a 15 mL centrifuge tube containing 5mL pre-warmed DMEM-20. Centrifuge for 5 minutes at 1,000 rpm. (Controlling the temperature is not necessary)
5) Remove supernatant.
6) Vigorously resuspend Caco-2 cell pellet by vortex with 5mL DMEM-20.
7) Pipette the whole cells into a 25 cm^2 flask containing 10 mL pre-warmed DMEM-20.
8) Incubate at 37 ºC and 5% CO_2.

- Day 4-11:

9) Feed cells by changing DMEM-20 approximately every three days until the cells reach 80-90% confluence (the 12-15th day). For details about feeding cells refer to the f cells maintenance section. (Section 5.1.11)

- Day 12:

10) Heat trypsin-versene solution and DMEM-10 to 37 ºC in water bath.
11) In laminar flow hood, aspirate media with glass Pasteur pipette connected to house vacuum line.
12) Rinse with PBS (5 mL per 25 cm^2 flask), incubate for 5 minutes and decant PBS.
13) Add the trypsin-versene solution (3 mL per 25 cm^2 flask); rinse the cells and pour off the majority of the trypsin-versene so that only about 500 μL remains in the flask; the

remaining trypsin-versene must wet the entire cell layer. Incubate the flask (closed lid) at 37 ºC for 5–10 minutes (use the shortest time possible).

14) Check the detachment of the cells from the plastic surface by mildly knocking the sidewall of the flask with your palm.

15) As soon as the cells are detached, immediately stop trypsinization by resuspending the cells in 5 mL DMEM-10. Reduce clumping by forcefully pipetting mixture against side of flask 4-5 times.

16) Add 15 mL DMEM-20 into a new 75 cm^2 flask.

17) Pipette whole Caco-2 cells to the75 cm^2 flask.

18) Label and incubate at 37 ºC.

- Day13-22:

19) Feed cells by changing medium approximately once every two days until the cells reach to 80-90% confluent (the 20th-22nd day)

- Day 23:

20) After day 23, the cell maintenance begins.

5.1.11 Caco-2 cells maintenance (Feeding cells)

5.1.11.1 Supplies

1) DMEM-10.

5.1.11.2 Method

Cells feeding is to take place approximately every two days.

1) Heat DMEM-10 to 37 ºC in water bath.

2) In laminar flow hood, aspirate media with glass Pasteur pipette connected to house vacuum line.
3) Add 20 mL of DMEM-10.
4) Tightly cap and return to 37 ºC incubator with 5% CO_2.

5.1.12 Passing Caco-2 cells

Trypsinize Caco-2 cells from maximally (80-90%) confluent Caco-2 cultures: about 6-7 days post previous passing.

5.1.12.1 Supplies

1) DMEM-10.
2) Trypsin-Versene mixture: 0.25% (w/v) Trypsin-0.03% (w/v) versene (EDTA). (BioWhittaker, cat. no. BW17-161F)
3) Phosphate Buffer Solution without calcium or magnesium (PBS, 1X). (BioWhittaker, cat.no. BW17-517Q)
4) 50 mL centrifuge tube. (Corning, cat.no.40290)
5) 75 cm^2 sterile tissue culture flasks (BD, cat. no. 13-680-65)

5.1.12.2 Method

1) Heat trypsin-versene solution and DMEM-10 to 37 ºC in water bath.
2) In laminar flow hood, aspirate media with glass Pasteur pipette connected to house vacuum line.
3) Rinse with PBS (10 mL per 75 cm^2 flask), incubate for 5 minutes and decant PBS.
4) Add the trypsin-versene solution (5mL per 75 cm^2 flask); rinse the cells and pour off the majority of the trypsin-versene so that only about 500 μl remains in the flask; the

remaining trypsin-versene must wet the entire cell layer. Incubate the flask (closed lid) at 37 ºC for 5–10 minutes (use the shortest time possible).

5) Check the detachment of the cells from the plastic surface by mildly knocking the sidewall of the flask with your palm.
6) As soon as the cells are detached, immediately stop trypsinization by resuspending the cells in 10 ml DMEM-10. Reduce clumping by forcefully pipetting mixture against side of flask 4-5 times.
7) Pipette total volume into 50 mL centrifuge tube and vortex.
8) Add 19 mL DMEM-10 to each new 75 cm^2 flask.
9) Add 1 mL of Caco-2 cells to each flask.
10) Label and incubate at 37 °C. Feed cells by changing medium approximately once every two days.

5.1.13 Caco-2 cells preservation

5.1.13.1 Supplies

1) DMEM-10.
2) Trypsin-Versene mixture: 0.25% (w/v) Trypsin-0.03% (w/v) versene (EDTA). (BioWhittaker, cat. no. BW17-161F)
3) Phosphate Buffer Solution without calcium or magnesium (PBS, 1X). (BioWhittaker, cat.no. BW17-517Q)
4) 15 mL centrifuge tube. (Corning, cat.no.430052)
5) Dimethyl sulfoxid (DMSO)
6) 1.5 mL ampoules. (Nunc, cat. no. 377224)

5.1.13.2 Method

1) Heat trypsin-versene solution and DMEM-10 to 37 ºC in water bath.
2) In laminar flow hood, aspirate media with glass Pasteur pipette connected to house vacuum line.
3) Rinse with PBS (10 mL per 75 cm^2 flask), incubate for 5 minutes and decant PBS.
4) Add the trypsin/ versene solution (5 mL per 75 cm^2 flask); rinse the cells and pour off the majority of the trypsin/ versene so that only about 500 μL to 1 mL remains in the flask; the remaining trypsin must wet the entire cell layer. Incubate the flask (closed lid) at 37 ºC for 5-10 minutes (use the shortest time possible).
5) Check the detachment of the cells from the plastic surface by mildly knocking the sidewall of the flask with your palm.
6) As soon as the cells are detached, immediately stop trypsinization by resuspending the cells in 5 mL DMEM-10. Reduce clumping by forcefully pipetting mixture against side of flask 4-5 times.
7) Pipette total volume into 15 mL centrifuge tube. Centrifuge for 5 minutes at 1,000 rpm.
8) Remove supernatant.
9) Vigorously resuspend Caco-2 cell pellet by vortexing with 2mL DMEM-10 containing 10 % DMSO (2ml for one 75 cm^2 flask).
10) Pipette 1 mL into each 1.5 mL ampoules.
11) Place ampoules in 4 ºC for 10 minutes, then move them into -20 ºC for 30 minutes, followed by -80 ºC for 16-24 hours. Cell can be stored in liquid nitrogen forever.

5.1.14 Cultivation of Caco-2 cells monolayer grown on permeable supports [3]

5.1.14.1 Timing

1) Start 21–29 day in advance of actual experiment.
2) Trypsinize Caco-2 cells from maximally (80-90%) confluent Caco-2 cultures: about 6-7 days post previous passing.

5.1.14.2 Supplies

1) DMEM-10.
2) DMEM-20.
3) Trypsin-Versene mixture: 0.25% (w/v) Trypsin-0.03% (w/v) versene (EDTA). (BioWhittaker, cat. no. BW17-161F)
4) Phosphate Buffer Solution without calcium or magnesium (PBS, 1X). (BioWhittaker, cat.no. BW17-517Q)
5) 50 mL centrifuge tube. (Corning, cat.no.40290)
6) 75 cm^2 sterile tissue culture flasks (BD, cat. no. 13-680-65)
7) Transwell permeable supports, polycarbonate membrane. (12-well plates, 12 mm diameter, 0.4μm pore size; Corning Costar Corp. Cambridge, MA. Cat. no. 3401)

5.1.14.3 Method

1) Heat trypsin-versene solution, DMEM-10 and DMEM-20 to 37 ºC in water bath.
2) In laminar flow hood, aspirate media with glass Pasteur pipette connected to house vacuum line. Alternatively, remove the medium by aseptical decantation.
3) Rinse with PBS (10 mL per 75 cm^2 flask), incubate for 5 minutes and decant PBS.
4) Add the trypsin-versene solution (5 mL per 75 cm^2 flask); rinse the cells and pour off the majority of the trypsin-versene solution so that only about 500 μl to 1 mL remains

in the flask; the remaining trypsin must wet the entire cell layer. Incubate the flask (closed lid) at 37 ºC for 5–10 minutes. Use the shortest time possible.

5) Check the detachment of the cells from the plastic surface by mildly knocking the sidewall of the flask with your palm.
6) As soon as the cells are detached, immediately stop trypsinization by resuspending the cells in 10 mL DMEM-10. Reduce clumping by forcefully pipetting mixture against side of flask 4-5 times.
7) Transfer total volume to a test tube and allow the debris and large cell aggregates (if any) to sediment.
8) Transfer the supernatant to a new test tube.
9) Take an aliquot and count the cells. The percentage of dead cells must not exceed 5%. This is assessed by counting the total number of cells as well as the number of non-viable cells.
10) Spin down the cells (5 minutes at 1,000 rpm) and remove the supernatant.
11) Resuspend the cells in DMEM-10 at a concentration of 5×10^5 cells/mL.
12) Pre-wet the filters (12 mm diameter) with about 0.1 mL of DMEM-20 (for at least 2 minutes) before seeding the cells.
13) Seed by dispensing 0.5 mL of the resuspended cell solution on each filter. Use a seeding density of 5×10^5 cells/ cm^2 (250,000 cells for each of the 12 mm diameter filter).
14) Fill the basolateral chamber with 1.5 mL DMEM-20
15) Incubate the plate with the filter supports at 37 ºC and 5% CO_2 in a humid atmosphere for 6 hours (if seeding is done in the morning) or overnight (16 hours) if seeding is done at the end of the day. The length of this incubation should not exceed 16 hours.

16) Remove the apical medium and replace with 0.5 mL of DMEM-20. This step is done to remove non-adherent cells and to reduce the risk of multilayer formation.
17) Maintain cells, every second day, as follows:
    a) Aspirate the medium from the basolateral side of all wells and then carefully and slowly from the apical side (of all filters in a plate).
    b) Replace the aspirated medium with fresh DMEM-20, first in the apical compartments and then in the basolateral compartments; the volumes used for 12-well plates are 1.5 ml for the basolateral side and 0.5 ml for the apical side. Do not touch the filter surface with the pipettes! Slow pipetting and avoiding physical contact between the pipette tip and the monolayer are essential for maintenance of monolayer integrity.
18) Repeat Step 17 every second day for a total of 21–29 days.
19) Change the culture medium 12–24 hours before the experiment. Longer periods without feeding before starting the experiment should be avoided because the cells may have consumed the essential nutrients and adapted to a more starved phenotype.

5.1.15 Testing the integrity of Caco-2 cells monolayer grown on permeable support

Before the transport experiments on Transwells, the integrity of the cell monolayer was tested by determining the diffusion of phenol red from the apical side to the basolateral side. The detail was described somewhere else [4].

5.1.16 Treating differentiated Caco-2 cells with vitamin K

Caco-2 cells (passages 20-40) were obtained from the American Type Culture Collection (Rockville, MD) and grown as described previously. Differentiated Caco-2 cells that were

grown to form a monolayer, were subjected to different treatments (MK-4, phylloquinone or menadione). Caco-2 cells were shown to secrete chylomicron-like particles after supplementation of oleic acid (OA) and Taurocholate (TA) [5].

5.1.16.1 Supplies

1) Phylloquinone (Other forms, such as MK-4 or menadione can be substituted based on the experiment).
   Concentration=10 mM phylloquinone in methanol.
2) Tween® 40 (Tween)(Sigma, P1504). Density: 1.09 g/mL at 20 °C.
3) Oleic acid (OA). (Sigma-Aldrich. Product # 01008). $C_{18}H_{34}O_2$. MW: 282.5 g/mole. Density: 0.891 gm/mL.
   Notice: Avoid multiple freezing/thawing of OA. Store OA in separate, single usage vial at -20 ℃.
4) Taurocholic acid sodium salt hydrate (TC). (Sigma. Product # T4009). $C_{26}H_{44}NO_7SNa\ xH_2O$. MW: 537.7 g/mol.
5) Autoclaved Di H_2O.
6) Serum-free media (SFM), preparation:
   i. 500 mL DMEM.
   ii. 5 mL non-essential amino acids (100X).
   iii. 5 mL PEST solution (100X).
7) Trypsin-Versene mixture: 0.25% (w/v) Trypsin-0.03% (w/v) versene (EDTA). (BioWhittaker, cat. no. BW17-161F)
8) Autoclaved Phosphate Buffer Solution without calcium or magnesium (PBS, 1X). (BioWhittaker, cat.no. BW17-517Q)
9) Sterile 0.22 μm disposable syringe filters (Millex-GV, Millipore)
10) Sterile Syringes.

5.1.16.2 Methods

1) Preparation of OA: TC Stock solution [6].

    a. Target concentration= 32:10 mM OA:TC in DI H_2O

    b. Weigh and dissolve 53.768 mg TC in 10 mL DI H_20

    Calculations: M= moles/L, MW=g/mole

    M= (g/MW)/ L = (53.768 mg/ 537.7 mg/mmol)/ 10 mL

    = 0.01 mmole/ mL = 0.01 mole/L = 10 mM

    c. Add 101.5 μL OA to TC solution (10mL)

    Calculations: M= moles/L, MW=g/mole, Density= g/L

- OA's density= 0.891 g/mL (0.891 mg/μL)

    Weight of OA used= 0.891 mg/μL X 101.5 μL = 90.44 mg

- OA's MW= 282.5 g/mole (mg/mmole)

    mmole of OA used= 90.44 mg / (282.5 mg/mmole)

    =0.320 mmole

- M=mole/L= mmole/mL= 0.320 mmole/10 mL= 0.032 mmole/ mL

    =32 mmole/L= 32 mM

    d. After adding OA, mix by gentle swirling and incubate at 37 ℃ until clear solution is achieved.

    e. Final concentrations of OA:TC Stock solution= 32:10 mM OA:TC in DI H_2O

2) Preparation of OA:TC solution in SFM:

    a. Dilute OA: TC Stock solution (32:10 mM OA:TC in Di H_2O) in SFM, 1:20 (v:v).

    b. Final concentration= 1.6:0.5 mM OA:TC in SFM.

3) Preparation of Tween solution in acetone [6].

    a. Final target concentration= 29μg/ mL SFM

b. Weigh and dissolve 29mg Tween in 1 mL Acetone. Volume of acetone used can be adjusted to accommodate amount of Tween weighed, giving that the final ratio remains 29mg Tween/ mL Acetone

4) Preparation of phylloquinone+ Tween solution in SFM

a. Pipette 400 μL of phylloquinone (concentration= 500 μM) + 20μL of Tween in acetone to a test tube. Mix and dry under a gentle stream of nitrogen.

b. Reconstitute with SFM. Final volume=20 mL.

c. Calculations:

i. Phylloquinone

$M_1V_1=M_2V_2$

(500 μM)*(0.4mL) = M_2*(20mL)

M_2= 10 μM phylloquinone in SFM

ii. Tween

29mg Tween/ 1000 μL= 580μg Tween/ 20μL

580μg Tween/20 mL= 29μg Tween/ mL SFM

5) Caco-2 cells treatment

a. Use differentiated Caco-2 cells grown on permeable support for 21 days.

b. Wash cells by cold PBS three times to remove any remaining FBS.

c. Sterilization: Use 0.22μm disposable syringe filters to sterilize different treatment solutions before adding them to the cells.

d. Add 0.5 mL of phylloquinone + Tween solution in SFM (10 μM phylloquinone + 29μg/mL Tween solution in SFM) to the apical side of differentiated Caco-2 cells.

e. To the basolateral side, add 1.5mL of 1.6:0.5 mM OA:TC in SFM

f. Treatment period: 24 hours

g. At certain indicated time points (1 hr, 2 hours, etc.), collect the basolateral medium (basolateral medium and filter insets should be transfer into new clusters containing the 1.5mL 1.6:0.5 mM OA:TC in SFM
h. At the end of experiments, wash cells by cold PBS three times, and then digest cells by 0.5mL Trpysin for 10 minutes at 37 ℃. S onicate cells for 1 minute on ice.
i. Store the homogenized cells and BM at -20 ℃ for further analysis. The protein concentration of the cell lysate is determined with a Coomassie Plus Assay Kit (Thermo).

5.2 Abstracts

5.2.1 Measurement of Menadione in Urine by HPLC

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Abstract

Mammals convert phylloquinone to MK-4, with menadione as a possible intermediate. We developed and validated a method measuring urinary menadione. A high performance liquid chromatography (HPLC) method with a C30 column, fluorescence detection and post-column zinc reduction was developed. The mobile phase was composed of 95 % methanol and 5 % DI H_2O. Aqueous solution (2 M zinc chloride, 1 M acetic acid, and 1 M sodium acetate) was added to both methanol and H_2O. MK-2 was used as an internal standard. The standard calibration curve was linear with a correlation coefficient (R^2) of 0.99 for both menadione and MK-2. Sample preparation involved hydrolysis of menadiol conjugates and oxidizing the released menadiol to menadione. Menadione was then extracted using iso-octane. Drying extraction solvent prior to HPLC injection resulted in high menadione losses. This was resolved by avoiding complete evaporation. We were able to detect < 0.05 pmole menadione/ injection. Menadione was detected in archived urine samples from subjects receiving 500 µg/d phylloquinone. The assay was validated by "spiking recovery" and by removing the zinc column and observing the disappearance of the menadione peak. This HPLC method presents a

sensitive and reproducible way to detect menadione in urine, and can be used to elucidate the role played by menadione in MK-4 formation.

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5.2.2 Menadione is an Intermediate in the Phylloquinone to Menaquinone-4 Conversion

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Abstract

Objective: Phylloquinone (phylloquinone; MW 451) is converted into menaquinone-4 (MK-4; MW 445) via side- chain removal. Direct evidence that menadione (MD; MW 172), the naphthoquinone ring without the side chain, is the conversion intermediate is lacking.

Methods: Following a 14 day phylloquinone-deficient diet, male Fischer 344 rats (8-mo; n=15) were fed deuterium-labeled (-d) collard greens as source of 1.6 mg phylloquinone-d [MW 459-463 (64% of total)] per kg diet for 0 (control), 1 and 7 days.

Results: Using LC/MS, phylloquinone-d and MK-4-d (MW 446-449) were detected in tissues after 1 and 7 days. The liver contains phylloquinone-d (209.7±111.8 pmol/g on day 7) but no MK-4-d; in contrast, the salivary gland and brain contained MK-4-d (757.2±111.8 and 38.6±8.3 pmol/g, respectively on day 7) but no phylloquinone-d. MK-4-d carried the d-label on the naphthoquinone ring, but not on the side chain, confirming the need for side chain removal for its formation. Using LC/MS, unconjugated MD-d (MW 173-174) was detected in urine. Using LC-MS/MS, unconjugated MD-d was detected in

serum of rats (n=4/5 at day 1; n= 3/5 at day 7). There was no detected phylloquinone-d, MK-4-d, or MD-d in the controls.

Conclusions: This is the first time unconjugated MD in biological materials has been detected. This is the first direct evidence that MD is an intermediate in the phylloquinone to MK-4 conversion. Future studies are required to determine the function of this conversion.

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