Vitamin K1 pharmacokinetics in a clinical study and VKORC1 enzyme kinetics using HPLC methodology

Inaugural-Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

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Bonn, Juni 2012

Diese Arbeit entstand in der Arbeitsgruppe von Herrn Prof. Dr. Johannes Oldenburg am Institut für Experimentelle Hämatologie und Transfusionsmedizin mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn.

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Tag der Disputaion: 12. Februar 2013
Erscheinungsjahr: 2013

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“Nihil tam difficile est,
quin quaerendo investigari possit.“

Nichts ist so schwierig, dass es nicht erforscht werden könnte.

Terenz

für meinen Bruder

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LIST OF ABBREVIATIONS

7αOH	7α-hydroxycholesterol
ABCC6	ATP-binding cassette sub-family C member 6
ALAT	Alanin-aminotransferase
ALTM	All laboratory trimmed mean
ANOVA	Analysis of variance
AP	Alkaline phosphatase
ApoA	Apolipoprotein A
ApoB-100	Apolipoprotein B-100
ApoB-48	Apolipoprotein B-48
ApoC	Apolipoprotein C
ApoE	Apolipoprotein E
APTT	Activated partial thromboplastin time
ASAT	Aspartat-aminotransferase
AUC	Area under the curve
AUMC	Area under the first moment curve
BfArM	Bundesinstitut für Arzneimittel und Medizinprodukte
BHT	Butylated hydroxytoluene
BMD	Bone mineral density
BMI	Body mass index
BSA	Bovine serum albumin
Ch	Cholesterol
CL	Clearance
CM	Chylomicrons
C_{max}	Maximum serum/ plasma concentration
C_{nom}	Nominal concentration
CR	Chylomicron remnants
CV	Coefficient of variation
CYP4F2	Cytochrome P450 4F2
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleotide acid
dNTP	Deoxynucleotide-triphosphate
EMEA	European Medicines Agency
FBS	Fetal bovine serum
FL	Fluorescence
F	Bioavailability
GC-FID	Gas chromatography-flame ionization detection
GC-MS	Gas chromatography-mass spectrometry
GGCX	γ-glutamyl carboxylase
Gla	γ-carboxyglutamate
Glu	Glutamate
HCC	Hepatocellular carcinoma

HDL	High density lipoprotein
HDL-C	High density lipoprotein-cholesterol
HEK cells	Human embryonic kidney cells
HPLC	High performance liquid chromatography
i.v.	intravenous
ICH	International Conference of Harmonization
IDL	Intermediate-density lipoprotein
INR	International Normalized Ratio
ISTD	Internal Standard
K1O	Vitamin K1 epoxide
K2O	Vitamin K2 epoxide
KEQAS	Vitamin K Quality Assurance Scheme
KH_2	Vitamin K hydroquinone
K_m	Michaelis-Menten constant
KO	Vitamin K epoxide
LB	Lysogeny broth
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein-cholesterol
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
LPL	Lipoprotein lipase
MANOVA	Multivariate analysis of variance
MEM	Minimal essential medium
MGP	Matrix Gla protein
MK	Menaquinone
MK4	Menaquinone-4
MK4O	Menaquinone-4 epoxide
MK7	Menaquinone-7
MK8	Menaquinone-8
MM	Mixed micelles
MRT	Mean residence time
MSD	Mass selective detector
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
p.o.	per os
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIVKA-II	Proteins induced in the vitamin K absence or antagonism-factor II
PT	Prothrombin time
PXE	Pseudoxanthoma elastica
QR	Quinone reductase
RP	Reversed phase

Rpm	Revolutions per minute
SIM	Selected ion monitoring
SNP	Single nucleotide polimorphism
SPE	Solide phase extraction
$t_{1/2}$	Elimination half-life
TG	Triglycerides
t_{max}	Time of the maximum serum/ plasma concentration
TMS	Trimethylsilyl
tOC	Total osteocalcin
TRL	Triglyceride-rich lipoproteins
ucOC	Uncarboxilated osteocalcin
UV	Ultraviolet
VKD	Vitamin K-dependent
VKDB	Vitamin K deficiency bleeding
VKDPs	Vitamin K-dependent proteins
VKOR	Vitamin K epoxide reductase
VKORC1	Vitamin K epoxide reductase complex subunit 1
VKORC1L1	Vitamin K epoxide reductase complex subunit 1-like 1
v/v	Volume per volume
VLDL	Very low density lipoproteins
V_{max}	Maximum enzyme velocity
V_{ss}	Volume of distribution at steady state
w/v	Weight per volume
w/w	Weight per weight
γ-GT	γ-glutamyl transferase

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1. INTRODUCTION

1.1. Biological role of vitamin K

Vitamin K is an essential group of fat-soluble vitamins needed for a unique posttranslational chemical modification of proteins with calcium-binding properties, collectively known as vitamin K-dependent proteins (VKDPs) or Gla-proteins. Vitamin K is mostly required for blood coagulation but is also involved in metabolic pathways in bone and other tissue.

1.1.1 Discovery, structure

Discovery

Vitamin K was discovered in the early 1930s by the Danish biochemist Henrik Dam by investigating the role of cholesterol by feeding chickens a cholesterol-depleted diet. After several weeks, the animals developed haemorrhages and started bleeding which could not be restored by adding purified cholesterol to the diet. In 1935, Dam was able to describe the nature of this antihaemorrhagic factor whose absence was responsible for bleeding [1], and he proposed to name the new factor vitamin K (for Koagulation from the German and Scandinavian languages). Structure determination of vitamin K1 was performed by Doisy [2,3]. Dam was awarded the Novel Prize in Physiology or Medicine in 1943 for the discovery of vitamin K; together with Doisy for the purification, characterization and synthesis of the molecule.

Structure

Compounds possessing the classical vitamin K cofactor activity for the conversion of specific peptide-bound glutamate (Glu) residues to γ-carboxyglutamate (Gla) share a common methylated naphthoquinone ring structure (2-methyl-1,4-naphthoquinone) called *menadione* or *vitamin K3* (Fig.1; I) and vary in the aliphatic side chain attached at position C3 (e.g. Fig.1; II, IV, V). In nature, this 3-substituent has an isoprenoid structure with varying length and degrees of saturation. Plants and cyanobacteria synthesize only one chemical form called phylloquinone or vitamin K1 containing four isoprenoid residues, one of which is unsaturated and three are saturated (Fig.1; II). All other bacteria that possess the ability for vitamin K synthesis produce a variety of isoprenologues called *menaquinones* or *vitamin K2* [4]. Menaquinones (MK) have side chains composed of a variable number of unsaturated isoprenoid residues; generally

these are designated as MK-n according to the number of prenyl units. Bacterially synthesized menaquinones (Fig.1; IV, V) that may contribute to human vitamin K requirements, either synthesized by the gut flora or present in foods, generally have side chains with 4-12 prenyl units [5-8].

Naphthoquinone is generally considered as the functional group, so that the mechanism of action is similar for all K vitamins. Substantial differences may be expected, however, with respect to intestinal absorption, transport, tissue distribution and bio-availability, caused by the different lipophilicity of the various side chains and by the different food matrices in which they occur.

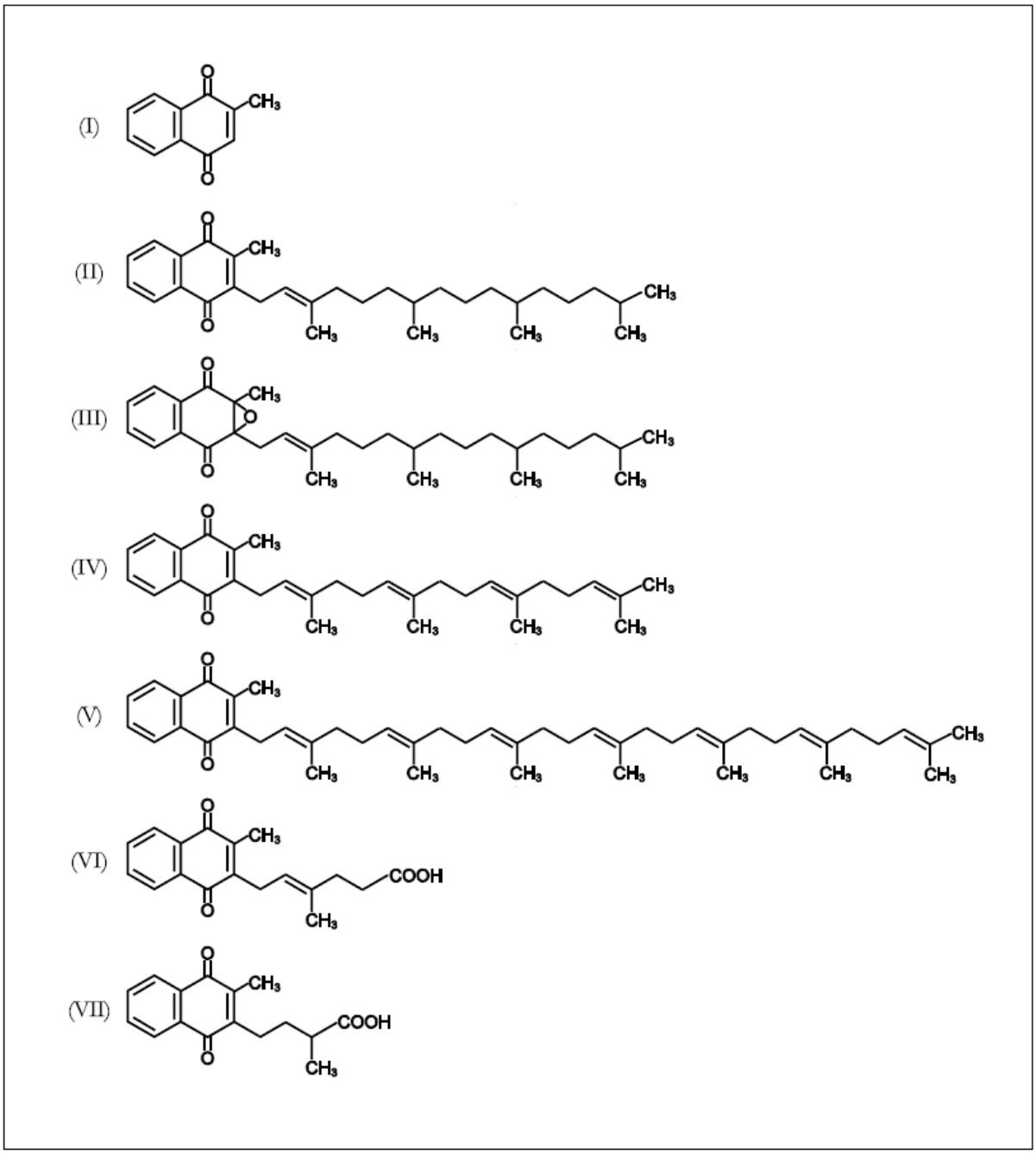

Figure 1 Chemical structure of vitamin K and metabolites [9]

(I) **Menadione; K3** (2-methyl-1,4-naphthoquinone)

(II) **Phylloquinone; K1** (2-methyl-3-phythyl-1,4-naphthoquinone)

(III) **Phylloquinone epoxide; K1O** (2-methyl-3-phythyl-1,4-naphthoquinone-2,3-epoxide)

(IV) **Menaquinone-4; MK4** (2-methyl-3-geranyl-geranyl-1,4-naphthoquinone)

(V) **Menaquinone-7; MK7** (2-methyl-3-farnesylgeranyl-geranyl-1,4-naphthoquinone)

(VI) 2-methyl-3-(5`-carboxy-3`-methyl-2`-pentenyl)-1,4-naphthoquinone

(VII) 2-methyl-3-(3`-carboxy-3`-methyl propyl)-1,4-naphthoquinone

1.1.2 Vitamin K dependent proteins

Vitamin K was discovered in association with a bleeding defect, about half of the known human vitamin K-dependent (VKD) proteins are circulating haemostatic blood proteins (factors VII, IX, X, prothrombin, proteins C, S and Z). Factor II, VII, IX and X are VKD procoagulant serin proteases while Protein C and S play a regulatory role in the inhibition of coagulation. Protein Z is suspected to have a haemostatic role as well. Haemostatic VKD proteins are mainly synthesized in the liver, however, several of these proteins are also expressed in other tissues and some have been shown to have additional roles beside haemostasis, e.g. protein C and S in inflammation [10]. Nonhaemostatic VKD proteins include: osteocalcin, an abundant bone matrix protein; Gas6, a ligand for tyrosine kinase receptors with multiple functions including growth control, chemotaxis and apoptosis [11-14]. Another nonhaemostatic VKD protein is matrix Gla protein, which inhibits calcification. Mice bearing a deletion of matrix Gla protein gene develop severe arterial calcification leading ultimately to death [15]. Humans lacking functional matrix Gla protein show calcification of cartilage without exhibiting the gross vascular calcification observed in mice [16]. A group of four transmembrane Gla proteins, homologue to VKD proteins by their structure, are suggested to have a role in signal transduction [17,18]. Moreover, the carboxylase itself is also carboxylated [19], which may regulate the carboxylation of other VKD proteins. Many of the VKD proteins show widespread tissue distribution, virtually every tissue expresses VKD carboxylase activity. Therefore, a broad biological impact of vitamin K is implicated by such a distribution and the various range of functions for VKD proteins. The VKD carboxylase is responsible for carboxylating all VKD proteins, and deletion of its gene leads to embryonic lethality in mice [20].

1.1.3 Vitamin K cycle

The biological role of vitamin K is to act as a cofactor for specific post-translational carboxylation that transforms selective glutamate (Glu) residues to γ-carboxyglutamate (Gla) residues [21,22] and is required for the activity of VKD proteins. The conversion of peptide-bound Glu to Gla residues in VKD proteins is linked to an enzymatic cycle, denoted by vitamin K cycle (Fig.2), which carries out both γ-glutamyl carboxylation and serves as a salvage pathway to recover vitamin K from its epoxide metabolite for reuse in carboxylation. The following enzymes are involved: γ-

glutamyl carboxylase (GGCX), vitamin K epoxide reductase (VKOR) and NAD(P)H-dependent quinone reductase (DT-diaphorase). The active form of vitamin K needed by the GGCX is the reduced form vitamin K hydroquinone. An obligatory metabolic consequence of γ- carboxylation is that hydroquinone is oxidased to epoxide which in turn undergoes reductive recycling, first to quinone and then to hydroquinone.

The activity of the VKOR can be blocked by warfarin and other coumarin-based drugs [23] which are synthetic derivatives of dicoumarol, a 4-hydroxycoumarin-derived mycotoxin anticoagulant originally discovered in spoiled sweet clover-based animal feeds. Dicoumarol, in turn, is derived from coumarin, a coagulation-inactive chemical found in sweet clover and many other plants. Warfarin and related coumarin-based drugs decrease blood coagulation by inhibiting the VKOR. This results in decreased concentrations of vitamin K and vitamin K hydroquinone in the tissues, such that the carboxylation reaction catalyzed by the GGCX and the production of active VKD clotting factors is inefficient as well as inadequate.

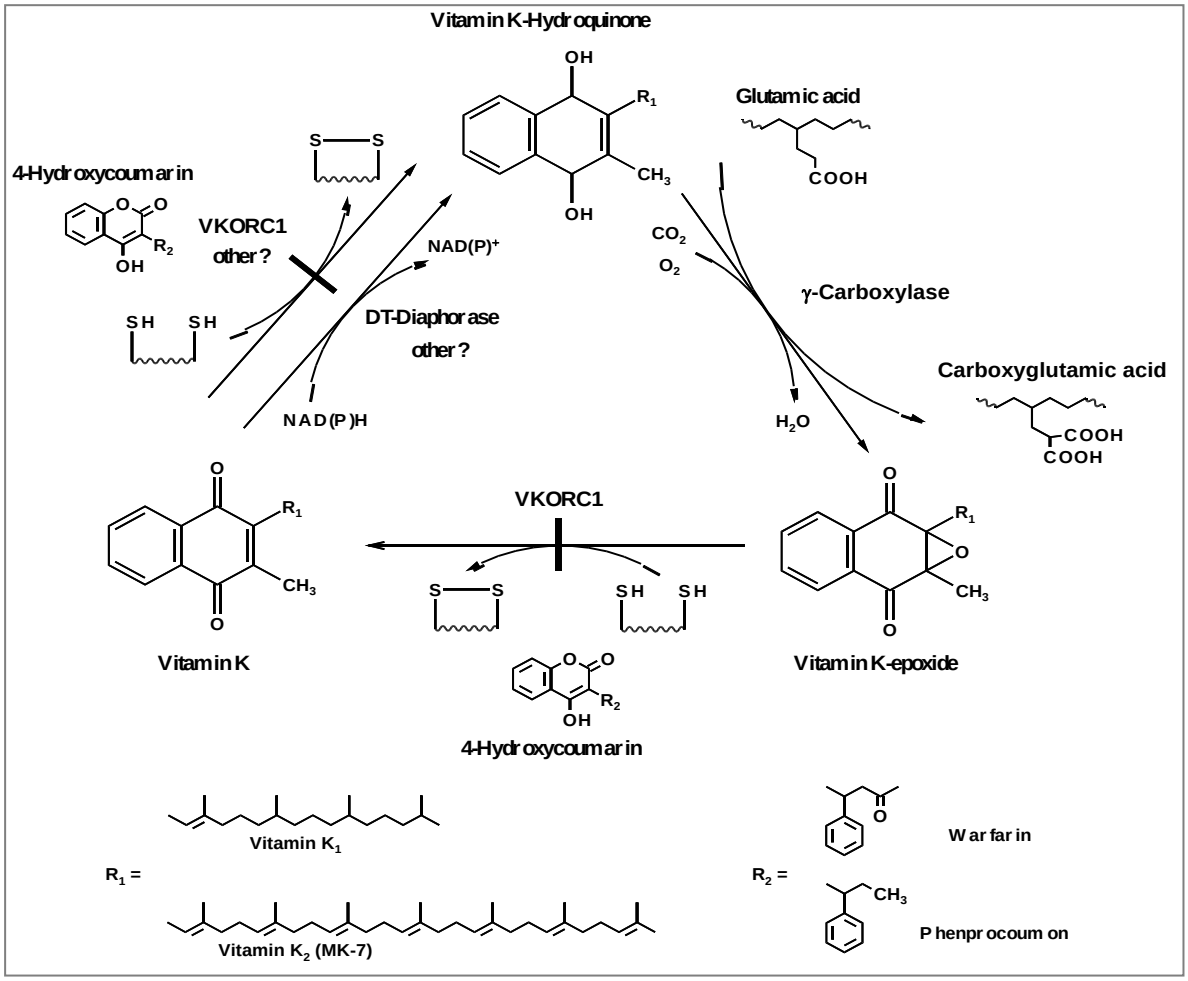

Figure 2 Vitamin K cycle [24]

1.1.4 Biochemical measures

Several determinants of the vitamin K status in the human body are known, though biochemical markers are more conclusive than dietary assessment alone.

- *Circulating concentrations of vitamin K*

Circulating phylloquinone concentrations in human plasma reflect dietary intake over the previous 24 h, and have high intra- and interindividual variation compared to other fat-soluble vitamins [25]. Less is known about the diet-plasma associations for menaquinones.

- *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 - prolongation is also indicative of hepatic dysfunction, a haematologic disease unrelated to vitamin K deficiency, as well as other acute or chronic conditions. PT becomes prolonged only when the prothrombin concentration drops below 50% of normal and therefore has a low sensitivity for detecting vitamin K deficiency [26].

- *Undercarboxylated VKD proteins*

Measurement of circulating undercarboxylated VKD proteins is currently considered to be a more sensitive indicator of vitamin K deficiency. Undercarboxylated prothrombin known as PIVKA-II (*p*roteins *i*nduced in the *v*itamin *K a*bsence or *a*ntagonism-factor *II*) detects abnormalities in prothrombin before the prolongation of PT. Most infants with vitamin K deficiency have elevated PIVKA-II concentrations. PIVKA-II has also been reported to increase in response to low-dose (1mg) warfarin [27] and vitamin K dietary restriction [28,29]. Another marker used for assessment of vitamin K status, presents the proportion of serum osteocalcin that is not carboxylated expressed as either % ucOC (uncarboxylated osteocalcin) or ucOC/ tOC (total osteocalcin). The high proportion of ucOC is indicative of poor vitamin K status.

- *Urinary measures*

Metabolites of vitamin K can be measured in urine and respond to dietary manipulation of vitamin K [30]. Measurement of urinary Gla excretion is an overall measure of VKD proteins because Gla cannot be recycled and is excreted in urine during the turnover of individual VKD proteins [31].

1.2. Determinants of vitamin K serum levels

To gain an insight into the multiple functions and impact of vitamin K in human health, it is important to identify determinants of the vitamin K status and its interindividual variation with regard to the current understanding of vitamin K physiology and metabolism. 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 haemorrhage due to vitamin K deficiency bleeding (VKDB) within the first few months of life. In contrast, dietary vitamin K deficiency in adults, without accompanying illness or predisposing factors, is rare.

1.2.1. Nongenetic determinants

1.2.1.1. Dietary intake of vitamin K

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 and flower vegetables including broccoli, spinach and certain lettuces contain the highest known phylloquinone concentrations and contribute ~60% of total phylloquinone intake [32,33]. Certain plant oils and margarine, spreads and salad dressings derived from these plant oils are also important dietary sources of phylloquinone [34]. Menaquinones (vitamin K2) are primarily of bacterial origin. Vitamin K2 is found in chicken egg yolk, butter, cow liver and cheese. Menaquinone-4 (MK4) 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 [35,36]. Menaquinone-7 (MK7) is primarily found in natto, a soybean product that is fermented using *Bacillus natto*.

The recommended daily intake for vitamin K according to the "*WHO expert consultation on human vitamins and mineral requirements"* is 100 µg/ day for males and 90 µg/ day for females.

Biomarkers of vitamin K status respond to phylloquinone restriction and supplementation. Interestingly, dietary restriction of phylloquinone to 35 µg/ day causes rapid decreases in plasma phylloquinone and urinary excretion of Gla residues and increases in the undercarboxylated forms of the VKD proteins, osteocalcin (% ucOC) and prothrombin (PIVKA-II), without affecting classic measurements of blood coagulation [29,37].

1.2.1.2. Absorption, transport and cellular uptake of vitamin K

Most diseases influencing vitamin K status are related to malabsorption or other gastrointestinal disorders including biliary atresia, cystic fibrosis, celiac disease and short bowel syndrome [38]. In common with other fat-soluble vitamins, phylloquinone is absorbed from the proximal intestine and the absorption is dependent on bile and pancreatic secretion [39]. In healthy adults the efficiency of absorption of phylloquinone in its free form is about 80% [39]. Vitamin K is not known to have a carrier protein; instead triglyceride-rich lipoproteins (TRL), primarily chylomicrons remnants and very low density lipoproteins (VLDL) are thought to be the main phylloquinone transporters [40-42]. Absolute plasma phylloquinone concentrations are higher among individuals with elevated triglyceride concentrations. Fasting plasma phylloquinone and triglyceride (TG) concentrations are both higher in older adults compared to younger adults [43,44], however, when phylloquinone concentrations are adjusted for TG, these are lower in the older adults compared to the younger individuals [44].

Most of the knowledge regarding intestinal absorption, cellular uptake and metabolic fate relates to phylloquinone with less known about menaquinones. Figure 3 shows a schematic illustration of the known metabolic processes that lead from the intestinal absorption of dietary vitamin K to its entry into cells [9] analogue to cholesterol transport. The following are key processes:

- ***Intestinal absorption and entry into circulation***

After digestion, dietary vitamin K in its quinone form and the products of triglycerides (TG) are emulsified by bile salts, mono – and diglycerides and lysolecithin to form mixed micelles. These are taken up by the enterocytes of the intestinal epithelium and incorporated into nascent chylomicrons (CM) containing apoA and apoB-48. CM are then secreted into the lymph lacteals within the intestinal villi. The lacteals drain into larger lymphatic vessels emptying into the blood circulation via the thoracic duct [45]. Once in the blood, CM acquire apoC and apoE from HDL. In the capillaries of muscle, adipose tissue etc., CM are stripped of their TG by lipoprotein lipase (LPL) which lines the capillaries. The resultant smaller CM remnants (CR) re-enter the circulation having lost much apoA and apoC (apolipoproteins) but retaining vitamin K in the lipophilic core.

- ***Uptake by liver***

In the liver CR enter hepatocytes by binding to LDLR and LRP followed by receptor mediated endocytosis. Their lipids are repackaged into VLDL containing apoB-100

and return to the circulation where they acquire apoC and apoE. Further TG is removed by LPL in the capillaries resulting in VLDL remnants called IDL. Subsequent metabolism and loss of apoC and apoE from IDL gives rise to smaller LDL particles containing almost exclusively apoB-100. Vitamin K is presumed to still be located in the lipophilic core.

- ***Uptake by bone***

Circulating lipoproteins such as CR and LDL can deliver lipids to osteoblasts which are attached to the surfaces of bone matrix. Osteoblasts express lipoprotein receptors such as LDL receptor (LDLR) and LDL receptor-related protein 1 (LRPI) which can interact with CR and LDL allowing receptor mediated endocytosis of the particles and their incorporated vitamin K. Evidence suggests that osteoblasts obtain most of their K1 via the CR pathway and most of their MK7 via the LDL pathway.

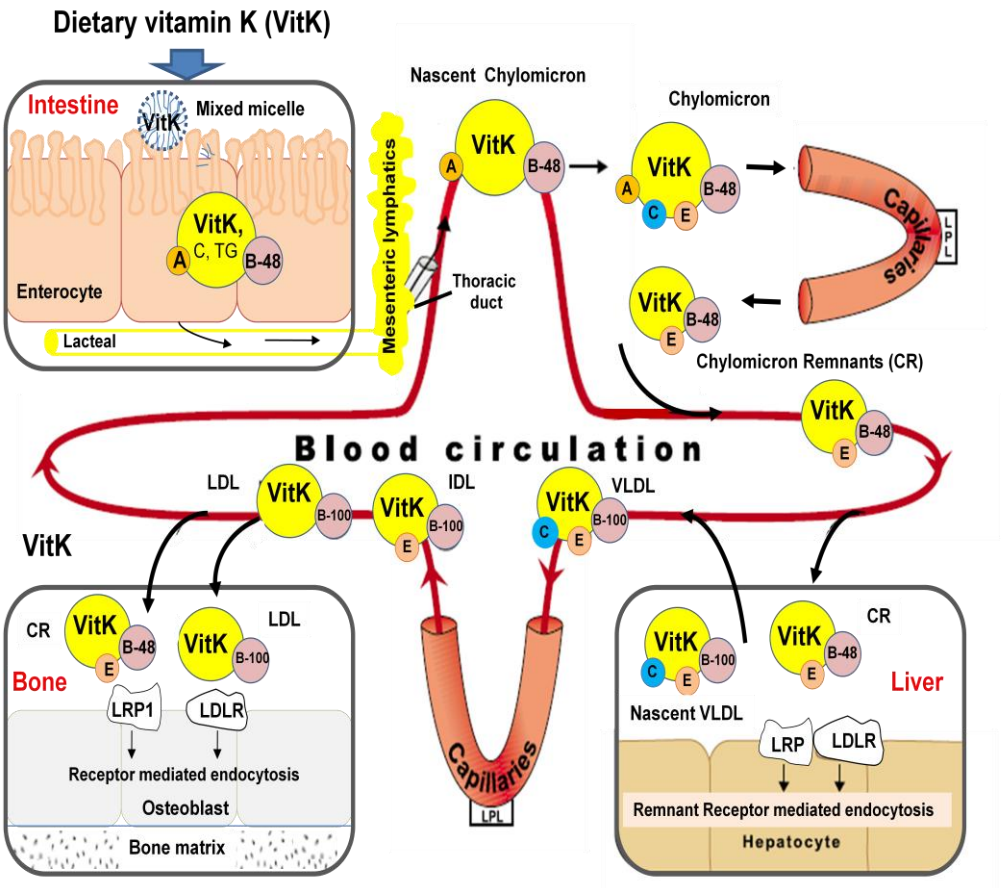

Figure 3 **Schematic representation of the absorption, transport and cellular uptake of dietary vitamin K (phylloquinone, MK7*)*** (modified from [9])

1.2.1.3. Tissue stores of vitamin K and distribution

Until the 1970s, the liver was the only known site of synthesis for VKD proteins and hence was presumed to be the only significant storage site for vitamin K. However, the discovery of VKD processes and proteins in a number of extra-hepatic tissues suggests that this may not be the case.

Human liver stores normally comprise about 90% menaquinones and 10% phylloquinone [46,47]. There is evidence that the phylloquinone liver stores are very labile: under conditions of severe dietary depletion liver concentrations were reduced to about 25% of initial levels after only 3 days [47]. This high turnover of hepatic reserves of phylloquinone corresponds with the high losses of vitamin K through excretion [39]. In contrast to the hepatic preference of long-chain menaquinones, the major circulatory form of vitamin K is invariably phylloquinone. The menaquinones MK7 and possibly MK8 are also present but the common hepatic forms MK 9-13 are not detectable in blood plasma [7,46]. This might be a consequence of a different route of absorption (a portal route for long-chain MKs *versus* the established lymphatic route for phylloquinone).

With the widespread occurrence of extrahepatic Gla proteins there is a need for knowledge of the extrahepatic distribution of K vitamins. In a study of human tissues obtained at autopsy, Thijssen *et al.* found that heart and pancreas contained phylloquinone at comparable or higher levels than found in the liver [48]. An intriguing finding was that MK4 was ubiquitously present in extrahepatic tissues with particularly high levels, often exceeding that of phylloquinone, in the brain, kidney and pancreas. The origin of tissue MK4 has yet to be exclusively defined. It is postulated that phylloquinone is locally converted into MK4 with menadione as an intermediate and accumulates in extrahepatic tissues [49].

1.2.1.4. Metabolism and excretion of vitamin K

Vitamin K is extensively metabolized in the liver and excreted in the urine and bile. Following scheme (Fig.4) presents hepatic metabolism of vitamin K [9]. Under usual physiological conditions vitamin K is mainly recycled by VKOR. However, in the presence of vitamin K antagonists such as warfarin the activity of the VKOR is blocked. This leads to a build-up of vitamin K epoxide (KO) in the cell. An alternative hepatic quinone reductase activity can bypass the warfarin inhibition of the VKOR to provide vitamin K hydroquinone (KH_2) substrate for the GGCX. The liver is also the site of a catabolic pathway, where the side chains of phylloquinone and MKs undergo

ω-oxidation followed by β-oxidation resulting in two major aglycone metabolites with side chain lengths of five and seven carbon atoms respectively (5C and 7C metabolites). After conjugation, the glucoronides are excreted in the bile and urine. Phylloquinone, the major dietary form, is rapidly and extensively catabolised in humans with about 40% of a daily physiological dose being excreted via the bile and 20% via the urine.

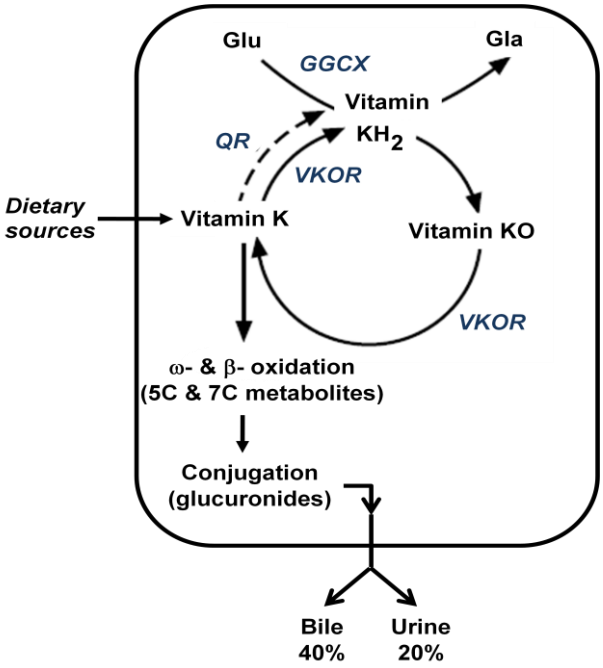

Figure 4 **Hepatic metabolism of vitamin K** (modified from [9])

Vitamin KH2	Vitamin K hydroquinone
Vitamin KO	Vitamin K epoxide
VKOR	Vitamin K epoxide reductase
GGCX	Gamma glutamyl carboxylase
QR	Quinone reductase

Further, in tracer experiments it was found that approximately 20 % of an injected dose of phylloquinone was recovered in the urine whereas 40 - 50 % was excreted in the faeces via the bile [39]. It seems, therefore, likely that approximately 60 – 70 % of the phylloquinone amounts absorbed from the diet will ultimately be lost from the body by excretion and the body stores of phylloquinone are being constantly replenished.

1.2.1.5. Age and gender

Infants are born deficient in vitamin K because of poor maternal-fetal transfer across the placenta [50]. 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, which could otherwise produce endogenous menaquinones. In addition, coagulation factor concentrations are low at the time of birth, which increases the risk of hemorrhagic bleeding (VKDB). Vitamin K prophylaxis in a form of a single oral dose of 2 mg Konakion® MM (phytomenadione, vitamin K1) is routinely administered as an effective intervention against VKDB.

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 the age groups. 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 [29]. It has been proposed that there are age-related differences in absorption and catabolism of phylloquinone, and the turnover of VKD proteins is lower in older adults. Further, Booth *et al.* [51] reported differences in vitamin K status among women of different estrogen status from the Framingham Offspring Study and suggested that estrogen levels may be an important determinant of vitamin K status, independent of diet.

1.2.1.6. Xenobiotics

Warfarin and other coumarin-based drugs block the activity of the Vitamin K epoxide reductase. This results in decreased concentrations of vitamin K quinone and hydroquinone in the tissue and increased levels of vitamin K epoxide. An oral therapeutic dose of warfarin produces a profound change in the plasma kinetics of an intravenous dose of tritiated vitamin K1 and its urinary metabolites [39,52]. It was found that warfarin produced an apparent delay in the clearance from the plasma of the injected radioactive substance due to the rapid accumulation of vitamin K epoxide in the plasma, but the rate of clearance of injected phylloquinone was not different compared to that in warfarin-untreated subjects. In addition, the urinal excretion of radioactive metabolites was increased about twofold. Furthermore, vitamin K deficiency is often attributed to broad-spectrum antibiotic use, presumably by a reduction in the endogenous menaquinone production by intestinal bacteria [53]. Salicylates and anticonvulsants are also associated with vitamin K deficiency in some individuals with coexisting compromised nutritional status.

1.2.1.7. Life style

Different observational studies examined intake and sources of vitamin K according to sociodemographic and lifestyle factors. 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 [54]. This study suggested that high phylloquinone intake may be a marker for low coronary heart disease risk. Thane *et al.* [33] showed positive correlation between plasma phylloquinone concentrations and vitamin K intake from the diet in free-living British people in comparison to hospitalized groups. Further, the participants living in private households showed a seasonal dependence of plasma phylloquinone levels which were significantly lower in the time period from October to March. In conclusion, a high phylloquinone intake may be a marker for an overall heart-healthy dietary pattern [55]. Furthermore, results from the Rotterdam study showed that dietary intake of menaquinone is associated with a reduced risk of coronary heart disease [56]. Intake of menaquinone was also inversely related to all-cause mortality and severe aortic calcification.

1.2.2. Genetic Determinants

There has been a recent interest in the role of genetics as a determinant of the interindividual variation in vitamin K status. The response to dietary vitamin K supplementation under controlled conditions has been shown to widely vary among healthy individuals [57]. Nongenetic determinants account for ~20% of the interindividual variation in vitamin K status in Caucasian adults [58]. Potential genetic determinants of vitamin K status include variation in the genes involved in the transport or uptake of vitamin K into the tissue and the genes involved in the tissue specific availability and recycling of vitamin K.

1.2.2.1. VKOR, gamma glutamyl carboxylase

Vitamin K epoxide reductase (VKOR) is involved in the vitamin K cycle. In 2004, the gene encoding vitamin K epoxide reductase complex subunit 1 (VKORC1), the key protein in the vitamin K cycle, was identified and characterized [59,60]. Common polymorphisms and haplotypes within *VKORC1* gene have been associated with interindividual variability in warfarin dose [61-63]. Because warfarin acts as a vitamin K

anatagonist and 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 study in a Chinese cohort has shown that single nucleotide polymorphisms and haplotypes within the *VKORC1* gene were associated with ucOC and PIVKA-II concentrations [64]. 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. In another study, Watzka *et al.* showed that functional promoter polymorphism in the *VKORC1* gene is no major genetic determinant for coronary heart disease (CHD) in Northern Germans [65]. The discrepant association findings in Chinese and German populations may be explained by ethnic differences in genetic and perhaps environmental predisposition, modifying the polygenic CHD phenotype by interacting with VKORC1 variants and thus conferring disease susceptibility in some populations, but not in others.

Gamma glutamyl carboxylase (GGCX) is necessary for the carboxylation of VKD proteins. Common variations within the *GGCX* locus have been analyzed for association with transcriptional activity and required warfarin dose [66]. However, little is known about the effect of these polymorphisms on direct measures of vitamin K or γ-carboxylation of extrahepatic proteins such as osteocalcin.

1.2.2.2. CYP4F2, ABCC6

Anticoagulant drugs work by directly inhibiting VKOR and thus the recycling of vitamin K. Therefore, polymorphisms affecting vitamin K recycling may modulate vitamin K status. McDonald *et al.* screened various cytochrome P450 enzymes and showed that CYP4F2 was capable of metabolizing vitamin K1 to a single product acting as a vitamin K1 oxidase [67]. The V433M polymorphism in CYP4F2 (haplotype CYP4F2*3) has been linked to altered vitamin K1 metabolism. The data demonstrate that CYP4F2 is a vitamin K1 oxidase and that carriers of the CYP4F2 V433M allele have a reduced capacity to metabolize vitamin K1 as well as decreased steady-state hepatic concentrations of the enzyme. Therefore, the presence of this polymorphism indicates elevated hepatic vitamin K1 levels, necessitating a higher warfarin dose to reach the same anticoagulant response.

ABCC6 represents a transmembrane efflux transporter expressed primarily in the liver. Mutations in the *ABCC6* gene cause an autosomal recessive disease, Pseudoxanthoma elasticum (PXE), characterized by a progressive mineralization of

connective tissue and resulting in skin, arterial and eye disease. Recent studies on *Abcc6*$^{-/-}$ mice show that the absence of ABCC6 in the liver is crucial for PXE and confirm the “metabolic disease hypothesis” that tissue calcification is caused by the absence of a plasma factor secreted from the basolateral hepatocyte membrane [68]. It was proposed that this plasma factor is vitamin K precursor secreted by ABCC6 from the liver as a glutathione-(or glucoronide)-conjugate and that this supplements the vitamin K need of peripheral tissue receiving insufficient vitamin from the diet due to ineffective extraction of dietary vitamin K from blood by the liver. Peripheral tissue vitamin K is needed for the γ-carboxylation in proteins required for calcification of connective tissue throughout the body. Vanakker *et al.* found significantly decreased vitamin K serum concentrations in PXE patients compared with controls [69]. The findings assumed that ectopic mineralization in the PXE-like syndrome and in PXE results from a deficient protein carboxylation of VKD inhibitors of calcification (e.g. MGP). In PXE-like patients this is caused by mutations in the GGXE gene, whereas a deficiency of the carboxylation cofactor vitamin K is crucial for the decreased activity of calcification inhibitors in PXE.

1.2.2.3. Apolipoprotein E

The gene encoding apolipoprotein E (ApoE) codes for a 34-kDa lipoprotein primarily found on the triglyceride rich lipoproteins (TRL;VLDL and chylomicrons) and HDL [70]. ApoE is a ligand for the LDL receptor and other TRL receptors and therefore it is responsible for cellular uptake of TRL. Three common alleles (amino acid substitutions at positions 112 and 158) are of particular importance: E2, E3 and E4 [71]. The various ApoE isoforms interact differently with the LDL receptors, altering circulating levels of cholesterol and triglycerides [71]. The ability of ApoE to clear vitamin K-rich intestinal lipoproteins from circulation is greatest with E4 and least with E2 [40,72]. Lower concentrations of LDL cholesterol were found in patients with the E3/4 or E4/4 genotypes compared to levels in patients carrying the E3/3 genotype. Those with the genotype E2/3 or E2/2 had the highest serum phylloquinone concentrations [40]. It is thought that carriers of the E4 allele may have increased hepatic vitamin K uptake and decreased vitamin K in circulation, hence decreased levels of vitamin K available for γ-carboxylation of osteocalcin in the bone [73]. This is supported by the observation that E4 homozygotes require, on average, a higher dose of warfarin in comparison to non-E4 carriers [74]; a higher warfarin dose in E4 homozygotes would

be necessary to compensate for the increased hepatic vitamin K uptake. In contrast, another study showed carriers of the E4 allele have higher plasma phylloquinone concentrations and lower % ucOC [75] which suggested that the E4 carriers had slower clearance of the TRL remnants from circulation and subsequently more phylloquinone available for uptake in the bone. Clearly, more research is required to determine the direction and magnitude of the ApoE genotype effect on vitamin K status.

1.3. Pharmacokinetics

Pharmacokinetics can be defined as the study of the fate of drugs in the body or as the study of the influence of the body on drugs. It includes the study of the mechanisms of absorption and distribution of an administered drug, the rate at which a drug action begins and the duration of the effect, the chemical changes of the substance in the body (e.g. by enzymes) and the effects and routes of excretion of the metabolites of the drug. The aim of pharmacokinetics is to provide the knowledge needed to understand how to adjust the dosage of a drug to obtain the plasma concentration including the optimum effect, i.e. the best efficacy with the minimum of adverse effects [76]. ***Pharmacodynamics*** is the study of the various effects of drugs. ***Pharmacogenetics*** is generally regarded as the study or clinical testing of genetic variations in metabolic pathways which can affect individual responses to drugs, both in terms of therapeutic effect as well as adverse effects.

In clinical pharmacology, the only directly accessible parameter is the concentration of drugs in plasma or serum. All pharmacokinetics is based on the variations of plasma concentrations. The pharmacokinetic features of a drug result primarily from their facility to cross biological membranes and from the rate of their inactivation.

To construct a concentration-time profile, valid bioanalytical methods are needed to measure the concentration of drugs and substances in biological matrix, most often plasma or serum. Proper bioanalytical methods such as high performance liquid chromatography with fluorescence detection (HPLC-FL) or liquid chromatography using mass spectrometry (LC-MS) should be selective and highly sensitive due to the complex nature of the matrix. Standard curves and internal standards are used for quantification in the samples. The samples represent different time points as a drug is administered and then metabolized or eliminated from the body.

Pharmacokinetic analysis is performed by non-compartmental (model independent) or compartmental methods [76]. Non-compartmental data analysis evaluates the exposure to a pharmaceutical compound by estimating the area under the curve (AUC) of a concentration-time graph and subsequent calculation of various pharmacokinetic parameters. Compartmental pharmacokinetic analysis uses kinetic models to describe and predict the concentration-time curve. Pharmacokinetic models are relatively simple mathematical schemes representing complex physiological spaces or processes.

Non-compartmental pharmacokinetic analysis is highly dependent on estimation of total drug exposure most often expressed by AUC methods as well as on the blood/ plasma sampling schedule. A large number of data points are necessary to reflect the actual shape of the concentration-time curve more precisely.

Population pharmacokinetics studies the various sources and correlations of variability in drug concentration among individuals who represent the target patient population [77].

1.4. Objectives

The main objective of the present work by means of a phase I clinical study was to determine inter-individual variance in pharmacokinetics of intravenous and oral phylloquinone (vitamin K1) mixed micelles formulation as well as to explore a possible effect of the *VKORC1* promoter polymorphism c.-1639 G>A on the metabolism of phylloquinone and its pharmacokinetics in humans. Examining the phenotype-genotype relationship in the clinical study facilitates to gain a deeper insight into potential determinants of inter-individual variation in vitamin K status. In this regard, the phenotype refers to physiological processes like absorption, distribution, metabolism and elimination of the vitamin K. Pharmacokinetic parameters including *maximum serum concentration* (C_{max}), *time of the maximum serum concentration* (t_{max}), *area under the curve* (AUC), *bioavailabilty* ($F_{p.o.}$), *clearance* (CL), *volume of distribution at steady state* (V_{ss}) and *terminal half-life* ($t_{1/2}$) based on serum concentration-time data of vitamin K1 were considered as primary variables.

To ensure accurate, precise and reliable measurements of plasma phylloquinone concentrations, our aim was also to obtain a simple and fast method, suitable for the routine analysis of plasma or serum vitamin K as well as to improve the sensitivity of

the determination assay. Validation evidence and quality assurance performance were required in order to prove and warrant the practical applicability of vitamin K serum determination. As first application of the HPLC method served examining of the vitamin K1 pharmacokinetics in several human volunteers.

Further aspect of the present work was to gain an insight into the enzymatic characteristics of VKORC1 by studying its enzyme kinetics. The enzyme activity was characterized after chromatographic separation and quantification of the substrates and the correspondent products. After calculation of the enzymatic kinetic constants K_m and V_{max}, the reaction velocity as well as the affinity of the substrate (vitamin K1 and vitamin K2) for the VKORC1 enzyme could be assessed to a certain extent. These experiments helped to explain how the enzyme works and to predict its behaviour *in vivo*.

2. MATERIALS AND METHODS

2.1. Materials

The described experiments were carried out in molecular biological laboratories of the security level S1 using common routine equipment. Basic equipment chemicals and devices are not separately listed. Chemicals of daily use were gained in highly purity degree, aqueous solutions were prepared using deionized water (Millipore).

2.1.1. Equipment

Name	***Purpose***	***Manufacturer***
ABI Prism 3130xl DNA Sequencer	DNA Sequencing	Applied Biosystems
ANTAES 48/72	Laminar airflow	Biohit
Axiovert 25	Microscope	Zeiss
ChemiDoc	UV gel documentation	Biorad
Cooling/Freezing Unit		Liebherr
CO_2 Incubator	Incubator	Nunc
Dispensette Organic		VWR
Elite LaChrom - Autosampler L-2200 - Column oven L-2300 - Fluorescence detector L-2485 - Pump L-2130	HPLC system	Hitachi/ VWR
Eppendorf Pipettes		Eppendorf
Gas Chromatograph-FID: 6890 GC -Injector : 7683 Autosampler	GC-FID system	Hewlett Packard/ Agilent Technologies
Gas Chromatograph-MS: 5890 Series II Plus - Injector: 7673 Autosampler - Detector: MSD 5972, 5973	GC-MS system	Hewlett Packard/ Agilent Technologies
Heating Block incl. N_2 Evaporation System		Labomedic
Heraeus Multifuge 4 KR		Heraeus
NanoDrop ND-1000	Spectrophotometer	Peqlab
Savant	Vacuum Dryer	Thermo Scientific
Speedvac		Savant

Thermomixer comfort	Thermomixer	Eppendorf
Vacuum Chamber	Chromabond	Macherey-Nagel
Vacuum Pump		Vacuubrand
Vortex Mixer		Heidolph

2.1.2. Reagents, disposables and standards

2.1.2.1. List of chemicals

Name/ Acronym	***Chemical/ Solution***	***Manufacturer***
Acetic acid, water-free	CH_3COOH>99 %, glacial acetic acid, ethanoic acid, ethylic acid, ice-vinegar	VWR/ Merck
Acetonitrile	CH_3CN, methyl cyanide LiChrosolv Acetonitrile gradient grade for liquid chromatography	VWR/ Merck
Agar		BD Biosiences
Agarose	Agarose polymer	Biozym
Amp	Ampicillin (beta-lactam antibiotic)	Sigma-Aldrich
AmphoB	Amphotericin B (polyene antifungal drug)	PAA
Aqua destillata	Distilled water	VWR/ Merck
Bacto-Yeast Extract		Becton Dickinson
BHT	3,5-di-tert.-Butyl-4-hydroxy-toluene	VWR/ Merck
BSA	Bovine serum albumin	Sigma-Aldrich
Calcium chloride	$CaCl_2$	Sigma-Aldrich
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate	Sigma-Aldrich
Chenodeoxycholic acid	Bile acid	Sigma-Aldrich
Cholic acid	Bile acid	Serva
Chloroform	$CHCl_3$	VWR/ Merck
5α-Cholestane		Sigma-Aldrich
Cholesterol		Sigma-Aldrich
Cupric sulfate	$CuSO_4$	Sigma-Aldrich
Cyclohexane		VWR/ Merck
n-Decane	HPLC-grade	VWR/ Merck
Deoxycholic acid	Bile acid	Sigma-Aldrich
dH_2O	Deionized water	VWR/Merck
Diethyl ether	$(C_2H_5)_2O$, ethoxyethane	VWR/ Merck
Dimethoxypropan		VWR/ Merck
DMEM High Glc 4,5 g/ L	Dulbecco/ Vogt modified Eagle´s minimal essential medium without	PAA

with L-Gln	phenol red	
DMSO	$(CH_3)_2SO$, dimethyl sulfoxide	Sigma-Aldrich
dNTPs		Fermentas
DTT	Dithiothreitol, Cleland´s reagent	Sigma-Aldrich
Dulbecco´s PBS with Ca & Mg	Phosphate buffer	PAA
Dulbecco´s PBS without Ca & Mg	Phosphate buffer	PAA
EDTA	Ethylenediaminetetraacetic acid	Sigma-Aldrich
Epicoprostanol	ISTD	Sigma-Aldrich
Ethanol	C_2H_5OH, EtOH, ethyl alcohol LiChrosolv gradient grade for liquid chromatography	VWR/ Merck
Ethidium bromide		Sigma-Aldrich
Ethyl acetate		VWR/ Merck
FBS	Fetal bovine serum	PAA
Folin-Ciocalteau-Reagent	Folin´s phenol reagent (mixture of phosphomolybdate and phosphotungstate)	Sigma-Aldrich
Gases: Helium Hydrogen Nitrogen	for GC and evaporation	Linde AG
GeneRuler DNA Mix	Length standard 100 – 10,000 bp	Fermentas
Hexamethyldisilazane		VWR/ Merck
n-Hexane	C_6H_{14}, hexane LiChrosolv n-Hexane for liquid chromatography	VWR/ Merck
HiDi Formamide	Carbamaldehyde	Applied Biosystems
Hydrochloric acid	HCl	Sigma-Aldrich
7α-Hydroxycholesterol	Bile acid precursor Oxysterol	Sigma-Aldrich
Hydrogen peroxide	H_2O_2, 30 % (w/ w)	Sigma-Aldrich
Hyodeoxycholic acid	Bile acid	Sigma-Aldrich
Imidazole	$C_3H_4N_2$, 1,3-diazole	Sigma-Aldrich
Isopropanol	$(CH_3)_2CHOH$, isopropyl alcohol, 2-propanol, IPA LiChrosolv 2-Propanol gradient grade for liquid chromatography	VWR/Merck
Kana	Kanamycin (aminoglycoside antibiotic)	Sigma-Aldrich
Lathosterol	Cholesterol precursor	Sigma-Aldrich
Lithocholic acid	Bile acid	Sigma-Aldrich
Magnesium chloride	$MgCl_2$	Sigma-Aldrich
MEM with Earls Salts	Minimal Essential Medium	PAA

Methanol	CH_3OH, MeOH, methyl alcohol LiChrosolv Methanol gradient grade for liquid chromatography	VWR/ Merck
NEAA	Non-essential amino acid	PAA
PBS	Phosphate buffered saline	PAA
PenStrep	Penicillin/ Streptomycin 100 x Concentrate	PAA
Peptone		Becton Dickinson
PFA	Paraformaldehyde, polyoxymethylene	Sigma-Aldrich
Potassium chloride	KCl	Sigma-Aldrich
Pyridine		VWR/ Merck
Sodium acetate	CH_3COONa	Sigma-Aldrich
Sodium carbonate	Na_2CO_3	Sigma-Aldrich
Sodium chloride	NaCl	Sigma-Aldrich
Sodium citrate		Sigma-Aldrich
Sodium hydroxide	NaOH	Sigma-Aldrich
Trimethylchlorosilane		VWR/ Merck
Tris	$(HOCH_2)_3CNH_2$ Tris(hydroxymethyl)aminomethane	Sigma-Aldrich
Tryptone		Becton Dickinson
Ursodeoxycholic acid	Bile acid	Sigma-Aldrich
Vitamin K_1	Phylloquinone	Sigma-Aldrich
Vitamin K_2	Menaquinone-4	Sigma-Aldrich
Warfarin	Vitamin K antagonist	Roche
Water for the chromatography	LiChrosolv	VWR/ Merck
Zinc Acetate	99.99 % metalics basis Acetic acid zinc salt	Sigma-Aldrich
Zinc Dust	< 10 µ, 98+ %	Sigma-Aldrich
Zinc Powder	< 150 µ, 99.995 %	Sigma-Aldrich

2.1.2.2. List of consumable material

Material	***Manufacturer***
Adjustable pipettes: 100 µL, 1000 µL	Eppendorf
Analytical balance	Sartorius MC-5, Sartorius AG
Cartridges Chromabond SiOH 3 mL/ 500 mg	Macherey-Nagel
Cell scraper, 23 cm	Nunc
Cellstar 1 mL Pipette	Greiner Bio One
Cellstar 12 Well Cell Culture Plate	Greiner Bio One

Cellstar 6 Well Cell Culture Plate	Greiner Bio One
Cryo tubes Nalgene	Nunc
Culture Test Tubes	VWR/ Duran
Glass Pasteur pipettes	Labomedic
Glass vials for auto-sampler, 2 mL	VWR
Inserts for glass vials 300 µL	VWR
Optical-8 Tube Strip + Cap Strip	Applied Biosystems
pH- indicator strips 0 – 6	
Pipette Tips	Eppendorf
Reaction glass tubes with Teflon® lined septa and screw caps	Macherey-Nagel
Reaction tubes 1.5 mL/ 2 mL	Eppendorf
Reaction tubes 15 mL/ 50 mL	Greiner Bio One
Spatula	Macherey-Nagel
Stripette 10 mL	Costar
Test Tube Racks	Roth
Tissue Culture Dish 100 mm	Sarstedt
Tissue Culture Dish 60 mm	Sarstedt
Tweezers	Roth
Vial Inserts	Macherey-Nagel
Vials With Slotted Cap	Macherey-Nagel

2.1.2.3. HPLC columns, lamps, GC columns

Column / Lamp	***Purpose of use***	***Manufacturer***
BDS Hypersil C18, 3 µ, 100 x 3 mm, reversed phase	Vitamin K serum determination assay by HPLC	ThermoScientific
D2 long Life Lamp	VKOR enzyme activity assay by HPLC	VWR/ Hitachi
DB-XLB 122-1232 30 m x 0.25 mm x 0.25 µm	Vitamin K, bile acids, 7α-hydroxycholesterol determination by GC-MS and cholesterol by GC-FID	J&W Scientific
Nucleodur C18 Gravity, 3 µ, 100 x 3 mm, reversed phase	Vitamin K serum determination assay by HPLC	Macherey-Nagel
Supelcosil TM LC-18-S 15 cm x 4,6 mm, 5 µm	HPLC enzyme activity assay	Sigma-Aldrich
Xenon Lamp Ushio Uxl 157	HPLC vitamin K serum determination assay	VWR/ Hitachi

2.1.2.4. Commercial kits

Name	***Purpose of use***	***Manufacturer***
BigDye Terminator v3.1 Cycle Sequencing Kit	Sequencing	Applied Biosystems
Fugene HD Reagent	Transfection	Roche
QIAprep Maxiprep Kit	Plasmid preparation	QIAGEN
QIAprep Midiprep Kit	Plasmid preparation	QIAGEN
QIAprep Spin Miniprep Kit	Plasmid preparation	QIAGEN
QIAquick Gel Extraction Kit	DNA gel extraction	QIAGEN

2.1.2.5. Plasmids

Name	***Purpose of use***	***Manufacturer***
peGFP-N2	eGFP plasmid expression in mammalian cells	Invitrogen
pcep4-VKORC1	VKORC1 expression in mammalian cells	Invitrogen self-production
pcep4-VKORC1L1	VKORC1L1 expression in mammalian cells	Invitrogen self-production

2.1.2.6. Buffers

10x PCR Buffer	0.5 M KCL 0.2 M Tris HCl (pH 8.4)	
1x PCR Buffer	100 µL 10 x PCR Buffer 0.22 mM dNTPs 1.67 mM $MgCl_2$ Aqua dest.	
10x TE-Puffer	0.1 M Tris HCl 10 mM EDTA (pH 8.0) Aqua dest.	
50x TAE	0.5 M EDTA pH 8.0 2 M Tris Base 1 M Glacial acetic acid	
LB Medium (Lysogeny Broth Medium)	Tryptone/ Peptone Bacto Yeast Extract NaCl Agar (optional) antibiotics (50 µg/ mL)	10 g/ L 5 g/ L 5 g/ L 15 g/ L
4x Loading Buffer for agarose gels	40 % w/ v Saccharose	

	0.1 % w /v Xylene cyanol 0.1 % w/ v Bromphenol blue TAE Buffer
EtOH Acetate Solution	0.1 M NaAc pH 4.6 75 % v/ v EtOH
Buffer for *VKOR Enzyme Activity Assay*	25 mM Imidazole pH 7.6 0.5 % w/ w CHAPS
DTT Stock Solution	125 mM
CHAPS Stock Solution	2 % w/ w
$CaCl_2$ Stock Solution	400 mM

2.1.2.7. Primer sequences

Name	***Sequence (5´- 3´)***	***Use***
M13-F M13-R	GTA AAA CGA CGG CCA G CAG GAA ACA GCT ATG AC	Plasmid Sequencing
VKORC1-Promoter F	CAA GTT CCA GGG ATT CAT GC	PCR VKORC1
VKORC1-Promoter R	CCA AGA CGC TAG ACC CAA TG	PCR VKORC1
Cyp4F2 Ex11-F	TGA GGG AGG TGA TGT TGG AT	PCR CYP4F2
Cyp4F2 Ex11-R	GTC TCC TGG GTA GGA AGA GG	PCR CYP4F2
SQ_Cyp4F2 Ex11-R	TGA AGG AGG CCT TCT CCT GAC	Seq CYP4F2

2.1.2.8. Biological material and enzymes

Name	***Use***	***Firm***
HEK 293T (human embryonic kidney cells)	Cell culture experiments	DMSZ
E. coli DH5α (competent)	Cloning, plasmid transformation	Invitrogen
BamHI, *NotI*	Restriction digestion	Fermentas
Taq Polymerase	PCR	Roche

2.1.2.9. Animals

Tissue from mice of the laboratory bred CD1 at the age of 7 weeks was used for determination of tissue vitamin K.

Following tissues were dissected and prepared for analysis:

lung, bone, pancreas, kidney, tongue, uterus, muscle, heart, brain, liver, ovary, testis

2.1.3. Standards and vitamin K-spiked serum preparation

2.1.3.1. Vitamin K epoxides

Vitamin K epoxides were performed as described by Fieser *et al.* [78].

100 mg vitamin K was dissolved in 10 mL of ethanol (100 %) and after addition of 100 mg sodium carbonate and 1.2 mL hydrogen peroxide 30 %, the mixture was warmed for 2.5 h at 70 °C with occasional agitation. Complete decolouration of the yellow mixture occurred after about 50 minutes. After thoroughly cooling, 10 mL water and 20 mL diethyl ether were added. The mixture was vortexed for 5 min followed by a brief centrifugation step (5 min at 3000 rpm). The upper diethyl ether phase was collected containing vitamin K epoxide (colourless). To remove traces of water from the organic phase, anhydrous sodium sulfate was added until the crystals no longer clumped together. Afterwards the ether extract was evaporated to dryness under a gentle stream of nitrogen at 50°C. The residue was being weighed and redissolved in a known volume of methanol. The epoxide solutions can be kept for a long period of time at 4 °C in the dark.

Vitamin K1 epoxide has a molar mass of 466 g mol^{-1}, menaquinone-4 epoxide of 460 g mol^{-1}.

2.1.3.2. Solutions and dilutions

All solutions and dilutions including K vitamins were prepared using 100 % HPLC grade solvents and were shielded from UV light.

Standards for VKOR Enzyme Activity Assay

Stock solutions of vitamin K1 and vitamin K2 (menaquinone-4) epoxide, with the molar mass of 466 g mol^{-1} and 460 g mol^{-1} respectively, were prepared in µM concentrations. For this adequate amounts (calculated by using molecular weight of each compound) of the epoxides were dissolved in ethanol. Initially, 8500 µM

solution of each epoxide was prepared. To create accurately diluted solutions, a constant one-fold serial dilution at each step was carried out nine times. Finally an appropriate amount of the epoxide stock solution was used while performing the enzyme assay to gain the designated end concentration of the substance - 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 µM. In addition, 400 µM vitamin K1 and K2 epoxide stock solutions were prepared for use as extraction standards.

Standards for vitamin K determination assay from serum

All standards – vitamin K1, vitamin K2 (MK-4), vitamin K1(25) ISTD - were dissolved in 100% HPLC-grade isopropanol.

Solvents:	*MK-4*	→	weigh 10 mg of MK-4 and dissolve in 1 mL of isopropanol
	K1	→	weigh 10 mg of K1 and dissolve in 1 mL isopropanol
	ISTD	→	weigh 10 mg of K1(25) and dissolve in 1 mL isopropanol

Stock Solution MK-4: Dilute the solution 10 mg/ mL MK-4 at the ratio 1:1000
= 10 µg/ mL of MK-4
= Primary Stock Solution MK-4

Stock Solution K1: Dilute the solution 10 mg/ mL K1 at the ratio 1:1000
= 10 µg/ mL of K1
= Primary Stock Solution K1

Stock Solution ISTD: Dilute the solution 10 mg/ mL K1(25) at the ratio 1:1000
= 10 µg/ mL of ISTD
Dilute the solution 10 µg/ mL K1(25) at the ratio 1:10
= 1000 ng/ mL of ISTD
= Primary Stock Solution ISTD

Preparation of K1 and MK-4 calibration curves using K1(25) as internal standard

For the calibration curves, working solutions of K1 and MK4 were prepared at concentrations ranging from 0.0625 to 20 ng per absolute injection. The primary stock solution of the concentration 10 µg/ mL was diluted at the ratio 1:20 to gain the calibration point 10 (0.5 µg/ mL equating to 20 ng per 40 µL absolute injection) which was further used to prepare the remaining calibration points by dilution as described below:

Calibration point	**Vitamin K** per absolute injection **[ng]**	**Diluted calibration point**	**Dilution ratio**
1	0.0625	2	1:1
2	0.125	3	1:1
3	0.25	4	1:1
4	0.5	5	1:1
5	1	6	1:1
6	2	8	1:4
7	5	8	1:1
8	10	10	1:1
9	15	10	2:1
10	20		

The internal standard K1(25) was kindly provided by Dr. Schurgers (University of Maastricht, the Netherlands). The primary stock solutions (1000 ng/ mL) of the internal standard were prepared in isopropanol. Dilutions of the primary stock were prepared at concentrations ranging between 25 and 100 ng/ mL. An appropriate amount of the defined secondary solution was added to the calibration solutions of vitamin K1 and MK4 to gain a final concentration of 0.5, 1 or 2 ng internal standard per absolute injection depending on the expected vitamin K concentration.

2.1.3.3. Vitamin K-spiked human serum

A serum pool was obtained from healthy volunteers. For validation studies and as qualifiers for performing the vitamin K serum determination assay, pool samples were enriched to increase the basal concentration of phylloquinone and MK4 by 0.1, 1, 10 and 100 ng per absolute injection.

Sample name	**Concentration of K1, MK4**
QM1	basal serum concentration
QM2	basal serum concentration + 0.1 ng/ inj.
QM3	basal serum concentration + 1 ng/ inj.
QM4	basal serum concentration + 10 ng/ inj.
QM5	basal serum concentration + 100 ng/ inj.

Prior to performing the vitamin K determination assay, an adequate amount of internal standard was added to each serum sample.

2.1.3.4. Bile acid calibration curves

Different concentrations of the bile acids were dissolved in 10 mL ethylacetate (stock solution). 100 µL of this solution was taken for quantification via GC-FID by adding 1 µg hyodeoxycholic acid as an internal standard. The ethylacetate was evaporated under nitrogen at 65 °C. The carboxylic group was methylated by addition of 2 mL MeOH, 1.5 mL 2.2-dimethoxypropane and 20 µL $HCl_{conc.}$ at room temperature over night. After solvent evaporation, hydroxy-groups of the bile acids were derivatisized to trimethylsilyl (TMSi)- ethers by adding 0.6 mL TMSi-reagent (pyridine:hexamethyldisilazane-trimethylcholorsilane; [9:3:1, v/v/v]) to the residue and incubation for 1 h at 65 °C. After evaporation under nitrogen at 65 °C, the residue was dissolved in 50 µL n-decane + 20 µL TMSi-reagent and transferred into micro-vials for GC-FID analysis.

The stock solution was diluted 5 times with ethylacetate at the ratio of 1:3. For the calibration curve 100 µL of each dilution were used. As internal standards for the preparation of standard curves 0.1 µg hyodeoxycholic acid and 0.1 µg [2,2,4,4-d_4] ursodeoxycholic acid were added. After methylation and silylation at 65 °C for 1 h and evaporation, the residue was dissolved in 50 µL n-decane + 20 µL TMSi-reagent and transferred into micro-vials for GC-MS analysis.

2.1.3.5. Cholesterol and 7α-hydroxycholesterol calibration curves

80 mg of cholesterol was dissolved in 100 mL cyclohexane, 5 mg of 7α-hydroxycholesterol was dissolved in 100 mL methanol. 100 µL of each solution was taken to quantify sterol content by adding 50 µg of 5α-cholestane and 1 µg of epicoprostanol as internal standards via GC-FID. Six samples were prepared and cyclohexane was evaporated under nitrogen by 65 °C. The residual sterols were trimethylsilylated by addition of 200 µL of a mixture of pyridine-hexamethyldisilazane-trimethylcholorsilane; (9:3:1, v/v/v) and incubated at 65 °C for 1 h. After evaporation, the residual TMSi-ethers were dissolved in 100 µL n-decane for GC-FID analysis.

Sterols were identified via retention time defined via individual measurement of prepared stock solutions. Peak integration was performed manually. The ratios of the sterols areas to the area of internal standard were calculated and multiplied by the added amount of the internal standard 5α-cholestane to reveal absolute concentrations of cholesterol and multiplied by the added amount of the second internal standard epicoprostanol to reveal absolute concentrations of 7α-

hydroxycholesterol in the stock solution. The stock solution was diluted seven times with cyclohexane at a ratio of 1:3 to gain eight calibration points. The sterol amount was calculated by dividing per three for each of the seven dilutions.

2.2. Molecular biological methods

2.2.1. PCR performance

2.2.1.1. Creation of oligo-nucleotides for the PCR

Oligo-nucleotides for the amplification of particular DNA fragments were ascertained by means of web-based program Primer3 (http://frodo.wi.mit.edu/primer3/). Synthesis and purification were carried out by Eurofins/ MWG (Cologne, Germany). Primer selection was guided by GC-content within 40-60 %, annealing temperature exceeding 55 °C and minimum length of 20 bases.

2.2.1.2. Polymerase chain reaction

The polymerase chain reaction (PCR) is a technique in molecular biology to amplify copies of appointed base sequences, generating thousands to millions of copies of a particular DNA sequence. Developed in 1983 by Kary B. Mullis, PCR was first described by Saiki et al. [79].

PCR initialization and DNA denaturation occur by heating the reaction to a temperature of 95°C which causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA. To enable annealing of the primers to the single-stranded DNA, the reaction temperature is lowered to 50-62°C depending on the primer sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis. At this extension/ elongation step at 72 °C the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs in 5` to 3` direction. Under optimum conditions at each extension step the amount of DNA target is doubled, leading to exponential amplification of the specific DNA fragment.

PCR Master-Mix:	**Temperature profile (35 cycles):**		
25 µL 1x PCR Buffer	95 °C	5 min	
0.5 µL Primer (5´-3´; 3´-5´)	95 °C	30 s	
0.3 µL *Taq* Polymerase	50-62 °C	30 s	35x
20-100 ng DNA	72 °C	30 s	
	72 °C	5 min	
	4 °C	∞	

2.2.1.3. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments by length and size with the help of defined length standard (DNA ladder). In the context of this work, DNA was separated electrophoretically using 1 % (w/ v) agarose gel in 1x TAE buffer. To produce the gel, the agarose was solved in 1x TAE buffer and boilt up. Ethidium bromide was added in the end concentration of 10 µg/ mL to allow later DNA detection. The DNA solution was injected into the gel slots; the particular DNA fragments were separated electrophoretically at 150 V for 20-30 min whereas the DNA ladder of defined length was injected into a separate gel slot. The following DNA detection was carried out in a ChemiDoc system (Biorad, Germany).

2.2.1.4. DNA isolation from agarose gels

DNA molecules were isolated from the agarose gel after electrophoretic separation using Qiaquick Gel Extraction Kits (Qiagen). The required gel band was cut out while illuminating with an ultraviolet lamp followed by a DNA purification on Qiaquick membrane columns and dissolving in 30 µL elution buffer.

2.2.1.5. Preparative plasmid isolation

The plasmid preparation from bacteria occurred by using Qiagen Plasmid Mini, Midi or Maxi Kits. Therefore, a predetermined volume of LB medium was inoculated with a particular bacterial strain (in glycerine) and incubated over night at 37 °C in a shaker. The bacterial lysis and subsequent plasmid purification was carried out regarding the kit protocol. Separated plasmids were held in dH_2O. Both concentration and grade of purification were determined photometrically.

2.2.1.6. DNA digestion by endonuclease restriction enzymes

Restriction assays of plasmids and DNA molecules were conducted in an end volume of 20 µL which contained the recommended enzyme concentration in an appropriate buffer depending on the used DNA amount as per manufacturer. All reactions were incubated at 4 °C over night and verified using an electrophoretic separation (see section 3.2.1.3.). If required, specific DNA bands were extracted from the gel for further use.

2.2.1.7. Plasmid transformation in E.coli DH5α

For transformation, 50 µL competent *E.coli* DH5α (Invitrogen) bacteria were carefully mixed with 2 µL ligation mix, incubated for 30 min on ice followed by heat shock at 42 °C for 45 sec in water bath. 250 µL LB medium (room temperature) were added and the reaction was agitated at 37 °C for 1.5 h in heating block. After incubation, 50-150 µL transformation mix was placed on antibiotic-rich LB agar plates and selected over night at 37 °C in an incubator. Transformed plasmids of grown colonies were verified via colony PCR and sequencing.

2.2.1.8. DNA sequencing and ethanol-acetate-precipitation

DNA sequencing is the process of determining the accurate nucleotide order of a given DNA fragment. DNA sequencing has been performed using the chain termination method with dideoxynucleotides developed by Sanger [80]. DNA sequencing was carried out by using ABI Prism big-Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 3130xl DNA Sequencer. Sequencing reactions contain DNA or purified PCR products regarding schema and temperature profile as follows:

Mix		Temperature profile (45 cycles)		
2 µL	DNA	1.)	96 °C	1 min
0.5 µL	Ready Mix	2.)	96 °C	10 s
1.5 µL	5 x Sequencing buffer	3.)	50 °C	5 s
0.5 µL	For or Rev Primer 3.2 pmol/ µL	4.)	60 °C	4 min
6.5 µL	Aqua dest.	5.)	4 °C	∞
10 µL	Total volume			

45x

After the sequencing reaction, DNA fragments were purified by *ethanol-acetate-precipitation.* 10 µL dH_2O, 2 µL 3M sodium acetate (pH 4.6) and 50 µL ethanol 95 %

were added to the sequencing product and centrifuged at 4°C and 4000 rpm for 45 min. After discarding the supernatant, the samples were centrifuged again with 200 µL of ethanol 70% (4 °C, 4000 rpm, 10 min). The supernatant was discarded again, the dried samples were dissolved in 15 µL HiDi formamide and sequenced.

2.3. Cell biological and enzymological methods

Cell culture experiments were performed under sterile laminar flow conditions using autoclaved or aseptic packed disposable material. All mediums and buffers, unless otherwise stated, were warmed to 37°C before use.

2.3.1. Cultivation of mammalian cell lines

Adherent (sticky) growing mammalian cells *HEK* 293T (human embryonic kidney 293T cells) were cultivated under controlled conditions at 37 °C, 5 % CO_2 in a cell incubator. MEM (minimal essential medium) containing 10 % FBS (fetal bovine serum) was used as growth medium. Penicillin-streptomycin solution and non-essential amino acid mixture were used to supplement the medium. Medium was changed every 3-4 days.

2.3.2. Subcultivation of cell cultures

Passaging also known as subculture or splitting cells involves transferring a small number of cells into a new vessel and occurred with 90 % confluent cell culture plates. After the medium was removed, cells were washed with PBS (phosphate buffered saline) and detached from the bottom of the plate with a mixture of trypsin-EDTA for 3 min at 37 °C. Care was taken not to overexpose cells to trypsin, as it can damage important cell surface proteins, channels and receptors. Growth medium was added (proteins present in growth medium essentially absorb the action of trypsin) and the cells and medium were mixed with. Then, the cell suspension was transferred to new plates, fresh medium was added to each plate.

2.3.3. Long-term storage of cells

After detaching from the plate with trypsin, the cells were resuspended in 1.7 mL medium containing 5 % DMSO (dimethyl sulfoxide) and 20 % FBS. Then the stocks were stored on ice for 30 min and at -20 °C for 24 h. After further storage at -80 °C for three days, the stocks were preserved at -196 °C in liquid nitrogen.

2.3.4. Cell transfection

Transient transfection for gene expressions in mammalian cell cultures was conducted with Fugene HD Reagent (Roche). The transfection suspension contained 500 µL FBS-free medium, 10 µg DNA and 30 mL Fugene HD transfection reagent and was incubated at room temperature for 15 min. For transfection, 80 % confluent cell plates were used. During the incubation of the transfection suspension, cell medium was replaced by FBS-free medium. DNA-Fugene suspension was distributed equally on the plate. After a period of 24 h, the cells could be further cultivated with FBS-containing medium.

2.3.5. Harvesting of full-cell homogenate

The preparation of cell extracts was required for enzymatic analysis of VKOR activity in HEK cells. Cells were washed with 4 °C cooled PBS followed by scraping these from the plate surface and transferring to 6 mL PBS. After centrifugation at 1000 g, 4 °C for 5 min, the supernatant was discarded. The cells were resuspended in 100 µL 2 % CHAPS (w/w) and incubated on ice for 10 minutes. Then 40 µL 250 mM imidazole was added to the suspension and incubated on ice for further 10 min. 230 µL glycerine (87 %, v/v) and 20 µL dH_2O were added prior to using the cell extracts for VKOR activity determination. The homogenates were stored at -80 °C.

2.3.6. Lowry protein assay

The Lowry protein assay was used for determining the total level of protein colorimetrically as described by Lowry et al. [81]. The method combines the reactions of cupric ions with the peptide bonds under alkaline conditions with the oxidation of aromatic protein residues. Cell extracts were diluted with dH_2O in the ratio 1: 100 in order to reach a protein concentration in the determination range of BSA (bovine

serum albumin) standard curve. Protein standards were generated with defined BSA concentrations in the range between 0 and 1 µg/ mL using 2 % BSA stock solution in dH_2O. Following reagents were used:

Reagent “A”: 2 % Na_2CO_3 in 0.1 N NaOH

Reagent “B”: 0.5 % $CuSO_4$ in 1% sodium citrate

Reagent “C”: 1 mL “B” to 50 mL “A”

Folin-Ciocalteu reagent: a mixture of phosphotungistic acid and phosphomolybdic acid in the Folin-Ciocalteu reaction

Protein determination was carried out in microtiter plates with sample volume of 10 µL. After addition of 100 µL reagent “C” and incubation for 10 min in the dark at room temperature, 10 µL Folin-Ciocalteu reagent was added. The plates were incubated for 30 min at room temperature in the dark. The absorption was photometrically measured at 690 nm. On the basis of the standard curve, the protein concentrations of the samples were determined.

2.3.7. VKORC1 enzymatic activity assay

The VKOR is featured to reduce vitamin K epoxide to quinone which is used to determine VKOR enzymatic activity and further for estimation of enzymatic constants as K_m and V_{max} for both epoxides. VKOR enzymatic activity was measured in whole-cell extracts according to published procedures [82]. Unless otherwise stated, all steps of the reaction were performed on ice. For each reaction, 30 µL HEK cell homogenate (VKORC1 transfected cells) was suspended in 470 µL on ice cooled reaction buffer. After addition of 20 µL dithiothreitol providing reducing equivalents (end concentration in the mixture 5 mM), the suspension was incubated for 1 min at room temperature and 5 µL $CaCl_2$ (end concentration 5 mM) was then added. An appropriate amount of vitamin K epoxide solution in ethanol was used to gain the designated substrate end concentration in the range 0.5 to 256 µM. After incubation at 30 °C for 1 h, the reaction was stopped with 1 mL isopropanol/ hexane (3:2, v/v). As an extraction in-process control step, the other epoxide was added in the end concentration of 8 µM. The remaining substrate (epoxide) and the product (corresponding quinone) contained in the hexane phase were extracted, dried under vacuum and redissolved in 50 µL methanol, 40 µL of which were used for injection in the HPLC. Subsequently, vitamin K epoxides and quinones were separated by isocratic HPLC on a reverse phase C18 column (oven temperature 45 °C) using

methanol as a mobile phase (flow rate 1 mL/ min). The detection was carried out by DAD at 254 nm [83]. The quantification of the generated quinone was adjusted according to the extraction standard and the total protein concentration in the sample. Some parameters were varied in the different trials while performing the VKOR enzymatic activity assay. The concentration of vitamin K epoxide as substrate was in the range between 0.5 and 256 µM. Moreover, the enzyme reaction was incubated for different time intervals: 5, 10, 30 or 60 min. Besides the regular experiments with vitamin K1 or K2 as substrate, some experiments were conducted in addition using both epoxides as substrate in varying ratios with the total concentration of 8 µM. More than 400 reactions were performed in triplicate.

2.3.8. Determination of kinetic constants

The most common kind of enzyme kinetic experiment is to vary the concentration of substrate and measure enzyme velocity. The main goal is to determine the enzyme´s kinetic constants K_m and V_{max} [84]. ***K_m*** is the Michaelis-Menten constant and describes the substrate concentration needed to achieve a half-maximum enzyme velocity. ***V_{max}*** is the maximum velocity that can be reached and it is the velocity of the enzyme at very high concentrations of substrate. In our experiments, the Y-axis of the typical Michaelis-Menten saturation curve presents the formation rate of vitamin K quinone in nmol per mg protein (reaction rate), the X-axis shows the substrate concentration in the range between 0.5 and 256 µM.

To calculate the apparent kinetic constants K_m and V_{max}, Lineweaver-Burk plot was used which is a common way of illustrating kinetic data. This model plots the reciprocal of substrate concentration versus the reciprocal of enzyme velocity (double-reciprocal plot), thereby curve data are transformed into straight lines. In this way, the slope and intercept of a linear regression line are used to determine values for K_m and V_{max}.

2.4. Bioanalytical methods

2.4.1. High-Performance Liquid Chromatography

Vitamin K1 (phylloquinone), vitamin K2 (menaquinone-4) and their epoxides were quantified via HPLC [85] with fluorescence detection according to their chromatographic features and the resultant specific retention times. A modified HPLC method, based on Davidson and Sadowski [86], with fluorescence detection after zinc post-column reduction was developed and validated for the analysis of K vitamins in plasma and serum samples. All solvents and chemicals were of the highest purity commercially available.

2.4.1.1. HPLC instrumentation

The isocratic HPLC system (VWR, Hitachi) consisted of separation modules with in-line vacuum degasser, L-2130 Pump, L-2200 Autosampler, L-2300 Column Oven and L-2485 Fluorescence Detector, all controlled by Elite LaChrom software (V.3.1.7.). A BDS Hypersil C18 (3x 100 mm) or Nucleodur C18 Gravity (3x 100 mm), both of 3 µm particle size, were used. During analysis the column temperature was maintained at 22 °C, the autosampler temperature at 15 °C. A 10 cm long, post-column peek tubing was dry-packed with zinc powder (<150 µ) and connected between the analytical column and fluorescence detector.

2.4.1.2. Sample preparation and hexane extraction of vitamin K from serum

Blood samples were obtained by venipuncture from German volunteers. Blood was collected into serum tubes and serum was separated by centrifugation, transferred to plastic cryotubes and stored at -80 °C for around 1 year with no further freeze-thaw cycles prior to analysis. Samples were protected from light immediately after blood withdrawal.

Vitamin K compounds were purified from serum by liquid-liquid extraction. Aliquots of 0.5 mL serum samples were transferred to a disposable glass centrifuge tube (16x 100 mm) containing 0.5 mL dH_2O. Two mL ethanol 100 % was added to precipitate the proteins with brief agitation. Then internal standard (0.5, 1 or 2 ng per absolute injection) was added and the mixture was vortexed for 30 sec. After this 4 mL n-hexane were added and the contents were mixed vigorously. Then the mixture was

vortexed again for 1 min and centrifuged at 1500 g for 10 min at room temperature to separate an upper hexane layer from a lower aqueous-ethanolic layer and precipitated proteins. The upper hexane layer was quantitatively transferred to a disposable glass tube. The lower layer was additionally re-extracted with 4 mL hexane as described above and the upper organic layer was pooled with the first hexane extract. The hexane pool was evaporated to dryness under a stream of nitrogen at 50 °C.

2.4.1.3. Isolation of vitamin K fraction from lipid extracts using Sep-Pak silica cartridges

The isolation of the vitamin K fraction from lipid extracts was carried out by using solid phase extraction (SPE) system. SPE Sep-Pak™ silica cartridges (Chromabond® SiOH 3 mL/ 500 mg, Macherey-Nagel) were placed in a SPE manifold and preconditioned with 9 mL of hexane (3 x 3 mL). The prewashing step with hexane removes materials from the cartridge which may otherwise interfere with the vitamin K assay. The dried lipid extract was dissolved in 2 mL of n-hexane, agitated briefly and introduced into the cartridge using Pasteur pipette. The hexane solution was pushed through the cartridge to load the extract at the head of the Sep-Pak. The tube that contained the lipid extract was rinsed with 2 mL hexane and loaded onto the cartridge in the same way. Nine mL of hexane (3 x 3 mL) were drawn through each cartridge under vacuum to separate the hydrocarbon lipid fraction and the eluate was discarded. Furthermore, the retained vitamin K compounds were eluted with 9 mL of 3 % (v/v) diethylether in hexane and collected in disposable glass tubes (16x 100 mm). Eluates were evaporated to dryness under a stream of nitrogen at 50 °C.

2.4.1.4. Final analytical stage of the vitamin K determination assay

The dried vitamin K fraction was dissolved in 50 µL isopropanol and transferred to 200-µL glass vials for HPLC analysis. A volume of 40 µL was injected onto the column via a valve injector.

The separation of the vitamin K compounds was carried out under the following HPLC conditions:

Column: Nucleodur C18 Gravity MN 3 µ, 100x 3.0 mm (RP, MN)

or

	BDS Hypersil C18 3 µ, 100x 3.0 mm (RP, TS)
Mobile phase:	100 mL Acetonitrile
	1.1 g Zinc acetate
	10 mL dH_2O
	10 mL Acetic acid
	in Methanol, final volume 1L
Flow-rate:	0.8 mL/ min
Detector:	Fluorescence detector
	Excitation wavelength: 246 nm
	Emission wavelength: 430 nm
Zinc-reduction:	100x 2 mm peek tubing filled with zinc powder (<150 µ)

2.4.1.4.1. Separation on RP-18 column

The isocratic separation of vitamin K compounds occurred on Reversed Phase-C18 column.

Reversed phase HPLC has a non-polar stationary phase and a moderately polar mobile phase. With this stationary phase, retention time is longer for molecules which are more non-polar, while polar molecules elute more readily. According to this, vitamin K epoxides elute faster than corresponding quinones. Furthermore, vitamin K2 (menaquinone-4) containing four isoprenoid residues in the side chain is considered as more polar than phyloquinone and elutes faster while vitamin K1 undergoes stronger interactions with the non-polar stationary phase and elutes at a slower rate from the column.

2.4.1.4.2. Post-column zinc reduction

The HPLC assays used for the determination of endogenous phylloquinone (vitamin K1), menaquinones (vitamin K2) and their epoxides, have evolved from the postcolumn reduction and fluorimetric detection methodology originally described by Haroon *et al.* [87-89]. The analytical system utilized to reduce nonfluorescent vitamin K compounds to their fluorescent hydroquinone forms consists of a postcolumn dry-packed with zinc powder (<150 µ). Hydroquinones are produced by chemical reduction over zinc in the presence of zinc ions, which are provided by the mobile phase. This on-line reduction process forms the core of the chromatographic systems used for the determination of vitamin K.

Vitamin K and the internal standard, all in the quinone or epoxide forms, were separated on the HPLC column and were then reduced to their hydroquinones in the post-column zinc reduction step.

2.4.1.4.3. Fluorescence detection

The chromatographic peaks of vitamin K hydroquinones and the appropriate internal standard (vitamin K1(25) eluting after phylloquinone) were detected and measured via fluorescence detector. The fluorescence detector is one of the most sensitive liquid chromatography detectors and senses only those substances that fluoresce i.e. compounds with conjugated double bonds (delocalised π-system). An excitation light of 246 nm was used for vitamin K detection. The emission wavelength was 430 nm.

2.4.1.5. Calibration and calibration curve of vitamin K

Calibration solutions (calibration point 1 – 10) of phylloquinone and menaquinone-4 were prepared as described above (3.1.3.2.). Calibration curves were constructed by plotting the weight ratio of vitamin K to internal standard on the Y-axis against the peak area ratios of vitamin K to internal standard on the X-axis. Tables 1 shows the calculated weight ratios used for construction of the calibration curves.

Table 1 Calibration points of K1 or MK4 using K1(25) as internal standard (1 or 2 ng/ absolute injection) and corresponding weight ratios

Calibration point	**K1** or **MK4** per absolute injection **[ng]**	**Internal Standard** per absolute injection **[ng]**		*Weight ratio* **MK4 / ISTD**	
1	0.0625	1	2	0.0625	0.03125
2	0.125	1	2	0.125	0.0625
3	0.25	1	2	0.25	0.125
4	0.5	1	2	0.5	0.25
5	1	1	2	1	0.5
6	2	1	2	2	1
7	5	1	2	5	2.5
8	10	1	2	10	5
9	15	1	2	15	7.5
10	20	1	2	20	10

After construction of the calibration curve, the linear fit (y=ax+b) and the goodness of fit (R^2) were determined. The linear equation was further used to calculate the vitamin K concentration in samples of interest with unknown substance concentration.

Theoretically, the peak area ratio (vitamin K/ ISTD) was calculated. Then, the corresponding weight ratio in the calibration curve was read and the amount of the unknown vitamin K in the sample calculated.

Practically, the linear equation was used to calculate the unknown vitamin K amount in the sample:

y = ax + b

a = *slope*

b = *axis intercept*

$$x = \frac{\textbf{Peak area Vitamin K}}{\textbf{Peak area ISTD}}$$

$$y = \frac{\textbf{Weight Vitamin K}}{\textbf{Weight ISTD}}$$

$$\textbf{Weight Vitamin K} = \left(a \times \frac{\textbf{Peak area Vitamin K}}{\textbf{Peak area ISTD}} + b \right) \times \textbf{Weight ISTD}$$

2.4.2. Gas-Chromatography Mass-Spectrometry

2.4.2.1. Gas-Chromatography

Gas chromatography identifies the individual substances that make up an unknown sample by separating those substances utilizing the basic chromatographic principles of mobile and stationary phases [85,90].

2.4.2.2. Mass-Spectrometry

MS is an analytical technique used to measure the mass to charge ratio of ions. It is most generally used to find the composition of a physical sample by generating a mass spectrum representing the masses of sample components. A typical mass spectrometer comprises three modules: an *ion source* (convert gas phase sample molecules into ions), a *mass analyzer* (sorts the ions by their masses by applying

electromagnetic fields), and a *detector* (measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present) [91].

2.4.2.3. GC-MS and GC-FID analyses

Authenticity of vitamin K1, MK4 and the corresponding epoxides was proven by comparison of the spectra with the literature using GC-MS analytics in Scan mode (m/z 50-600). Cholesterol was measured by gas-liquid chromatography-flame ionization detection (GLC-FID). Bile acids and the bile acid precursor 7α-hydroxycholesterol were analysed using gas-chromatography mass-spectrometry-selected ion monitoring (GLC-MS-SIM) in the splitless mode using helium (1mL/ min) as carrier gas.

2.4.2.3.1. Determination of vitamin K in human serum by GC-MS

Vitamin K was extracted from human serum and subsequently subjected to GC-MS analysis. A HP 5890 Series II Model gas chromatograph coupled to HP 5972 mass spectrometer was used. The gas chromatograph was equipped with a cross-linked methyl silicone DB-XLB 122-1232 fused silica capillary column (J&W Scientific), 30 m x 0.25 mm i.d. x 0.25 µm film thickness. Helium was used as the carrier gas. The initial column temperature was 70 °C for 1 min, followed by an increase of 30 °C min^{-1} to 150 °C, an isothermal hold for 3 min, followed by another increase of 30 °C min^{-1} to 180 °C and a final isothermal hold of 45 min. One mL serum sample, diluted with 1 mL of water, was mixed thoroughly, and kept in the dark for 10 min at room temperature. A 2 mL volume of methanol was added and the mixture left another 10 min in the dark. After the addition of 4 mL of hexane, the mixture was extracted for 15 min. After centrifugation, the supernatant was decanted into glass vials and the solvent was evaporated under a stream of nitrogen. After evaporation of the solvent, the sample was dissolved in hexane, transferred to autosampler vials and an aliquot of 2 µL was subjected to GC-MS analysis.

2.4.2.3.2. GC-MS bile acid analysis

0.1 µg of hyodeoxycholic acid and 0.1 µg of [2.2,4.4-d_4] ursodeoxycholic acid, as internal standards, were added to 500 µL of human serum (at room temperature). Alkaline hydrolysis was performed at 90 °C for 1 h after addition of 1 mL 10 M sodium hydroxide solution. After cooling to room temperature and addition of 500 µL distilled water and 500 µL ethanol the unsaponified lipids were extracted twice with 3

mL cyclohexane and discharged. After adjusting the pH to 1-2 by adding 1mL conc. HCl, the bile acids were extracted twice with 3 mL diethylether and transferred into a new scew-capped 10 mL glass-tube. The combined organic phases were evaporated at 30 °C under nitrogen. The carboxylic group was methylated by addition of 2 mL methanol, 1.5 mL 2.2-dimethoxypropane and 20 µL $HCl_{conc.}$ at room temperature over night. The samples were incubated for 30 min at 30 °C. The solvent was evaporated at 65 °C under nitrogen. The hydroxyl-groups of the bile acids were derivatisized to trimethylsilyl (TMSi)-ethers by adding 0.6 mL TMSi- reagent (pyridine-hexamethyldisilazan-trimethylchlorsilane 9:3:1, v/v/v) to the residue and incubated for 1 h at 65 °C followed by evaporation under nitrogen at 65 °C. The residue was dissolved in 50 µL n-decane + 20 µL TMS and transferred to micro- vials for GC-MS-analysis. Bile acids from the samples were analyzed by GC-MS on HP gas chromatograph 6890 combined with an HP 5973 quadrupole mass selective detector (MSD). Splitless injection was performed with an HP 7673 automatic sample injector plus tray. The GC-MSD instrument is controlled by a computer with the HP ChemStation Software G1701DA revision D.03.00. The chromatographic separation of bile acids was performed on a 30 m DB-XLB capillary column (J&W, Folsom, USA) with a 250 µm film of crosslinked methyl silicone with helium as carrier gas. Initial pressure was set to 0.765 bar, the pulse pressure to 2 bar. The measurement took place in constant flow mode at an initial flow of 0.8 mL/ min. The temperature program started at 150 °C and raised after 3 min to 290 °C with a rate of 30 °C/ min. The final temperature was maintained for 23 min. The injector and MSD transfer line heater temperature were set to 280 °C. The MS source was set to 230 °C. Multiplier voltage was set to ~ 1400 V. Electron impact ionisation was employed at 70 eV ionisation energy. Selected ion monitoring (SIM) was performed by cycling the quadrupole mass filter between the chosen m/z values at a rate of 2.0 cycles/ s. Autotune was performed before a sequence in order to check the condition of the mass selective detector. SIM mode was started after a solvent delay of 12 min. Table 2 lists the retention times and specific masses (m/z values) of each measured compounds.

Table 2 Chromatographic and mass selective detection parameters for trimethylsilyl-ethers of bile acids in human serum or plasma

No.	*Derivatives of*	*Category*	*Retention Time [min]*	*m/z-value(s)*
1	Deoxycholic acid	Bile acid	17.25	255/370
2	Cholic acid	Bile acid	17.56	458/368
3	Lithocholic acid	Bile acid	17.61	372
4	Chenodeoxycholic acid	Bile acid	18.03	370
5	Hyodeoxycholic acid	Internal standard	18.27	370
6	d_4-Ursodeoxycholic acid	Internal standard	18.85	464/374
7	Ursodeoxycholic acid	Bile acid	18.94	460/370

2.4.2.3.3. GC-FID analysis of cholesterol and GC-MS analysis of 7α-hydroxycholesterol

Ten mg tertiary butylated hydroxytoluene (BHT) as antioxidant, 50 µg 5α-cholestane and 100 ng d_6-7α-OH-Chol as internal standards were added to 100 µL serum sample. After alkaline hydrolysis with 1 mL 1N sodium hydroxide in 90 % ethanol for 1 h at 68 °C and addition of 500 µL of distilled water the unsaponified lipids were extracted twice into 3 mL of cyclohexane. The combined organic phases were dried under nitrogen at 65 °C. The hydroxyl groups of the sterols and oxysterols were derivatisized to trimethylsilyl (TMSi)-ethers by adding 1 mL TMSi-reagent (pyridine-hexamethyldisilazan-trimethylchlorosilane 9:3:1, v/v/v) to the residue and incubation for 1 h at 65 °C. After evaporation under nitrogen at 65 °C, the residue was dissolved in 160 µL n-decane. 80 µL were transferred to micro-vials for GC-MS-analysis. The residual 80 µL were diluted by addition of 500 µL n-decane for GC-analysis.

Cholesterol was measured after separation on a 30 m DB-XLB capillary column (J&W, Folsom, USA) with a 0.250 µm film of crosslinked methyl silicone in an Agilent Technologies 6890 GC using flame ionization detection. Splitless injection was performed with an HP 7683 automatic sample injector plus tray. Initial pressure was set to 3.0 psi. and column head pressure was set to 13.4 psi. Carrier gas flow was 1 mL/ min. and velocity is 37.8 cm/ s. The temperature program started at 150 °C and raised after 1 min to 290 °C with a rate of 30 °C/ min. The final temperature was maintained for 30 min. The injector and detector temperature were set to 280 °C.

The oxysterol 7α-hydroxycholesterol from the same sample for GC analysis was analyzed by GC-MS on HP gas chromatograph 6890 serie N combined with HP 5973 quadrupole mass selective detector (MSD). Splitless injection was performed with HP 7683 automatic sample injector plus tray. The GC-MSD instrument was controlled by HP ChemStation Software G1701DA revision D.03.00. The chromatographic separation of sterols and oxysterols was performed on a 30 m DB-XLB capillary column (J&W, Folsom, USA) with a 0.250 µm film of crosslinked methyl silicone with helium as carrier gas. Initial pressure was set to 3.0 psi. and column head pressure was set to 13.4 psi. Carrier gas flow was 1 mL/ min and velocity is 37.8 cm/ s. The temperature program started at 150 °C and raised after 1 min to 290 °C with a rate of 30 °C/ min. The final temperature was maintained for 30 min. The injector and detector temperature were set to 280 °C. Multiplier voltage was set to 2700 V. The emission current was 220 µA. Electron impact ionisation was employed at 70 eV ionisation energy. SIM was performed by cycling the quadrupole mass filter between the chosen m/z values at a rate of 2.0 cycles/ s. SIM mode was started after a solvent delay of 14 min with the ion m/z 370 for epicoprostanol. Table 3 lists the retention times and specific masses (m/z values) of the measured compounds.

Table 3 Chromatographic and mass selective detection parameters for trimethylsilyl-ethers of sterols (except cholesterol) and stanols in human serum or plasma

No.	***TMSi-Ether of***	***Category***	***Retention Time [min]***	***m/z-value(s)***
1	D6-7α-OH-Cholesterol	Oxysterol	16.80	462
2	7α-OH-Cholesterol	Internal standard	16.88	456

2.5. Bioanalytical Method Validation

The modified HPLC method for vitamin K analysis in serum samples with fluorescence detection after post-column zinc reduction was validated according to the guidances for Bioanalytical Method Validation [92,93]. The fundamental parameters to ensure the acceptability in performance are accuracy, precision, selectivity, sensitivity, reproducibility and stability. Whenever possible, the same biological matrix as that in the intended samples should be used for validation purposes.

Measurements for each analyte in the biological matrix should be validated. In addition, the stability of the analyte in spiked samples should be determined.

2.5.1. Selectivity

Selectivity is the ability of an analytical method to measure unequivocally and differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples without vitamin K and pool serum samples containing only the endogenous vitamin K basal concentration were conducted.

2.5.2. Range of determination; Limit of detection (LOD); Limit of quantification (LOQ)

The concentration range over which the analyte will be determined must be defined in the bioanalytical method, based on the evaluation of actual standard samples over the range, including their statistical variation (***range of determination***). This range of concentration should be reliably and reproducibly quantifiable with acceptable accuracy and precision through the use of a concentration response relationship. The ***limit of detection*** (LOD, the detection limit or lower limit of detection) is the lowest quantity at which a substance can be distinguished from the absence of that substance (a blank value) within a stated confidence limit. The ***limit of quantification*** (LOQ) is the analyte amount in a sample that can be quantitatively determined with suitable precision and accuracy.

2.5.3. Calibration/ standard curve

A calibration curve should be generated for each analyte in the sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/ response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without

internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ.

2.5.3.1. Lower limit of quantification (LLOQ)

The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met:

- The analyte response at the LLOQ should be at least 5 times the response compared to blank response.
- Analyte peak (response) should be identifiable, discrete and reproducible with a precision of 20 % and accuracy of 80-120 %.

2.5.3.2. Concentration-response

The simplest model that adequately describes the concentration-response relationship should be used. The following conditions should be met in developing a calibration curve:

- 20 % deviation of the LLOQ from nominal concentration
- 15 % deviation of standards other than LLOQ from nominal concentration

At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

2.5.4. Accuracy, precision

The ***accuracy*** of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte and should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15 % of the actual value except at LLOQ, where it should not deviate by more than 20 %. The deviation of the mean from the true value serves as the measure of accuracy.

The ***precision*** of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three

concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15 % of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20 % of the CV.

2.5.5. Recovery

The ***recovery*** of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100 %, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100 % recovery.

2.5.6. Quality Assurance

Quality assurance or *quality control* is warranted by using a sample, most commonly used a spiked sample, to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

2.5.7. Stability

Substance ***stability*** in a biological fluid is a function of the storage conditions, the chemical properties of the substance, the matrix and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also

include an evaluation of analyte stability in stock solution. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

2.5.7.1. Freeze and thaw stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 h and thawed at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 h under the same conditions. The freeze-thaw cycle should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at the intended temperature, the stability sample should be frozen at -70 °C during the three freeze and thaw cycles.

2.5.7.2. Short-term stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 h (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

2.5.7.3. Long-term stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared by means of back-calculated values to the standards at the appropriate concentrations from the first day of long-term stability testing.

2.5.7.4. Stock solution stability

The stability of the relevant stock solutions should be evaluated at room temperature for at least 6 h. Whether the stock solutions are refrigerated or frozen for a relevant period, the stability should be documented. After completion of the desired storage

time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

2.5.7.5. Post-preparative stability

The stability of processed samples including the resident time in the autosampler should be determined. The stability of the substance of interest and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

2.6. Vitamin K in mouse tissue

Organ phylloquinone and MK4 and the respective epoxides were quantified using vitamin K determination assay. Briefly, organs were dried in vaccum dryer for 24 h. After visual controlling if the drying process was satisfactory, organs were weighed. The organs were transferred to disposable glass tubes (16 x 100 mm) and chloroform-methanol mixture (2:1 v/v) was added to gain an end concentration of 5 mg/ mL. After storing the tubes at 4 °C for 24 h, 100 to 500 µL of the mixture was transferred to new glass tubes and internal standard K1(25) was added (1 to 5 ng per absolute injection). The mixture was evaporated to dryness under stream of nitrogen at 50 °C and redissolved in 2 mL ethanol 100 % and 1 mL dH_2O. The components of interest were extracted twice with 4 mL of n-hexane. The organic phase was pooled and evaporated to dryness followed by isolation of the vitamin K fraction from lipid extracts using Sep-Pak silica cartridges as described in 3.4.1.3. The vitamin K compounds were quantified by fluorometric detection upon HPLC separation on RP-18 column and post-column zinc reduction under the same conditions as noted above (3.4.).

2.7. “Influence of VKORC1 promoter polymorphism c.-1639 G>A on the vitamin K1 pharmacokinetics”- phase I clinical study

This chapter outlines briefly the main components of developing and preparing of a phase I clinical study that regards the **“Influence of *VKORC1* promoter polymorphism c.-1639 G>A on the vitamin K1 pharmacokinetics”**.

The study protocol and trial design were worked out according to the current requirements of EMEA (European Medicines Agency) [94] to enable getting the required permissions from the different authorities. The EudraCT number (2008-003643-36) of the proposed clinical investigation was issued from EMEA. The responsible local ethic committee of the Medical Faculty of the Friedrich-Wilhelm University Bonn gave its affirmative assessment. Moreover, the clinical trial was approved by the Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, BfArM), the Regulatory Agency for medicines in Germany. Each volunteer participating in this study was informed by the clinical investigator on the modality and the possible risks of the trial and gave written consent. Prior to and after the trial, volunteers underwent safety clinical and laboratory screening. Excluded from the study were: women of child bearing potential without reliable contraception, pregnant or lactating women, persons with any disease likely to disturb the vitamin K metabolism such as bleeding or thromboembolic history (acute or persistent) or dysfunctions of intestinal lipid absorption (Morbus Crohn, cholestatic liver diseases).

2.7.1. Study period and subject definition

The intended study period covered 18 days per test person. Furthermore, 18 months were scheduled for planning, trial design, analysis of the samples and pharmacokinetic and statistical evaluation.

Thirty subjects of Caucasian race, aged 22-46 years, participated in this study. Fifteen were males (28 ± 6 years, BMI 24 ± 2 kg/ m^2) and 15 were females (29 ± 7 years, BMI 21 ± 2 kg/ m^2). Thereof, 5 females and 5 males belong to each genotype specific group of interest regarding *VKORC1* promoter polymorphism c.-1639 G>A [62]. Confirmatory genotyping was performed to ensure that the study subjects are equally distributed in the three genotype groups of interest regarding *VKORC1*:c.-1639 G>A promoter polymorphism: group GG (carriers of GG wild-type genotype), group AG (heterozygous individuals), group AA (homozygous AA carriers). Furthermore, confirmatory genotyping was performed retrospectively with regard to SNPs in the *CYP4F2* (V433M polymorphism; haplotype CYP4F2*3 [67]) and *ABCC6* (promoter polymorphism c.-127 C>T [68,69,95]) genes involved in vitamin K metabolism. All subjects were healthy according to medical history, physical examination and standard laboratory procedures.

2.7.2. Trial design

The clinical study is classified as a *phase I* trial by reason of testing the pharmacokinetics of a drug in human subjects. Furthermore, it is considered as *open* (each test person obtains the same drug in the same application-way), *"single center"* (conducted in one trial centre - the Institute of Experimental Haematology and Transfusion Medicine) and genotype specific (exact genotype specificity of the subjects is required).

2.7.3. Investigational product

Vitamin K1 acts as an investigational product in its synthesized form phytomenadione.

KONAKION is the trade name of the medicine and contains phytomenadione as the active ingredient. Phytomenadione is a clear, yellow, very viscous, odourless oil.

Konakion® MM 2 mg is available in ampoules as an oral liquid. The ampoule contains the active ingredient phytomenadione 2 mg/ 200 µL in a mixed micelles (MM) solution (the micelles are composed of glycocholic acid and lecithin in an aqueous solution). The MM ampoule also contains sodium hydroxide, hydrochloric acid and water for injection [96]. Konakion® MM 2 mg is approved for prophylaxis and therapy of vitamin K deficiency bleeding in newborns. In this clinical trial vitamin K pharmacokinetics are investigated in healthy volunteers.

2.7.4. Indication

The indication for the accomplishment of the clinical investigation is to examine a possible effect of the *VKORC1* promoter polymorphism c.-1639 G>A on the metabolism of vitamin K1 and its pharmacokinetics in humans.

2.7.5. Trial procedure

The trial procedure started with a single oral intake of 2 mg Konakion® MM followed by a wash-out period of seven days and continued with a single intravenous administration of the same dosage. The i.v. injection was carried out into a vein of

one forearm, opposite to the one from which blood samples were taken. Systemic and local tolerance of the test medication were carefully checked by the clinical investigator. Blood samples (à 7.5 mL) were collected into serum gel tubes to 24 h upon p.o. and i.v. vitamin K1 administration: at 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, 10, 24 h after p.o. intake (Flow chart I), and at 2, 5, 10, 20, 30, 40, 50 min and at 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10, 24 h after i.v. injection (Flow chart II). During the first ten hours blood was collected through a peripherous vein catheter. Serum was separated immediately by centrifugation at 2250 g for 10 min at 18 °C and stored protected from light at -20 °C prior to analysis.

Flow charts – blood withdrawals after Konakion® MM 2 mg administration

Flow chart I: **p.o.** (oral administration)

Blood withdrawal à 7.5 ml (serum tube)

Date	Time Set	Time Actual	Point	Comments
Day -7			Safety laboratory	1 serum, 1 EDTA and 1 citrate tube
Day 1	7.45		- 15 min	
	8.00		0 min	**p.o.** administration of Konakion® MM 2 mg
	9.00		1 h	
	10.00		2 h	
	10.30		2 h 30 min	
	11.00		3 h	
	11.30		3 h 30 min	
	12.00		4 h	
	12.30		4 h 30 min	
	13.00		5 h	
	13.30		5 h 30 min	
	14.00		6 h	
	14.30		6 h 30 min	
	15.00		7 h	
	15.30		7 h 30 min	
	16.00		8 h	
	17.00		9 h	
	18.00		10 h	
Day 2	8.00		24 h	24 h value

Flow chart II: i.v. (intravenous administration)

Blood withdrawal à 7.5 ml (serum tube)

Date	Time Set	Time Actual	Point	Comments
Day 8	7.45		- 15 min	
	8.00		0 min	**i.v.** administration of Konakion® MM 2 mg
	8.02		2 min	
	8.05		5 min	
	8.10		10 min	
	8.20		20 min	
	8.30		30 min	
	8.40		40 min	
	8.50		50 min	
	9.00		1 h	
	9.20		1 h 20 min	
	9.40		1 h 40 min	
	10.00		2 h	
	10.20		2 h 20 min	
	10.40		2 h 40 min	
	11.00		3 h	
	11.30		3 h 30 min	
	12.00		4 h	
	12.30		4 h 30 min	
	13.00		5 h	
	13.30		5 h 30 min	
	14.00		6 h	
	15.00		7 h	
	16.00		8 h	
	17.00		9 h	
	18.00		10 h	
Day 10	8.00		24 h	24 h value
			Safety laboratory	1 serum and 1 citrate tube

2.7.6. Trial schedule

Visit	*Day*	
I	**-7**	**Screening; Safety laboratory; Medical checkup**
	0	**VKORC1 specific genotype assignment**
II	**1**	***Investigation Part I*** **→ blood collection after oral administration of Konakion®MM 2 mg**
III	**2**	**Controlling; Adverse events**
	3-9	**Wash-out period**
IV	**10**	***Investigation Part II*** **→ blood collection after intravenous administration of Konakion®MM 2 mg**
V	**11**	**Controlling; Adverse events; Medical checkup**

The clinical trial was completed when 30 patients (assigned to the genotype specific groups) finished all visits (18 days per test person).

Due to the fact that this trial was an explorative clinical trial with healthy volunteers and no patients were included, there was no treatment planned after the end of the clinical trial. The family doctor was informed about pathological blood values.

2.7.7. Primary aim, primary endpoint

The ***primary aim*** consisted in exploring the vitamin K1 pharmacokinetics depending on VKORC1 genotype, the so-called phenotype-genotype relationship. In this regard, the phenotype refers to physiological processes such as absorption, distribution, metabolism, elimination of the drug of interest.

Serum concentration of vitamin K1 was considered as a ***primary endpoint*** as well as the evaluation of some pharmacokinetic parameters including *Area under the curve* (AUC), *half-life time* ($t_{1/2}$), *maximal serum concentration* (C_{max}), *time of the maximal serum concentration* (T_{max}), *bioequivalence* (F), *clearance* (CL), *distribution volume* (V_d).

2.7.8. Secondary aims

Following ***secondary aims*** were defined:

- serum concentration of vitamin K2 (MK4), vitamin K1 and K2 epoxide

- dimension of the phenotype variability
- serum level of precursors and metabolites of cholesterol and bile acid metabolism

2.7.9. Inclusion and exclusion criteria, criteria for stopping trial

Certain requirements had to be fulfilled in order to participate in clinical trials. Defined inclusion and exclusion criteria should be met to enable enrolling of test persons.

2.7.9.1. Inclusion criteria

- Healthy volunteers, male and female
- Age: 18 to 60 years
- Full written *Informed Consent* to participating
- *VKORC1* genotype specific assignment (group A, B or C)
- No blood clotting disorder
- Normal values of safety laboratory parameters:

Quick	70-130 %	
INR	0.9-1.1	
PTT	22-36 s	
ALAT	<34 U/ L	
ASAT	<31 U /L	
γ-GT	<38 U/ L	
AP	34-131 U/ L	
LDL-C	<150 mg/ dL	
HDL-C	m	27-65 mg/ dL
	f	33-77 mg/ dL
Triglycerides	<200 mg/ dL	
Creatinine	0.5-1.4 mg/ dL	

2.7.9.2. Exclusion criteria

- Participation in another clinical trial
- Bleeding or thromboembolic history (acute or persistent)
- Dysfunctions of intestinal lipid resorption (M.Crohn, cholestatic liver diseases)
- Pregnancy or lactation
- Intake of following medication 10 days before starting the trial:

Acetylsalicylic acid and other salicylates
Anticoagulants
Cephalosporines
Sulfonamides
Anticonvulsants (Phenobarbital, Diphenylhydantoin)
Antituberculotic drugs (Isoniazid, Rifampicin)
Lipid lowering drugs

2.7.9.3. Criteria for stopping trial

- Proband absence
- Insufficient compliance
- Acute hazard of the test person during the trial (investigator´s decision)
- Withdrawal of consent (proband´s decision)

2.8. First approaches in human volunteers

Some preliminary experiments to the phase I clinical study served as first relevant application of the vitamin K serum determination assay. Four human volunteers were investigated as described in section 3.4. With two of them the experiment was repeated a year later to gain insight into possible intra-individual differences in vitamin K1 pharmacokinetics. Collected blood samples were used for vitamin K determination from serum directly after the experiment was performed and a year later. In this way, possible differences in vitamin K serum level depending on the storage time were distinguished.

2.9. Statistical and pharmacokinetic analysis

Linear regression, mean, median, standard deviation (S.D.), standard error of the mean (S.E.M.) etc. were calculated by using Microsoft Excel and SPSS. Statistical evaluation was performed using SPSS (IBM SPSS Statistics 19.0). Various univariate analyses of variance (*uANOVA*) were performed which generally included gender as a factor and Tukey post-hoc testing.

The precision of the assay was defined as the coefficient of variation (CV) of at least six repeats. The limit of detection (LOD) and limit of quantification (LOQ) was based on signal-to-noise ratio. A signal-to-noise ratio of 3:1 was considered acceptable for estimating the LOD wheares the LOQ was based on signal-to-noise ratio of at least 10:1.

Pharmacokinetic evaluation was carried out on the basis of the data from the experiments on vitamin K1 pharmacokinetics using WinNonlin (version 5.2.1, Pharsight Corp.) as a standard program. Non-compartmental analysis of the serum concentration *vs* time profiles after oral and intravenous administration of 2 mg Konakion® MM was done for all volunteers and all experiments. Serum concentration-time data of vitamin K1 was considered as the primary variable as well as the evaluation of pharmacokinetic parameters including *maximum serum concentration* (C_{max}), *time of the maximum serum concentration* (t_{max}), *area under the curve* (AUC), *bioavailabilty* ($F_{p.o.}$), *clearance* (CL), *volume of distribution at steady state* (V_{ss}), *terminal half-life* ($t_{1/2}$). Some of the main pharmacokinetic parameters could be directly taken from the data such as C_{max} and t_{max}. The area under the plasma concentration *vs* time curve from time zero extrapolated to infinity was calculated by:

$AUC = AUC_{0-t} + C_{last} / \lambda_z$

AUC_{0-t} is the area under the plasma concentration versus time curve from time zero to the last sampling time at which the plasma concentration is at, or above, the limit of quantification (LOQ). The calculation was performed by using the lin-log trapezoidal method. C_{last} is the observed plasma concentration at the last sampling time at which plasma concentrations are at, or above, the LOQ.

The terminal (first-order) elimination rate constant (λ_z) was calculated from the slope of the terminal linear portion of the log concentration *vs* time curve by linear regression analysis.

The volume of distribution at steady-state was calculated as:

$V_{ss} = MRT \times CL$

where MRT is the mean residence time calculated by $AUMC_{i.v.}/ AUC_{i.v.}$, and $AUMC_{i.v.}$ is the area under the first moment curve from zero extrapolated to infinity following intravenous administration. AUMC was calculated as:

$AUMC = AUMC_{0-t} + C_{last} \times t_{last} / \lambda_z + C_{last} / \lambda_z^2$.

The absolute bioavailability upon oral administration of Konakion® MM was calculated as:

$F_{p.o.} = AUC_{p.o.} / AUC_{i.v.} \times Dose_{i.v.} / Dose_{p.o.} \times 100\ \%$

The systemic clearance was calculated according to the equation:

$CL = Dose_{i.v.} / AUC_{i.v.}$

The terminal half-lives ($t_{1/2}$) were calculated using the terminal elimination rate constant as:

$t_{1/2} = 0.693 / \lambda_z$.

The plasma concentration values were corrected for endogenous vitamin K levels in each subject by subtraction of individual pre-dose value from all post-dose values. The plasma concentration was set to zero on some instances were such correction resulted in small negative values.

3. RESULTS

3.1. Enzymological characterization of the VKORC1

3.1.1. Separation and quantification of K vitamins

Separation of the substances of interest was performed by reversed-phase chromatography. Phylloquinone (K1), menaquinone-4 (MK4) and their epoxides were quantified via HPLC with DAD detection according to their specific chromatographic features and the resultant retention times (RT).

Figure 5 represents an authentic chromatogram of the vitamin K1 kinetics. MK4 epoxide (RT 3.3 min) was used as an extraction standard. K1 epoxide (RT 4.7 min) served as a substrate for the VKORC1 which catalyzed its conversion to the product K1 quinone (RT 6.6 min).

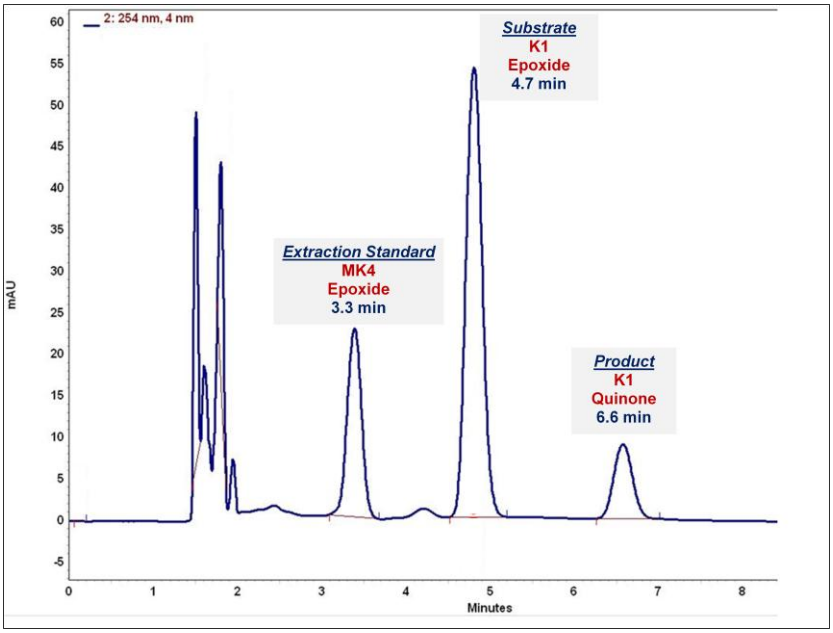

Figure 5 Chromatogram of vitamin K1 kinetics

Figure 6 shows an authentic chromatogram of the vitamin K2 (MK4) kinetics. K1 epoxide (RT 4.7 min) was used as an extraction standard. MK4 epoxide (RT 3.3 min) acts as a substrate for the VKORC1 which catalyzed its conversion to the product MK4 quinone (RT 4.2 min).

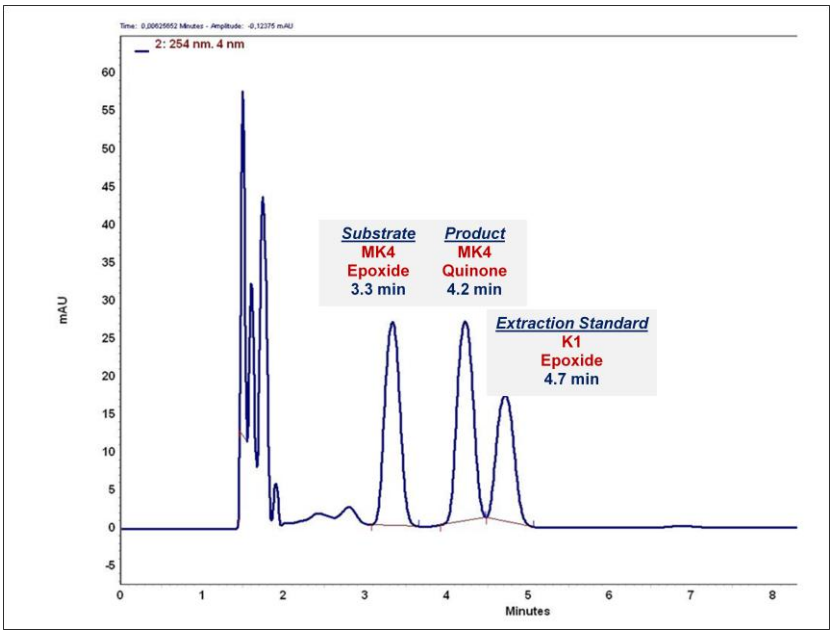

Figure 6 Chromatogram of MK4 kinetics

3.1.2. VKORC1 enzyme kinetics: determination of kinetic constants

To gain an insight into the enzymatic characteristics of VKORC1, a number of *in vitro* experiments (described in 3.3.7.) were performed. These reproduced the step of quinone formation by the VKORC1 using the corresponding epoxide as substrate.

3.1.2.1. Michaelis-Menten curves

The Michaelis-Menten curves represent saturation curves for the VKORC1 displaying the relation between substrate concentration and the rate of product formation. The concentration of vitamin K epoxide as substrate was in the range between 0.5 and 125 µM. Figures 7-10 show that VKORC1 velocity followed the Michaelis-Menten equation and display Michaelis-Menten curves of the enzyme reaction when incubated for different time intervals 5, 10, 30 or 60 min, respectively. Each figure shows the typical curves for both vitamin K1 and K2 kinetics of the VKORC1.

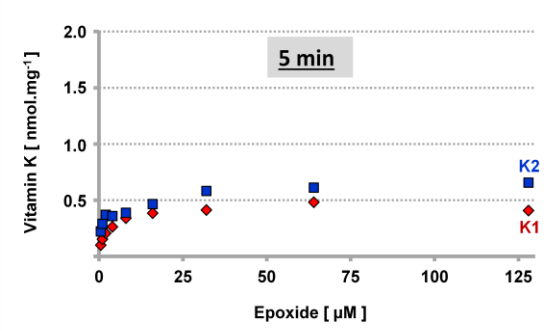

Fig.7 VKORC1 kinetics: 5 min incubation time

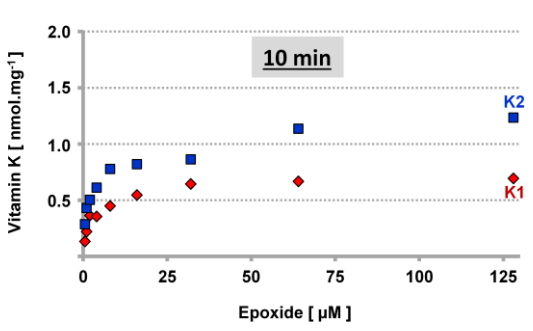

Fig.8 VKORC1 kinetics: 10 min incubation time

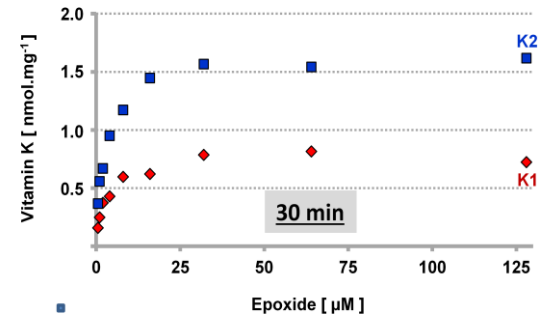

Fig.9 VKORC1 kinetics: 30 min incubation time

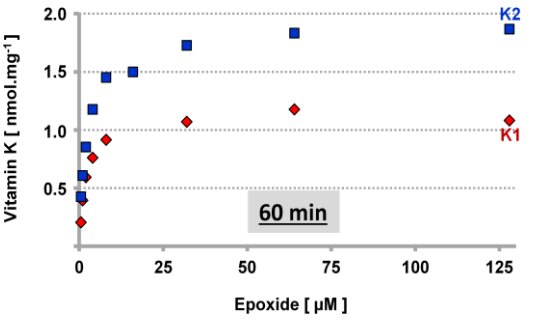

Fig.10 VKORC1 kinetics: 60 min incubation time

The same course of the curves was found for each incubation time. The quinone formation rate was lowest at 5 min and highest at 60 min incubation for both vitamin K1 and K2. Furthermore, the generation of K2 quinone was faster than the formation of K1 quinone. This difference is most obvious for incubation time of 30 and 60 min. Vitamin K1 time kinetics for each incubation time of 5, 10, 30 and 60 min is presented separately in figure 11. The appropriate vitamin K2 time kinetic curves are given in figure 12. Indeed, the formation of vitamin K2 quinone occurred significantly faster at 30 and 60 min comparing to the generation of vitamin K1 quinone.

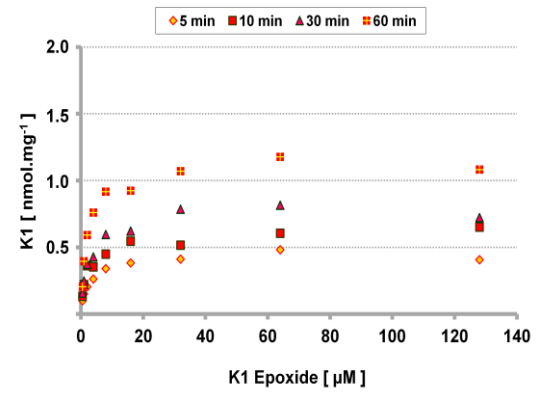

Figure 11 K1 time kinetics

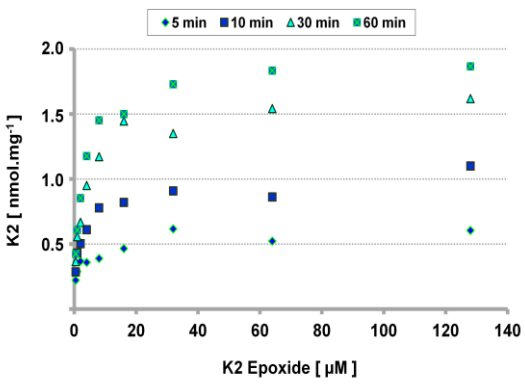

Figure 12 K2 time kinetics

3.1.2.2. Lineweaver-Burk graphs

Lineweaver-Burk plot as a common model of illustrating kinetic data was used to calculate the apparent kinetic constants K_m and V_{max}.

Figure 13 shows the Lineweaver-Burk graphs for vitamin K1 and vitamin K2 for incubation time of 60 min produced by taking the reciprocal of both sides of the Michaelis-Menten equation and transforming the curve data into straight lines.

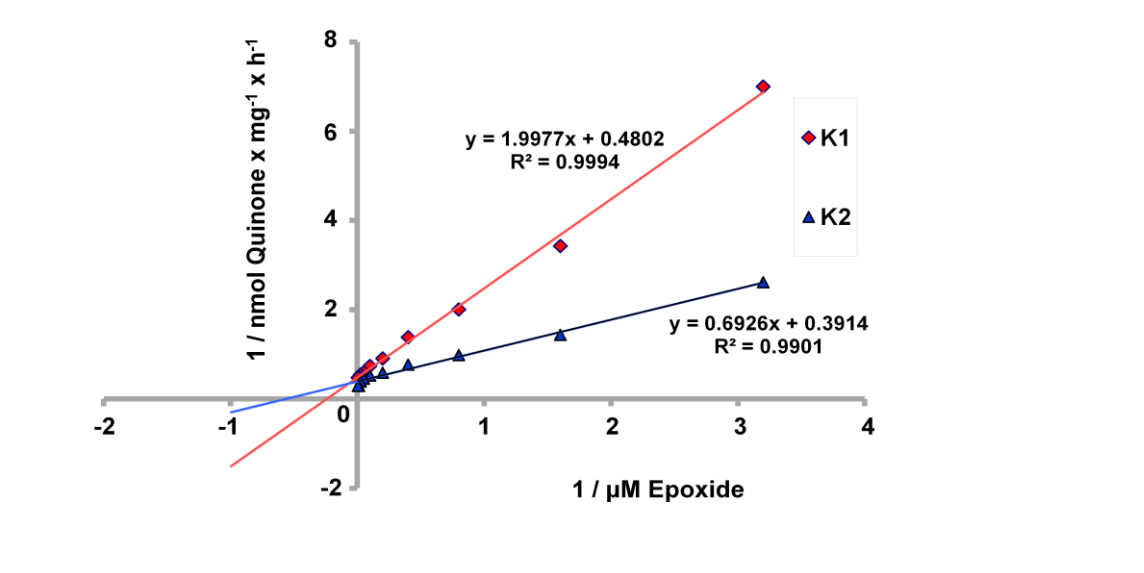

Figure 13 Lineweaver-Burk Graph

3.1.2.3. K_m and V_{max} determination

Based on the Lineweaver-Burk graphs for K1 and K2, the following K_m and V_{max} values were obtained shown in figure 14 and figure 15, respectively.

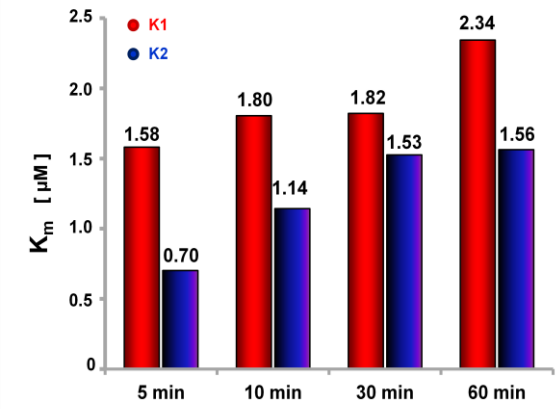

Figure 14 K_m values

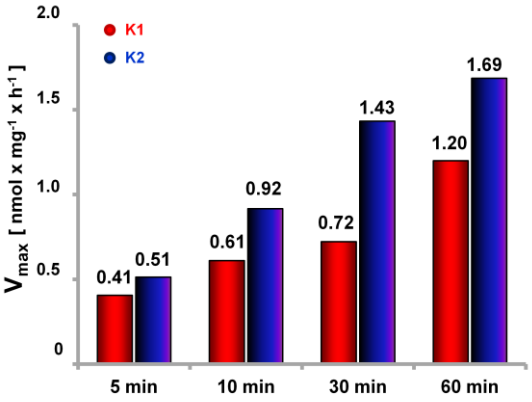

Figure 15 V_{max} values

Differences were observed comparing K_m and V_{max} values for both substrates, K1 and K2. 60 min K_m values of 2.34 and 1.56 µM were calculated for K1 and K2, respectively. The K_m for K1 was 1.5-fold higher than for K2. Therefore, the binding affinity of vitamin K2 epoxide to VKORC1 appears to be higher while vitamin K1 epoxide seems to bind in a weaker manner to the enzyme.

Differences were also identified when comparing the V_{max} values 1.20 nmol mg^{-1} h^{-1} for K1 and 1.69 nmol mg^{-1} h^{-1} for at the incubation time of 60 min. The 1.4 fold higher V_{max} value for K2 indicates faster generation of the K2 quinone by VKORC1 compared with the formation of vitamin K1.

3.1.2.4. Quinone generation during K1O and K2O substrate competition

In addition, differences in quinone generation were also observed when K1 and K2 epoxide was added as substrate in a varying ratio to the reaction. Single and concurrent K1 and K2 quinone production rate was distinguished. Starting with 100 % K1 epoxide, K2 epoxide was added in the different trials to K1 epoxide at increasing ratios up to 100 % K2 epoxide as substrate.

Using single epoxide as substrate, the VKORC1 showed a 1.5-fold higher preference for the reduction of K2 epoxide to the corresponding quinone compared to K1. The K2 quinone formation rate was found to be continuously 2.5 to 3.7-fold higher when K1 and K2 epoxides were added as substrate in various ratios to explore the competititve conversion to the corresponding epoxides by VKORC1. Figures 16 and 17 below show the results for quinone production rate during substrate competititon (K1 and K2 epoxide). The quinone generation is expressed as percent of vitamin K epoxide converted into the appropriate quinone (Fig.16) and as µM quionone (Fig.17).

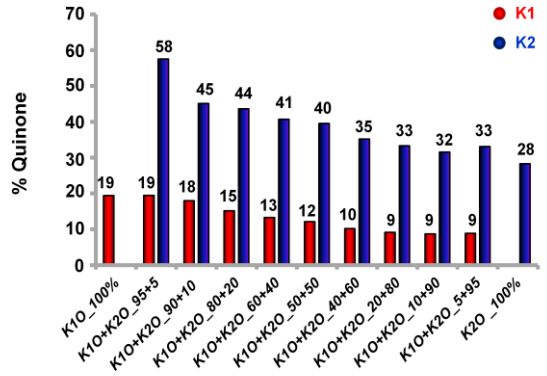

Figure 16 % Quinone generation

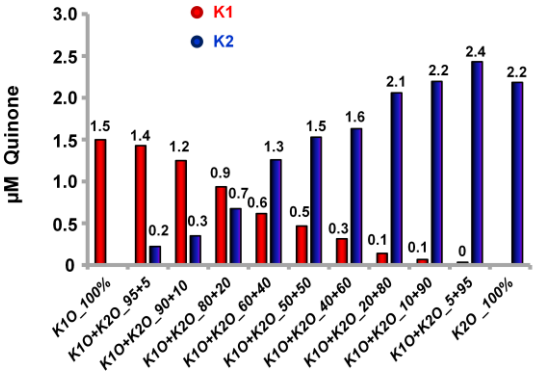

Figure 17 µM Quinone generation

When K1 and K2 epoxide were added as substrate in the ratio 1:1, simultaneously 12 % of K1 epoxide and 40 % of K2 epoxide was converted into quinone. The corresponding absolute values were 0.5 µM K1 and 1.5 µM K2. These results support what the VKORC1 kinetic constants indicate: higher binding affinity of VKORC1 to K2 epoxide and faster production of K2, as well as confirming the VKORC1 ability to reduce K2 epoxide significantly more efficiently than K1 epoxide *in vitro*.

3.2. Validation of the quantitative analysis of vitamin K1 (phylloquinone) and vitamin K2 (menaquinone-4) in human serum by HPLC with fluorescence detection after solid-phase extraction

The modified HPLC method for vitamin K determination in human serum or plasma was validated according to the guidances for Bioanalytical Method Validation of the International Conference of Harmonization. Main parameters were proved to ensure the acceptability of the performance of the method.

3.2.1. Specificity, selectivity

Selectivity and ***specificity*** express the ability of an analytical method to differentiate and quantify unequivocally the analyte in presence of other components in the biological sample. For instance, the retention time under particular conditions is considered to be reasonably unique to identify a given analyte.

Figure 18 shows an exemplary authentic chromatogram of the separation of vitamin K compounds MK4 (RT 2.6 min) and phylloquinone (RT 4.9 min) from the appropriate internal standard K1(25) (RT 10.5 min).

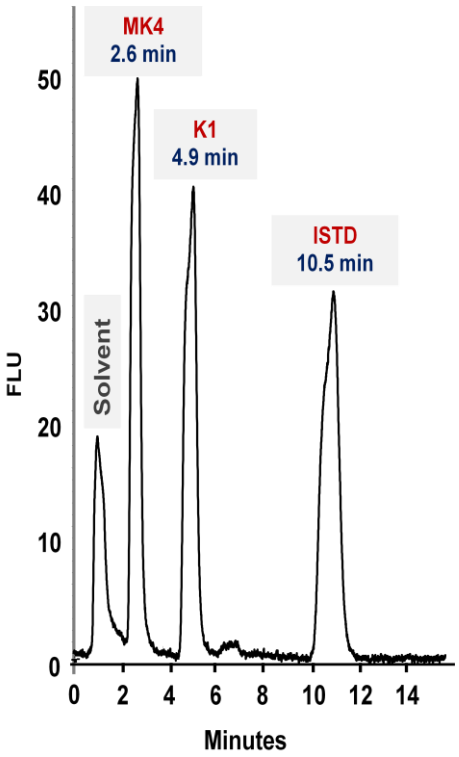

Figure 18 HPLC chromatogram of K1, MK4, and K1(25) (ISTD = Internal Standard)

The authenticity of K vitamins was proved by GC-MS by using scan mode. Figure 19, 20, 21, 22 and 23 present the characteristic spectra specific for K1, MK4, K1O, MK4O and K1(25) respectively.

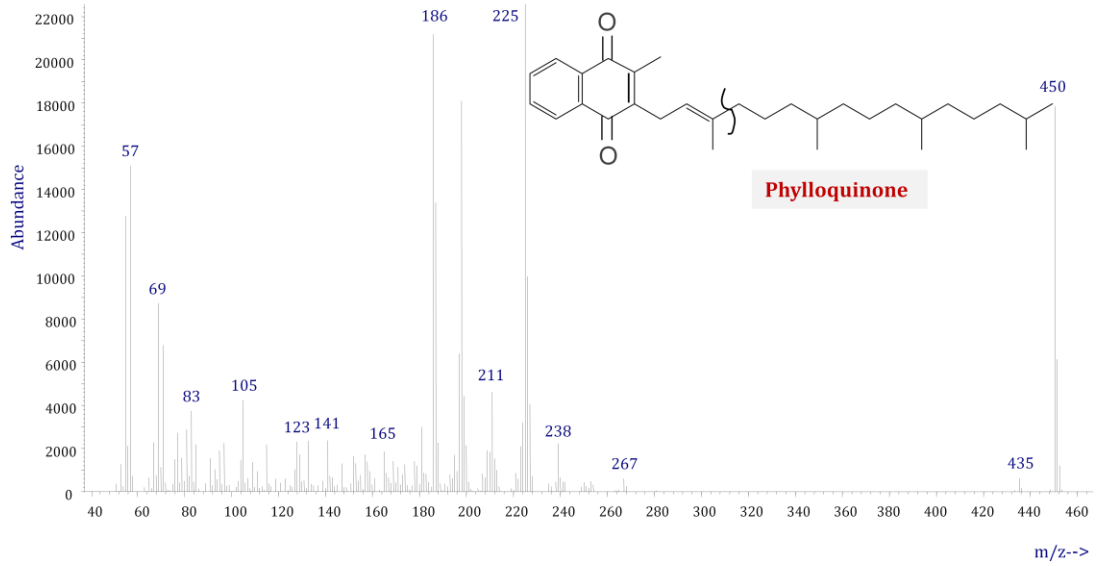

Figure 19 Mass spectrum of phylloquinone (vitamin K1)

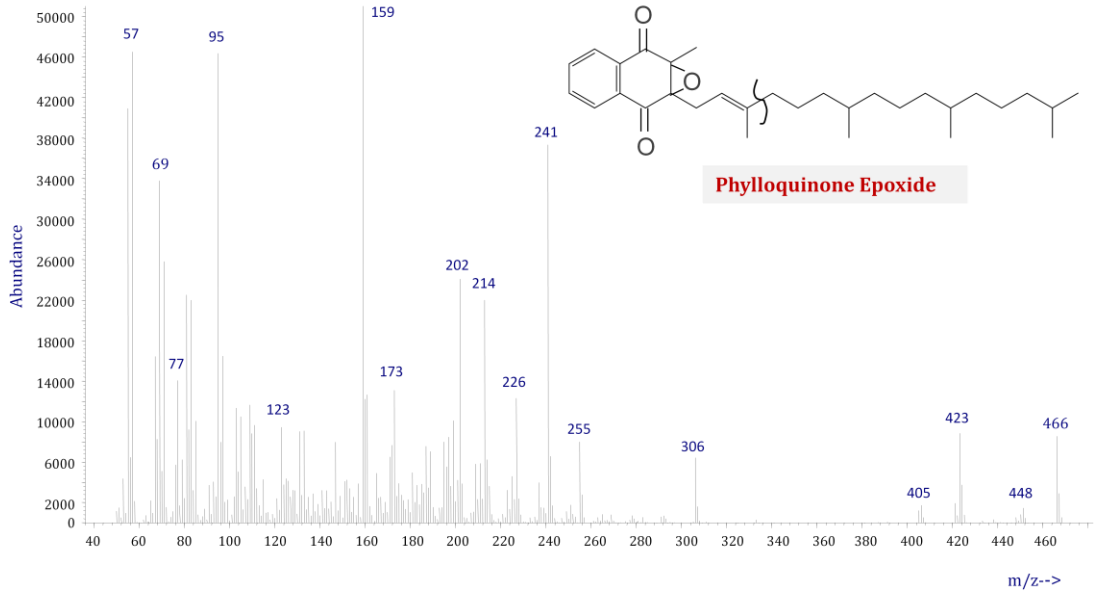

Figure 20 Mass spectrum of vitamin K1 epoxide

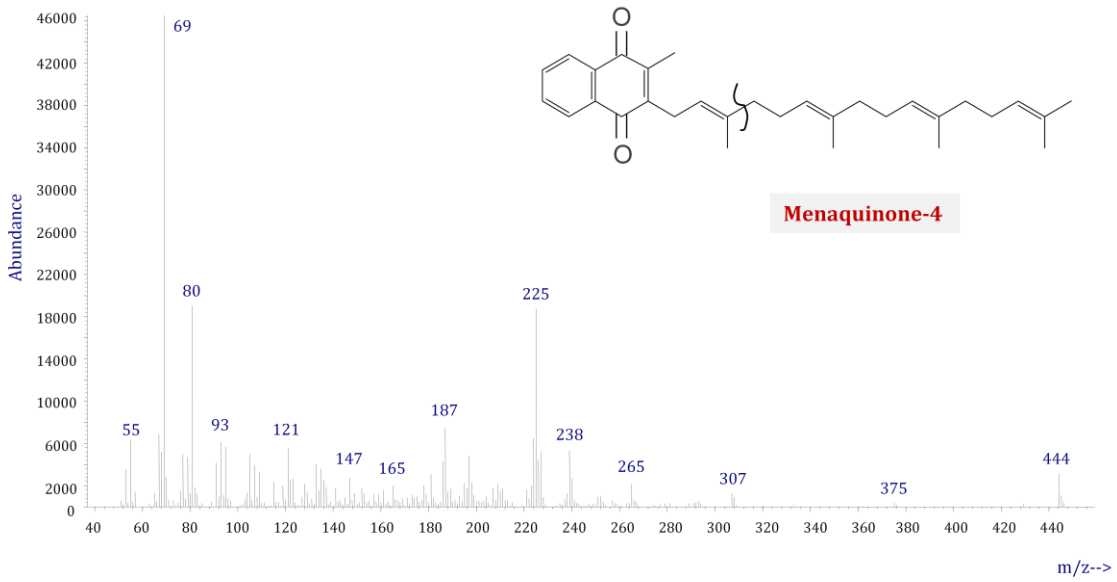

Figure 21 Mass spectrum of menaquinone-4 (MK4; vitamin K2)

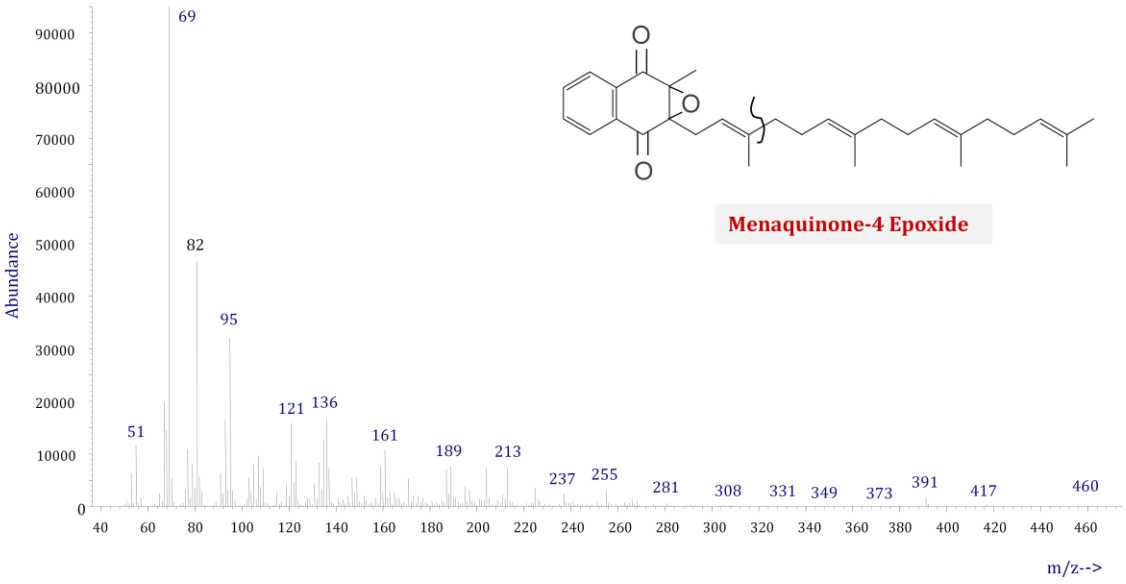

Figure 22 Mass spectrum of menaquinone-4 epoxide (MK4O)

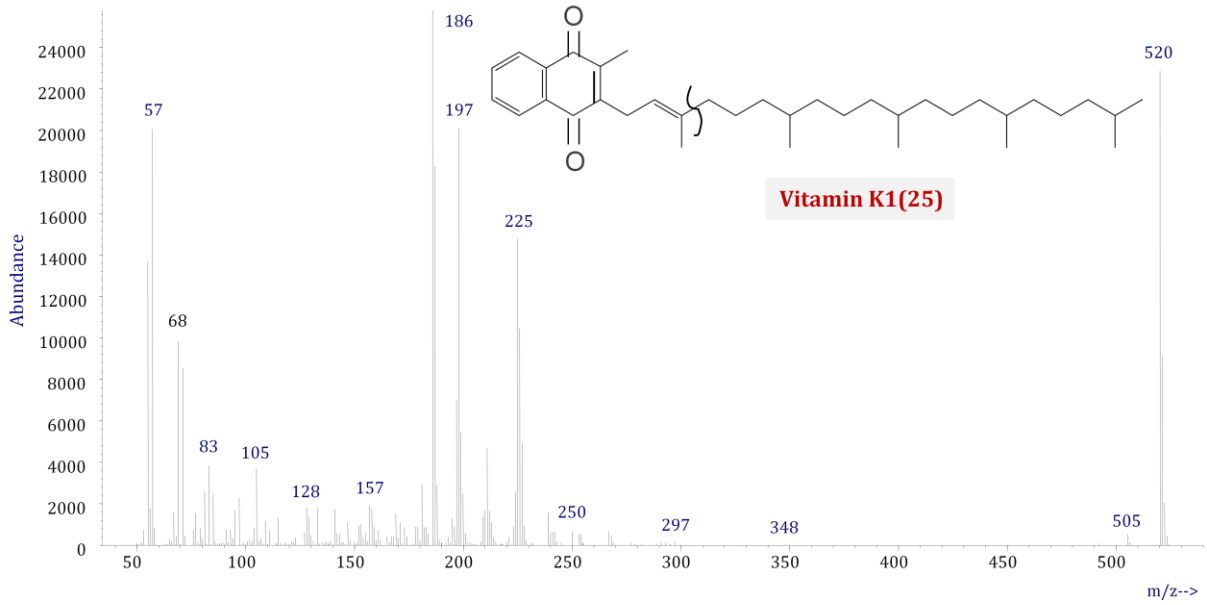

Figure 23 Mass spectrum of vitamin K1(25) – ISTD = Internal Standard
(2-Methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4-naphtalenedione)

Specificity

The specificity of the method for the substances of interest, vitamin K1 (phylloquinone) and vitamin K2 (menaquionone-4), was proved by testing several human serum samples from different healthy subjects for the absence of interfering compounds.

The retention times of the substances under the conditions of the assay are given in Table 4.

Table 4 Retention times of K vitamins

No.	*Substance*	*Category*	*Retention Time [min]*	*HPLC column**
1	**MK4**	Vitamin K2; Menaquinone-4	**2.6**	BDS Hypersil C18
2	**K1**	Vitamin K1; Phylloquinone	**4.9**	BDS Hypersil C18
3	**K1(25)**	Internal Standard	**10.5**	BDS Hypersil C18
1	**MK4O**	Vitamin K2 Epoxide	**3.1**	Nucleodur C18
2	**MK4**	Vitamin K2	**4.3**	Nucleodur C18
3	**K1O**	Vitamin K1 Epoxide	**5.3**	Nucleodur C18
4	**K1**	Vitamin K1	**8.9**	Nucleodur C18
5	**K1(25)**	Internal Standard	**21.6**	Nucleodur C18

***** described in detail in 3.1.2.3.

Two concentration ranges were used for quantification during validation analyses:

Concentration range I → 0.0625 – 2 ng per absolute injection
(calibration points 1-6, described in 3.4.1.5.)

Concentration range II → 1 – 20 ng per absolute injection
(calibration points 5-10, described in 3.4.1.5.)

Samples containing vitamin K concentration higher than 20 ng per absolute injection were further diluted to gain substance concentration in the range described above.

Selectivity

To prove the ***selectivity*** of the modified method for vitamin K determination, analyses of blank samples without vitamin K and of pool serum samples containing only the endogenous vitamin K basal concentration were conducted (n= 6). Concentration range I was used for precise quantification of low vitamin K concentrations. The peak areas of vitamin K1 and MK4 in spiked serum differed clearly and precisely from the peak areas of the lowest calibration point at the same retention times and were at least 5-fold greater than those of the blank values in pure isopropanol samples.

3.2.2. Range of determination

The ***limit of detection*** (LOD, the detection limit or lower limit of detection) as well as the ***limit of quantification*** (LOQ) could be determined.

3.2.2.1. Limit of detection (LOD)

The detection limit was based on signal-to-noise ratio. Determination of the signal-to-noise ratio was performed by comparing measured signals (abundance) from samples with the lowest concentration of an analyte with the abundance of the baseline surrounding of vitamin K peak. A signal-to-noise ratio of 3:1 was considered acceptable for estimating the detection limit according to the following guidelines.

$$\text{detection limit} = [\,(3 \times \Delta_{baseline}) / \Delta_{analyte}\,] \times c_{analyte}$$

$\Delta_{baseline}$ = difference in the abundance for the baseline

Δ_{analyt} = difference in the abundance for the analyte

$c_{analyte}$ = absolute concentration of the analyte

The LOD that could be distinguished from the absence of the substances was 0.006 ng K1 and MK4 per absolute injection. An exemplary chromatogram for the LOD estimation of phylloquinone is shown in Fig.24.

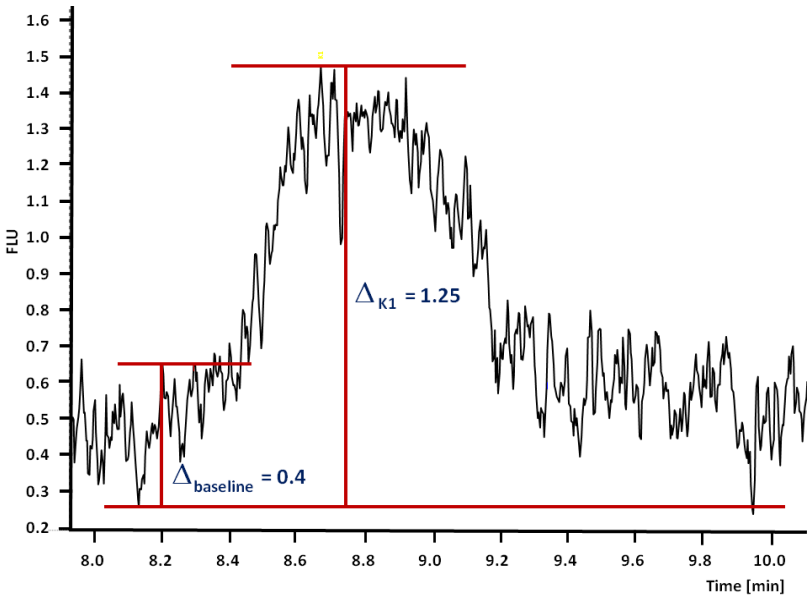

Figure 24 LOD of K1, signal-to-noise ratio 3:1

3.2.2.2. Limit of quantification (LOQ), lower limit of quantification (LLOQ)

The LOQ averaged 0.0625 ng K1 and MK4 per absolute injection (10-fold higher than LOD). The LOQ was based on signal-to-noise ratio of at least 10:1 as shown in Fig.25 for phylloquinone where signal-to-noise ratio of 12:1 was accepted.

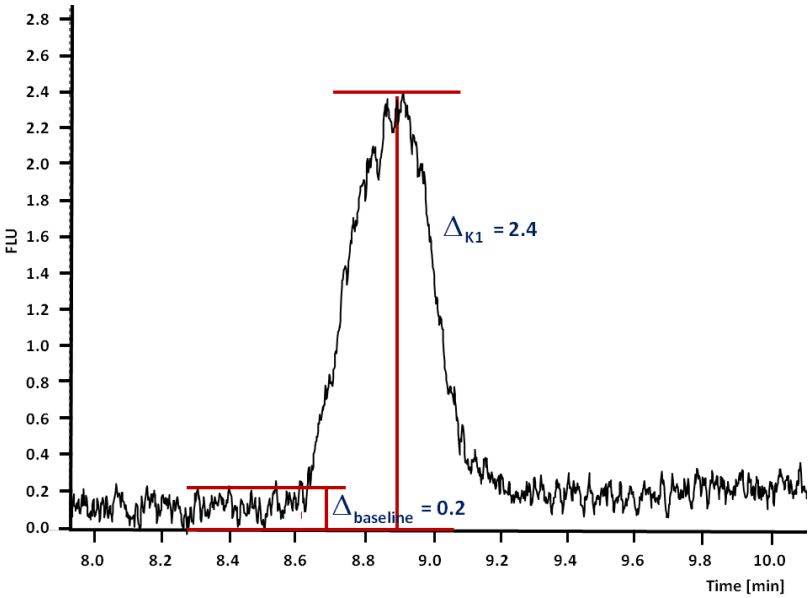

Figure 25 LOQ of K1, signal-to-noise ratio 12:1

Lower limit of quantification (LLOQ) also met the required conditions since the analyte response at the LLOQ was at least 5 times the response compared to blank response and the analyte peak was identifiable, discrete and reproducible with 20% precision and 80-120% accuracy.

The lowest standard on the calibration curve equal to 0.0625 ng K1 and MK4 per absolute injection was accepted as the LLOQ.

Concentration range I was used for precise quantification of vitamin K concentration.

The signals of blank isopropanol samples were compared to the signals of calibration point 1 and 2 containing 0.0625 and 0.125 ng K1 and MK4 per absolute injection, respectively, and the amount of vitamin K was evaluated. Additionally, a solution of 0.1 ng/ absolute injection of both substances (solution LQ) was analyzed quantitatively in the same way. The analysis of K1 and MK4 of all three solutions was carried out both with and without solid-phase extraction in order to get insight into possible substance during the extraction procedure.

Beside serum pool without any supplemental K1 or MK4 (QM1 with basal serum concentration of K1 and without any detectable MK4), pool samples enriched by 0.1 ng K1 and MK4 per absolute injection (QM2 containing basal serum concentration of K1 plus 0.1 ng/ absolute injection and MK4 of 0.1 ng/ absolute injection) were analyzed. Table 5 lists relevant parameters of the quantitative K1 and MK4 analysis regarding LOQ.

Table 5 Limit of quantification of the method for MK4 and K1

Sample	***Solide-phase extraction***	***Nominal value*** [ng/ abs.inj.]		***Actual value*** **Mean** [ng/ abs.inj.]		***Standard deviation SD***		***Variation coefficient*** **CV %**		***Recovery*** **%**	
		MK4	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
Isopropanol	+	0	0	n.d.	n.d.						
Cal.point 1	+	0.0625	0.0625	0.0611	0.0602	0.014	0.009	22.5	14.4	97.7	96.2
Cal.point 2	+	0.125	0.125	0.133	0.121	0.036	0.008	27.3	6.4	106.4	96.9
Solution LQ	+	0.100	0.100	0.096	0.115	0.010	0.001	10.7	0.7	96.2	114.9
Isopropanol	-	0	0	n.d.	n.d.						
Cal.point 1	-	0.0625	0.0625	0.0716	0.0720	0.002	0.004	3.3	5.0	114.6	115.2
Cal.point 2	-	0.125	0.125	0.136	0.128	0.009	0.017	6.4	13.2	108.8	102.3
Solution LQ	-	0.100	0.100	0.098	0.108	0.004	0.003	4.4	3.1	97.5	108.4
QM1	+	n.d	*	n.d.	0.268		0.004		1.6		
QM2	+	0.100	**	0.138	0.332	0.003	0.134	1.8	40.4	138.2	112.3

n= 6 for each sample
n.d.= non-detectable
* basal serum concentration
** basal serum concentration + 0.1 ng/ absolute injection

3.2.3. Calibration/ standard curve

Two concentration ranges of vitamin K1 and MK4 were used for quantification during validation analyses:

Concentration range I → 0.0625 – 2 ng per absolute injection
(calibration points 1-6, described in 3.4.1.5.)

Concentration range II → 1 – 20 ng per absolute injection
(calibration points 5-10, described in 3.4.1.5.)

Samples containing vitamin K concentration higher than 20 ng per absolute injection were further diluted to gain substance concentration in the range described above.

To evaluate linearity of the calibration curves, several calibration curves were prepared and analyzed. The curves were judged linear if the correlation coefficient r^2 was > 0.99 as calculated by weighted linear regression. The concentrations of the calibration standards were back-calculated in order to confirm the suitability of the used calibration model of linear regression.

In developing a calibration curve, following conditions were met for every concentration range:

- 20 % deviation of the LLOQ from nominal concentration
- 15 % deviation of standards other than LLOQ from nominal concentration
- correlation coefficient of the regression line ≥ 0.99

At least four out of six non-zero standards met the above criteria, including the LLOQ and the calibration standard at the highest concentration.

The standard curves for K1 and MK4 over range I (0.0625-2 ng/ abs.inj.) and range II (1-20 ng/ abs.inj.) exhibited good linearity with average correlation coefficient (r^2) equal to 0.9996 and 0.9965 for MK4 and range I and II, respectively and equal to 0.9992 and 0.9968 for K1 and range I and II, respectively. The deviation of the interpolated concentrations of standards in the daily calibration curves of K1 and MK4 were within the acceptable range of 85 - 115 % (80 - 120 % for LLOQ).

Table 6 and 7 give an overview of the results.

Table 6 Concentration range I – calibration/ standard curve

Calibration point	*Cal.curve r^2*	*Nominal conc.*		*Mean* [ng/ abs.inj.]		*SD*		*CV %*		*Recovery %*	
	MK4 r^2:0.9996 K1 r^2:0.9995	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
1	1a-6a	0.0625	0.0625	0.058	0.059	0.001	0.001	0.1	2.4	92.3	94.3
2	1a-6a	0.125	0.125	0.115	0.123	0.001	0.006	0.6	5.3	91.7	98.4
3	1a-6a	0.25	0.25	0.240	0.257	0.011	0.011	4.5	4.4	96.0	102.8
4	1a-6a	0.5	0.5	0.475	0.491	0.012	0.016	2.5	3.2	95.0	98.2
5	1a-6a	1	1	0.888	0.942	0.009	0.017	1.0	1.8	88.6	94.2
6	1a-6a	2	2	1.786	1.957	0.056	0.104	3.2	5.3	89.3	97.9
	MK4 r^2:0.9996 K1 r^2:0.9993	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
1	1b-6b	0.0625	0.0625	0.051	0.051	0.003	0.001	5.8	2.7	82.2	81.8
2	1b-6b	0.125	0.125	0.108	0.116	0.006	0.015	6.0	12.9	86.5	92.7
3	1b-6b	0.25	0.25	0.259	0.256	0.011	0.024	4.4	9.4	103.4	102.5
4	1b-6b	0.5	0.5	0.528	0.512	0.013	0.014	2.4	2.7	105.6	102.5
5	1b-6b	1	1	1.080	1.055	0.100	0.065	9.2	6.2	108.0	105.5
6	1b-6b	2	2	2.096	2.008	0.016	0.008	0.1	0.1	104.8	100.4
	MK4 r^2:0.9997 K1 r^2: 0.9988	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
1	1c-6c	0.0625	0.0625	0.060	0.068	0.004	0.003	6.5	4.0	95.8	108.2
2	1c-6c	0.125	0.125	0.116	0.122	0.007	0.007	6.0	6.1	92.4	97.8
3	1c-6c	0.25	0.25	0.249	0.248	0.003	0.010	1.0	4.0	99.8	99.2
4	1c-6c	0.5	0.5	0.526	0.505	0.001	0.003	0.2	0.5	105.2	101.1
5	1c-6c	1	1	1.039	0.985	0.105	0.077	10.1	7.8	103.9	98.5
6	1c-6c	2	2	1.974	1.875	0.077	0.001	3.9	0.1	98.7	93.8

n= 3 for each calibration point

Table 7 Concentration range II – calibration/ standard curve

Calibration point	*Cal.curve r^2*	*Nominal conc.*		*Mean* [ng/ abs.inj.]		*SD*		*CV %*		*Recovery %*	
	MK4 r^2:0.9974 K1 r^2:0.9976	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
5	5a-10a	1	1	0.807	0.880	0.004	0.007	0.5	0.8	80.7	88.0
6	5a-10a	2	2	1.732	1.915	0.088	0.067	5.1	3.5	86.6	95.8
7	5a-10a	5	5	5.150	5.374	0.108	0.096	2.1	1.8	103.0	107.5
8	5a-10a	10	10	10.342	10.633	0.098	0.313	1.0	2.9	103.4	106.3
9	5a-10a	15	15	13.914	14.501	0.278	0.359	2.1	2.5	92.8	96.7
10	5a-10a	20	20	19.548	20.439	0.422	0.502	2.2	2.5	97.7	102.2
	MK4 r^2:0.9957 K1 r^2:0.9961	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
5	5b-10b	1	1	0.868	0.844	0.004	0.071	0.4	8.4	86.8	84.8
6	5b-10b	2	2	1.838	1.888	0.127	0.122	6.9	6.5	91.9	94.4
7	5b-10b	5	5	5.429	5.394	0.087	0.092	1.6	1.7	108.6	107.9
8	5b-10b	10	10	10.455	10.387	0.593	0.700	5.7	6.7	104.6	103.9
9	5b-10b	15	15	14.429	14.415	0.406	0.528	2.8	3.7	96.2	96.1
10	5b-10b	20	20	20.203	20.295	0.661	0.768	3.3	3.8	101.0	101.5
	MK4 r^2:0.9965 K1 r^2:0.9968	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
5	5c-10c	1	1	0.816	0.880	0.054	0.010	6.6	1.2	81.6	88.0
6	5c-10c	2	2	1.735	1.798	0.034	0.053	2.0	3.0	86.7	89.9
7	5c-10c	5	5	5.212	5.166	0.025	0.003	0.5	0.1	104.2	103.3
8	5c-10c	10	10	10.105	9.928	0.477	0.497	4.7	5.0	101.0	99.3
9	5c-10c	15	15	13.833	13.694	0.104	0.153	0.8	1.1	92.2	91.3
10	5c-10c	20	20	19.351	19.283	0.216	0.256	1.1	1.3	96.8	96.4

n= 3 for each calibration point
* basal serum concentration
** basal serum concentration + 0.1 ng/ absolute injection

3.2.4. Accuracy – robin round tests, KEQAS participation

The ***accuracy*** describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. To assess the ***accuracy*** of the method by examining vitamin K presented in human serum at basal level, our lab participated in the vitamin K quality assurance scheme (KEQAS), the so-called robin-round tests. The performance of such tests aims to assist in the development and harmonisation of vitamin K analysis in order to improve the comparability of clinical and nutritional studies [97] as well as to improve the quality of vitamin K analysis.

Aliquots from a serum pool including spiked samples (vitamin K spiked within an endogenous range) as well as vitamin K ethanolic standard solutions were distributed for multiple replicate analyses within a period of one year by a number of participants including our lab (specified as group 44). All samples had to be analyzed according to the scheme schedule.

Three batches per year (batch 43, 44 and 45 in 2010 and batch 46, 47 and 48 in 2011) were sent out to be measured in February, June and October respectively. Thereby each batch contained two plasma samples (serum A and serum B) and one ethanolic standard (EtOH std.). The target for results was ±20 % deviation from the ALTM (*all laboratory trimmed mean* representing the target concentration) which was based on previous performance and designed to be attainable and yet indicative of poor performance according to participants´ manuals.

A guide to performance was shown using a Z score system where:

$$Z = \frac{X_i - X}{\sigma}$$

Z : Z score

X_i : Result

X : Mean result from all labs

Σ : Target standard deviation

The target standard deviation was 20% from the mean:

$$Z = \frac{5 \,.\, (X_i - X)}{X}$$

Z scores were divided into the following categories:

Z < 1	Good - *GOLD*
1 < Z < 2	Satisfactory - *SILVER*
2 < Z < 3	Questionable - *BRONZE*
Z > 3	Unsatisfactory - *RED*

Table 8 and 9 summarized the results achieved from our laboratory from the robin round tests in 2010 and 2011, respectively. The international requirements for harmonisation and quality assurance of vitamin K analysis were herewith certified successfully.

Table 8 KEQAS results 2010

	Serum A	Serum B	EtOH std.	Serum A	Serum B	EtOH std.	Serum A	Serum B	EtOH std.
	Batch 43 K1 [µg/ L]			***Batch 44*** K1 [µg/ L]			***Batch 45*** K1 [µg/ L]		
Result	0.25	2.06	1.41	0.26	0.16	1.69	2.06	0.16	4.06
ALTM	0.32	2.91	2.16	0.31	0.19	2.49	2.85	0.16	5.38
Z score	1.1	1.5	1.7	0.7	0.8	1.6	1.4	0.1	1.2
Category	*SILVER*	*SILVER*	*SILVER*	*GOLD*	*GOLD*	*SILVER*	*SILVER*	*GOLD*	*SILVER*
Outlier?	no	no	no	no	no	no	no	no	no

Table 9 KEQAS results 2011

	Serum A	Serum B	EtOH std.	Serum A	Serum B	EtOH std.	Serum A	Serum B	EtOH std.
	Batch 46 K1 [µg/ L]			***Batch 47*** K1 [µg/ L]			***Batch 48*** K1 [µg/ L]		
Result	0.53	0.97	1.40	0.74	3.47	2.07	3.11	-	2.24
ALTM	0.53	0.86	1.26	0.86	3.92	1.52	3.64	-	2.51
Z score	0.0	0.6	0.6	0.7	0.6	1.8	0.7	-	0.5
Category	*GOLD*	*GOLD*	*GOLD*	*GOLD*	*GOLD*	*SILVER*	*GOLD*	-	*GOLD*
Outlier?	no	no	no	no	no	no	no		no

3.2.5. Precision

To assess the ***precision*** of the method, six replicates per concentration for QMs (described in 3.1.3.3.) were analyzed three times on the same day and once a day during three days within a week in order to determine the intra- and interday-reproducibility, respectively.

The ***precision*** of the method at each concentration was calculated as the coefficient of variation (CV). A CV of less than 15 % was accepted, except for the LLOQ, where it should not be more than 20 % as required in the guidelines (s. 3.5.4.). The precision determined at each concentration level did not exceed 15 % of the CV except for the LLOQ, where it did not exceed 20 % of the CV.

The data for the validation of intra- and inter-day accuracy and precision are presented in table 10 and 12 for K1 and table 11 and 13 for MK4. The results showed CVs within the acceptable range.

Table 10 Intra-day reproducibility for K1

Sample	*Nominal value* [ng/ inj.]	*Actual value* **Mean** [ng/ inj.]			*Standard deviation SD*			*Variation coefficient* **CV %**			*Recovery* **%**		
Run		**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
Cal.point 3	0.25	0.27	0.28	0.26	0.01	0.01	0.01	3.9	2.9	2.0	106	107	105
Cal.point 5	1	0.99	0.92	1.00	0.02	0.01	0.04	2.4	1.5	4.1	99	92	86
Cal.point 8	10	10.0	10.2	10.8	0.25	1.2	1.31	2.5	11.7	9.5	100	102	108
QM1	*	0.24	0.14	0.16	0.22	0.01	0.02	9.0	6.6	8.8			
QM3	***	1.08	1.12	1.09	0.05	0.06	0.06	4.4	5.0	5.8	95	98	95
QM4	****	10.2	10.7	10.9	0.56	0.46	1.11	5.5	4.2	7.9	101	106	108
QM5	*****	107	105	110	9.01	3.97	5.99	8.4	3.8	5.5	107	105	109

n= 6 for each sample
* basal serum concentration
** basal serum concentration + 0.1 ng/ absolute injection
*** basal serum concentration + 1 ng / absolute injection
**** basal serum concentration + 10 ng/ absolute injection
***** basal serum concentration + 100 ng/ absolute injection

Table 11 Intra-day reproducibility for MK4

Sample	*Nominal value* [ng/ inj.]	*Actual value* **Mean** [ng/ inj.]			*Standard deviation SD*			*Variation coefficient* **CV %**			*Recovery* **%**		
Run		**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
Cal.point 3	0.25	0.22	0.22	0.21	0.02	0.03	0.02	6.7	7.2	9.4	89	90	105
Cal.point 5	1	0.90	0.98	0.90	0.06	0.03	0.06	7.1	2.6	6.9	90	98	90
Cal.point 8	10	9.9	9.6	11.0	0.30	1.11	2.04	3.0	11.6	13.6	99	96	110
QM1	n.d.	n.d.	n.d.	n.d.									
QM3	1	1.00	0.97	0.98	0.05	0.04	0.06	4.6	3.6	5.9	100	97	98
QM4	10	11.2	11.4	11.3	0.98	0.53	1.41	8.8	4.6	9.2	112	114	113
QM5	100	110	110	112	15.9	2.92	6.57	14.4	2.7	5.9	111	110	112

n= 6 for each sample n.d.= non-detectable

Table 12 Inter-day reproducibility for K1

Sample	*Nominal value* [ng/ inj.]	*Actual value* **Mean** [ng/ inj.]			*Standard deviation SD*			*Variation coefficient* **CV %**			*Recovery* **%**		
Day		**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
Cal.point 3	0.25	0.27	0.24	0.26	0.03	0.01	0.02	10.1	4.8	4.9	109	97	105
Cal.point 4	0.5	0.49	0.48	0.49	0.03	0.01	0.02	5.2	2.7	4.2	99	96	98
Cal.point 5	1	0.96	1.05	1.01	0.03	0.05	0.06	2.9	4.2	5.2	96	105	100
Cal.point 8	10	10.4	10.1	9.9	0.56	0.32	0.76	4.4	3.2	7.6	104	100	100
QM1	*	0.24	0.22	0.23	0.01	0.01	0.03	5.6	2.6	12.6			
QM2	**	0.32	0.30	0.31	0.02	0.01	0.02	6.5	3.2	6.0	99	91	100
QM3	***	1.31	1.29	1.36	0.07	0.08	0.06	5.4	5.9	4.6	107	106	114
QM4	****	11.5	11.4	10.7	0.42	0.32	0.62	3.6	2.8	5.8	113	112	105
QM5	*****	105	107	103	4.4	1.51	1.36	4.2	1.4	1.3	105	108	103

n= 6 for each sample
* basal serum concentration
** basal serum concentration + 0.1 ng/ absolute injection
*** basal serum concentration + 1 ng/ absolute injection
**** basal serum concentration + 10 ng/ absolute injection
***** basal serum concentration + 100 ng/ absolute injection

Table 13 Inter-day reproducibility for MK4

Sample	*Nominal value* [ng/ inj.]	*Actual value* **Mean** [ng/ inj.]			*Standard deviation SD*			*Variation coefficient* **CV %**			*Recovery* **%**		
Day		**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
Cal.point 3	0.25	0.23	0.24	0.22	0.02	0.02	0.01	4.9	7.7	3.4	92	95	89
Cal.point 5	1	0.86	0.89	0.88	0.02	0.07	0.10	2.7	6.8	11.6	86	89	88
Cal.point 8	10	9.53	9.39	10.9	0.94	0.19	1.50	9.9	2.8	13.6	95	94	109
QM1	n.d.	n.d.	n.d.	n.d.									
QM3	1	1.09	1.02	1.09	0.11	0.07	0.04	9.7	7.3	3.8	109	102	109
QM4	10	10.9	11.3	11.2	1.21	0.38	0.20	11.2	2.0	1.8	109	113	112
QM5	100	105	99.1	114	4.71	1.86	4.29	4.2	2.4	3.8	105	99	114

n= 6 for each sample n.d.= non-detectable

3.2.6. Recovery

Recovery experiments were performed by comparing analytical results for extracted samples at different concentrations (low, medium, high) with unextracted samples that represent 100 % recovery.

Recovery rate

MK4 → MK4 amount quantified from a spiked serum samples compared to MK4 solutions in isopropanol of the same concentration

MK4 in QM2 *vs* MK4 in solution LQ (0.1 ng/ inj.)

MK4 in QM3 *vs* MK4 in Cal.5 (1 ng/ inj.)

MK4 in QM4 *vs* MK4 in Cal.8 (10 ng/ inj.)

K1 → K1 amount quantified from a spiked serum samples *minus* measured K1 amount in QM1 (K1 mean from minimum 3 QM1 samples) compared to K1 solutions in isopropanol of the same concentration

K1 in QM2 *minus* K1 in QM1 *vs* K1 in solution LQ (0.1 ng/ inj.)

K1 in QM3 *minus* K1 in QM1 *vs* K1 in Cal.5 (1 ng/ inj.)

K1 in QM4 *minus* K1 in QM1 *vs* K1 in Cal.8 (10 ng/ inj.)

The ratio of the estimated concentration from matrix-containing samples ($C_{matrix,SPE}$) to that from the solutions in isopropanol ($C_{isopropanol}$) yielded the recovery rate.

Recovery rate $$Rec\ 1\ [\%] = \frac{C_{matrix,SPE}}{C_{isopropanol}} \times 100$$

These experiments allowed an insight into the influence of the matrix (serum) and the extraction procedure on the signal areas to be gained.

In addition, the influence of the matrix and the extraction procedure respectively were investigated separately.

Recovery rate $$Rec\ 2\ [\%] = \frac{C_{matrix,SPE}}{C_{isopropanol,SPE}} \times 100$$

Recovery rate $$Rec\ 3\ [\%] = \frac{C_{isopropanol,SPE}}{C_{isopropanol}} \times 100$$

Calculated recovery rates (*Rec 1, Rec 2, Rec 3*) are summarized in table 14.

Table 14 Recovery rates of MK4 and K1

Sample	*SPE* *Solide-phase extraction*	*Nominal value* [ng/ abs.inj.]		*Actual value* Mean [ng/ abs.inj.]		*Rec 1* %		*Rec 2* %		*Rec 3* %	
		MK4	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
Isopropanol	+	0	0	n.d.	n.d.						
Isopropanol	-	0	0	n.d.	n.d.						
Solution LQ	+	0.1	0.1	0.07	0.12					**70**	**109**
Solution LQ	-	0.1	0.1	0.10	0.11						
Cal.point 5	+	1	1	0.90	0.99					**89**	**95**
Cal.point 5	-	1	1	1.01	1.04						
Cal.point 8	+	10	10	9.92	10.2					**100**	**101**
Cal.point 8	-	10	10	9.88	10.1						
QM1	+	n.d	*	n.d.	0.27						
QM2	+	0.1	**	0.09[(1)]	0.40[(1)]	**90**	**118**	**129**	**108**		
QM3	+	1	***	0.98	1.12	**97**	**82**	**109**	**86**		
QM4	+	10	****	11.2	10.7	**113**	**103**	**113**	**102**		

n= 6 for each sample n.d.= non-detectable
* basal serum concentration
** basal serum concentration + 0.1 ng/ absolute injection
*** basal serum concentration + 1 ng/ absolute injection
**** basal serum concentration + 10 ng/ absolute injection
(1) n= 4

3.2.7. Quality assurance

For quality assurance in analysis, the intraday and interday CVs were evaluated (shown in table 10-13; see section 3.2.5.).

Spiked samples and solutions in isopropanol of the substances of interest (e.g. QM3, QM4, Cal.5, Cal.8) were used to monitor the performance of the bioanalytical method for vitamin K determination and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

A batch run was accepted and the data underwent further analysis when following conditions were met:

- The relative error does not exceed 15 % (20 % for LLOQ)
- 2/3 of all quality assurance samples should meet this condition

The relative error *RE* is calculated as follows:

$$RE\ [\%]\ = \frac{(MC_{act} - C_{nom})}{C_{nom}} \times 100$$

MC_{act} = mean of actual concentration

C_{nom} = nominal concentration

3.2.8. Stability

Stability testing of K vitamins (MK4, K1) was performed on both in serum and stock solution. For stability in serum, QMs were analyzed in triplicate for determination of freeze and thaw stability; short-term stability; long-term stability and post-preparative stability. The substances of interest were considered stable if more than 85 % were retained at the end of the study period; for the lowest and highest quantifiable concentration 80 - 120 % recovery were accepted. CV values of lower than 20 % were required.

3.2.8.1. Freeze and thaw stability

The freeze-thaw stability was determined after 3 refreeze and thaw cycles. Three aliquots of the low (solution LQ, QM1) and high (cal. solution 10, QM4) concentration were stored at -80 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 hours under the same conditions. The freeze-thaw cycles were repeated twice and then analyzed immediately on the third cycle. The results are summarized in table 15.

Table 15 Freeze and thaw stability

Sample	***Nominal value*** [ng/ abs.inj.]		***Actual value*** **Mean** [ng/ abs.inj.]		***Standard deviation SD***		***Variation coefficient*** **CV %**		***Recovery*** **%**	
	MK4	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
Solution LQ	0.1	0.1	0.081	0.091	0.001	0.001	1.4	0.9	80.9	91.4
Cal.point 10	20	20	22.175	23.395	2.281	2.438	10.3	10.4	110.9	117.0
QM1	n.d.	*	n.d.	0.120		0.006		4.7		
QM4	10	****	9.004	8.826	1.125	0.852	14.1	9.7	90.0	87.1

n= 3 for each sample n.d.= non-detectable
* basal serum concentration
**** basal serum concentration + 10 ng/ absolute injection

3.2.8.2. Short-term stability

Three aliquots of each of the low (solution LQ, QM1) and high concentrations (cal. solution 10, QM4) were thawed at room temperature, kept at this temperature for five hours (based on the expected duration that samples will be maintained at room

temperature in the intended study) and analyzed. Results are summarized in table 16.

Table 16 Short-term stability

Sample	***Nominal value*** [ng/ abs.inj.]		***Actual value*** **Mean** [ng/ abs.inj.]		***Standard deviation SD***		***Variation coefficient*** **CV %**		***Recovery*** **%**	
	MK4	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**	**MK4**	**K1**
Solution LQ	0.1	0.1	0.083	0.092	0.010	0.005	7.9	5.3	83.2	92.0
Cal.point 10	20	20	22.486	22.405	2.455	1.948	10.9	8.7	112.4	112.0
QM1	n.d.	*		0.155		0.043		7.7		
QM4	10	****	9.627	9.058	1.291	0.543	13.4	6.0	96.3	89.1

n= 3 for each sample n.d.= non-detectable
* basal serum concentration
**** basal serum concentration + 10 ng/absolute injection

3.2.8.3. Long-term stability

The long-term stability in frozen human serum (-80 °C) was determined by storing three aliquots of each of the low (solution LQ, QM1) and high concentrations (cal. solution 10, QM4) under the same conditions as the study samples (at -80 °C) and periodic analysis over six months. Prior to their analysis, samples were brought to room temperature and vortex-mixed well. The following batches of serum samples were prepared:

Batch I → analysis immediately
Batch II → analysis after 1 month storage at -80 °C
Batch III → analysis after 3 months storage at -80 °C
Batch IV → analysis after 6 months storage at -80 °C

Table 17 and 18 present the results for the long-term stability of K1 and MK4 respectively. The corresponding CV and recovery values were within the acceptable range (CV< 20 %, Recovery 80-120 %) during performed periodic analysis over six months.

Table 17 Long-term stability of K1

Sample	***Nominal value*** [ng/ inj.]	***Actual value*** **Mean** [ng/ inj.]				***Variation coefficient*** **CV %**				***Recovery*** **%**			
Batch		**I**	**II**	**III**	**IV**	**I**	**II**	**III**	**IV**	**I**	**II**	**III**	**IV**
Solution LQ	0.1	0.09	0.09	0.10	0.08	0.9	5.2	2.9	2.1	91	92	103	84
Cal.point 10	20	23.4	19.1	21.4	17.5	10.4	3.5	1.7	3.5	117	96	107	88
QM1	*	0.12	0.12	0.13	0.18	4.7	8.5	7.5	3.5				
QM4	****	8.83	9.72	8.16	10.1	9.7	3.4	4.7	16.1	87	96	81	99

n= 3 for each sample and batch
* basal serum concentration
**** basal serum concentration + 10 ng/ absolute injection

Table 18 Long-term stability of MK4

Sample	***Nominal value*** [ng/ inj.]	***Actual value*** **Mean** [ng/ inj.]				***Variation coefficient*** **CV %**				***Recovery*** **%**			
Batch		**I**	**II**	**III**	**IV**	**I**	**II**	**III**	**IV**	**I**	**II**	**III**	**IV**
Solution LQ	0.1	0.08	0.08	0.08	0.08	1.4	6.5	5.5	2.6	81	85	82	81
Cal.point 10	20	22.2	19.2	20.6	17.0	10.3	5.1	1.3	7.6	111	96	103	85
QM1	n.d.	n.d.	n.d.	n.d.	n.d.								
QM4	10	8.01	9.8	8.56	10.7	14.1	3.4	2.4	12.8	80	98	86	107

n= 3 for each sample and batch n.d.= non-detectable

3.2.8.4. Stock solution stability

The stability of stock solutions of the substances of interest and the internal standard were evaluated by storing the solutions 1-10 under defined conditions for a designated period of time. Thereafter, analysis of the samples was carried out and vitamin K concentration was calculated.

Batch I	→	at room temperature + daylight for 6 h	→	analysis
Batch II	→	at room temperature + in the dark for 6 h	→	analysis
Batch III	→	frozen at -20 °C for 6 h,		
		thawed at room temperature for 1 h	→	analysis

Table 19 Stock solution stability for K1

Sample	***Nominal value*** [ng/ inj.]	***Actual value*** **Mean** [ng/ inj.]			***I,II,III*** **Mean** [ng/ inj.]	***SD***	**CV %**	***Recovery*** **%**
Batch		**I***	**II ***	**III***				
Cal.point 1	0.0625	0.056	0.055	0.058	0.056	0.002	2.9	90.2
Cal.point 2	0.125	0.116	0.119	0.113	0.116	0.003	2.4	92.7
Cal.point 3	0.25	0.251	0.291	0.255	0.266	0.022	8.2	106.3
Cal.point 4	0.5	0.456	0.498	0.510	0.488	0.029	5.9	97.6
Cal.point 5	1	0.971	1.054	1.088	1.038	0.060	5.8	103.8
Cal.point 6	2	2.043	2.429	2.062	2.178	0.218	10.0	108.9
Cal.point 7	5	4.849	5.082	5.199	5.044	0.178	3.5	100.9
Cal.point 8	10	9.455	10.609	9.203	9.755	0.750	7.7	97.6
Cal.point 9	15	16.595	14.973	13.899	15.156	1.357	9.0	101.0
Cal.point 10	20	18.520	22.122	23.341	21.328	2.507	11.8	106.6

n= 3 for each sample
I* batch I (room temperature + daylight for 6 h before analysis)
II* batch II (room temperature + in the dark for 6 h before analysis)
III* batch III (frozen at -20°C for 6 h, thawed at room temperature for 1 h before analysis)

Table 20 Stock solution stability for MK4

Sample	***Nominal value*** [ng/ inj.]	***Actual value*** **Mean** [ng/ inj.]			***I,II,III*** **Mean** [ng/ inj.]	***SD***	**CV %**	***Recovery*** **%**
Batch		**I***	**II ***	**III***				
Cal.point 1	0.0625	0.044	0.052	0.054	0.050	0.005	10.8	79.9
Cal.point 2	0.125	0.106	0.116	0.118	0.113	0.007	5.9	90.5
Cal.point 3	0.25	0.263	0.254	0.243	0.244	0.009	3.7	97.7
Cal.point 4	0.5	0.439	0.496	0.495	0.477	0.033	6.9	95.3
Cal.point 5	1	0.949	1.083	1.076	1.036	0.076	7.3	103.6
Cal.point 6	2	2.328	2.410	1.972	2.237	0.232	10.4	111.8
Cal.point 7	5	4.478	4.808	4.857	4.715	0.206	4.4	94.3
Cal.point 8	10	9.063	10.157	8.844	9.355	0.704	7.5	93.5
Cal.point 9	15	15.600	14.101	13.618	14.440	1.034	7.2	96.3
Cal.point 10	20	17.726	21.082	22.068	20.292	2.276	11.2	101.5

n= 3 for each sample
I* batch I (room temperature + daylight for 6 h before analysis)
II* batch II (room temperature + in the dark for 6 h before analysis)
III* batch III (frozen at -20°C for 6 h, thawed at room temperature for 1 h before analysis)

3.2.8.5. Post-preparative stability

The stability of the substance of interest and the internal standard were assessed over a period of 24 h run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

Processed serum samples and stock solutions containing MK4 and K1 were found to be stable at 10° C for 24 h in the autosampler. CV and recovery values were within the acceptable range as stated above.

3.3. Vitamin K levels in mouse tissue

Organ phylloquinone and MK4 were assayed as previously described in 3.6. The following mice tissues were dissected and prepared for analysis:

lung, bone, pancreas, kidney, tongue, muscle, heart, brain, liver, ovary, uterus, testis, salivary gland

The calculated concentrations of vitamin K1 and MK4 varied per tissue and are shown in table 21. Values are represented as means in pmol per g dry weight tissue as well as medians for both genders separately. Additionally, K1 to MK4 ratio was calculated for each tissue.

Phylloquinone (K1) was recovered in all tissues with highest levels found in *liver*, *ovary* and *tongue*. Menaquinone-4 (MK4) was also present in all tissues. Remarkably high tissue concentrations of MK4 exceeding significantly the K1 levels were found in *pancreas*, *brain*, *heart*, *kidney* and *testis*. K1 status nearly equalled MK4 levels in *lung* and *uterus*. Relatively high tissue concentrations of K1 and MK4 were measured in *bone*, *muscle* and *salivary gland*. Thereby, the MK4 values exceeded the corresponding K1 levels.

Table 21 Tissue distribution of vitamin K in mice [pmol/ g dry tissue]

Organ	*Vit. K*	*Mean ± S.E.*		*Median*	
		Male ♂	*Female* ♀	*Male*	*Female*
Lung	**K1**	200.5 ± 60.5 n=6	361.3 ± 137.6 n=7	198.6	342.5
	MK4	290.2 ± 53.9 n=8	398.0 ± 69.5 n=9	262.7	288.8
	$^{K1}/_{MK4}$	0.69	0.91	0.76	1.19
Bone	**K1**	156.8 ± 88.5 n=7	314.7 ± 127.7 n=7	29.9	237.2
	MK4	304.3 ± 44.1 n=9	591.6 ± 183.8 n=9	305.4	550.5
	$^{K1}/_{MK4}$	0.52	0.53	0.10	0.43
Pancreas	**K1**	191.5 ± 92.7 n=7	319.8 ± 130.7 n=6	93.3	190.2
	MK4	3609.1 ± 389.0 n=9	3746.6 ± 444.3 n=9	4140.2	4110.3
	$^{K1}/_{MK4}$	0.05	0.09	0.02	0.05
Kidney	**K1**	171.0 ± 78.4 n=7	373.9 ± 245.8 n=5	56.5	180.6
	MK4	1270.8 ± 216.7 n=8	2605.5 ± 665.4 n=8	1210.9	2030.9
	$^{K1}/_{MK4}$	0.13	0.14	0.05	0.09
Tongue	**K1**	889.9 ± 531.2 n=7	1747.7 ± 760.3 n=7	191.5	903.2
	MK4	572.3 ± 62.1 n=9	1120.0 ± 86.6 n=9	624.2	1105.6
	$^{K1}/_{MK4}$	1.55	1.56	0.31	0.82

Organ	*Vit. K*	*Mean ± S.E.*		*Median*	
Muscle	**K1**	72.8 ± 28.0 n=7	153.0 ± 54.3 n=8	35.9	111.3
	MK4	439.7 ± 93.1 n=9	589.4 ± 18.9 n=9	380.9	589.8
	$^{K1}/_{MK4}$	0.17	0.26	0.09	0.19
Heart	**K1**	148.1 ± 53.6 n=7	175.9 ± 59.8 n=7	86.6	186.2
	MK4	979.0 ± 104.5 n=9	1576.0 ± 135.4 n=9	1151.4	1499.6
	$^{K1}/_{MK4}$	0.15	0.11	0.08	0.12
Brain	**K1**	161.8 ± 87.0 n=6	483.8 ± 296.5 n=5	99.9	225.2
	MK4	2328.8 ± 307.7 n=9	3559.9 ± 148.8 n=8	2352.7	3521.1
	$^{K1}/_{MK4}$	0.07	0.14	0.04	0.06
Liver	**K1**	210.4 ± 134.3 n=8	1054.5 ± 839.8 n=8	25.9	105.2
	MK4	94.9 ± 14.9 n=9	232.0 ± 29.3 n=9	102.5	199.7
	$^{K1}/_{MK4}$	2.22	4.55	0.25	0.53
Ovary	**K1**		2384.0 ± 1465.4 n=8		699.5
	MK4		912.2 ± 97.7 n=9		819.5
	$^{K1}/_{MK4}$		2.61		0.85
Uterus	**K1**		890.1 ± 547.9 n=8		376.5
	MK4		892.6 ± 83.1 n=9		891.5
	$^{K1}/_{MK4}$		1.00		0.42
Testis	**K1**	259.9 ± 119.3 n=8		79.5	
	MK4	2983.8 ± 360.7 n=9		3278.5	
	$^{K1}/_{MK4}$	0.09		0.02	
Salivary gland	**K1**	348.6 ± 231.0 n=4		154.2	
	MK4	514.3 ± 77.3 n=4		480.9	
	$^{K1}/_{MK4}$	0.68		0.32	

3.4. Vitamin K1 determination assay and pharmacokinetic analysis - preliminary studies in human volunteers

Some preliminary experiments were performed over a two-year period and served as first relevant application of the vitamin K determination assay from human serum.

Four human volunteers A, B, C and D were investigated (*experiment 1*) starting with a single oral administration of 2 mg Konakion® MM (p.o.). A wash-out period of 7 days was followed by a single intravenous administration (i.v.) of the same dosage, except for volunteer C and D where a period of more than two weeks reclined between both experiments. Blood samples were collected within 24 h following each Konakion® MM intake.

The experiment was repeated two years later with two of the volunteers (A and B) (*experiment 2*). In addition the same experiment , however, without any Konakion® MM intake (*experiment 3*) was performed to gain the so-called “zero” kinetics when only the endogenous amount of vitamin K1 was present and measured.

After centrifugation collected blood samples were kept frozen until analysis. The samples from each experiment were analyzed directly after the investigation was performed (A_1, B_1, A_2, B_2, C_1, D_1) and a determined period of time later (two years for A_{1R}, B_{1R}, and one year for C_{1R}, D_{1R}).

	A	B	C	D
p.o. ***experiment 1***	**$A_{1,p.o.}$** ▪ $A_{1,p.o.}$ ▪ $A_{1R,p.o.}$	**$B_{1,p.o.}$** ▪ $B_{1,p.o.}$ ▪ $B_{1R,p.o.}$	**$C_{1,p.o.}$** ▪ $C_{1,p.o.}$ ▪ $C_{1R,p.o.}$	**$D_{1,p.o.}$** ▪ $D_{1,p.o.}$ ▪ $D_{1R,p.o.}$
i.v. ***experiment 1***	**$A_{1,i.v.}$** ▪ $A_{1,i.v.}$ ▪ $A_{1R,i.v.}$	**$B_{1,i.v.}$** ▪ $B_{1,i.v.}$ ▪ $B_{1R,i.v.}$	**$C_{1,i.v.}$** ▪ $C_{1,i.v.}$ ▪ $C_{1R,i.v.}$	**$D_{1,i.v.}$** ▪ $D_{1,i.v.}$ ▪ $D_{1R,i.v.}$
p.o. ***experiment 2***	**$A_{2,p.o.}$** ▪ $A_{2,p.o.}$	**$B_{2,p.o.}$** ▪ $B_{2,p.o.}$		
i.v. ***experiment 2***	**$A_{2,i.v.}$** ▪ $A_{2,i.v.}$	**$B_{2,i.v.}$** ▪ $B_{2,i.v.}$		
0 kinetics ***experiment 3***	**A_3**	**B_3**		

Furthermore, the *VKORC1* promoter polymorphism c.-1639 G>A of the four volunteers was analyzed. A and B are homozygous AA carriers, C is heterozygous (AG) and D is a carrier of GG genotype (wild-type).

In addition, serum bile acids levels and the serum concentrations of cholesterol and the immediate cholesterol metabolite and bile acid precursor 7α-hydroxycholesterol were determined from the kinetic samples of volunteers A and B.

3.4.1. Raw concentration – time data after/ without administration of Konakion® MM 2 mg

The following tables present mean values of minimum two measurements of vitamin K1 serum concentrations for each volunteer (A, B, C, D). Additionally, the mean values are plotted in figures/ graphs against the time points of blood collection after vitamin K1 administration.

A1,p.o., A1R,p.o., A2,p.o.

Tables 22 and 23 present mean values of vitamin K1 serum concentrations in ng/ mL after 2 mg Konakion® MM were administered ***orally*** to volunteer **A**.

$\mathbf{A_{1,p.o.}}$ refers to the experiment which was carried out in 2008, while $\mathbf{A_{2,p.o.}}$ relates to the investigation repeated two years later. $\mathbf{A_{1R,p.o.}}$ column shows the vitamin K1 values of the same blood samples collected in 2008 but measured two years later. There was a notable decrease of detectable vitamin K1 by 39.5 % in the mean between the measurements directly after blood collection and two years later.

The mean vitamin K1 serum values obtained from both experiments (*experiment 1* in 2008 and *experiment 2* in 2010, respectively) are plotted against the time in figure 26 and 27.

Table 22 Serum concentration of K1

	$A_{1,p.o.}$	$A_{1R,p.o.}$
Point [min]	K1 [ng/ mL]	K1 [ng/ mL]
-15	0.850	0.186
20	0.582	0.024
63	1.068	0.449
120	54.302	40.591
149	44.503	29.369
183	32.086	20.503
210	22.052	14.793
250	23.915	14.881
270	23.607	9.904
302	17.792	11.980
340	17.661	12.851
362	14.183	9.642
393	10.804	8.007
420	10.244	6.359
480	12.479	8.542
538	11.271	9.549
600	8.841	6.885
1455	2.258	1.580

Table 23 Serum concentration of K1

	$A_{2,p.o.}$
Point [min]	K1 [ng/ mL]
-15	0.548
46	0.721
106	42.211
135	46.711
165	28.625
197	19.086
231	18.267
253	18.496
289	34.964
316	18.098
345	15.793
375	10.151
406	9.863
439	9.767
470	9.316
498	7.463
555	5.180
1436	3.404

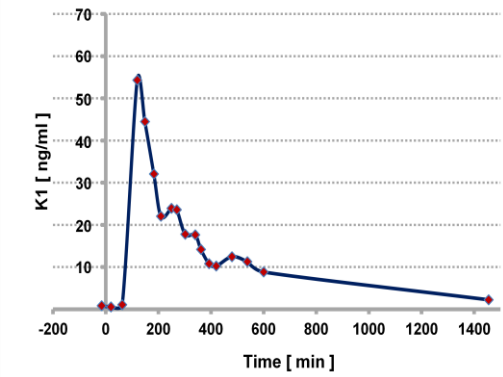

Fig. 26 Vitamin K1 *vs* time- $A_{1,p.o.}$

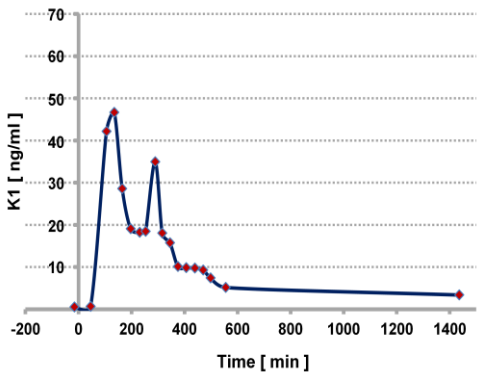

Fig. 27 Vitamin K1 *vs* time- $A_{2,p.o.}$

A1,i.v., A1R,i.v., A2,i.v.

Tables 24 and 25 show mean values of vitamin K1 serum concentrations in ng/ mL after 2 mg Konakion® MM were administered ***intravenously*** to volunteer **A**.

$A_{1,i.v.}$ refers to the experiment which was performed in 2008 while $A_{2,i.v.}$ to the two years later repeated investigation. $A_{1R,i.v.}$ column shows the vitamin K1 values of the same blood samples collected in 2008 but measured two years later. There was a considerable decrease of detectable vitamin K1 by 12.3 % in the mean between the measurements directly after blood samples were collected and two years later.

The mean vitamin K1 serum values obtained from both experiments (*experiment 1* in 2008 and *experiment 2* in 2010, respectively) are plotted against the time in figure 28 and 29.

Table 24 Serum concentration of K1

	$A_{1,i.v.}$	$A_{1R,i.v.}$
Point [min]	**K1 [ng/ mL]**	**K1 [ng/ mL]**
-15	**1.219**	0.670
3	**662.504**	453.300
6	**485.355**	331.060
10	**456.886**	289.700
21	**202.571**	139.120
29	**148.213**	129.063
40	**121.644**	96.684
50	**103.964**	89.375
60	**90.133**	87.488
80	**75.969**	77.369
100	**69.491**	71.150
124	**56.857**	54.703
139	**42.185**	43.752
160	**41.850**	32.281
180	**36.096**	23.517
210	**27.265**	22.366
242	**25.670**	26.142
270	**21.526**	16.200
302	**16.292**	15.603
331	**13.693**	13.472
361	**14.416**	10.686
422	**7.478**	10.428
480	**6.675**	6.331
540	**5.993**	6.701
610	**4.816**	4.301
1455	**1.375**	1.343

Table 25 Serum concentration of K1

	$A_{2,i.v.}$
Point [min]	**K1 [ng/ mL]**
-15	**0.918**
3	**430.192**
5	**365.483**
10	**274.372**
23	**137.205**
32	**85.652**
42	**69.141**
55	**58.044**
62	**52.046**
80	**42.623**
97	**40.123**
129	**35.032**
143	**27.635**
157	**27.001**
183	**19.734**
209	**17.768**
242	**16.613**
270	**13.580**
300	**12.693**
365	**7.324**
420	**8.253**
482	**5.241**
539	**4.808**
600	**3.356**
1442	**1.184**

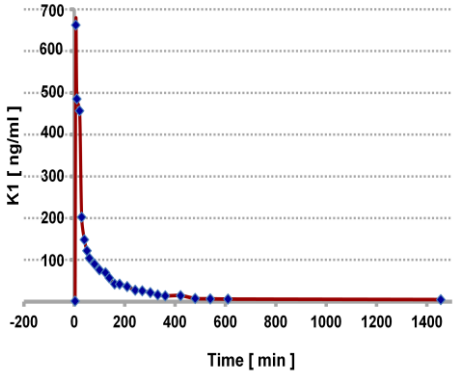

Fig. 28 Vitamin K1 *vs* time- $A_{1,i.v.}$

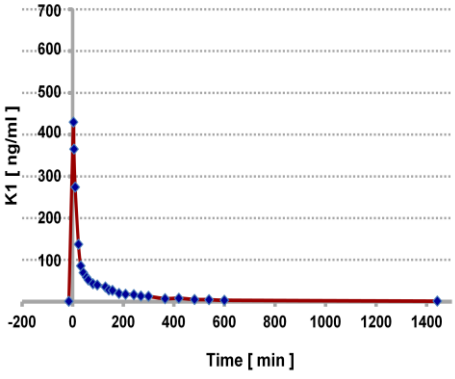

Fig. 29 Vitamin K1 *vs* time- $A_{2,i.v.}$

$B_{1,p.o.}$, $B_{1R,p.o.}$, $B_{2,p.o.}$

Tables 26 and 27 present mean values of vitamin K1 serum concentrations in ng/ mL after 2 mg Konakion® MM were administered ***orally*** to volunteer **B**.

$B_{1,p.o.}$ refers to the experiment which was carried out in 2008, while **$B_{2,p.o.}$** relates to the investigation repeated two years later. **$B_{1R,p.o.}$** column shows the vitamin K1 values of the same blood samples collected in 2008 but measured two years later. There was a notable decrease of detected vitamin K1 by 27.8 % in the mean between the measurements directly after blood collection and two years later. The mean vitamin K1 serum values obtained from both experiments (*experiment 1* in 2008 and *experiment 2* in 2010, respectively) are plotted against the time in figure 30 and 31.

Table 26 Serum concentration of K1

	$B_{1,p.o.}$	$B_{1R,p.o.}$
Point [min]	**K1 [ng/ mL]**	**K1 [ng/ mL]**
0	**0.585**	0.582
60	**0.583**	0.543
120	**13.527**	8.900
180	**53.673**	34.461
210	**59.931**	44.065
240	**48.211**	44.690
270	**42.221**	31.226
300	**30.271**	27.860
330	**33.002**	22.408
360	**36.638**	25.679
390	**39.998**	21.105
420	**40.963**	22.721
450	**41.676**	24.427
480	**38.005**	12.390
520	**27.689**	23.426
580	**24.273**	16.374
1440	**3.800**	3.188

Table 27 Serum concentration of K1

	$B_{2,p.o.}$
Point [min]	**K1 [ng/ mL]**
-15	**0.325**
60	**0.393**
119	**7.386**
150	**20.926**
179	**19.829**
210	**14.386**
242	**12.231**
271	**10.401**
300	**16.728**
330	**18.649**
360	**14.425**
390	**9.737**
423	**7.960**
450	**7.172**
482	**6.768**
541	**3.977**
596	**3.645**
1440	**0.956**

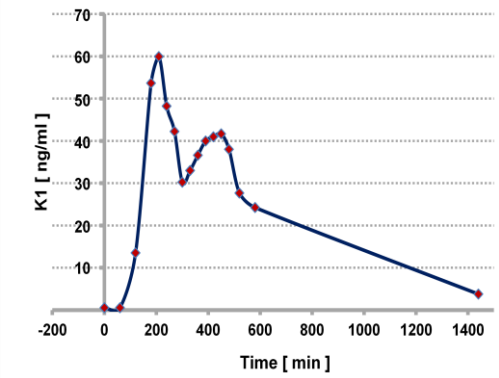

Fig. 30 Vitamin K1 *vs* time- $B_{1,p.o.}$

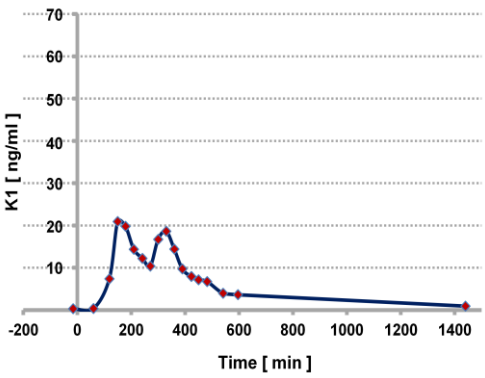

Fig. 31 Vitamin K1 *vs* time- $B_{2,p.o.}$

$B_{1,i.v.}$, $B_{1R,i.v.}$, $B_{2,i.v.}$

Tables 28 and 29 show mean values of vitamin K1 serum concentrations in ng/ mL after 2 mg Konakion® MM were administered ***intravenously*** to volunteer **B**.

$B_{1,i.v.}$ refers to the experiment which was performed in 2008, while **$B_{2,i.v.}$** relates to the two years later repeated investigation. **$B_{1R,i.v.}$** column presents the vitamin K1 values of the same blood samples collected in 2008 but measured two years later. The decrease of detectable vitamin K1 averaged 28.0 % between the measurements directly after blood sampling and after two year storage.

The mean vitamin K1 serum values obtained from both experiments (*experiment 1* in 2008 and *experiment 2* in 2010, respectively) are plotted against the time in figure 32 and 33.

Table 28 Serum concentration of K1

	$B_{1,i.v.}$	$B_{1R,i.v.}$
Point [min]	**K1 [ng/ mL]**	**K1 [ng/ mL]**
0	**0.699**	0.288
5	**674.960**	512.590
10	**352.920**	233.620
20	**202.830**	159.380
30	**115.467**	87.645
40	**130.153**	90.953
50	**97.485**	54.473
60	**73.544**	51.635
80	**76.022**	45.661
100	**62.457**	40.161
120	**53.321**	32.075
140	**38.511**	25.235
160	**35.262**	26.846
180	**30.516**	23.451
210	**25.766**	18.725
240	**22.921**	18.320
270	**17.772**	16.269
300	**21.682**	13.794
330	**18.838**	12.045
360	**13.957**	9.667
420	**9.682**	8.005
480	**8.860**	7.250
540	**6.504**	5.754
600	**6.631**	5.883
1440	**1.295**	1.045

Table 29 Serum concentration of K1

	$B_{2,i.v.}$
Point [min]	**K1 [ng/ mL]**
-8	**0.593**
2	**167.422**
5	**348.069**
10	**245.194**
20	**155.593**
30	**82.831**
40	**61.731**
51	**50.453**
65	**39.170**
80	**34.030**
100	**27.605**
124	**21.890**
142	**17.736**
159	**16.961**
185	**11.782**
209	**9.950**
239	**6.913**
264	**6.199**
298	**5.248**
327	**4.797**
359	**4.109**
418	**3.410**
473	**2.390**
531	**2.093**
590	**1.625**
1498	**0.665**

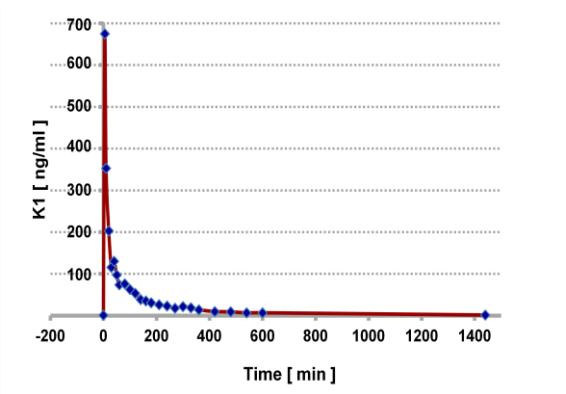

Fig. 32 Vitamin K1 *vs* time- $B_{1,i.v.}$

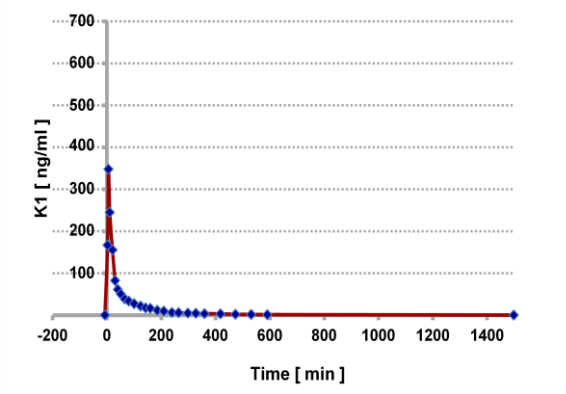

Fig. 33 Vitamin K1 *vs* time- $B_{2,i.v.}$

A_3, B_3

Tables 30 and 31 present mean values, standard deviation and coefficient of variation of vitamin K1 serum concentrations ***without*** any Konakion® MM intake. A_3 shows mean baseline K1 serum concentrations for volunteer A, while B_3 presents mean baseline K1 serum concentrations for volunteer B.

Table 30 Endogenous vitamin K1

	A_3
Point [min]	**K1 [ng/ mL]**
-16	0.431
7	0.484
38	0.549
58	0.490
88	0.488
121	0.518
149	0.524
179	0.538
210	0.457
240	0.589
268	0.505
300	0.333
331	0.589
394	0.355
457	0.608
1321	0.865
mean	***0.520***
sd	***0.116***
cv	***22.29 %***

Table 31 Endogenous vitamin K1

	B_3
Point [min]	**K1 [ng/ mL]**
-35	0.296
4	0.284
32	0.487
60	0.450
91	1.077
122	0.309
152	0.772
182	0.675
212	0.458
246	0.233
269	0.896
300	0.837
326	0.817
357	0.646
385	0.694
413	0.672
447	0.467
475	0.581
504	0.428
535	0.414
1356	1.408
mean	***0.614***
sd	***0.282***
cv	***45.86 %***

There is no significant difference between the individual vitamin K1 serum concentrations for volunteer A and B (p= 0,188; unpaired t-test), though the coefficient of variation within this time interval in volunteer B appears to be as twice as high in comparison to the variation in volunteer A.

$C_{1,p.o.}$, $C_{1R,p.o.}$

Table 32 presents mean values of vitamin K1 serum concentrations in ng/ mL after 2 mg Konakion® MM were administered ***orally*** to volunteer **C**.

$C_{1,p.o.}$ refers to the experiment which was performed in 2009. **$C_{1R,p.o.}$** column shows the vitamin K1 values of the same blood samples collected in 2009 but measured a year later. There was a notable decrease of detectable vitamin K1 by 30.6 % in the mean. The mean vitamin K1 serum values are plotted against the time in figure 34.

Table 32 Serum concentration of K1

	$C_{1,p.o.}$	$C_{1R,p.o.}$
Point [min]	**K1 [ng/ mL]**	**K1 [ng/ mL]**
0	**1.477**	0.516
60	**1.401**	0.787
120	**49.234**	33.596
180	**44.561**	30.711
210	**31.753**	23.698
240	**20.095**	16.210
270	**17.510**	9.742
300	**19.507**	13.357
330	**16.324**	14.029
360	**13.025**	12.821
390	**17.650**	10.603
420	**13.724**	9.671
450	**18.087**	13.812
480	**24.014**	19.819
520	**21.463**	13.269
580	**18.562**	14.156
1440	**2.396**	1.464

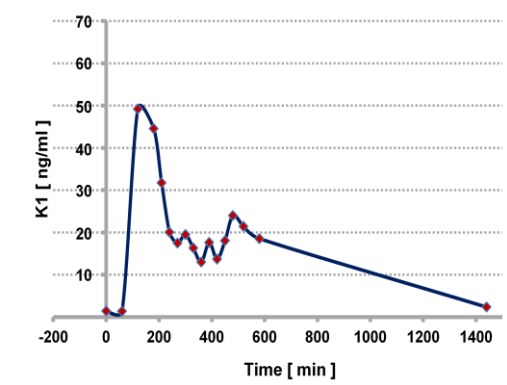

Fig. 34 Vitamin K1 *vs* time- $C_{1,p.o.}$

$C_{1,i.v.}$, $C_{1R,i.v.}$

Table 33 presents mean values of vitamin K1 serum concentrations in ng/ mL after 2 mg Konakion® MM were administered ***intravenously*** to volunteer **C**.

$C_{1,i.v.}$ refers to the experiment which was performed in 2009. **$C_{1R,i.v.}$** column shows the vitamin K1 values of the same blood samples collected in 2009 but measured a year later. There was a slight increase of detectable vitamin K1 by 1.1 % in the mean between the measurement directly after blood samples were collected and a year later. The mean vitamin K1 serum values are plotted against the time in figure 35.

Table 33 Serum concentration of K1

	$C_{1,i.v.}$	$C_{1R,i.v.}$
Point [min]	**K1 [ng/ mL]**	**K1 [ng/ mL]**
-15	**0.804**	0.960
3.8	**430.145**	348.900
5	**354.580**	352.800
11	**237.275**	189.100
21	**134.510**	123.800
28	**103.401**	104.400
40	**77.413**	69.775
51	**63.261**	58.263
65	**54.724**	49.763
80	**45.757**	41.450
101	**35.145**	34.325
120	**30.558**	28.338
142	**26.541**	25.635
160	**21.748**	21.580
182	**17.589**	18.950
212	**15.737**	17.445
243	**12.124**	13.400
275	**10.079**	12.455
301	**8.585**	10.993
330	**7.352**	8.790
361	**6.800**	5.805
423	**5.523**	6.848
480	**4.574**	4.365
543	**3.640**	4.285
600	**3.149**	4.410
1460	**0.463**	1.253

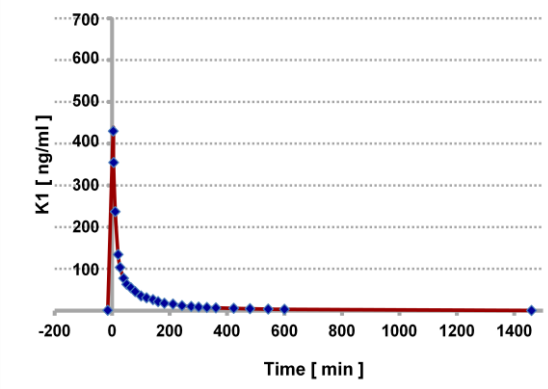

Fig. 35 Vitamin K1 *vs* time- $C_{1,i.v.}$

$D_{1,p.o.}$, $D_{1R,p.o.}$

Table 34 presents mean values of vitamin K1 serum concentrations in ng/ mL after 2 mg Konakion® MM were administered ***orally*** to volunteer **D**.

$D_{1,p.o.}$ refers to the experiment which was performed in 2009. **$D_{1R,p.o.}$** column shows the vitamin K1 values of the same blood samples collected in 2009 but measured a year later. The decrease of detectable vitamin K1 averaged 6.5 % between the measurements directly after blood sampling and a year later. The mean vitamin K1 serum values are plotted against the time in figure 36.

Table 34 Serum concentration of K1

	$D_{1,p.o.}$	$D_{1R,p.o.}$
Point [min]	**K1 [ng/ mL]**	**K1 [ng/ mL]**
0	**0.520**	0.796
60	**2.640**	0.858
120	**4.409**	3.184
180	**35.375**	23.985
210	**21.253**	18.295
240	**28.338**	21.555
270	**25.638**	21.101
300	**22.466**	18.795
330	**17.355**	14.947
360	**15.922**	13.506
390	**12.773**	10.606
420	**9.169**	8.612
450	**6.353**	6.842
480	**5.264**	5.846
520	**4.835**	5.527
580	**3.308**	4.034
1440	**1.037**	1.376

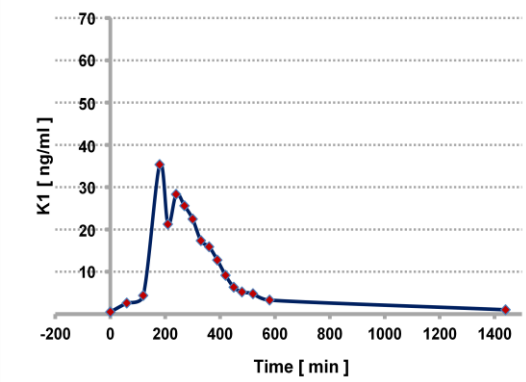

Fig. 36 Vitamin K1 *vs* time- $D_{1,p.o.}$

$D_{1,i.v.}$, $D_{1R,i.v.}$

Table 35 presents mean values of vitamin K1 serum concentrations in ng/ mL after 2 mg Konakion® MM were administered ***intravenously*** to volunteer **D**.

$D_{1,i.v.}$ refers to the experiment which was performed in 2009. **$D_{1R,i.v.}$** column shows the vitamin K1 values of the same blood samples collected in 2009 but measured a year later. The decrease of detected vitamin K1 averaged 10.9 % between the measurements directly after blood collection and a year later. The mean vitamin K1 serum values are plotted against the time in figure 37.

Table 35 Serum concentration of K1

	$D_{1,i.v.}$	$D_{1R,i.v.}$
Point [min]	**K1 [ng/ mL]**	**K1 [ng/ mL]**
-15	**0.140**	0.628
2	**327.505**	279.123
5	**309.950**	273.200
10	**210.688**	212.500
20	**130.853**	101.700
30	**97.505**	99.038
40	**74.609**	78.563
50	**73.168**	67.363
30	**60.180**	56.563
80	**44.635**	43.875
100	**34.039**	33.150
120	**28.328**	25.375
140	**27.666**	24.050
160	**23.745**	20.615
180	**21.363**	14.230
210	**14.629**	13.205
240	**12.645**	11.770
270	**10.333**	10.693
300	**8.737**	7.605
330	**6.643**	5.158
360	**5.500**	5.675
420	**4.558**	4.888
480	**3.455**	3.308
540	**2.693**	2.870
600	**2.100**	1.873
1440	**0.292**	1.323

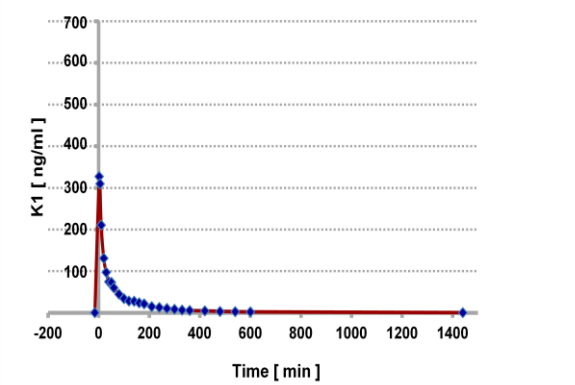

Fig. 37 Vitamin K1 *vs* time- $D_{1,i.v.}$

3.4.2. Pharmacokinetic analysis

Pharmacokinetic analysis after p.o. and i.v. administration of 2 mg Konakion® MM was performed for all participants A, B, C, and D.

3.4.2.1. Graphic presentation of the data

By using y-axis scaled logarithmically, vitamin K1 serum concentrations were plotted against time profiles in order to elucidate pharmacokinetic data more clearly.

For each curve and each volunteer, the baseline serum concentration of vitamin K1 (the vitamin K1 value in serum before any Konakion® MM administration) was subtracted from the vitamin K1 serum value at the corresponding time point. Hereby, negative values were equated with zero. In this way calculated values were further used to generate the appropriate graphs.

3.4.2.1.1. Serum concentration vs time profiles of vitamin K1 following p.o. administration of Konakion® MM 2 mg

The following figures (38 – 43) show vitamin K1 serum concentration *vs* time profiles obtained from each volunteer (A, B, C, and D) after 2 mg Konakion® MM were administered ***orally***.

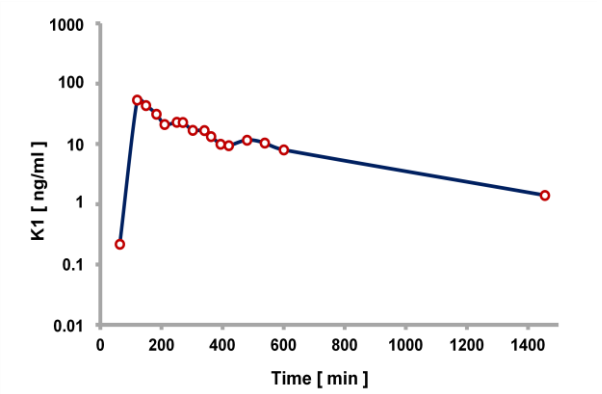

Fig. 38 LOG-Vitamin K1 *vs* time- $A_{1,p.o.}$

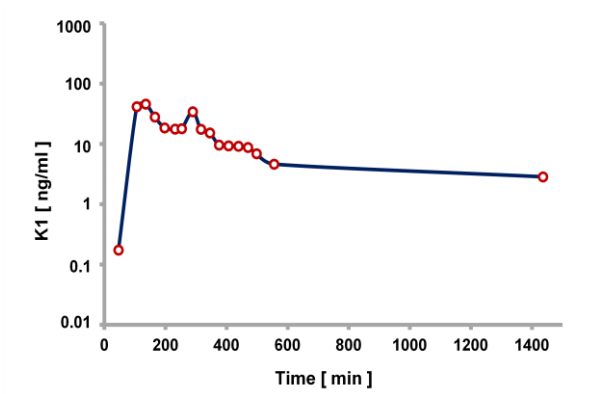

Fig. 39 LOG-Vitamin K1 *vs* time- $A_{2,p.o.}$

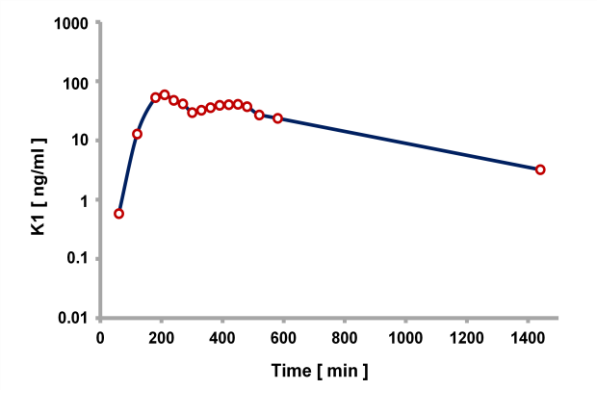

Fig. 40 LOG-Vitamin K1 *vs* time- $B_{1,p.o.}$

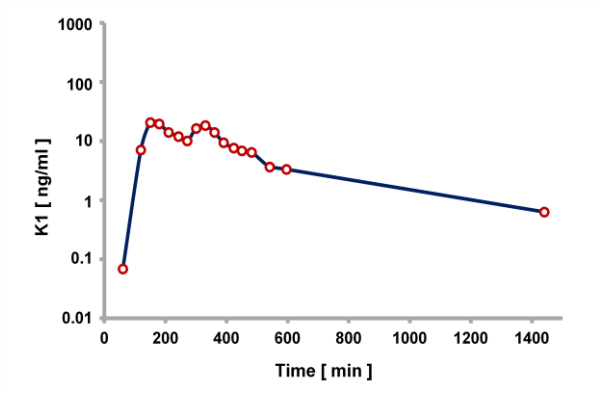

Fig. 41 LOG-Vitamin K1 *vs* time- $B_{2,p.o.}$

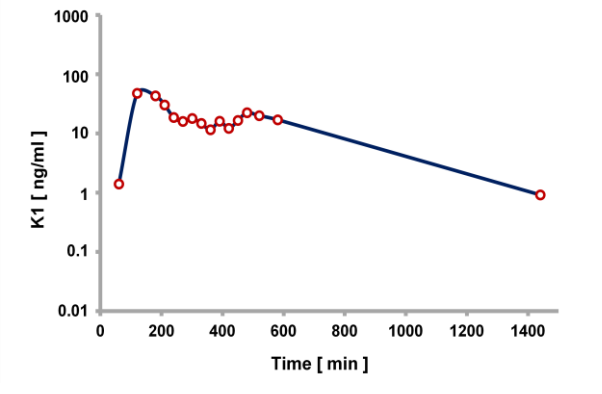

Fig. 42 LOG-Vitamin K1 *vs* time- $C_{1,p.o.}$

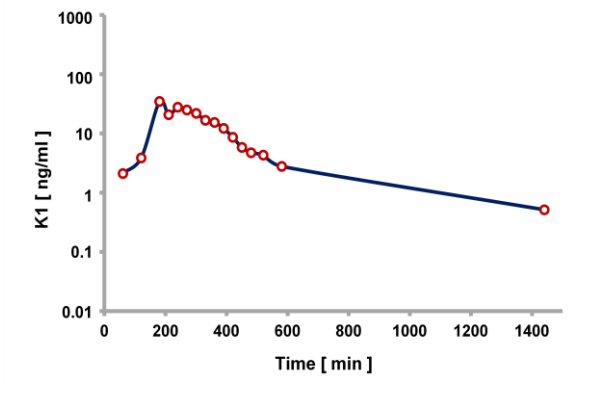

Fig. 43 LOG-Vitamin K1 *vs* time- $D_{1,p.o.}$

3.4.2.1.2. Serum concentration vs time profiles of vitamin K1 following i.v. administration of Konakion® MM 2 mg

The figures below (44 – 49) present vitamin K1 serum concentration *vs* time profiles obtained from each volunteer (A, B, C, and D) after 2 mg Konakion® MM were administered ***intravenously***.

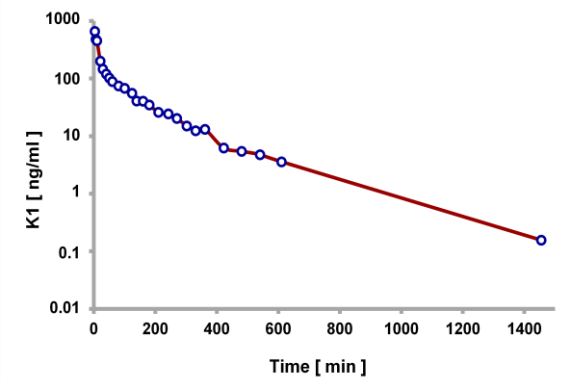

Fig. 44 LOG-Vitamin K1 *vs* time- $A_{1,i.v.}$

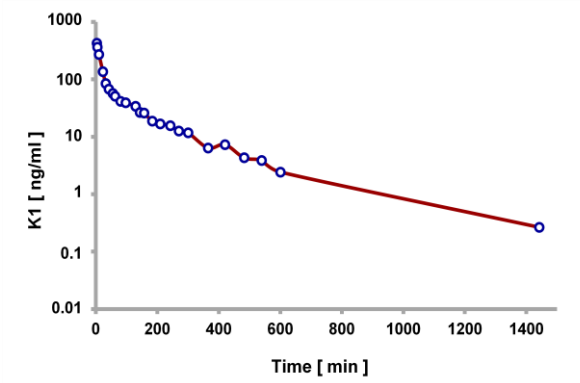

Fig. 45 LOG-Vitamin K1 *vs* time- $A_{2,i.v.}$

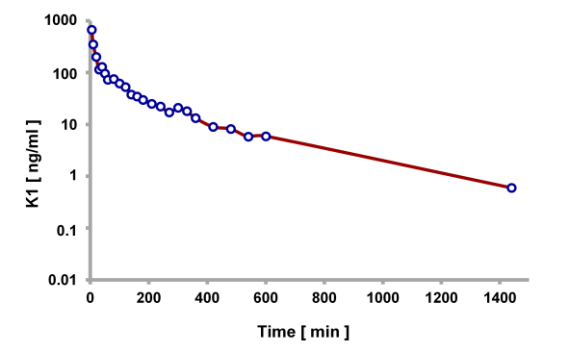

Fig. 46 LOG-Vitamin K1 *vs* time- $B_{1,i.v.}$

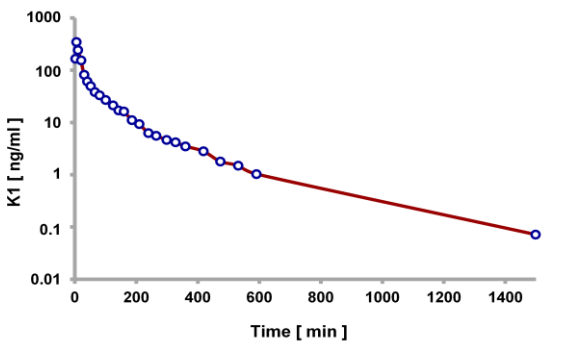

Fig. 47 LOG-Vitamin K1 *vs* time- $B_{2,i.v.}$

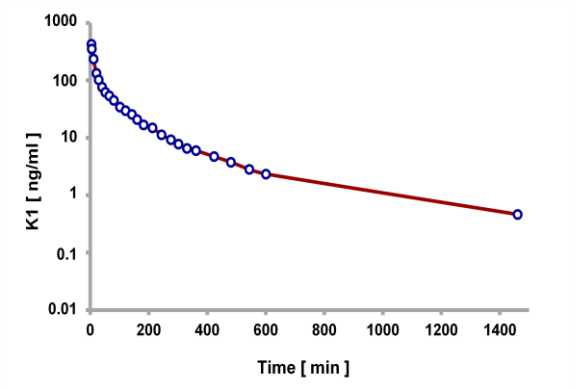

Fig. 48 LOG-Vitamin K1 *vs* time- $C_{1,i.v.}$

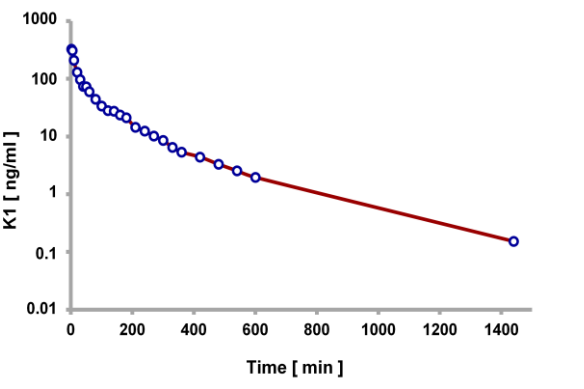

Fig. 49 LOG-Vitamin K1 *vs* time- $D_{1,i.v.}$

3.4.2.2. Pharmacokinetic parameters after p.o./ i.v. administration of Konakion® MM 2 mg

The pharmacokinetic evaluation was carried out for all participants (A, B, C, and D).

Following pharmacokinetic parameters were estimated on the basis of the data:

Symbol	*Unit*	*Definition*	*Calculation*
t_{max}	min	Time of maximum plasma concentration	directly taken from analytical data
C_{max}	ng mL^{-1}	Maximum plasma concentration	directly taken from analytical data
C_{max_D}	L^{-1}	C_{max} divided by dose	$= C\ max / D$
C_0	ng mL^{-1}	Back extrapolated concentration at time = 0	
T_{last}	min	Time of last observation where C is positive	
C_{last}	ng mL^{-1}	Concentration at T_{last}	
AUC_{last}	ng h mL^{-1}	Area under the curve from 0 to T_{last}	
AUC_{all}	ng h mL^{-1}	Area under the curve from 0 to time of last observation	
AUC_{0_600}	ng h mL^{-1}	Area under the curve from 0 to 600 min	
F_{0_600}		Bioavailability as calculated from IV and PO $AUC_{0\text{-}600}$	$= \frac{[AUC]_{oral} \times dose_{iv}}{[AUC]_{iv} \times dose_{oral}}$
AUC_{INF_obs}	ng h mL^{-1}	Area under the curve from 0 to infinity, where the extrapolation is based on C_{last}	$= \int_0^\infty C_p dt$
F_{inf}		Bioavailability as calculated from IV and PO $AUC_{INF\text{-}obs}$	$= \frac{[AUC]_{oral} \times dose_{iv}}{[AUC]_{iv} \times dose_{oral}}$
$AUC_{INF\_D\_obs}$	min L^{-1}	$AUC_{INF\text{-}obs}$ divided by dose	$= AUC\ INF\_obs\ /\ D$
$AUC_{\%Extrap\_obs}$		Percentage of total AUC which is extrapolated	
$AUC_{\%Back\_Ext\_obs}$		Percentage of total AUC which is back extrapolated from first observation to time 0	
V_{ss_obs}	L	Volume of distribution at steady-state, calculated from mean residence time and clearance	
V_{z_obs}	L	Volume of distribution based on the terminal phase → i.v. administration	$= D_{iv} / (AUC \times \lambda_z)$
$V_{z\_F\_obs}$	L	Volume of distribution based on the terminal phase divided by bioavailability → p.o. administration	$= f \times D_{po} / (AUC \times \lambda_z)$
Cl_{obs}	L min^{-1}	Clearance calculated from AUC INF_obs and Dose → i.v. administration	$= D_{iv} / AUC$
Cl_{F_obs}	L min^{-1}	Clearance calculated from $AUC_{INF\text{-}obs}$ and Dose divided by bioavailability → p.o. administration	$= f \times (D_{po} / AUC)$
Rsq		Goodness of fit statistic for the terminal phase	
Rsq adjusted		Goodness of fit statistic for the terminal phase	
$Corr_{XY}$		Correlation between time and log concentration for the observations used in λ_z (terminal slope) estimation	

$\lambda_{z\ No\_points}$		Number of observations used in computation of λ_z	
λ_z	min^{-1}	Terminal elimination slope	
$\lambda_{z\ lower}$		Lower limit of time time values for calculation of l λ_z	
$\lambda_{z\ upper}$		Upper limit of time time values for calculation of λ_z	
$t_{½}\ \lambda_z$	min	Terminal half life as calcualted from λ_z	$= ln\,2/\lambda_z$

3.4.2.2.1. Pharmacokinetic parameters of volunteer A

Table 36 below presents pharmacokinetic parameters of volunteer **A** starting with data from the experiment performed in 2008. Thereby, data after intravenous administration of 2 mg Konakion® MM are listed followed by these after oral application of the drug. Subsequently, the parameters from the same experiment in 2010 are shown in the same order – i.v. data first followed by p.o. values.

The maximum concentration (**C_{max}**) and the time point of C_{max} (**t_{max}**) of the K1 serum concentration after oral and intravenous administration of Konakion® MM 2 mg were determined from the raw data.

t_{max} was 6 and 5 minutes for **$A_{1,i.v.}$** and **$A_{2,i.v.}$**, respectively, while t_{max} amounted to 120 min for **$A_{1,p.o.}$** and to 135 min for **$A_{2,p.o.}$**.

C_{max} reached 484 and 365 ng mL^{-1} for **$A_{1,i.v.}$** and **$A_{2,i.v.}$**, respectively. **C_{max}** obtained after p.o. administration showed 9.1 and 7.9-fold lower values for the experiment in 2008 and 2010, respectively compared to the i.v. data and equaled 53 ng mL^{-1} for **$A_{1,p.o.}$** and 46 ng mL^{-1} for **$A_{2,p.o.}$**.

The area under the curve **AUC** was calculated using the linear-logarithmic trapezoidal rule.

AUC_{all} was after i.v. administration of 2 mg Konakion® MM in 2008 1.9-fold higher compared to AUC_{all} after p.o. application of the same dosage. For the experiment in 2010, AUC_{all} was 1.4-fold higher after i.v. dose than the corresponding p.o. value.

Bioavailability F_{0_600} as calculated from AUC_{0_600} i.v. and p.o. seemed to be 1.4-fold higher when the experiment was repeated two years later while **F_{inf}** as calculated from AUC_{INF_obs} i.v. and p.o. showed 1.5- fold higher value by comparing **$A_{2,p.o.}$** and **$A_{1,p.o.}$**.

The observed **volume of distribution** at steady-state **V_{ss}** was 10 and 17 L after i.v. dose in 2008 and 2010, respectively and therewith 1.7-fold lower for the first experiment.

Clearance Cl_{obs} calculated from AUC_{INF_obs} and Dose (mL min^{-1}) was 1.5-fold higher for **$A_{2,i.v.}$** compared to **$A_{1,i.v.}$** while **Cl_{F_obs}** with regard to biovailability was nearly equal for both p.o. experiments.

Elimination half-lives $t_{½}$ λ_z ranged from 175 to 386 min. Comparing both p.o. experiments in 2008 and 2010, $t_{½}$ λ_z was 1.2-fold lower in the first test. Comparing both i.v. experiments in 2008 and 2010, $t_{½}$ λ_z was also 1.2-fold lower in the first test.

Table 36 Pharmacokinetic parameters of volunteer A

	$A_{1,i.v.}$	$A_{1,p.o.}$	$A_{2,i.v.}$	$A_{2,p.o.}$	*Units*
t_{max}	**6**	**120**	**5**	**135**	**min**
C_{max}	**484**	**53**	**365**	**46**	**ng mL^{-1}**
C_{max_D}	0.24	0.03	0.18	0.02	L^{-1}
C_0	530		486		ng mL^{-1}
T_{last}	1455	1455	1442	1436	min
C_{last}	0.16	1.41	0.27	2.86	ng mL^{-1}
AUC_{last}	438	226	293	208	ng h mL^{-1}
AUC_{all}	**438**	**226**	**293**	**208**	**ng h mL^{-1}**
AUC_{0_600}	421	172	279	157	ng h mL^{-1}
F_{0_600}		**41**		**56**	**%**
AUC_{INF_obs}	438	237	294	234	ng h mL^{-1}
F_{inf}		**54**		**80**	**%**
$AUC_{INF\_D\_obs}$	13.15	7.10	8.82	7.02	min L^{-1}
$AUC_{\%Extrap\_obs}$	0.15	4.62	0.47	11.331	
$AUC_{\%Back\_Ext\_obs}$	11.57		11.97		
V_{ss_obs}	**10**		**17**		**L**
V_{z_obs}	19		35		L
$V_{z\_F\_obs}$		66		79	L μg^{-1}
Cl_{obs}	**76**		**113**		**mL min^{-1}**
Cl_{F_obs}		**141**		**142**	**mL min^{-1}**
Rsq	0.983	0.948	0.968	0.674	
Rsq adjusted	0.980	0.941	0.962	0.647	
$Corr_{XY}$	-0.991	-0.973	-0.984	-0.821	
$\lambda_{z\ No\_points}$	9	10	8	14	
λ_z	0.0040	0.0021	0.0032	0.0018	
$\lambda_{z\ lower}$	270	270	270	165	
$\lambda_{z\ upper}$	1455	1455	1442	1436	
$t_{½}\lambda_z$	**175**	**323**	**215**	**386**	**min**

3.4.2.2.2. Pharmakocinetic parameters of volunteer B

Table 37 below presents pharmacokinetic parameters of volunteer **B** starting with data from the intravenous experiment performed in 2008 and followed by these obtained after oral administration of 2 mg Konakion® MM. Subsequently, the parameters from the same experiment in 2010 are shown in the same order – i.v. data first followed by p.o. values.

t_{max} was 5 minutes for **$B_{1,i.v.}$** and **$B_{2,i.v.}$** while t_{max} amounted to 210 min for **$B_{1,p.o.}$** and to 150 min for **$B_{2,p.o.}$**.

C_{max} reached 674 and 347 ng mL^{-1} for **$B_{1,i.v.}$** and **$B_{2,i.v.}$**, respectively. C_{max} obtained after p.o. administration showed 11.4 and 16.9-fold lower values for the experiment in 2008 and 2010, respectively compared to the i.v. data and equaled 59 ng mL^{-1} for **$B_{1,p.o.}$** and 21 ng mL^{-1} for **$B_{2,p.o.}$**.

AUC_{all} was after i.v. administration of 2 mg Konakion® MM in 2008 1.1-fold higher compared to AUC_{all} after p.o. application of the same dosage. For the experiment in 2010, AUC_{all} was 2.0-fold higher after i.v. dose than the corresponding p.o. value.

Bioavailability F_{0_600} as calculated from AUC_{0_600} i.v. and p.o. was 1.6-fold lower when the experiment was repeated two years later while **F_{inf}** as calculated from AUC_{INF_obs} i.v. and p.o. showed 1.8-fold higher value for the first experiment **$B_{1,p.o.}$** compared to **$B_{2,p.o.}$**.

The observed **volume of distribution** at steady-state **V_{ss}** was 12 and 15 L after i.v. dose in 2008 and 2010, respectively and 1.3-fold lower for the first experiment.

Clearance Cl_{obs} calculated from AUC_{INF_obs} and Dose (mL min^{-1}) was 2.2-fold higher for **$B_{2,i.v.}$** compared to **$B_{1,i.v.}$** while **Cl_{F_obs}** with regard to biovailability was nearly 4-fold higher for **$B_{2,p.o.}$**.

Elimination half-lives $t_{½}$ $ƛ_z$ ranged from 201 to 316 min. Comparing both p.o. experiments in 2008 and 2010, $t_{½}$ $ƛ_z$ was 1.2-fold higher in the first test. Comparing both i.v. experiments in 2008 and 2010, $t_{½}$ $ƛ_z$ was also 1.2-fold higher in the first test.

Table 37 Pharmacokinetic parameters of volunteer B

	$B_{1,i.v.}$	$B_{1,p.o.}$	$B_{2,i.v.}$	$B_{2,p.o.}$	*Units*
t_{max}	**5**	**210**	**5**	**150**	**min**
C_{max}	**674**	**59**	**347**	**21**	**ng mL^{-1}**
C_{max_D}	0.34	0.03	0.17	0.01	L^{-1}
C_0	1291		494		ng mL^{-1}
T_{last}	1440	1440	1509	1440	min
C_{last}	0.60	3.22	0.07	0.63	ng mL^{-1}
AUC_{last}	476	439	219	112	ng h mL^{-1}
AUC_{all}	**476**	**439**	**219**	**112**	**ng h mL^{-1}**
AUC_{0_600}	443	300	214	90	ng h mL^{-1}
F_{0_600}		**68**		**42**	**%**
AUC_{INF_obs}	479	464	220	116	ng h mL^{-1}
F_{inf}		**97**		**53**	**%**
$AUC_{INF\_D\_obs}$	14.37	13.91	6.59	3.49	min L^{-1}
$AUC_{\%Extrap\_obs}$	0.69	5.27	0.16	3.34	
$AUC_{\%Back\_Ext\_obs}$	16.52		15.79		
V_{ss_obs}	**12**		**15**		**L**
V_{z_obs}	23		44		L
$V_{z\_F\_obs}$		33		106	L μg^{-1}
Cl_{obs}	**70**		**152**		**mL min^{-1}**
Cl_{F_obs}		**72**		**287**	**mL min^{-1}**
Rsq	0.969	0.935	0.978	0.887	
Rsq adjusted	0.965	0.930	0.975	0.876	
$Corr_{XY}$	-0.984	-0.967	-0.989	-0.942	
λ_z No_points	10	14	9	13	
λ_z	0.0030	0.0022	0.0035	0.0027	
λ_z lower	240	180	264	185	
λ_z upper	1440	1440	1509	1509	
$t_{½}\lambda_z$	**231**	**316**	**201**	**255**	**min**

3.4.2.2.3. Pharmakocinetic parameters of volunteer C

Table 38 presents pharmacokinetic parameters of volunteer **C**. Calculated values from the intravenous experiment are listed first followed by these obtained after oral administration of 2 mg Konakion® MM.

t_{max} was 5 min for **$C_{1,i.v.}$** while t_{max} amounted to 120 min for **$C_{1,p.o.}$**.

C_{max} reached 354 and 48 ng mL^{-1} after intravenous and oral administration, respectively. Therewith, C_{max} obtained after p.o. intake showed 7.4-fold lower value compared to the i.v. value.

AUC_{all} after i.v. and p.o. administration of 2 mg Konakion® MM was nearly equal.

Clearance Cl_{obs} as calculated from AUC_{INF_obs} and Dose was 126 mL min^{-1} while **Cl_{F_obs}** with regard to biovailability was 119 mL min^{-1}.

Elimination half-lives $t_{½}$ λ_z 129 min was calculated for the i.v. experiment and 280 min for p.o. experiment.

Finally, it should be noted that bioavailability could not be calculated for volunteer C due to the fact that there was a period of more than two weeks between the p.o. and i.v. experiment.

Table 38 Pharmacokinetic parameters of volunteer C

	$C_{1,i.v.}$	$C_{1,p.o.}$	*Units*
t_{max}	**5**	**120**	**min**
C_{max}	**354**	**48**	**ng mL^{-1}**
C_{max_D}	0.18	0.02	L^{-1}
C_0	495		ng mL^{-1}
T_{last}	600	1440	min
C_{last}	2.35	0.92	ng mL^{-1}
AUC_{last}	258	274	ng h mL^{-1}
AUC_{all}	**275**	**274**	**ng h mL^{-1}**
AUC_{INF_obs}	265	280	ng h mL^{-1}
$AUC_{INF\_D\_obs}$	7.96	8.41	min L^{-1}
$AUC_{\%Extrap\_obs}$	2.732	2.21	
$AUC_{\%Back\_Ext\_obs}$	13.20		
V_{z_obs}	23		L
$V_{z\_F\_obs}$		48	L μg^{-1}
Cl_{obs}	**126**		**mL min^{-1}**
Cl_{F_obs}		**119**	**mL min^{-1}**
$\lambda_{z\ No\_points}$	15	13	
λ_z	0.0054	0.0025	
$\lambda_{z\ lower}$	101	210	
$\lambda_{z\ upper}$	600	1440	
$t_{½}\lambda_z$	**129**	**280**	**min**

3.4.2.2.4. Pharmakocinetic parameters of volunteer D

Table 39 presents pharmacokinetic parameters of volunteer **D**. Calculated values from the intravenous experiment are listed at first followed by these obtained after p.o. intake of 2 mg Konakion® MM.

t_{max} was 5 minutes for **$D_{1,i.v.}$** while t_{max} amounted to 180 min for **$D_{1,p.o.}$**.

C_{max} reached 310 and 35 ng mL^{-1} after intravenous and oral administration, respectively. Therewith, C_{max} obtained after p.o. intake showed 8.9-fold lower value compared to the i.v. value.

AUC_{all} was after i.v. administration of 2 mg Konakion® MM 1.9-fold higher compared to AUC_{all} after p.o. application of the same dosage.

Clearance Cl_{obs} as calculated from AUC_{INF_obs} and Dose was 128 mL min^{-1} while **Cl_{F_obs}** with regard to biovailability was 239 mL min^{-1}.

Elimination half-lives $t_{½}$ λ_z 200 min was calculated for the i.v. experiment and 301 min for p.o. experiment.

Finally, it should be noted that bioavailability could not be calculated for volunteer D due to the fact that there was a period of more than two weeks between the p.o. and i.v. experiment.

Table 39 Pharmacokinetic parameters of volunteer D

	$D_{1,i.v.}$	$D_{1,p.o.}$	Units
t_{max}	**5**	**180**	**min**
C_{max}	**310**	**35**	**ng mL^{-1}**
C_{max_D}	0.16	0.02	L^{-1}
C_0	456		ng mL^{-1}
T_{last}	1440	1440	Min
C_{last}	0.152	0.517	ng mL^{-1}
AUC_{last}	260	136	ng h mL^{-1}
AUC_{all}	**260**	**136**	**ng h mL^{-1}**
AUC_{INF_obs}	260	140	ng h mL^{-1}
$AUC_{INF\_D\_obs}$	7.81	4.19	min L^{-1}
$AUC_{\%Extrap\_obs}$	0.28	2.68	
$AUC_{\%Back\_Ext\_obs}$	12.11		
V_{z_obs}	36.86		L
$V_{z\_F\_obs}$		103.52	L μg^{-1}
Cl_{obs}	**128**		**mL min^{-1}**
Cl_{F_obs}		**239**	**mL min^{-1}**
$\lambda_{z\ No\_points}$	9	5	
λ_z	0.035	0.0023	
$\lambda_{z\ lower}$	270	450	
$\lambda_{z\ upper}$	1440	1440	
$t_{½}\lambda_z$	**200**	**301**	**min**

3.4.3. Bile acid, cholesterol and 7α-hydroxycholesterol concentrations

Bile acid levels and the serum concentrations of cholesterol and the immediate cholesterol metabolite and bile acid precursor 7α-hydroxycholesterol were determined from the kinetic samples of volunteers A and B, i.e. in specific: $A_{2,p.o.}$, $A_{2,i.v.}$, A_3 and $B_{2,p.o.}$, $B_{2,i.v.}$, B_3.

The following tables present values of bile acids, cholesterol and 7α-hydroxycholesterol serum concentrations for volunteer A and B. The values for cholic acid (contained in each ampoule Konakion® MM 2 mg) and total bile acids as well are plotted in figures against the time points of blood collection after vitamin K1 administration.

3.4.3.1. Bile acid concentrations

3.4.3.1.1. A2,p.o., A2,i.v., A3

Tables 40 and 41 present values of bile acids serum concentration in µg/ mL after 2 mg Konakion® MM were administered ***orally*** ($A_{2,p.o.}$) and ***intravenously*** ($A_{2,i.v.}$) respectively or without any vitamin K1 administration (A_3) to volunteer **A** (table 42).

The figures below (50 – 55) present cholic acid and total bile acid concentration plotted against time.

Table 40 Serum concentration of bile acids $A_{2,p.o.}$

Point [min]	DCA [µg/ mL]	CA [µg/ mL]	LICA [µg/ mL]	CDCA [µg/ mL]	UDCA [µg/ mL]	Total Bile Acids [µg/ mL]
-15	0.202	**0.108**	0.031	0.143	0.003	**0.487**
46	0.331	**0.244**	0.060	0.273	0.006	**0.913**
106	0.369	**0.286**	0.090	0.327	0.004	**1.078**
135	0.577	**0.563**	0.084	0.651	0.009	**1.883**
165	0.541	**0.418**	0.085	0.566	0.006	**1.615**
197	0.563	**0.541**	0.064	0.676	0.009	**1.853**
231	0.579	**0.436**	0.065	0.702	0.008	**1.790**
253	0.449	**0.271**	0.075	0.510	0.006	**1.312**
289	0.861	**1.091**	0.058	1.281	0.011	**3.302**
316	0.787	**0.617**	0.097	0.976	0.011	**2.488**
345	0.705	**0.418**	0.087	0.871	0.011	**2.091**
375	0.725	**0.413**	0.093	0.819	0.008	**2.058**
406	0.510	**0.198**	0.053	0.477	0.004	**1.242**
439	0.393	**0.200**	0.049	0.424	0.005	**1.072**
470	0.650	**0.418**	0.058	0.733	0.007	**1.866**
498	0.739	**0.350**	0.080	0.763	0.008	**1.940**
555	0.341	**0.127**	0.034	0.332	0.003	**0.837**
1436	1.660	**1.124**	0.101	1.789	0.011	**4.685**

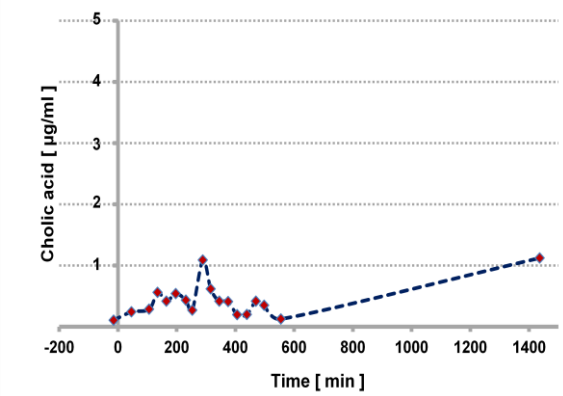

Fig. 50 Cholic acid *vs* time- $A_{2,p.o.}$

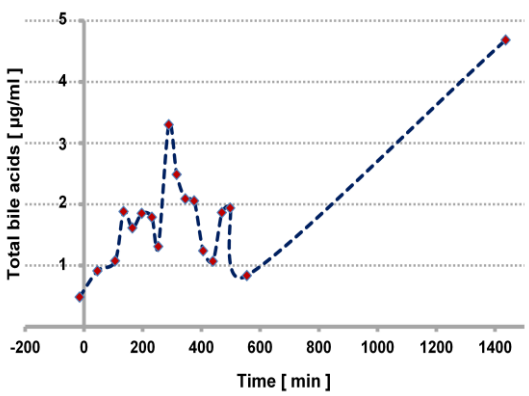

Fig. 51 Total bile acids *vs* time- $A_{2,p.o.}$

Table 41 Serum concentration of bile acids $A_{2,i.v.}$

Point [min]	DCA [µg/ mL]	CA [µg/ mL]	LICA [µg/ mL]	CDCA [µg/ mL]	UDCA [µg/ mL]	Total Bile Acids [µg/ mL]
-15	0.312	**0.118**	0.084	0.260	0.006	**0.780**
3	0.290	**0.866**	0.097	0.278	0.005	**1.536**
5	0.320	**0.552**	0.096	0.263	0.005	**1.236**
10	0.295	**0.320**	0.087	0.251	0.005	**0.958**
23	0.374	**0.153**	0.071	0.252	0.006	**0.856**
32	0.342	**0.169**	0.080	0.292	0.004	**0.888**
42	0.375	**0.138**	0.077	0.270	0.009	**0.869**
55	0.356	**0.220**	0.067	0.384	0.007	**1.034**
62	0.559	**0.280**	0.065	0.444	0.008	**1.356**
80	0.690	**0.673**	0.136	0.656	0.010	**2.166**
97	0.442	**0.219**	0.157	0.374	0.007	**1.199**
129	1.019	**0.066**	0.434	0.607	0.008	**2.134**
143	0.452	**0.142**	0.151	0.341	0.006	**1.092**
157	0.577	**0.212**	0.156	0.546	0.007	**1.498**
183	0.514	**0.189**	0.144	0.475	0.009	**1.331**
209	0.454	**0.165**	0.114	0.396	0.007	**1.136**
242	0.629	**0.323**	0.132	0.635	0.009	**1.729**
270	0.509	**0.183**	0.152	0.438	0.007	**1.289**
300	0.561	**0.172**	0.142	0.459	0.007	**1.341**
365	0.859	**0.302**	0.123	0.776	0.009	**2.069**
420	0.951	**0.458**	0.171	0.930	0.012	**2.522**
482	1.976	**0.060**	0.420	1.803	0.020	**4.279**
539	0.587	**0.164**	0.104	0.500	0.010	**1.365**
600	0.433	**0.285**	0.067	0.407	0.006	**1.198**
1442	0.902	**0.195**	0.104	0.576	0.014	**1.791**

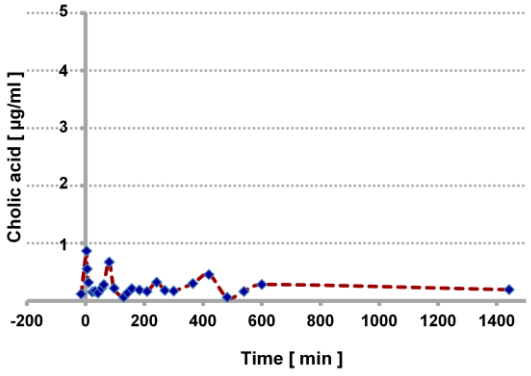

Fig. 52 Cholic acid *vs* time- $A_{2,i.v.}$

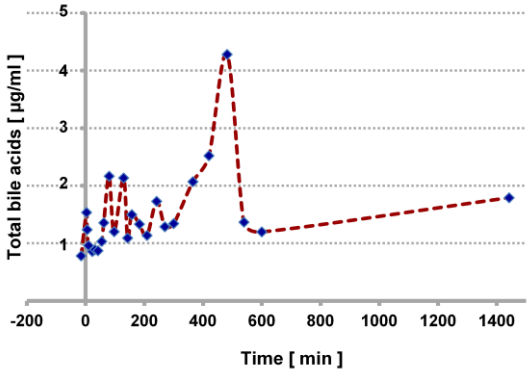

Fig. 53 Total bile acids *vs* time- $A_{2,i.v.}$

Table 42 Serum concentration of bile acids A_3

Point [min]	DCA [µg/ mL]	CA [µg/ mL]	LICA [µg/ mL]	CDCA [µg/ mL]	UDCA [µg/ mL]	Total Bile Acids [µg/ mL]
-16	0.433	**0.101**	0.150	0.305	0.005	**0.993**
7	0.226	**0.036**	0.083	0.133	0.004	**0.482**
38	0.511	**0.195**	0.110	0.432	0.008	**1.255**
58	0.710	**0.184**	0.198	0.429	0.011	**1.532**
88	0.449	**0.081**	0.133	0.258	0.006	**0.926**
121	0.413	**0.092**	0.111	0.298	0.005	**0.919**
149	0.314	**0.050**	0.120	0.188	0.005	**0.677**
179	0.274	**0.047**	0.095	0.195	0.005	**0.617**
210	0.217	**0.027**	0.081	0.124	0.003	**0.452**
240	0.624	**0.316**	0.091	0.696	0.009	**1.736**
268	0.547	**0.245**	0.116	0.491	0.009	**1.409**
300	0.455	**0.084**	0.105	0.351	0.007	**1.002**
331	0.432	**0.085**	0.089	0.296	0.005	**0.907**
394	0.422	**0.060**	0.073	0.235	0.004	**0.795**
457	0.270	**0.037**	0.055	0.167	0.004	**0.534**
1321	0.812	**0.130**	0.115	0.461	0.009	**1.527**

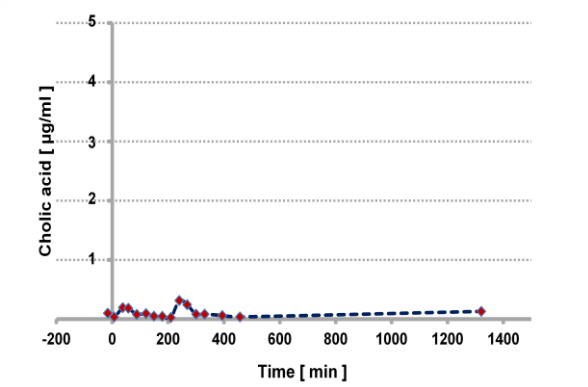

Fig. 54 Cholic acid *vs* time- A_3

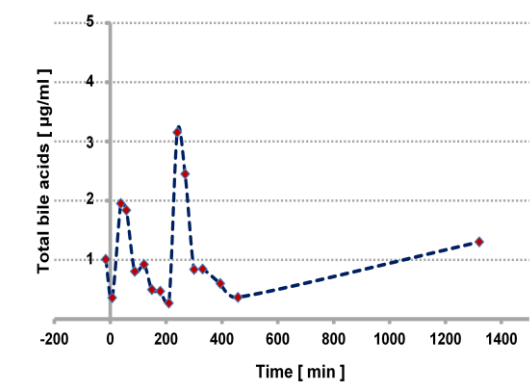

Fig. 55 Total bile acids *vs* time- A_3

3.4.3.1.2. B2,p.o., B2,i.v., B3

Tables 43 and 44 present values of bile acids serum concentration in µg/ mL after 2 mg Konakion® MM were administered ***orally*** ($B_{2,p.o.}$) and ***intravenously*** ($B_{2,i.v.}$) respectively or without any vitamin K1 administration (**B_3**) to volunteer **B** (table 45).

The figures below (56 – 61) present cholic acid and total bile acid concentration plotted against time.

Table 43 Serum concentration of bile acids $B_{2,p.o.}$

Point [min]	DCA [µg/ mL]	CA [µg/ mL]	LICA [µg/ mL]	CDCA [µg/ mL]	UDCA [µg/ mL]	Total Bile Acids [µg/ mL]
-15	0.571	**0.170**	0.323	0.256	0.011	**1.331**
60	0.358	**0.121**	0.223	0.183	0.006	**0.890**
119	0.418	**0.131**	0.215	0.193	0.009	**0.966**
150	0.564	**0.371**	0.252	0.349	0.008	**1.544**
179	0.596	**0.538**	0.222	0.439	0.018	**1.814**
210	0.730	**0.740**	0.311	0.562	0.014	**2.357**
242	1.289	**0.787**	0.349	0.911	0.025	**3.361**
271	1.214	**1.173**	0.284	1.104	0.030	**3.805**
300	1.150	**1.012**	0.339	0.941	0.025	**3.468**
330	1.133	**0.991**	0.298	0.975	0.020	**3.418**
360	0.908	**0.516**	0.300	0.605	0.017	**2.345**
390	0.800	**0.697**	0.241	0.573	0.017	**2.328**
423	0.664	**0.503**	0.217	0.576	0.020	**1.981**
450	1.157	**0.969**	0.269	0.985	0.026	**3.406**
482	0.877	**0.686**	0.198	0.686	0.018	**2.465**
541	1.183	**0.873**	0.277	0.996	0.021	**3.350**
596	0.612	**0.297**	0.178	0.450	0.016	**1.553**
1440	1.414	**1.938**	0.355	0.538	0.020	**4.265**

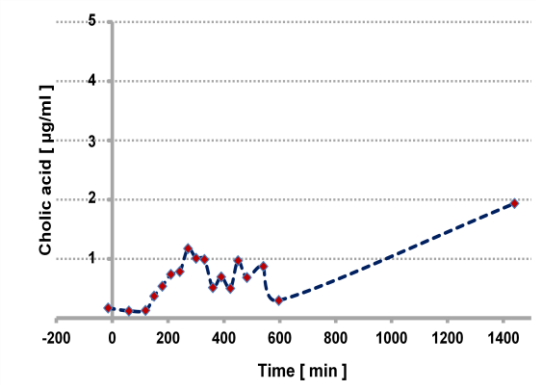

Fig. 56 Cholic acid *vs* time- $B_{2,p.o.}$

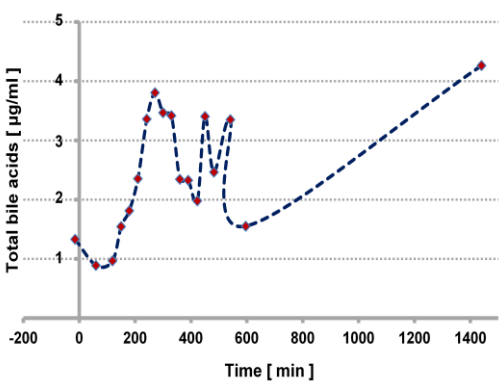

Fig. 57 Total bile acids *vs* time- $B_{2,p.o.}$

Table 44 Serum concentration of bile acids $B_{2,i.v.}$

Point [min]	DCA [µg/ mL]	CA [µg/ mL]	LICA [µg/ mL]	CDCA [µg/ mL]	UDCA [µg/ mL]	Total Bile Acids [µg/ mL]
-8	0.981	**0.151**	0.303	0.273	0.032	**1.739**
2	0.555	**0.989**	0.183	0.136	0.017	**1.880**
5	0.598	**1.303**	0.177	0.145	0.015	**2.236**
10	0.791	**0.885**	0.261	0.255	0.026	**2.219**
20	0.536	**0.185**	0.181	0.154	0.012	**1.069**
30	0.814	**0.182**	0.183	0.185	0.027	**1.391**
40	0.588	**0.215**	0.180	0.211	0.025	**1.219**
51	0.989	**0.274**	0.222	0.256	0.036	**1.778**
65	0.883	**0.141**	0.280	0.274	0.033	**1.611**
80	0.866	**0.100**	0.283	0.240	0.024	**1.513**
100	0.852	**0.125**	0.313	0.259	0.020	**1.569**
124	0.845	**0.161**	0.338	0.306	0.020	**1.671**
142	0.738	**0.070**	0.263	0.200	0.017	**1.288**
159	0.712	**0.044**	0.264	0.216	0.020	**1.256**
185	0.986	**0.124**	0.295	0.412	0.030	**1.846**
209	0.671	**0.102**	0.302	0.287	0.021	**1.383**
239	0.743	**0.162**	0.251	0.466	0.030	**1.651**
264	0.997	**0.169**	0.286	0.349	0.025	**1.827**
298	0.924	**0.175**	0.265	0.387	0.026	**1.776**
327	1.208	**0.375**	0.266	0.692	0.040	**2.581**
359	1.123	**0.406**	0.343	0.546	0.040	**2.458**
418	0.999	**0.205**	0.332	0.432	0.030	**1.997**
473	1.198	**0.331**	0.328	0.672	0.040	**2.569**
531	1.076	**0.189**	0.300	0.620	0.026	**2.210**
590	0.651	**0.083**	0.244	0.342	0.025	**1.345**
1498	1.283	**0.115**	0.373	0.435	0.028	**2.234**

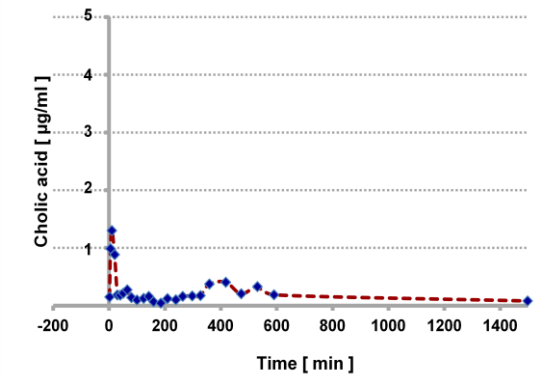

Fig. 58 Cholic acid *vs* time- $B_{2,i.v.}$

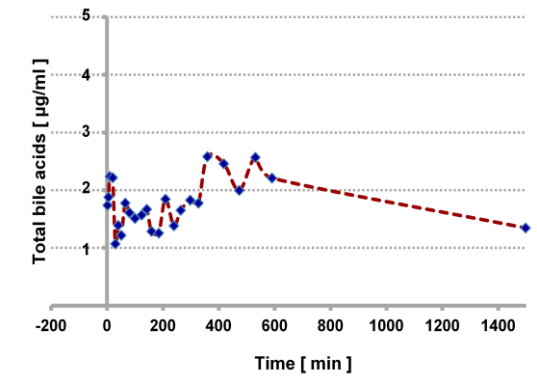

Fig. 59 Total bile acids *vs* time- $B_{2,i.v.}$

Table 45 Serum concentration of bile acids B_3

Point [min]	DCA [µg/ mL]	CA [µg/ mL]	LICA [µg/ mL]	CDCA [µg/ mL]	UDCA [µg/ mL]	Total Bile Acids [µg/ mL]
-35	0.197	**0.023**	0.100	0.095	0.004	**0.418**
4	0.123	**0.013**	0.070	0.041	0.003	**0.250**
32	0.216	**0.181**	0.094	0.157	0.008	**0.656**
60	0.286	**0.112**	0.124	0.159	0.009	**0.691**
91	0.243	**0.037**	0.107	0.111	0.008	**0.507**
122	0.647	**0.423**	0.231	0.425	0.015	**1.741**
152	0.824	**0.347**	0.261	0.660	0.018	**2.110**
182	0.305	**0.065**	0.114	0.175	0.014	**0.673**
212	0.538	**0.170**	0.164	0.356	0.015	**1.243**
246	0.664	**0.456**	0.159	0.503	0.027	**1.808**
269	1.220	**1.240**	0.237	0.843	0.026	**3.566**
300	1.071	**0.879**	0.232	0.746	0.017	**2.945**
326	0.468	**0.128**	0.186	0.264	0.019	**1.065**
357	0.469	**0.167**	0.154	0.302	0.018	**1.111**
385	0.435	**0.104**	0.155	0.265	0.013	**0.973**
413	0.486	**0.152**	0.169	0.306	0.014	**1.127**
447	0.944	**0.315**	0.326	0.685	0.020	**2.290**
475	0.806	**0.201**	0.282	0.476	0.013	**1.779**
504	0.456	**0.179**	0.124	0.338	0.017	**1.114**
535	0.922	**0.544**	0.244	0.571	0.015	**2.295**
1356	1.861	**0.724**	0.355	0.971	0.032	**3.942**

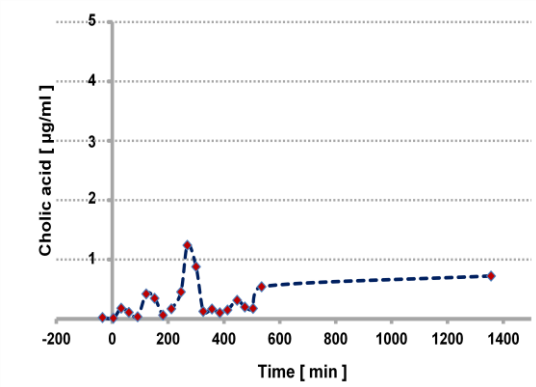

Fig. 60 Cholic acid *vs* time- B_3

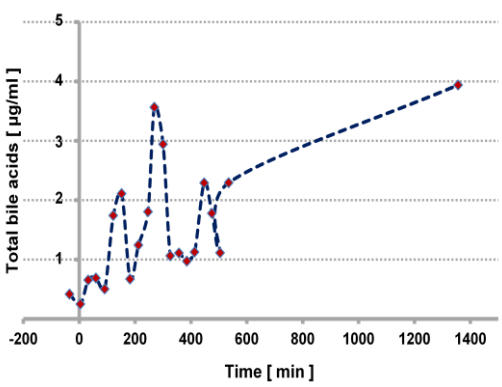

Fig. 61 Total bile acids *vs* time- B_3

3.4.3.2. Cholesterol and 7α-hydroxycholesterol concentrations

3.4.3.2.1. A2,p.o., A2,i.v., A3

Table 46 presents values of 7α-hydroxycholesterol (7αOH) and cholesterol (Ch) serum concentrations in ng/ mL and mg/ dL respectively as well as the ratio 7αOH to Ch in ng/ mg after 2 mg Konakion® MM were administered ***orally*** ($A_{2,p.o.}$) to volunteer **A**.

Table 46 Serum concentration of 7α-hydroxycholesterol and cholesterol $A_{2,p.o.}$

Point [min]	7αOH [ng/ mL]	Cholesterol [mg/ dL]	7αOH / Ch [ng/ mg]
-15	54.8	214.3	25.6
46	53.9	232.3	23.2
106	51.0	219.8	23.2
135	56.3	225.4	25.0
165	48.1	225.5	21.3
197	50.8	219.8	23.1
231	51.9	230.4	22.5
253	48.8	226.5	21.5
289	49.5	233.8	21.2
316	51.5	227.6	22.6
345	52.0	220.9	23.5
375	48.0	218.7	22.0
406	46.2	218.0	21.2
439	45.7	223.4	20.5
470	42.1	226.5	18.6
498	44.5	226.5	19.8
555	42.9	213.7	20.1
1436	41.6	229.8	18.1
mean	**48.9**	**224.0**	**21.8**
sd	**4.4**	**5.8**	**2.0**
cv	**8.9 %**	**2.6 %**	**9.2 %**

Table 47 presents values of 7αOH and Ch serum concentrations in ng/ mL and mg/ dL respectively as well as the ratio 7αOH to Ch in ng/ mg after 2 mg Konakion® MM were administered ***intravenously*** ($A_{2,i.v.}$) to volunteer **A**.

Table 47 Serum concentration of 7α-hydroxycholesterol and cholesterol $A_{2,i.v.}$

Point [min]	7αOH [ng/ mL]	Cholesterol [mg/ dL]	7αOH / Ch [ng/ mg]
-15	42.9	170.5	25.2
3	35.6	186.9	19.1
5	83.7	134.4	62.3
10	33.7	182.8	18.4
23	32.1	178.1	18.0
32	27.9	179.9	15.5
42	36.4	175.0	20.8
55	33.2	173.5	19.2
62	32.5	181.9	17.9
80	32.7	180.1	18.1
97	37.3	174.3	21.4
129	35.8	181.3	19.7
143	30.5	181.6	16.8
157	30.6	181.1	16.9
183	35.1	179.7	19.5
209	31.6	181.7	17.4
242	33.9	181.5	18.7
270	30.3	176.8	17.1
300	32.4	184.6	17.6
365	30.4	179.2	17.0
420	29.7	181.9	16.3
482	35.2	166.9	21.1
539	26.4	171.4	15.4
600	33.4	176.2	19.0
1442	30.8	184.1	16.7
mean	**33.2**	**177.0**	**18.9**
sd	**3.5**	**10.1**	**3.0**
cv	**10.6 %**	**5.7 %**	**15.7 %**

Table 48 presents values of 7αOH and Ch serum concentrations in ng/ mL and mg/ dL respectively as well as the ratio 7αOH to Ch in ng/ mg without any vitamin K1 administration (A_3) to volunteer **A**.

Table 48 Serum concentration of 7α-hydroxycholesterol and cholesterol A_3

Point [min]	7αOH [ng/ mL]	Cholesterol [mg/ dL]	7αOH / Ch [ng/ mg]
-16	41.2	191.1	21.5
7	42.0	202.6	20.7
38	30.6	189.2	16.2
58	36.0	193.2	18.6
88	37.2	185.7	20.1
121	42.9	176.0	24.4
149	29.8	191.0	15.6
179	34.7	179.0	19.4
210	32.9	185.6	17.7
240	39.5	191.7	20.6
268	31.5	187.2	16.8
300	34.1	187.6	18.2
331	35.3	184.5	19.1
394	30.5	196.7	15.5
457	37.9	184.0	20.6
1321	34.2	193.2	17.7
mean	**35.6**	**188.6**	**18.9**
sd	**4.2**	**6.5**	**2.4**
cv	**11.7 %**	**3.4 %**	**12.6 %**

3.4.3.2.2. B2,p.o., B2,i.v., B3

Table 49 presents values of 7αOH and Ch serum concentrations in ng/ mL and mg/ dL respectively as well as the ratio 7αOH to Ch in ng/ mg after 2 mg Konakion® MM were administered ***orally*** ($B_{2,p.o.}$) to volunteer **B**.

Table 49 Serum concentration of 7α-hydroxycholesterol and cholesterol $B_{2,p.o.}$

Point [min]	7αOH [ng/ mL]	Cholesterol [mg/ dL]	7αOH / Ch [ng/ mg]
-15	26.0	131.6	19.8
60	25.5	127.2	20.0
119	23.3	130.8	17.8
150	31.8	125.8	25.3
179	25.7	127.8	20.1
210	25.6	122.0	21.0
242	31.2	125.8	24.8
271	29.7	125.6	23.7
300	26.2	127.8	20.5
330	28.7	127.4	22.6
360	25.1	126.7	19.8
390	25.8	127.7	20.2
423	33.5	111.3	30.1
450	26.2	129.3	20.3
482	25.4	131.4	19.3
541	24.7	123.3	20.0
596	25.8	126.4	20.4
1440	28.5	131.8	21.6
mean	**27.1**	**126.6**	**21.5**
sd	**2.8**	**4.7**	**2.9**
cv	**10.2 %**	**3.7 %**	**13.4 %**

Table 50 presents values of 7αOH and Ch serum concentrations in ng/ mL and mg/ dL respectively as well as the ratio 7αOH to Ch in ng/ mg after 2 mg Konakion® MM were administered ***intravenously*** ($B_{2,i.v.}$) to volunteer B.

Table 50 Serum concentration of 7α-hydroxycholesterol and cholesterol $B_{2,i.v.}$

Point [min]	7αOH [ng /mL]	Cholesterol [mg/ dL]	7αOH / Ch [ng/ mg]
-8	21.0	120.5	17.4
2	19.9	117.5	16.9
5	22.1	117.5	18.8
10	25.5	114.5	22.3
20	21.0	124.9	16.8
30	24.8	119.4	20.8
40	19.4	114.9	16.9
51	20.3	121.0	16.8
65	20.0	121.2	16.5
80	22.3	121.3	18.4
100	23.1	121.7	19.0
124	24.3	122.0	19.9
142	20.9	117.9	17.7
159	20.7	123.1	16.8
185	23.3	114.7	20.3
209	21.4	121.7	17.6
239	21.0	117.6	17.9
264	21.7	119.8	18.2
298	19.5	116.2	16.7
327	18.0	121.0	14.9
359	21.3	116.9	18.2
418	17.9	121.5	14.7
473	20.6	120.9	17.1
531	18.9	123.9	15.3
590	17.9	126.3	14.2
1498	19.1	120.7	15.8
mean	**21.0**	**119.9**	**17.5**
sd	**2.0**	**3.1**	**1.9**
cv	**9.6 %**	**2.6 %**	**10.8 %**

Table 51 presents values of 7αOH and Ch serum concentrations in ng/ mL and mg/ dL respectively as well as the ratio 7αOH to Ch in ng/ mg without any vitamin K1 administration (B_3) to volunteer **B**.

Table 51 Serum concentration of 7α-hydroxycholesterol and cholesterol B_3

Point [min]	7αOH [ng/ mL]	Cholesterol [mg/ dL]	7αOH / Ch [ng/ mg]
-35	20.0	118.6	16.9
4	18.5	122.0	15.2
32	18.3	114.5	16.0
60	19.0	117.4	16.2
91	17.2	113.5	15.2
122	18.6	118.2	15.7
152	18.8	116.1	16.2
182	18.9	118.7	15.9
212	21.1	123.8	17.1
246	20.7	122.7	16.8
269	19.4	119.1	16.3
300	19.2	121.1	15.9
326	20.6	122.5	16.8
357	18.9	122.0	15.5
385	20.3	118.7	17.1
413	19.6	121.1	16.2
447	21.0	124.6	16.8
475	21.4	118.8	18.0
504	19.3	122.4	15.8
535	18.4	115.9	15.9
1356	18.1	118.00	15.3
mean	**19.4**	**119.5**	**16.2**
sd	**1.1**	**3.0**	**0.7**
cv	**5.7 %**	**2.5 %**	**4.5 %**

3.5. Phase I clinical study –

3.5.1. "Influence of VKORC1 promoter polymorphism c.-1639 G>A on the vitamin K1 pharmacokinetics"

The study was conducted in accordance with Good Clinical Practice (GCP), the current requirements of EMEA (European Medicines Agency) [94] and the Helsinki Declaration. Extensive pharmacokinetic evaluation was carried out for all participants in the clinical trial. Pharmacokinetic parameters were determined using non-compartmental analysis since a large number of data points were collected and were estimated on the basis of the data for each route of administration of 2 mg Konakion® MM (oral and intravenous). These data are presented in Table I – XII (appendix).

Thirty healthy volunteers were included in the clinical study on the pharmacokinetics of vitamin K1. Demographic data of these consecutive subjects are presented in Table 52. Baseline characteristics were generally balanced across *VKORC1* genotype groups except for triglycerides significantly differing between GG and AG carriers and GG and AA group (p = 0.008) but not between AG and AA carriers. However, all triglycerides levels were in the acceptable range (< 200 mg/ dL).

Table 52 Demographics of the study subjects

Parameter	*Genotype group VKORC1*:c.-1639 G>A			*P*-value *uANOVA*
	GG	AG	AA	
Age [years]	28.3 ± 4.6[a] (22 – 39)	29.1 ± 8.7 (20 – 46)	28.4 ± 6.1 (22 – 42)	n.s.
Body mass index [kg/m^2]	22.8 ± 2.2 (20 – 28)	22.9 ± 2.8 (19 – 28)	22.4 ± 2.7 (19 – 27)	n.s.
Triglycerides [mg/ dL]	58.8 ± 13.4***** (36 – 74)	99.1 ± 30.2 (67 – 151)	86.9 ± 32.0 (44 – 133)	0.008
LDL-Cholesterol [mg/ dL]	101.1 ± 26.4 (74 – 150)	99.2 ± 30.7 (37 – 137)	102.6 ± 26.2 (65 – 134)	n.s.
HDL-Cholesterol [mg/ dL]	62.5 ± 8.5 (49 – 77)	56.4 ± 17.1 (37 – 93)	60.6 ± 16.4 (40 – 87)	n.s.
Creatinine [mg/ dL]	0.9 ± 0.2 (0.6 – 1.2)	0.9 ± 0.1 (0.8 – 1.2)	1.0 ± 0.2 (0.8 – 1.3)	n.s.

a mean values ± standard deviation with range in parenthesis; n = 10 in each group

***** p= 0.007; significantly different from AG; Post-hoc with Turkey

The plasma-concentration-time profiles of vitamin K1 were obtained by HPLC-RP18 with fluorescence detection. All evaluated pharmacokinetic parameters using non-compartmental analysis were normally distributed. Pharmacokinetic data for each genotype GG, AG and AA *VKORC1*:c.-1639 G>A are summarized in following Table 53.

Table 53 Differences in vitamin K1 pharmacokinetic parameters between *VKORC1*:c.-1639 G>A groups (*uANOVA* with regard to gender)

Pharmacokinetic parameter		Genotype *VKORC1*:c.-1639 G>A			*P*-value
		GG	AG	AA	*uANOVA*
p.o.					
t_{max}	[min]	181 ± 67[a] (120 – 362)	210 ± 119 (119 – 481)	217 ± 132 (122 – 542)	n.s.
C_{max}	[ng mL^{-1}]	29 ± 11 (15 – 55)	30 ± 10 (14 – 44)	27 ± 12 (6 – 46)	n.s.
AUC	[ng h mL^{-1}]	145 ± 68 (94 – 278)	206 ± 79*,§ (92 – 364)	132 ± 50 (45 – 211)	0.015
$F_{p.o.}$	[%]	51 ± 20 (23 – 90)	73 ± 33§ (38 – 150)	43 ± 13 (27 – 70)	0.011
CL[b]	[mL min^{-1}]	253 ± 80 (116 – 331)	165 ± 76 (72 – 338)	286 ± 160 (154 – 679)	n.s.
$t_{1/2}$	[min]	311 ± 54 (250 – 394)	457 ± 141$,§ (313 – 787)	332 ± 48 (261 – 429)	0.003
i.v.					
AUC	[ng h mL^{-1}]	298 ± 97 (204 – 510)	318 ± 48 (238 – 390)	319 ± 78 (177 – 453)	n.s.
V_{ss}	[L]	23 ± 8 (11 – 35)	25 ± 13 (10 – 58)	19 ± 5 (12 – 27)	n.s.
CL	[mL min^{-1}]	118 ± 32 (64 – 164)	104 ± 19 (70 – 136)	109 ± 33 (71 – 187)	n.s.
$t_{1/2}$	[min]	436 ± 186 (133 – 727)	492 ± 385 (194 – 1543)	383 ± 114 (158 – 548)	n.s.

[a]Mean values ± standard deviation with range in parenthesis; n= 10 in each group
[b]Clearance following oral (p.o.) administration CL/F
*$p < 0.05$; significantly different from GG (Post-hoc with Tukey)
§$p < 0.05$; significantly different from AA (Post-hoc with Tukey)
$$p < 0.01$; significantly different from GG (Post-hoc with Tukey)

Some of the main parameters such as C_{max} and t_{max} were taken directly from the data. Phylloquinone concentration-time curves varied between the volunteers as well as when p.o. and i.v. experiments were compared although the general curve shape following each route of administration remained similar. The i.v. plasma concentration-time curves showed a biexponential decline, suggesting a short initial distribution phase followed by an elimination phase. The curves obtained after p.o.

route of administration showed an initial absorption phase with a roughly monoexponential decay after C_{max} was reached. However, there was also a second and a third distinct maximum following C_{max} which is typical for substances undergoing enterohepatic circulation.

Following oral vitamin K1 administration, pharmacokinetic parameters such as AUC, $F_{p.o.}$ and $t_{1/2}$ showed statistically significant differences between the *VKORC1* genotype groups (*uANOVA* model; correspondent *p*-values are listed in table 53). AG subjects exhibited 30 % (p=0.042) and 36 % (p=0.021) higher mean AUC compared to GG and AA individuals, respectively. The mean bioavailability for AG carriers was 41 % higher (p=0.01; Fig.62) than for homozygous AA individuals. Statistically significant differences in terminal half-life between groups were also observed. AG genotype carriers showed 32 % and 27 % longer $t_{1/2}$ to GG (p=0.004) and AA (p=0.015) genotype carriers, respectively.

Interestingly, statistically significant differences in bioavailability (Fig.62) and terminal half-life (Fig.63) were also found when the results from both groups of homozygous individuals (GG + AA) were pooled and compared to those of heterozygous subjects (AG). In addition, homozygote carriers of the A-allele showed lower $F_{p.o.}$ mean value compared to GG + AG genotype carriers (Fig.62), whereas homozygous carriers of the G-allele exhibited shorter $t_{1/2}$ compared to the carriers of at least one A allele (AG + AA) (Fig.63).

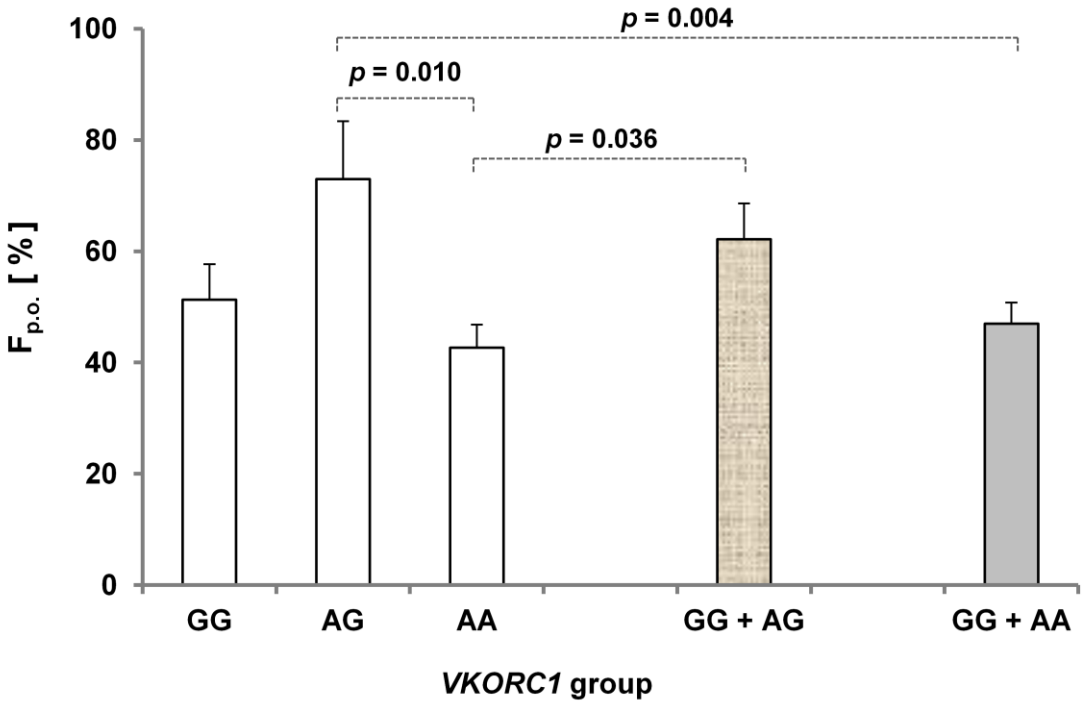

Figure 62 **Significant differences (*uANOVA* with regard to gender) in *Bioavailability* between *VKORC1* groups (AG to AA; AG to GG+AA; AA to GG+AG) following vitamin K1 p.o. administration**

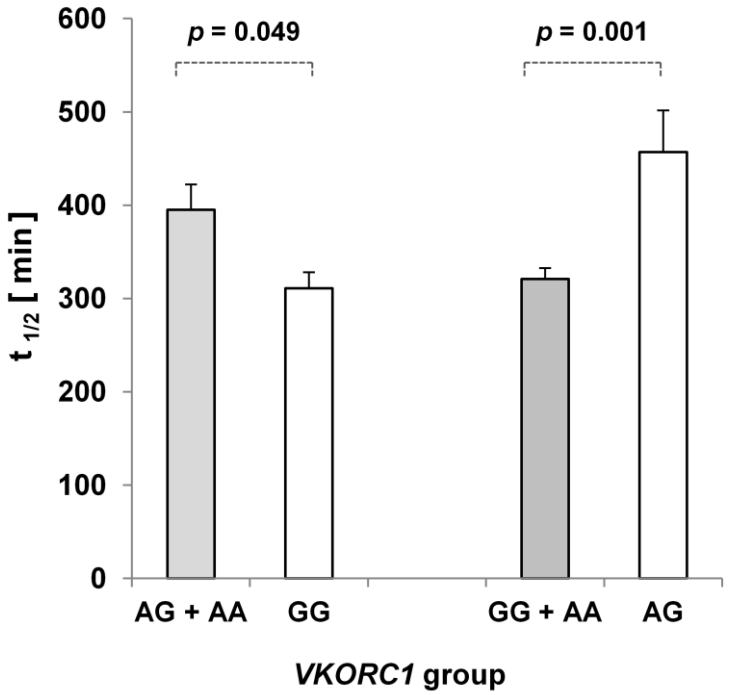

Figure 63 **Significant differences (*uANOVA* with regard to gender) in *Elimination half-time* between *VKORC1* groups (AG+AA to GG; GG+AA to AG)**

Following intravenous vitamin K1 administration, statistically significant differences between VKORC1 genotype groups were not observed for pharmacokinetic parameters AUC, V_{ss}, CL and $t_{1/2}$ by application of *uANOVA* analysis (mean values shown in Table 53).

To investigate the role of polymorphisms of other genes such as *CYP4F2* and *ABCC6* on vitamin K pharmacokinetics, differences in main pharmacokinetic parameters were assessed in relationship to vitamin K metabolizing enzyme genotypes (Table 54).

Table 54 Differences in vitamin K1 pharmacokinetic parameters between *CYP4F2* (V433M polymorphism; haplotype CYP4F2*3) and *ABCC6* (promoter polymorphism c.-127C>T) group CC and group CT+TT

Pharmacokinetic parameter		***CYP4F2***		***ABCC6***	
		CC (*n* = 20)	CT + TT (*n* = 10)	CC (*n* = 21)	CT + TT (*n* = 9)
p.o.					
t_{max}	[min]	209 ± 111[a] (119 – 542)	190 ± 104 (122 – 481)	211 ± 120 (119 – 542)	184 ± 69 (122 – 360)
C_{max}	[ng mL^{-1}]	29 ± 13 (6 – 55)	29 ± 8 (14 – 38)	29 ± 13 (6 – 55)	28 ± 6 (20 – 38)
AUC	[ng h mL^{-1}]	160 ± 82 (45 – 364)	163 ± 51 (96 – 262)	154 ± 81 (45 – 364)	176 ± 49 (106 – 262)
$F_{p.o.}$	[%]	56 ± 30 (23 – 150)	54 ± 17 (32 – 90)	52 ± 28 (23 – 150)	63 ± 19 (32 – 92)
CL[b]	[mL min^{-1}]	248 ± 138 (72 – 679)	208 ± 69 (116 – 331)	255 ± 133 (72 – 679)	187 ± 66 (116 – 307)
$t_{1/2}$	[min]	366 ± 126 (250 – 787)	367 ± 74 (285 – 524)	349 ± 76 (250 – 592)	409 ± 164 (259 - 787)
i.v.					
AUC	[ng h mL^{-1}]	310 ± 81 (177 – 510)	322 ± 63 (219 – 390)	315 ± 78 (177 – 510)	303 ± 71 (204 – 390)
V_{ss}	[L]	20 ± 6* (10 – 35)	27 ± 13 (12 – 58)	20 ± 6* (11 – 33)	28 ± 13 (10 – 58)
CL	[mL min^{-1}]	112 ± 30 (64 – 187)	106 ± 25 (70 – 148)	110 ± 28 (64 – 187)	112 ± 31 (70 – 164)
$t_{1/2}$	[min]	386 ± 135 (133 – 707)	539 ± 383 (158 – 1,543)	402 ± 120 (194 – 707)	519 ± 425 (133 – 1,543)

[a]Mean values ± standard deviation with range in parenthesis
[b]Clearance following oral (p.o.) administration CL/F
*$p < 0.05$; significantly different from CT+TT *ABCC6; uANOVA* (with regard to gender)

For both, *CYP4F2* and *ABCC6*, two groups were selected: one group of homozygous wild-type carriers CC and one group of individuals of at least one T allele (CT + TT). For the V433M polymorphism in *CYP4F2* gene, mean V_{ss} values differed significantly between both groups (*uANOVA*; p=0.036) and were lower for the wild-type individuals. Between *ABCC6* genotypes considerable differences in i.v. V_{ss} mean values were also observed (*uANOVA*; p=0.031), in that V_{ss} values were lower for the wild-type group (CC-genotype).

Correlation analysis between vitamin K1 pharmacokinetic parameters and fasting plasma triglycerides was performed without stratification for *VKORC1* genotypes. For each route of phylloquinone administration (p.o. and i.v), triglycerides levels inversely correlated significantly with CL (p<0.05), showing Pearson product-moment correlation coefficients of about -0.4 (data not shown).

4. DISCUSSION

4.1. Enzymological characterization of the VKORC1

To gain insight into the enzymatic characteristics of the VKORC1 with regard to its apparent kinetic constants K_m and V_{max}, various *in vitro* experiments were performed reproducing the step of quinone formation by the VKORC1 using the corresponding epoxide as substrate. The enzyme activity was characterized after chromatographic separation and quantification of the substrates, vitamin K1 and K2 epoxide, and of the corresponding quinone products.

After calculation of the enzymatic kinetic constants K_m and V_{max}, the reaction velocity as well as the affinity of the substrate for the VKORC1 enzyme could be assessed to a certain extent. The study of enzyme kinetics is important for two basic reasons. Firstly, it helps to explain how enzymes work, and secondly, it helps to predict how enzymes behave in living organisms. The kinetic constants, K_m and V_{max}, are critical to attempts to understand how enzymes work together to control metabolism [84].

Comparing both substrates and their constants, the K_m value i.e. the substrate concentration that yields a half-maximal velocity was 1.5 fold higher for K1 than for K2. Therefore, the binding affinity of vitamin K2 epoxide to the VKORC1 appears to be higher while vitamin K1 epoxide seems to bind in a weaker manner to the enzyme. However, K_m is not a binding constant that measures the strength of binding between the enzyme and substrate. Its value takes into account the affinity of substrate for enzyme, and also the rate at which the substrate bound to the enzyme is converted to product.

With regard to the enzyme velocity extrapolated to very high concentrations of substrate, the 1.4 fold higher V_{max} value for K2 indicated faster generation of K2 quinone by the VKORC1 compared with the formation of vitamin K1.

At this point, it is important to make a note of the gradually reduced VKORC1 activity during the process of quinone generation due to product inhibition. Therefore, the estimated enzyme constants have some to these experiments bounded validity [98].

Our measurements showed the higher binding affinity to the VKORC1 for vitamin K1 whereas vitamin K2 conversion occurs faster. However, a prediction of being a “better” and preferred substrate for the enzyme cannot be stated at this point neither for K1 nor for K2. To enable evaluation of the real catalytic efficacy, the use of

defined enzyme amount is required. Additionally, the generated quinone should be removed from the reaction in order to avoid possible product inhibition and masking of the estimated enzymatic constants. Until now, no group could either purify the VKORC1 or hold off the substrate to enable the estimation of the specific activity.

To gain deeper insight into the enzymatic characteristics of the VKOR, the VKORC1 and VKORC1L1, paralog enzymes coexpressed in all tissues at relatively uniform levels, and their apparent enzymatic parameters were compared. Both enzymes possess both vitamin K 2,3-epoxide reductase (VKOR) and vitamin K quinone reductase (VKR) activities, although VKORC1 appears to be specialized to perform the VKOR reaction at high turnover rates relative to VKR activity, while our group recently proposed that VKORC1L1 is specialized to carry out the VKR reaction at high turnover rates relative to measured VKORC1L1-specific VKOR activity [99]. Consistent with this view, we measured lower apparent K_m and V_{max} values for VKOR activity of VKORC1 compared to the values measured for VKORC1L1 (Table 55). VKORC1 showed K_m value which was 1.4 fold higher for K1 than for K2 while K_m value of VKORC1L1 was 5.2 fold lower for K1 compared to K2. This difference in determined K_m values indicated opposite binding affinity of the isoenzymes to both substrates. Considering the enzyme velocity, VKORC1L1 showed 2.1 and 8 fold higher turnover for K1 and K2 respectively in comparison to VKORC1.

Table 55 VKOR enzymatic parameters *in vitro*

Enzyme	Substrate	Km [µM]	Vmax [nmol.mg^{-1}.h^{-1}]
VKORC1	K1O	2.34	1.20
VKORC1	K2O	1.56	1.69
VKORC1L1	K1O	4.15*	2.57*
VKORC1L1	K2O	11.24*	13.46*

***** original data from Westhofen et al.

At this point, it could be referred to the assumption of different functions of both isoenzymes. VKORC1L1 was reported not to be able to support adequate VKOR activity to substitute for the function of VKORC1 in *VKORC1*$^{-/-}$ mice [100]. We hypothesize that VKOR enzymatic activity is the primary physiological enzymatic function of VKORC1L1 which is consistent with our findings that VKORC1L1 supports vitamin K-mediated intracellular antioxidative mechanisms [99].

4.2. Bioanalytical method validation of vitamin K determination

The modified HPLC method for vitamin K analysis in serum samples with fluorescence detection after post-column zinc reduction was validated according to the guidances for Bioanalytical Method Validation [92,93] of the *International Conference of Harmonization* for the intended purpose of use. The main purpose was to investigate the utility of the method to simultaneously define the kinetics of vitamin K1 and measure the extent to which oral doses are absorbed.

Concerning the fundamental parameters to ensure the acceptability of the performance such as accuracy, precision, selectivity, sensitivity, reproducibility, and stability, the method was tested thoroughly and it showed good reproducible results within the acceptable ranges regarding each particular parameter. Finally, all the required criteria were met.

To prove and warrant the practical applicability of the method for vitamin K determination in human serum, our laboratory participated in the vitamin K quality assurance scheme (KEQAS) representing robin-round tests and described as *External Quality Assurance*. The performance of these tests assisted in the development and harmonization of vitamin K analysis and tested the quality of vitamin K analysis.

Within KEQAS, the performance of participating groups was assessed by comparison of each group´s percentage absolute difference from the ALTM, i.e. *all laboratory trimmed mean* representing the target concentration, in consideration of the fact that there is no particular reference laboratory stated and no gold standard method for vitamin K analysis defined.

In the overall inter-laboratory assessment for low and high vitamin K serum concentrations, our group achieved *Z score* between 0.0 and 1.8. Our results, compared to the corresponding KEQAS consensus means, were indicative of good to very good performance. To what extent the other participating groups basically yield low- or high-leaning values, depended in the main on the used method for vitamin K analysis in human serum.

There is a perceived need to harmonize serum K measurements by quantifying inter-laboratory variation for vitamin K analysis in serum and developing an ongoing international scheme. Thereby, analytical performance can be gauged and participants encouraged to improve the accuracy of their analyses.

A number of HPLC assays using various detection systems have been reported. These include UV [101,102], electrochemical [101,103], electrofluorimetric [101], and postcolumn chemical reduction with fluorescence detection [86,87,104]. The latter technique has been shown to be inherently more selective, sensitive, and reproducible[86,87]. Postcolumn chemical reduction of K vitamins is usually achieved by using a zinc reducer system- metallic/ ionic zinc [86,87,104]. This system suffers from an impediment upon exhaustion of the supply of zinc in the reducer column. The shrinking zinc particles pass through the reducer column frits that limits the useful life of the reducer column and releases damaging particles into the HPLC system, leading to baseline shifts. In our experience, the zinc reducer column had to be replaced after every batch run of 40 samples to maintain optimal reduction and chromatographic performance which was proved to be sufficient for our purpose of use regarding pharmacokinetics of vitamin K1 within 24 hours after single-dose administration. The method allows analysis of up to 40 serum samples during a day and shows additionally the versatility by working with human serum or plasma as well as with organic tissues. The reported methods for the extraction of vitamin K from plasma or serum are labor intensive, all requiring multiple extraction procedures to remove lipophilic interferences. The single solid-phase extraction procedure for sample cleanup and concentration of vitamin K compounds, including the internal standard K1(25), showed good effectiveness with no interference from impurities and endogenous compounds. The internal standard is a structural analog of phylloquinone [86,87] and not present in biological material. The phylloquinone and menaquinone-4 peaks and that of internal standard were well separated during chromatographic analysis from matrix signals both in serum or plasma. The identity of the analyte was confirmed by the tests mentioned above. Intra- and interday repeatability was adequate as well as recovery testing. For the serum, a limit of detection of 0.03 nmol/ L (0.015 ng/ mL) was found, which indicates an about 3 fold higher sensitivity compared to assays, previously reported [86,87,103-105].

The method fulfilled all analytical standard criteria. It provided high specificity for K vitamins that could be directly detected without conversion to other derivatives. We examined recovery and accuracy using pool serum. Each peak of the vitamin K analogues was clearly resolved as a single peak within a separation time of 20 minutes. These results proved the system to be reliable and reproducible for the measurement of serum vitamin K. The reliability of the HPLC method was evaluated

based on the variation in retention times. The relative standard deviation, calculated from retention times obtained from over 40 injections, proved to be less than 1% for all compounds, indicating good chromatographic stability. The assay provides a rapid and relatively easy-to-use approach to the quantification of vitamin K analogues in human serum without compromising assay sensitivity and shows good reproducible results within the acceptable ranges regarding each particular validation parameter.

4.3. Vitamin K levels in mouse tissue

K vitamins were found in all mice tissues investigated, however their levels varied greatly (3.3., table 21). Both their absolute tissue concentrations and the ratio between K1 and MK4 were tissue-dependent. The tissues which were examined originated from mice fed a conventional laboratory chow diet.

Basically, tissue-distribution patterns of K vitamins did not differ highly between male and female mice, although the levels of vitamin K1 (phylloquinone) and vitamin K2 (menaquinone-4) were relatively higher in the females in comparison to those in the males. In general, tissue concentrations of MK4 exceed those of K1 in all tissues examined except for liver and ovary. Relatively high levels of phylloquinone were found in liver, testis and salivary gland (> 200 pmol/ g tissue), while very high levels were detected in ovary and uterus (> 800 pmol/ g tissue). Most other tissues contained less than 200 pmol/ g phylloquinone. In contrast, relatively high MK4 levels (> 200 pmol/ g tissue) were found in lung, bone, muscle and salivary gland, while very high MK4 concentrations (> 800 pmol/ g tissue) were measured in almost all the other tissues (pancreas, kidney, heart, brain, ovary, uterus and testis). The heart contained the lowest MK4 amount (< 200 pmol/ g tissue). There was no consistent correlation between the tissue levels of K1 and MK4.

Interestingly, the gender specific differences in vitamin K levels seemed to be higher for K1 compared to MK4 within the tissue. Thus, the ratio K1 to MK4 was 1.5-fold higher on the average for females and all tissues except for heart.

Okano et al. [49] described a similar tissue distribution of vitamin K in mice fed a normal diet. In general, higher K1 and MK4 levels were found in females compared to those in males. The absolute vitamin K values are not directly comparable with our results. This could be due to analytical differences such as varying preparation procedures of the tissues and different methods of detection. In contrast to our method, Okano et al. used LC-MS methodology and the vitamin K concentration was given in pmol per g wet weight tissue [49] while we indicate the levels in pmol per g dry weight tissue. However, it is possible to neglect these different ways of data demonstration and to draw comparative conclusions from the ratios K1 to MK4 in the mice tissues examined during the experiments. Interestingly, upon close inspection of the data from Okano it appears that the differences in vitamin K levels between males and females seemed to be lower for K1 compared to those for MK4 within the

tissue. It is therefore obvious that the ratio K1 to MK4 is 1.4-fold lower on the average for females and all tissues except for heart and pancreas.

The K1 to MK4 ratios we found in muscle and heart in males are comparable with those described by Okano et al. All the other ratios except for heart in females demonstrated in this study were nearly 10-fold lower in comparison to our results which remains unexplained. This difference may derive from the difference in the amount of vitamin K in the diets used as well as from the varying working procedures and detection methods, i.e. from the analytical procedure.

In view of the results from rats [106] and humans [48] regarding the tissue distribution of vitamin K, the presented K1 to MK4 ratio in the liver supported our measurement due to the fact that phylloquinone concentration in this tissue remarkably exceeded the level of MK4. In contrast, Okano et al. found lower K1 concentration in the liver compared to MK4.

The study from Ronden et al. [106] demonstrated tissue-dependent ratios between K1 and MK4 which are good comparable with our results except for heart. Consistent with this report, more K1 was found in heart in comparison to MK4 level. The significantly higher MK4 amount in diverse tissues may be explained by vitamin K accumulation in extrahepatic tissues and assuming that nutritional K1 had been converted into MK4. Tissue K1 and MK4 levels in the sum can be regarded as the accumulated vitamin K after a certain dietary regimen. Amounts of accumulated vitamin K differ widely from one tissue to another, thereby the liver is unique in its selective accumulation of K1. Other tissues (like pancreas and testis) seem to have a substantially higher preference for MK4 than for K1.

Thijssen et al. [48] showed that in man there are tissue-specific, vitamin K distribution patterns comparable to our results and to those in the rat except for pancreas and heart. This observational study on vitamin K contents in human tissues demonstrated the specific accumulation of K1 and/ or MK4 in the different human organs.

In conclusion, the comparison of our data with recently published vitamin K tissue levels in mice, rats and humans either using absolute values or the ratio of K1 to MK4 ranges from good agreement to poor accordance. These differences in storing of the samples, work-up procedures and detection methodologies could be balanced by harmonizing the procedures for analyses of vitamin K1 and MK4. A rational approach is the use of robin-round tests, spreading well defined samples containing K vitamins at a low and higher range. This should be done by reference laboratories,

which finally discuss the measured results from all the laboratories taking part in such a trial.

Accumulation of K1 and MK4 was found in all tissues investigated. Thereby, both the absolute tissue concentration and the ratio between K1 and MK4 were tissue-dependent. This may be linked to the fact that many of the vitamin K dependent (VKD) proteins (e.g., the transmembrane Gla proteins, matrix Gla protein and Gas6) show widespread tissue distribution, and virtually every tissue expresses vitamin K dependent carboxylase activity. This distribution and the diverse range of functions for VKD proteins in hemostasis, apoptosis, bone mineralization, calcium homeostasis, growth control, and signal transduction implicate a broad biological impact of K vitamins as reviewed by Berkner [107].

Many studies and overviews demonstrate the essential role of vitamin K and VKD proteins in the different tissues and organs which is especially investigated for humans. The liver with high hepatic accumulation of K1 is the main site of hemostatic VKD protein synthesis. Vitamin K is absolutely necessary as a co-factor of γ-carboxylase, an enzyme responsible for functional active blood coagulation factors. Due to hereditary conditions such as mutations in VKORC1 or γ-carboxylase (GGCX) or to acquired pathophysiological conditions like vitamin K deficiency due to malnutrition/malabsorption, coumarin poisoning, or autoimmune response to VKORC1/ GGCX, a severe bleeding phenotype is observed. In some cases, especially due to acquired pathophysiologies, high doses of vitamin K can reverse the bleeding phenotype.

Furthermore, in recent years the vitamin K antitumor effects have been closely examined. The suppressive effects of vitamin K2 on tumor recurrence after curative treatment for hepatocellular carcinoma (HCC) were clinically investigated on the basis of reports of the antitumor effects of vitamin K on various cancers [108]. Consistent with this report was showed that vitamin K2 administration significantly suppressed HCC recurrence by suppressing cyclin D1 expression through inhibition of NF-kappa B activation.

Moreover as described by Arai et al. [109], vitamin K-dependent growth factor (Gas6) has a significant role in the pathogenesis of diabetic nephropathy.

Bügel [110] reviews the role of K vitamins in bone health in adult humans with regard to osteocalcin, another VKD protein, and thereby in the prevention of osteoporosis. Several studies have demonstrated that vitamin K insufficiency is associated with low

bone mineral density (BMD) and increased fractures. Vitamin K supplementation, on the other hand, has been shown to improve the bone turnover profile.

Matrix Gla protein (MGP), another VKD protein in calcium homoeostasis [111], regulates calcium metabolism predominantly in the extracellular matrix of soft tissue. MGP is expressed in almost all soft tissues with highest levels of expression found in vascular tissue, heart, lung, kidney and cartilage [112], where MGP serves as a central inhibitor of tissues calcification . This can allude to the vitamin K presence in these tissues at high levels.

4.4. Pharmacokinetic analysis – preliminary studies in human volunteers

First application of the HPLC method in a pharmacological study on the pharmacokinetics of vitamin K1

As first application of the HPLC method for vitamin K serum determination served analysis of samples from an exploratory study on the pharmacokinetics of vitamin K1. There are only a few reports about direct measurements of serum concentrations of vitamin K after oral, intravenous or intramuscular single-dose administration [113-117]. As soon as sensitive analytical methods for vitamin K determination were introduced [86,101,102,104], information on the kinetics of phylloquinone became available. The methods showed sufficient sensitivity to enable detection of vitamin K1 in pg mL^{-1} range and thus allowed profiling of phylloquinone levels for many hours to days after administration.

Our study of the i.v. and p.o. pharmacokinetics of vitamin K1 as Konakion® MM 2 mg showed considerable differences between both repeated dosing occasions and between subjects. The pharmacokinetic results were indicative of intra- and inter-subject variability. The general shape of plasma concentration-time curves following p.o. and i.v (see section 3.4.2.1) administration remained similar during the two year interval although the systemic exposure in terms of C_{max} and AUC showed changes. The i.v. plasma concentration-time curves showed a biexponential decline, suggesting a short intial distribution phase followed by an elimination phase. The curves obtained after p.o. route of administration showed an intial absorption phase with a roughly monoexponential decay after C_{max} was reached. However, there was also a second distinct maximum following C_{max} which is typical for substances undergoing enterohepatic circulation.

The mean clearance rate of 95 and 111 mL min^{-1} for volunteer A and B, respectively, found in our experiments is within the range of those that were calculated from previous data of phylloquinone behavior upon injection which varied from 60 to 128 mL min^{-1} [113,118-120]. The corresponding mean terminal half-lives of 3.5 h after i.v. administration and 5.4 h after p.o. intake were also well comparable with previously published data [113]. Interestingly, phylloquinone serum half-lives in patients with severe acute liver disease were approximately two-fold longer than those found in

subjects with normal hepatic function [116]. Furthermore, terminal half-lives of vitamin K1 in newborns were also considerably longer compared to adults [115].

Oral administration of phylloquinone revealed substantial inter- and intra-individual variations in the bioavailability of this compound. Comparing both experiments, a considerable increase from 54 % to 80 % for volunteer A and a decrease from 97 % to 53 % for volunteer B was noted during the two-year interval. The differences in volunteer B might have been caused by significant loss of body weight and change in life-style. Hence, observed differences may be explained by intra- and inter-individual variation in dietary intake, transport and uptake of vitamin K as well as in lipoprotein metabolism, especially regarding triglyceride-enriched lipoproteins (VLDL)[9,41] and other determinants of vitamin K status. All these findings affirm the importance of standardizing trial procedures including co-medication when performing pharmacokinetic experiments.

4.5. Pharmacokinetic analysis – phase I clinical study

Inter-individual variance in pharmacokinetics of intravenous and oral phylloquinone (vitamin K1) mixed micelles formulation

The present exploratory phase I clinical study was designed to determine inter-individual variance in pharmacokinetics of intravenous and oral phylloquinone (vitamin K1) mixed micelles formulation as well as to explore a possible effect of the *VKORC1* promoter polymorphism c.-1639 G>A on the metabolism of phylloquinone, by means of correlating pharmacokinetic properties with the different genotypes in humans. There are only a few reports about the pharmacokinetics of vitamin K after oral, intravenous or intramuscular single-dose administration [113-117]. The investigation was performed in a representative group of healthy volunteers. The trial showed statistically significant difference in bioavailability after oral administration of phylloquinone between *VKORC1* genotype groups (particularly by comparison of AG individuals to AA carriers). $F_{p.o.}$ values were in accordance with data from previous studies [121] and revealed substantial inter-individual variability. It is noteworthy that the highest bioavailability was found in heterozygotes, carriers of the AG-genotype; furthermore, it was higher for males than to to females (data not shown). Therefore, this finding cannot be physiologically allegeable and might be indicative of certain heterozygote advantage in terms of phylloquinone availability in the human body where vitamin K is mostly required for blood coagulation but is also involved in metabolic pathways in bone and other tissue. In view of the multifunctional role of this vitamin in the human body, an evolutionary enrichment in individuals with a potential of high vitamin K exposure in the population seems reasonable. However, the mechanism by which the VKORC1 AG genotype would yield the highest bioavailability compared to the other polymorphisms remains to be elucidated. Moreover, it must be noted that AA genotype carriers showed the lowest phylloquinone bioavailability. This fact could play a role, for instance, when vitamin K adjustment in unstable patients under warfarin therapy is required or vitamin K has to be supplemented orally to antagonize intoxications with rodenticides.

Considering retrospectively other genes involved in vitamin K metabolism and tissue distribution, such as *CYP4F2* and *ABCC6* polymorphisms, considerable difference in intravenous V_{ss} between phenotypes was shown, whereas other pharmacokinetic

parameters seemed not to be influenced. However, to obtain a deeper insight into such a relationship further investigations with more subjects are needed.

The degree of considerable differences in terminal half-life, is also apparent from this study. Terminal half-lives following oral and intravenous phylloquinone administration were considerably longer for *VKORC1* heterozygotes compared to the homozygous carriers. This observation remained also valid after pooling both homozygous groups, GG and AA genotypes, together and comparing them to the AG genotype carriers. The estimated mean terminal half-lives of 6.1 h after oral phylloquinone administration were comparable with previously published data [113] . In contrast, the mean terminal half-life of 7.3 h following intravenous administration was longer than previous data, but can be explained by one subject showing an extremely long value. It should be noted that all observed significant differences between groups remain valid even after excluding this individual from the analysis.

The liver is the primary eliminating organ, as is evident from previously published studies in patients with severe acute liver disease, where phylloquinone serum half-lives were approximately twice longer than those found in subjects with normal hepatic function [116]. Moreover, terminal half-lives of vitamin K1 in newborns were also considerably longer compared to adults [115]. Based on our findings, it can be suggested that the highest phylloquinone bioavailability in *VKORC1* heterozygotes can be aligned with lower clearance and a potential of higher systemic exposure to vitamin K in these subjects. Furthermore, provided that hepatic metabolism is the major route of elimination, lower clearance indicates lower hepatic uptake and/ or vitamin K metabolism in VKORC1 AG genotype carriers. Theoretically, differences in body composition (fatty tissue) and transporting triglyceride-enriched lipoproteins (VLDL)[9,41] could affect tissue distribution and elimination rates for a lipid soluble compound such as vitamin K. However, in this study there were no significant differences in V_{ss} between groups, and inclusion of plasma triglyceride levels as a potential influencing factor in *uANOVA* did not diminish the significance of differences in $F_{p.o.}$ between groups.

In conclusion, significant inter-individual pharmacokinetic variance of vitamin K fate in the human body could be indicated. Further, an influence of the *VKORC1* promoter polymorphism c.-1639 G>A on the pharmacokinetic properties of phylloquinone in humans was shown. Significant differences in main pharmacokinetic parameters such as bioavailability and terminal half-life between groups suggest corresponding

differences in processing of vitamin K in the human body. The relevance of polymorphisms in *CYP4F2* and *ABCC6* in this regard must be further elucidated in an enlarged sampling. The clinical importance of potential genetic determinants of vitamin K status should be further investigated with respect to effects on absorption, distribution, metabolism and elimination of vitamin K.

5. SUMMARY

The main objective of the present work by means of a phase I clinical study was to determine inter-individual variance in pharmacokinetics of intravenous and oral phylloquinone (vitamin K1) mixed micelles formulation in humans as well as to explore a possible effect of the *VKORC1* promoter polymorphism c.-1639 G>A on the metabolism of phylloquinone. The pharmacokinetics of phylloquinone mixed micelles formulation (Konakion® MM 2 mg) were evaluated, in healthy human adult volunteers (n=30; 15 m, 15 f) using an open phase I design protocol upon oral and intravenous administration. The probands were subjected equally distributed (n=10; 5 m, 5 f) to three genotype-specific groups regarding *VKORC1* promoter polymorphism c.-1639 G>A (GG, AG and AA) to explore their relationship to specific pharmacokinetic parameters. Phylloquinone serum levels were determined by reversed phase HPLC with fluorometric detection after post-column zinc reduction. The method proved to be highly accurate, robust and reliable and showed a limit of detection and quantification of 0.015 ng mL^{-1} and 0.15 ng mL^{-1}, respectively. Measured phylloquinone serum concentrations were subjected to pharmacokinetic evaluation using a non-compartment analysis. Pharmacokinetic analysis of serum phylloquinone concentration versus time profiles revealed significant differences in main pharmacokinetic parameters. Significant inter-individual pharmacokinetic variance of vitamin K fate in the human body could be indicated. Further, an influence of the *VKORC1* promoter polymorphism c.-1639 G>A on the pharmacokinetic properties of phylloquinone in humans was shown. Significant differences in main pharmacokinetic parameters such as bioavailability and terminal half-life between groups suggest corresponding differences in processing of vitamin K in the human body. The relevance of polymorphisms in *CYP4F2* and *ABCC6* in this regard must be further elucidated in an enlarged sampling. The clinical importance of potential genetic determinants of vitamin K status should be further investigated with respect to effects on absorption, distribution, metabolism and elimination of vitamin K.

Furthermore, the enzymatic characteristics of the VKORC1 were examined by studying its enzyme kinetics. Comparing vitamin K1 and K2 as substrates and their apparent kinetic constants K_m and V_{max}, the binding affinity of vitamin K2 epoxide to the VKORC1 appears to be higher while vitamin K1 epoxide seems to bind in a weaker manner to the enzyme.

6. Appendix

Table I **Pharmacokinetic parameters of group GG, female, p.o.**

	GG_01_F_po	GG_02_F_po	GG_03_F_po	GG_04_F_po	GG_05_F_po	*Mean ± S.E.M.*
t_{max} [min]	150	362	183	151	120	193 ± 43
C_{max} [ng mL^{-1}]	27	28	30	26	42	31 ± 3
C_{max_D} [L^{-1}]	0.01	0.01	0.01	0.01	0.02	0.02 ± 0.001
T_{last} [min]	1445	1443	1447	1432	1440	1441 ± 3
C_{last} [ng mL^{-1}]	0.61	0.54	2.60	0.87	0.47	1.02 ± 0.40
AUC_{all} [ng h mL^{-1}]	96	103	262	111	127	140 ± 31
AUC_{0_600} [ng h mL^{-1}]	76	82	156	77	104	99 ± 15
F_{0_600} [%]	31	26	56	16	43	34 ± 7
AUC_{INFobs} [ng h mL^{-1}]	101	107	287	118	130	148 ± 35
F_{inf} [%]	40	32	90	23	50	47 ± 12
$V_{z\_F\_obs}$ [L µg^{-1}]	136	136	65	143	100	116 ± 15
Cl_{F_obs} [mL min^{-1}]	331	311	116	282	257	259 ± 38
Rsq	0.855	0.997	0.790	0.836	0.900	0.875 ± 0.035
$Corr_{XY}$	-0.925	-0.998	-0.889	-0.914	-0.949	-0.935 ± 0.019
$\lambda_{z\ No\_points}$	14	5	12	13	14	12 ± 1.7
λ_z	0.0024	0.0023	0.0018	0.0020	0.0026	0.0022 ± 0.0001
$\lambda_{z\ lower}$	180	461	212	213	180	249 ± 53
$\lambda_{z\ upper}$	1445	1443	1447	1432	1440	1441 ± 3
$t_{½\lambda_z}$ [min]	285	303	389	352	270	320 ± 22

Table II Pharmacokinetic parameters of group AG, female, p.o.

	AG_01_F_po	AG_02_F_po	AG_03_F_po	AG_04_F_po	AG_05_F_po	*Mean ± S.E.M.*
t_{max} [min]	122	119	151	120	360	174 ± 47
C_{max} [ng mL^{-1}]	20	30	37	42	20	30 ± 4
C_{max_D} [L^{-1}]	0.01	0.02	0.02	0.02	0.01	0.02 ± 0.002
T_{last} [min]	1440	1441	1453	1442	1435	1442 ± 3
C_{last} [ng mL^{-1}]	0.77	1.12	2.68	1.87	1.88	1.66 ± 0.33
AUC_{all} [ng h mL^{-1}]	92	132	219	239	176	172 ± 27
AUC_{0_600} [ng h mL^{-1}]	68	96	143	171	108	117 ± 18
F_{0_600} [%]	27	34	41	51	31	37 ± 4
AUC_{INFobs} [ng h mL^{-1}]	99	143	253	256	193	189 ± 31
F_{inf} [%]	38	46	53	68	55	52 ± 5
$V_{z\_F\_obs}$ [L µg^{-1}]	178	141	100	72	93	117 ± 19
Cl_{F_obs} [mL min^{-1}]	338	233	132	130	173	201 ± 39
Rsq	0.884	0.917	0.849	0.767	0.761	0.836 ± 0.031
$Corr_{XY}$	-0.940	-0.958	-0.921	-0.876	-0.873	-0.914 ± 0.017
$\lambda_{z\ No\_points}$	13	11	12	14	9	12 ± 0.9
λ_z	0.0019	0.0017	0.0013	0.0018	0.0019	0.0017 ± 0.0001
$\lambda_{z\ lower}$	212	284	242	183	330	250 ± 26
$\lambda_{z\ upper}$	1440	1441	1453	1442	1435	1442 ± 3
$t_{½\lambda z}$ [min]	364	419	524	382	373	412 ± 29

Table III Pharmacokinetic parameters of group AA, female, p.o.

	AA_01_F_po	AA_02_F_po	AA_03_F_po	AA_04_F_po	AA_05_F_po	*Mean ± S.E.M.*
t_{max} [min]	122	359	542	150	150	265 ± 81
C_{max} [ng mL^{-1}]	27	24	10	37	26	25 ± 4
C_{max_D} [L^{-1}]	0.01	0.01	0.01	0.02	0.01	0.01 ± 0.002
T_{last} [min]	1428	1433	1428	1440	1440	1434 ± 3
C_{last} [ng mL^{-1}]	0.82	1.34	1.31	0.95	0.37	0.96 ± 0.18
AUC_{all} [ng h mL^{-1}]	117	140	102	149	75	116 ± 13
AUC_{0_600} [ng h mL^{-1}]	87	87	57	108	60	80 ± 9
F_{0_600} [%]	27	28	20	30	23	26 ± 2
AUC_{INFobs} [ng h mL^{-1}]	124	150	113	156	77	124 ± 14
F_{inf} [%]	33	46	35	41	28	37 ± 3
$V_{z\_F\_obs}$ [L μg^{-1}]	133	96	153	103	183	134 ± 16
Cl_{F_obs} [mL min^{-1}]	269	223	295	213	4322	286 ± 39
Rsq	0.907	0.994	1.000	0.836	0.934	0.934 ± 0.030
$Corr_{XY}$	-0.953	-0.997	-1.000	-0.914	-0.967	-0.966 ± 0.016
$\lambda_{z\ No\_points}$	13	3	2	14	9	8 ± 2.5
λ_z	0.0020	0.0023	0.0019	0.0021	0.0024	0.0021 ± 0.0001
$\lambda_{z\ lower}$	219	540	600	180	326	373 ± 84
$\lambda_{z\ upper}$	1428	1433	1428	1440	1440	1434 ± 3
$t_{½\lambda z}$ [min]	342	298	360	336	294	326 ± 13

Table IV Pharmacokinetic parameters of group GG, male, p.o.

	GG_01_M_po	GG_02_M_po	GG_03_M_po	GG_04_M_po	GG_05_M_po	*Mean ± S.E.M.*
t_{max} [min]	180	150	180	182	150	168 ± 8
C_{max} [ng mL^{-1}]	55	23	23	15	22	28 ± 7
C_{max_D} [L^{-1}]	0.03	0.01	0.01	0.01	0.01	0.01 ± 0.004
T_{last} [min]	1438	1439	1433	1447	1462	1444 ± 5
C_{last} [ng mL^{-1}]	0.81	0.91	0.44	0.87	0.62	0.73 ± 0.09
AUC_{all} [ng h mL^{-1}]	278	120	106	94	148	149 ± 34
AUC_{0_600} [ng h mL^{-1}]	207	81	76	64	104	106 ± 26
F_{0_600} [%]	54	40	38	24	53	42 ± 5
AUC_{INFobs} [ng h mL^{-1}]	283	127	109	102	152	155 ± 33
F_{inf} [%]	68	56	53	34	68	56 ± 6
$V_{z\_F\_obs}$ [L μg^{-1}]	42	129	116	186	82	140 ± 52
Cl_{F_obs} [mL min^{-1}]	118	262	307	327	219	247 ± 37
Rsq	0.835	0.849	0.860	0.805	0.920	0.858 ± 0.018
$Corr_{XY}$	-0.924	-0.922	-0.927	-0.897	-0.959	-0.926 ± 0.010
$\lambda_{z\ No\_points}$	13	13	12	13	12	13 ± 0.2
λ_z	0.028	0.0020	0.0026	0.0018	0.0027	0.0024 ± 0.0002
$\lambda_{z\ lower}$	211	209	238	213	240	222 ± 7
$\lambda_{z\ upper}$	1438	1439	1433	1447	1462	1444 ± 5
$t_{½\lambda z}$ [min]	250	341	262	394	259	301 ± 28

Table V Pharmacokinetic parameters of group AG, male, p.o.

	AG_01_M_po	AG_02_M_po	AG_03_M_po	AG_04_M_po	AG_05_M_po	*Mean ± S.E.M.*
t_{max} [min]	210	180	149	481	210	246 ± 60
C_{max} [ng mL^{-1}]	44	25	30	14	39	31 ± 5
C_{max_D} [L^{-1}]	0.02	0.01	0.02	0.01	0.02	0.02 ± 0.003
T_{last} [min]	1441	1481	1440	1422	1440	1445 ± 10
C_{last} [ng mL^{-1}]	6.85	3.99	1.15	2.99	3.57	3.71 ± 0.92
AUC_{all} [ng h mL^{-1}]	364	193	169	177	296	240 ± 39
AUC_{0_600} [ng h mL^{-1}]	218	101	122	92	189	144 ± 25
F_{0_600} [%]	75	39	55	31	60	52 ± 8
AUC_{INFobs} [ng h mL^{-1}]	462	269	177	208	330	289 ± 51
F_{inf} [%]	150	92	72	60	97	94 ± 15
$V_{z\_F\_obs}$ [L μg^{-1}]	61	141	85	98	58	89 ± 15
Cl_{F_obs} [mL min^{-1}]	72	124	188	161	101	129 ± 21
Rsq	0.712	0.692	0.907	1.000	0.953	0.853 ± 0.063
$Corr_{XY}$	-0.844	-0.832	-0.952	-1.000	-0.976	-0.921 ± 0.035
$\lambda_{z\ No\_points}$	10	11	13	4	12	10 ± 1.6
λ_z	0.0012	0.0009	0.0022	0.0016	0.0017	0.0015 ± 0.0002
$\lambda_{z\ lower}$	299	274	211	481	240	301 ± 47
$\lambda_{z\ upper}$	1441	1481	1440	1422	1440	1445 ± 10
$t_{½\lambda_z}$ [min]	592	787	313	423	397	502 ± 84

Table VI Pharmacokinetic parameters of group AA, male, p.o.

	AA_01_M_po	AA_02_M_po	AA_03_M_po	AA_04_M_po	AA_05_M_po	*Mean ± S.E.M.*
t_{max} [min]	150	185	180	150	182	169 ± 8
C_{max} [ng mL^{-1}]	46	6	38	27	24	28 ± 7
C_{max_D} [L^{-1}]	0.02	0.01	0.02	0.01	0.01	0.01 ± 0.003
T_{last} [min]	1435	1438	1440	1440	1432	1437 ± 2
C_{last} [ng mL^{-1}]	0.87	0.47	2.02	1.98	0.84	1.23 ± 0.32
AUC_{all} [ng h mL^{-1}]	211	45	190	163	128	147 ± 29
AUC_{0_600} [ng h mL^{-1}]	163	32	127	93	101	103 ± 22
F_{0_600} [%]	40	19	39	38	36	34 ± 4
AUC_{INFobs} [ng h mL^{-1}]	216	49	210	180	134	158 ± 31
F_{inf} [%]	46	28	55	71	45	49 ± 7
$V_{z\_F\_obs}$ [L μg^{-1}]	58	344	98	95	105	140 ± 52
Cl_{F_obs} [mL min^{-1}]	154	679	158	186	248	285 ± 100
Rsq	0.950	0.870	0.888	1.000	0.950	0.932 ± 0.023
$Corr_{XY}$	-0.975	-0.933	-0.943	-1.000	-0.974	-0.965 ± 0.012
$\lambda_{z\ No\_points}$	13	11	8	2	11	9 ± 1.9
λ_z	0.0027	0.0020	0.0016	0.0019	0.0024	0.0021 ± 0.0002
$\lambda_{z\ lower}$	210	274	363	600	270	343 ± 69
$\lambda_{z\ upper}$	1435	1438	1440	1440	1432	1437 ± 2
$t_{½}\lambda_z$ [min]	261	351	429	357	294	338 ± 29

Table VII Pharmacokinetic parameters of group GG, female, i.v.

	GG_01_F_iv	GG_02_F_iv	GG_03_F_iv	GG_04_F_iv	GG_05_F_iv	*Mean ± S.E.M.*
t_{max} [min]	7	5	5	5	5	5 ± 0.4
C_{max} [ng mL^{-1}]	356	414	384	597	319	414 ± 48
C_{max_D} [L^{-1}]	0.18	0.21	0.19	0.30	0.16	0.21 ± 0.2
C_0 [ng mL^{-1}]	565	554	486	862	442	582 ± 73
T_{last} [min]	1440	1454	1443	1444	1458	1448 ± 3
C_{last} [ng mL^{-1}]	0.38	0.78	1.01	0.87	0.25	0.66 ± 0.15
AUC_{all} [ng h mL^{-1}]	250	329	301	510	257	330 ± 47
AUC_{0_600} [ng h mL^{-1}]	241	312	279	484	242	312 ± 45
AUC_{INFobs} [ng h mL^{-1}]	255	338	319	519	259	338 ± 48
V_{ss_obs} [L]	18	19	29	11	18	19 ± 3
V_{z_obs} [L]	89	73	110	39	41	70 ± 14
Cl_{obs} [mL min^{-1}]	131	99	105	64	129	105 ± 12
Rsq	0.953	0.878	1.000	0.981	1.000	0.962 ± 0.023
$Corr_{XY}$	-0.976	-0.937	-1.000	-0.990	-1.000	-0.981 ± 0.012
$\lambda_{z\ No\_points}$	4	4	3	4	2	3 ± 0.4
λ_z	0.0015	0.0014	0.0010	0.0016	0.0031	0.0017 ± 0.0004
$\lambda_{z\ lower}$	482	480	540	479	783	553 ± 59
$\lambda_{z\ upper}$	1440	1454	1443	1444	1458	1448 ± 3
$t_{½}\lambda_z$ [min]	469	511	727	423	222	470 ± 81

Table VIII Pharmacokinetic parameters of group AG, female, i.v.

	AG_01_F_iv	AG_02_F_iv	AG_03_F_iv	AG_04_F_iv	AG_05_F_iv	*Mean ± S.E.M.*
t_{max} [min]	5	6	5	5	5	5 ± 0.2
C_{max} [ng mL^{-1}]	320	301	430	425	485	392 ± 35
C_{max_D} [L^{-1}]	0.16	0.15	0.21	0.21	0.24	0.20 ± 0.02
C_0 [ng mL^{-1}]	447	333	505	566	600	490 ± 47
T_{last} [min]	1454	1436	1459	1448	1435	1446 ± 5
C_{last} [ng mL^{-1}]	0.21	0.82	2.38	0.94	0.26	0.92 ± 0.39
AUC_{all} [ng h mL^{-1}]	261	301	390	365	353	334 ± 23
AUC_{0_600} [ng h mL^{-1}]	251	281	353	337	343	313 ± 20
AUC_{INFobs} [ng h mL^{-1}]	263	311	478	376	355	356 ± 36
V_{ss_obs} [L]	16	23	58	20	10	25 ± 8
V_{z_obs} [L]	55	78	155	61	44	79 ± 20
Cl_{obs} [mL min^{-1}]	127	107	70	89	94	97 ± 10
Rsq	1.000	0.980	0.594	0.969	0.993	0.907 ± 0.079
$Corr_{XY}$	-1.000	-0.990	-0.771	-0.985	-0.997	-0.948 ± 0.045
$\lambda_{z\ No\_points}$	3	3	3	4	4	3 ± 0.2
λ_z	0.0023	0.0014	0.004	0.0014	0.0021	0.0015 ± 0.0003
$\lambda_{z\ lower}$	538	541	540	485	480	517 ± 14
$\lambda_{z\ upper}$	1454	1436	1459	1448	1435	1446 ± 5
$t_{½}\lambda_z$ [min]	298	505	1543	479	326	630 ± 232

Table IX Pharmacokinetic parameters of group AA, female, i.v.

	AA_01_F_iv	AA_02_F_iv	AA_03_F_iv	AA_04_F_iv	AA_05_F_iv	*Mean ± S.E.M.*
t_{max} [min]	5	5	6	5	5	5 ± 0.2
C_{max} [ng mL^{-1}]	369	423	431	489	392	421 ± 20
C_{max_D} [L^{-1}]	0.18	0.21	0.22	0.24	0.20	0.21 ± 0.01
C_0 [ng mL^{-1}]	536	523	868	730	542	640 ± 69
T_{last} [min]	1463	1437	1450	1454	1440	1449 ± 5
C_{last} [ng mL^{-1}]	1.31	0.47	1.15	0.40	0.35	0.74 ± 0.20
AUC_{all} [ng h mL^{-1}]	366	321	308	380	269	329 ± 20
AUC_{0_600} [ng h mL^{-1}]	330	304	280	365	259	308 ± 19
AUC_{INFobs} [ng h mL^{-1}]	382	324	323	383	272	337 ± 21
V_{ss_obs} [L]	24	15	27	12	16	19 ± 3
V_{z_obs} [L]	61	49	82	44	76	62 ± 7
Cl_{obs} [mL min^{-1}]	87	103	103	87	122	101 ± 7
Rsq	0.998	0.984	0.999	1.000	0.996	0.996 ± 0.003
$Corr_{XY}$	-0.999	-0.992	-1.000	-1.000	-0.998	-0.998 ± 0.001
$\lambda_{z\ No\_points}$	3	4	4	3	4	4 ± 0.2
λ_z	0.0014	0.0021	0.0013	0.0020	0.0016	0.0017 ± 0.0002
$\lambda_{z\ lower}$	543	479	474	553	480	506 ± 17
$\lambda_{z\ upper}$	1463	1437	1450	1454	1440	1449 ± 5
$t_{½\lambda z}$ [min]	480	328	548	354	433	428 ± 40

Table X Pharmacokinetic parameters of group GG, male, i.v.

	GG_01_M_iv	GG_02_M_iv	GG_03_M_iv	GG_04_M_iv	GG_05_M_iv	*Mean ± S.E.M.*
t_{max} [min]	5	5	7	5	5	5 ± 0.4
C_{max} [ng mL^{-1}]	369	262	231	336	230	286 ± 28
C_{max_D} [L^{-1}]	0.18	0.13	0.12	0.17	0.12	0.14 ± 0.01
C_0 [ng mL^{-1}]	470	334	384	426	313	385 ± 29
T_{last} [min]	1433	1440	1433	1453	1431	1438 ± 4
C_{last} [ng mL^{-1}]	1.02	0.62	0.02	0.95	0.66	0.65 ± 0.18
AUC_{all} [ng h mL^{-1}]	410	219	204	283	257	267 ± 38
AUC_{0_600} [ng h mL^{-1}]	381	201	199	263	197	248 ± 35
AUC_{INFobs} [ng h mL^{-1}]	419	225	205	299	224	274 ± 40
V_{ss_obs} [L]	17	33	17	30	35	26 ± 4
V_{z_obs} [L]	42	92	31	114	78	72 ± 15
Cl_{obs} [mL min^{-1}]	80	148	164	111	149	130 ± 15
Rsq	0.972	0.998	0.994	0.947	0.998	0.982 ± 0.010
$Corr_{XY}$	-0.986	-0.999	-0.997	-0.973	-0.999	-0.991 ± 0.005
$\lambda_{z\ No\_points}$	4	4	4	3	3	4 ± 0.2
λ_z	0.0019	0.0016	0.0052	0.0010	0.0019	0.0023 ± 0.0007
$\lambda_{z\ lower}$	479	482	477	538	540	503 ± 15
$\lambda_{z\ upper}$	1433	1440	1433	1453	1431	1438 ± 4
$t_{½}\lambda_z$ [min]	369	432	133	707	365	401 ± 92

Table XI Pharmacokinetic parameters of group AG, male, i.v.

	AG_01_M_iv	AG_02_M_iv	AG_03_M_iv	AG_04_M_iv	AG_05_M_iv	*Mean ± S.E.M.*
t_{max} [min]	6	5	5	5	5	5 ± 0.2
C_{max} [ng mL^{-1}]	296	296	258	244	385	296 ± 25
C_{max_D} [L^{-1}]	0.15	0.15	0.13	0.12	0.19	0.15 ± 0.01
C_0 [ng mL^{-1}]	459	371	324	367	608	426 ± 51
T_{last} [min]	1438	1470	1448	1528	1445	1466 ± 16
C_{last} [ng mL^{-1}]	0.19	0.74	0.64	0.99	0.50	0.61 ± 0.13
AUC_{all} [ng h mL^{-1}]	307	284	238	340	338	301 ± 19
AUC_{0_600} [ng h mL^{-1}]	291	259	223	294	316	277 ± 16
AUC_{INFobs} [ng h mL^{-1}]	308	291	246	348	342	307 ± 18
V_{ss_obs} [L]	17	26	30	28	18	24 ± 3
V_{z_obs} [L]	30	66	106	48	41	58 ± 13
Cl_{obs} [mL min^{-1}]	108	115	136	96	98	110 ± 7
Rsq	0.999	0.999	0.963	0.999	1.000	0.992 ± 0.007
$Corr_{XY}$	-1.000	-0.999	-0.982	-0.999	-1.000	-0.996 ± 0.004
$\lambda_{z\ No\_points}$	3	3	3	4	3	3 ± 0.2
λ_z	0.0036	0.0017	0.0013	0.0020	0.0024	0.0022 ± 0.0004
$\lambda_{z\ lower}$	542	540	543	483	544	530 ± 12
$\lambda_{z\ upper}$	1438	1470	1448	1528	1445	1466 ± 16
$t_{½}\lambda_z$ [min]	194	401	542	345	288	354 ± 58

Table XII Pharmacokinetic parameters of group AA, male, i.v.

	AA_01_M_iv	AA_02_M_iv	AA_03_M_iv	AA_04_M_iv	AA_05_M_iv	*Mean ± S.E.M.*
t_{max} [min]	5	5	5	6	6	5 ± 0.2
C_{max} [ng mL^{-1}]	414	217	386	362	294	335 ± 35
C_{max_D} [L^{-1}]	0.21	0.11	0.19	0.18	0.15	0.17 ± 0.02
C_0 [ng mL^{-1}]	629	267	485	490	436	461 ± 58
T_{last} [min]	1436	1440	1440	1438	1435	1438 ± 1
C_{last} [ng mL^{-1}]	1.57	0.18	3.11	0.26	0.46	1.12 ± 0.56
AUC_{all} [ng h mL^{-1}]	453	177	369	252	295	309 ± 47
AUC_{0_600} [ng h mL^{-1}]	412	169	329	244	278	287 ± 41
AUC_{INFobs} [ng h mL^{-1}]	472	179	381	255	299	317 ± 51
V_{ss_obs} [L]	19	25	22	15	19	20 ± 2
V_{z_obs} [L]	51	79	20	73	55	56 ± 10
Cl_{obs} [mL min^{-1}]	71	187	87	131	112	117 ± 20
Rsq	0.999	0.997	0.977	0.995	0.999	0.993 ± 0.004
$Corr_{XY}$	-0.999	-0.998	-0.988	-0.998	-0.999	0.997 ± 0.002
$\lambda_{z\ No\_points}$	3	4	5	4	4	4 ± 0.3
λ_z	0.0014	0.0024	0.0044	0.0018	0.0020	0.0024 ± 0.0005
$\lambda_{z\ lower}$	541	482	364	484	488	472 ± 29
$\lambda_{z\ upper}$	1436	1440	600	1438	1435	1270 ± 167
$t_{½}\lambda_z$ [min]	503	293	158	388	343	337 ± 57

LITERATURE

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Publikationen

1. Marinova, M.*, Lütjohann, D.*, Breuer, O., Kölsch, H., Westhofen, P., Watzka, M., Mengel, M., Stoffel-Wagner, B., Hartmann, G.,Coch, C., and Oldenburg, J. (2012) **Inter-individual variance in pharmacokinetics of intravenous and oral phylloquinone (vitamin K1) mixed micells formulation**, *Eur J Clin Pharmacol. 2012 Aug 5.*

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8. Oldenburg, J., Marinova, M., Müller-Reible, C., and Watzka, M. (2008) **The vitamin K cycle**, *Vitam Horm 78*, 35-62.

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