Determination of the urinary aglycone metabolites of vitamin K by HPLC with redox-mode electrochemical detection

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Running title: Determination of the Urinary Metabolites of Vitamin K

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Abbreviations: K_1, phylloquinone; MKs, menaquinones; 5C-metabolite, 2-Methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone; 7C-metabolite, 2-Methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone; EC, electrochemical; ECD, electrochemical detection; HPLC, High performance liquid chromatography; IS, Internal standard; SPE, Solid phase extraction.

Abstract

We describe a method for the determination of the two major urinary metabolites of vitamin K as the methyl esters of their agyclone structures, 2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone (5C-side-chain metabolite) and 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone (7C-side-chain metabolite), by HPLC with electrochemical detection (ECD) in the redox mode. Urinary salts were removed by reversed-phase (C_{18}) solid phase extraction (SPE) and the predominately conjugated vitamin K metabolites hydrolysed with methanolic HCl. The resultant carboxylic acid aglycones were quantitatively methylated with diazomethane and fractionated by normal-phase (silica) SPE. Final analysis was by reversed-phase (C_{18}) HPLC with a methanol-aqueous mobile phase. Metabolites were detected by amperometric, oxidative ECD of their quinol forms, which were generated by post-column coulometric reduction at an upstream electrode. The assay gave excellent linearity (r^2 typically ≥ 0.999) and high sensitivity with an on-column detection limit of <3.5 fmol (<1pg). The inter-assay precision was typically 10%. Metabolite recovery was compared to that of an internal standard (2-methyl-3-(7′-carboxy-heptyl)-1,4-naphthoquinone), added to urine samples just before analysis. Using this methodology we confirmed that the 5C- and 7C-metabolite were major catabolites of both phylloquinone (vitamin K_1) and menaquinones (vitamin K_2) in humans. We propose that the measurement of urinary vitamin K metabolite excretion is a candidate non-invasive marker of total vitamin K status.

Supplementary key words vitamin K, phylloquinone, menaquinones, urinary vitamin K metabolites, aglycones, HPLC-ECD

INTRODUCTION

Compounds with vitamin K activity have a common 2-methyl-1, 4-naphthoquinone nucleus and a variable alkyl substituent at the 3 position. The major naturally occurring K vitamins are the plant form phylloquinone (vitamin K_1) and multiple forms of menaquinones (vitamins K_2), predominately of bacterial origin. Phylloquinone (abbreviated K_1) has a 20-carbon, phytyl side chain whereas menaquinones have multi-prenyl side chains, their number being indicated by a suffix (i.e. menaquinone-n, abbreviated MK-n). In a typical Western diet, K_1 and MK-n account for about 90% and 10% of vitamin K intakes respectively (1). Dietary MK-n mainly comprise MK-4, MK-7, MK-8, and MK-9 (2), although MKs with longer side chains up to MK-13 are present in human liver (3,4). Menadione (abbreviated K_3) is a synthetic vitamin K homologue that lacks the side chain at the 3-position and which, despite toxicity issues and restricted biological activity, is still available in some countries as a pharmaceutical vitamin K preparation in the form of menadiol sodium phosphate (5) or similar water-soluble salt. The biological activity of K_3 *in vivo* depends entirely on its prenylation to MK-4 (6,7).

At the cellular level the cofactor role of vitamin K for the post-translational conversion of specific peptide-bound glutamate (Glu) to γ-carboxyglutamate (Gla) is well established as is the intimately associated metabolic cycle whereby the vitamin K 2,3 epoxide metabolite generated during γ-glutamyl carboxylation is salvaged (8) (9).

Other aspects of the intermediary metabolism of vitamin K, including the processes leading to vitamin K catabolism and excretion, are much less understood. During the 1970's human studies employing radio-labelled-tracer and unlabelled pharmacological doses showed that K_1 was rapidly and extensively catabolised via the urine and bile (10) (11) (12). These studies established that the major aglycone

metabolites of K_1 were two side-chain shortened carboxylic acids with the structures 2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone (side chain length 5 carbon atoms, structure IV in **Fig. 1**) and 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone (side chain length 7 carbon atoms, structure III in **Fig. 1**) respectively. (10) (11) (12). Both 5C- and 7C-aglycones were excreted as water-soluble conjugates, mainly with glucuronic acid.

At the time of isolation and characterization of these two metabolites (henceforward abbreviated to 5C- and 7C-aglycones respectively), the role of vitamin K was thought to be limited to its classical coagulation function, and human deficiency at the population level was only considered a problem during the first 6-months of life (13). However the subsequent discovery of many more vitamin K-dependent proteins (also called Gla proteins), with a widespread tissue distribution, has lead to a re-evaluation of the general physiological function and health roles of vitamin K. Putative roles of Gla proteins now extend to a diversity of functions such as the regulation of bone turnover and calcification (14) (15), inhibition of vascular calcification (16), and roles in vascular repair processes (17), cell cycle regulation, cell-cell adhesion, and signal transduction (18). Of particular note is the accumulating body of evidence that has linked a suboptimal vitamin K reserves in bone to an increased risk of osteoporotic fracture (19) (20) or to reduced bone mineral density (21). To address this issue, and other questions, there has been a need to develop new biochemical measures to monitor vitamin K status in human populations (22). Current measures include the direct measurement of circulating vitamin K (as an indicator of tissue stores) and functional assessments of the γ-carboxylation status of specific Gla proteins such as prothrombin and osteocalcin representing hepatic and bone γ-carboxylation capacity respectively. In addition, measurements of urinary free

Gla offer an overall assessment of the γ-carboxylation status of Gla proteins. Each of these status assessments has a number of methodological and interpretational drawbacks (23) (24) (25). Although useful in many situations, the measurement of circulating vitamin K to assess tissue stores has the major disadvantage that only K_1 is commonly measured to the detriment of MKs. MKs from the diet (1) (2) and possibly from intestinal synthesis (3) (4) may make a significant contribution to total daily vitamin K intake (1), and the liver stores of vitamin K are predominately long chain MKs (3) (4).

Here we describe the development of chromatographic techniques to quantify the two major urinary aglycones of vitamin K with high sensitivity, enabling their measurement at low physiological concentrations. We chose ECD in the redox mode for the final analytical HPLC stage as this method is especially suitable for quinone compounds and has been successfully used for the determination of vitamin K in serum (26) (27). To find out whether the 5C- and 7C-aglycones are common to all K vitamins, we obtained urine samples from adults before and after supplementation with different doses of K_1, MK-4, MK-7 and K_3. We also analysed urine samples from newborn infants who, as a group, are known to have precariously low vitamin K stores, and are routinely given vitamin K prophylaxis at birth.

MATERIALS AND METHODS

Reagents

The 5C-aglycone (2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone), 7C-aglycone (2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone) and the compound (2-methyl-3-(7′-carboxy-heptyl)-1,4-naphthoquinone) used for the internal standard (IS), are not commercially available and were synthesised for this study (28). The γ-lactone form of the 7C-aglycone (2-methyl-3-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-naphthoquinone lactone), also known as vitamin K γ-lactone, was a gift to MJS from Hoffmann-La Roche and Co., Basle, Switzerland. Organic solvents were of HPLC grade (Rathburns Chemicals, Walkerburn, Scotland). Water for ECD was purified using a Purite Neptune water purification system (Jencons-PLS, Leighton Buzzard, UK). Potassium hydroxide (Ultra grade), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), and ethylenediaminetetraacetic acid (disodium salt, dihydrate, Analar grade) were obtained from Sigma-Aldrich (Dorset, UK). Anhydrous sodium acetate, glacial acetic acid, and 3M HCl, all AristaR grade, were obtained from BDH (Lutterworth, Leics, UK). Nitrogen gas (oxygen free), for removal of solvents, was obtained from BOC (Guildford, Surrey, UK).

Apparatus

Solid phase extraction (SPE) procedures were performed using Isolute™ SPE C_{18} (100 mg, 1 ml reservoir) cartridges (Jones Chromatography, Mid Glamorgan, UK) and Sep-Pak™ silica plus cartridge (Waters Ltd, Elstree, Herts, UK) with a SPE vacuum manifold.

For HPLC we used a Gynkotek model 480 pump with pulse damper, a Waters™ 717 plus Autosampler, and an in-line De-Jour, X-Act HPLC degassing unit.

For on-line detection we used a DECADE electrochemical detector (Antec, Leyden, The Netherlands) equipped with an in-line cell (model 5011) containing dual porous graphite coulometric electrodes in series (ESA Analytical Limited, Aylesbury, Bucks, UK) and an amperometric, wall jet electrode situated immediately downstream (Antec model VT-03). Chromatographic data was captured using a data chromatography manager (Waters).

Urine collection and human subject protocols

Twenty four-hour and spot urine collections were made into plastic containers. Owing to the photosensitive nature of the metabolites, the urine containers were protected from sources of strong light. Urine collections were stored at room temperature for the duration of the collection period and aliquots frozen at -70°C until analysis. No significant metabolite loss occurred under these conditions.

We investigated urinary 5C- and 7C-aglycone excretion in healthy adult volunteers. Measurements were made in spot or 24-hour urine collections in both unsupplemented and vitamin K-supplemented subjects. For the supplementation studies the subjects took different forms of vitamin K orally at doses previously used for the treatment or prevention of several pathologies. The vitamin K compounds used were K_1 (2 mg and 50 mg) (29); two homologues representative of the menaquinone series, namely MK-4 (45 mg) (30), and MK-7 (1 mg) (31); and K_3 (20 mg) (5). The amounts of 5C- and 7C-aglycone excreted in the urine were measured pre- and post- supplementation.

A number of spot urines were also collected from newborn infants (2 males, 1 female) before and after intramuscular K_1 prophylaxis with 1 mg Konakion Neonatal (Roche, Basel, Switzerland).

All adult volunteers and guardians of newborns gave full written informed consent prior to participation in these investigations.

Assay Procedure

A flowchart showing the extraction and analytical procedures is shown in **Fig. 2**.

Extraction and hydrolysis of metabolites

A C_{18} SPE cartridge was pre-washed with 1ml of methanol followed by 1 ml of deionised water. Aliquots of 0.5 ml (unsupplemented subjects) or 0.05 ml (after vitamin K supplementation) of urine were loaded onto the SPE cartridge and allowed to elute to waste under gravity. To remove urinary salts, the SPE cartridge was washed with 1 ml of deionised water at a flow rate of ~0.5 ml/min and the eluent discarded (32). The conjugated vitamin K metabolites were then eluted into a clean tapered polypropylene test tube (12 ml capacity) with 2 ml of a methanolic stock solution containing 0.15 μg of the IS and the eluent evaporated to dryness under a stream of N_2 at 50°C.

Conjugated urinary vitamin K metabolites were hydrolysed overnight at room temperature (in the dark) with 1.1 ml of methanolic HCl (prepared by combining one volume of concentrated (35%) HCl with 4 volumes of methanol) (33). The metabolites, now as their aglycones, were extracted into chloroform by the addition of 1.1 ml chloroform and 1.0 ml water to the sample tube (34). The upper methanolic-aqueous layer was removed, and discarded. To prevent acid-catalysed lactonisation of the 7C-aglycone during subsequent drying procedures, the lower chloroform layer was washed with 10 ml of deionised water to remove any residual mineral acid. After brief shaking, and allowing the two phases to separate, the upper aqueous layer was discarded and the lower chloroform layer evaporated to dryness under a stream of N_2 at 50˚C.

Methylation of aglycones

The carboxylic acid forms of the vitamin K aglycones were converted to their methyl ester derivatives by reaction with freshly prepared ethereal diazomethane generated on a small scale by a modification of a method described elsewhere (35). In brief, a mixture of 5M KOH (~20 ml) and diethyl ether (~15 ml) are placed into a small, screw capped bottle (100 ml-capacity Duran) and solid MNNG gradually added until sufficient diazomethane has been generated to turn the upper ether layer yellow (Caution: only perform in well-ventilated fume hood). A volume of 0.5 ml of this ethereal diazomethane was then added to each urine extract. Complete methylation of the carboxylic acid group of the aglycone-metabolites was achieved at room temperature within 5 minutes after which the diethyl ether was removed under a stream of N_2 at 50°C.

Normal-phase SPE of methylated aglycones

Further fractionation of the urine extract was achieved by normal phase-SPE using Sep-Pak™ cartridges. Each cartridge was attached to a 10-ml glass syringe with a Luer end fitting and activated by drawing through 2 ml of *n*-hexane. Each extract, was dissolved in 2 ml of *n*-hexane, and then pipetted into the glass syringe attached to the activated cartridge. The *n*-hexane was drawn through the cartridge and the eluent discarded. A further volume of 8 mL of *n*-hexane was added to the sample tube, the washings drawn through the cartridge, and discarded as before. The vitamin K metabolites and IS were then eluted from the cartridge with 10 ml of *n*-hexane-diethyl ether (85:15, *v/v*), and collected into a fresh polypropylene, stoppered, tapered test tube. The solvent was removed under a stream of nitrogen at 50° C.

Reversed-phase HPLC-ECD

The final separation of the methylated aglycones was achieved by reversed-phase HPLC using a Thermo Hypersil-Keystone HyPURITY C_{18} column (3μm particle size, dimensions 100 x 4.4 mm supplied by Hichrom, Reading, Berks, UK) and a mobile phase consisting of 60: 40 (v/v) methanol–0.05M sodium acetate buffer (pH 3.0 and containing 0.1% EDTA) (26). The flow rate was 1.0 ml/min. Effluent from the column passed through the coulometric dual-electrode cell in which the twin, porous-graphite, electrodes were set at a negative potential (–1.2 V), thereby reducing the urinary vitamin K quinone metabolites and IS to their quinol states. The effluent then passed to the amperometric wall jet electrode set at +0.3 V which resulted in the re-oxidation of the quinol forms of metabolites and IS to their respective quinones. Chromatograms were generated by monitoring the current (nA) at the wall jet electrode.

Quantification of 5C- and 7C-aglycone metabolites

From methanolic stock solutions of the 5C- and 7C-aglycones and the IS (methyl ester forms) we prepared a series of standards containing all 3 analytes with spectrophotometrically determined (E mM = 18.9 at 248 nm) weight ratios of 5C-aglycone/IS and 7C-aglycone/IS ranging from 0.003 to 0.121. Each chromatographic run included the direct injection of 10 μl of each calibration standard. For routine analyses (i.e. for physiological urinary concentrations of metabolites) the SPE-fractionated extracts were reconstituted in 40 μl of methanol and a volume of 10 μl injected onto the RP-HPLC system. For the determination of the much higher levels after vitamin K supplementation, the extract was reconstituted in 100 μl of methanol and 10 μl injected. Metabolite concentrations were quantified by the method of peak-height ratios. For each chromatogram generated, the peak heights of 5C- and 7C-aglycones were expressed as a ratio to the IS peak. A calibration plot of peak height

ratios of the standards versus their weight ratios gave excellent linearity (r^2 typically $\geq$ 0.999) and was used to calculate the equivalent weight ratios of aglycone/IS for each unknown. Multiplication of this weight ratio by the amount of IS originally added gave the amounts of aglycones in the volume of urine processed.

RESULTS

Optimisation of extraction and conjugate-hydrolysis procedures

The initial SPE procedure using C_{18} cartridges was an effective strategy for desalting the urine and concentrating the urinary metabolites in their conjugated forms. True recovery experiments at this C_{18} SPE stage were not possible because there is limited information on the chemical nature and variety of conjugated metabolites *in vivo* and appropriate standards are unavailable. Nevertheless, recovery experiments using the synthetic 5C-aglycone, 7C-aglycone and IS showed that there was no significant loss of vitamin K moieties at this stage. Recoveries were reduced if lower eluting volumes (<2ml) of methanol were used, or if the urine was acidified (with HCl) to promote protonisation of the carboxylic acid metabolites. Trials with other SPE cartridge chemistries with side chain lengths $<C_{18}$ also resulted in a reduced recovery. Analyte recovery was found to vary inversely with the flow rates of both the first aqueous wash and second methanolic elution steps. Omission of this initial SPE stage increased the baseline current in the final electrochemical RP-HPLC analytical stage resulting in a decrease in assay sensitivity.

After the initial SPE stage, conjugated metabolites of vitamin K were hydrolysed with methanolic HCl. At room temperature, hydrolysis of conjugated metabolites with methanolic HCl was complete within 16 hours. After hydrolysis, the lipid-soluble aglycones and IS were efficiently extracted into chloroform after the addition of further volumes of chloroform and water to form a two-phase system as described for the final stage of the Bligh and Dyer total lipid extraction technique (34). A second extraction of the methanolic digest with chloroform did not improve the recovery of aglycones.

Methyl ester derivatisation of carboxylic acid aglycones

There were two main reasons for adopting a pre-HPLC methylation procedure to quantitatively convert the aglycone carboxylic acids to their respective methyl esters. Firstly, exposure to methanol during the chromatographic procedures resulted in some inadvertent methylation, which was enhanced by use of methanolic HCl to deconjugate the aglycones. Secondly, the reduced polarity of these methylated derivatives improved their retention in our RP-HPLC separation system.

SPE purification of methylated aglycones

An additional SPE purification stage using silica cartridges was introduced to remove interfering compounds, which prevented baseline resolution of the 5C- and 7C-aglycones in the final HPLC-ECD analytical stage. Optimisation of the composition of the eluting solvent was carried out using a standard mixture containing known concentrations of the 5C-aglycone, 7C-aglycone and IS. A diethyl ether concentration of 15% diethyl ether in *n*-hexane (v/v) was found to give quantitative recoveries of the methylated aglycones and the IS while producing good baseline resolution on HPLC-ECD. Recovery of the vitamin K aglycones and IS after this and previous purification stages was consistently >90% for each analyte with no inter-analyte bias.

Optimisation of HPLC-ECD Conditions

The hydrodynamic voltammograms of the 5C- and 7C-aglycone methyl esters together with the IS are shown in **Fig. 3**. A voltage of –1.2 V was selected as the applied potential in the upstream flow-through coulometric twin electrodes for quantitative reduction of the quinone forms of metabolites and IS to their corresponding quinols (this was the maximum possible voltage to the electrodes of the ESA coulometric cell when controlled by the DECADE instrument). In selecting

+0.3 V as the applied potential for the down stream amperometric electrode (for the re-oxidation of the respective quinols), a minimal degree of assay sensitivity was sacrificed. However this reduced interferences from co-extracted and co-chromatographed substances and resulted in a reduced background current.

The EC reduction of the quinone to the quinol moiety was temperature-dependent. Increasing the temperature in which the reductive EC cell was housed improved overall sensitivity of the assay and we routinely operated the EC cells in the temperature range of 37-42°C. The methanol component of the mobile phase and system temperature were adjusted until the retention times of the 5C-aglycone, 7C-aglycone and IS at a flow rate of 1 ml/min were *ca.* 8, 12 and 32 minutes respectively. Both reductive and oxidative EC cells were found to be robust and foul free after prolonged use.

Calibration, Sensitivity, and Precision of the HPLC – EC assay

The injection of varying amounts of the 5C- and 7C-aglycones and the IS gave good peak shapes and a linear detector response within and beyond the mass range used for quantification of the aglycones. Typical chromatograms of a calibration standard solution and urinary extracts from both unsupplemented and vitamin K-supplemented subjects are shown in **Fig. 4**. The 'on column' lower limit of detection of 5C- and 7C-aglycones (defined as 5 x baseline noise) was <3.5 fmol (<1 pg). The intra- and inter-assay co-efficients of variation (CV) for 5C- and 7C-aglycone determination in urine from a healthy, non-supplemented volunteer is shown in **Table 1.**

Mineral acid catalysed lactonization of 7C-aglycone side chain

The 7C-aglycone in its acid form differs from the 5C-aglycone in having a double bond at the γ-δ position relative to the carboxyl group of the side chain.

Organic acids with this γ,δ-unsaturated structure are unstable to mineral acids and undergo cyclization to a 5-membered γ-lactone ring structure. As has been previously demonstrated in radioisotopic studies, the 7C-aglycone in its carboxylic acid form is readily converted to 2-methyl-3-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-naphthoquinone lactone (vitamin K γ-lactone, structure V in **Fig. 1**) under aqueous acidic conditions such as when mineral acid is used to release the aglycones from their conjugated forms in urine (10) (11) (12). To find out whether we could demonstrate γ-lactonization by HPLC, we subjected 0.5 ml aliquots of urine (urine collected from a volunteer who had ingested 50 mg of K_1) to hydrolysis with 1ml of aqueous 3M HCl at 70°C for 60 minutes. After aqueous HCl hydrolysis the aglycones were extracted with chloroform and subjected to the usual assay procedure. Treatment with mineral acid completely abolished the peak that co-chromatographed with the 7C-aglycone standard and resulted instead in an extra peak that co-chromatographed with authentic vitamin K γ-lactone (data not shown). A peak of vitamin K γ-lactone was not found in urine samples after hydrolysis of the metabolite conjugates with methanolic HCl hydrolysis or enzyme preparations of β-glucuronidase (also containing sulfatase).

Confirmation of chromatographic peak identification

Identification of the 5C- and 7C-methyl ester aglycone chromatographic peaks isolated from urine was initially achieved by confirming that they had identical chromatographic and chemical properties to the authentic synthetic standards. The chemical similarities between analytes and standards included their hydrodynamic voltammograms, their sensitivity to light and, for the 7C-aglycone, the lability towards mineral acid and conversion to a compound chromatographically indistinguishable from vitamin K γ-lactone. Definitive confirmation of correct peak

assignment was provided by *in vivo* studies that confirmed that the chromatographic measurements responded to supplementation with different forms of vitamin K.

Typical concentrations (corrected for creatinine) of 5C- and 7C-aglycones in urine samples collected on two consecutive days from 5 healthy, fasting adults (subjects A-E) are shown in **Table 2**. **Table 2** also shows the increase in urinary concentrations of both aglycones in response to supplementation with K_1, MK-4, MK-7 and K_3 in a dose-dependent manner. These studies provided confirmation of the chromatographic peak identity for the respective aglycones.

DISCUSSION

As far as we are aware this is the first method to have been developed for the routine measurement of the two major urinary aglycones of vitamin K. The method is sensitive requiring small sample volumes and the inter-assay precision was about 10% for both 5C- and 7C-aglycones. Although we used the same principle of redox mode electrochemical detection as for a previous assay of the parent K vitamins (27) (36) the metabolite assay has proved to be much less susceptible to periodic loss of sensitivity. In the serum vitamin K assay, this often-rapid loss of sensitivity is thought to be due to passivation of the electrodes by adsorbed species of vitamin K and co-extracted non-polar lipids (27) (36). The lack of significant electrode passivation in the present assay may be related to the greater polarity of the metabolites and co-extracted urinary components, which makes them less likely to adsorb to the carbon electrodes than the highly lipophilic vitamin K and other lipids present in serum extracts.

The 5C- and 7C- aglycones measured in this assay were derived by the chemical hydrolysis of conjugates by methanolic HCl. This method of hydrolysis was previously employed for the isolation of the urinary aglycones of ubiquinone-7 (33) and phylloquinone (37) from rabbit urine. Previous work from our laboratory has suggested that the majority of the 5C- and 7C-metabolites in human urine are excreted as glucuronides (10) (11). We also confirmed in this study (data not shown) that incubation of urine with β-glucuronidase released the same two 5C- and 7C-aglycones and gave identical chromatograms to those after methanolic HCl hydrolysis. This does not exclude the possibility of other conjugates being present since 2-methyl-1, 4-naphthoquinone (menadione, vitamin K_3), which lacks the side chain, has been shown to be excreted as both glucuronide and the sulfate conjugates of the quinol form in rats

(38) (39) and rabbits (40). For routine quantitative analyses we chose methanolic HCl over enzymic hydrolysis for reasons of its greater convenience and efficiency, and to ensure complete hydrolysis of all conjugates present.

The results in **Table 2** show that in both unsupplemented and supplemented adults, the excretion of the 5C-aglycone is greater than the excretion of the 7C-aglycone. This is in agreement with early radioisotopic measurements of these two aglycones after the administration of tritium-labelled phylloquinone to adults (10) (11). In the latter studies, the intravenous administration of single doses of 45-1000 μg of labelled phylloquinone resulted in a gradually increasing proportion of the 7C-aglycone (assayed as vitamin K γ-lactone after acid hydrolysis) from an initial 13% to some 30-40% in successive urine collections made over the first 24 hours (10). In rabbits, the administration of a huge (91 mg) intravenous dose of phylloquinone changed the percentage proportion of 7C-aglycone (in 24-hour urine collections) from 13% in control animals to 57% post-supplementation (37). This data suggests that whereas physiological amounts of phylloquinone are largely metabolised to the terminal 5C-aglycone, vitamin K supplementation may lead to a greater urinary excretion of the less extensively metabolised 7C-aglycone. Interestingly, one of the infants studied here (Infant A, Table 2) excreted predominately the 7C-aglycone after supplementation with 1 mg K_1. Further support that large doses may overload the pathway comes from the previous isolation and definitive identification of a 10C-side chain length aglycone after a very large phylloquinone dose (12). No evidence for this 10C-aglycone was seen on our chromatograms. Although the preliminary supplementation studies shown in **Table 2** using the present assay showed dose-related increases in the urinary concentrations of both 5C- and 7C-aglycones, there was no consistent pattern in their relative concentrations. The supplementation

studies with K_1 and members of the menaquinone series (MK-4 and MK-7) clearly indicate that the 5C- and 7C-aglycones are common to the major naturally occurring forms of vitamin K **(Table 2)**. Although the excretion of different forms of vitamin K as these two common catabolites has not been formally demonstrated before, it was to be expected in view of previous work that has shown that the isoprenoid side chains of ubiquinone-7 (33), K_1 (11,37), α-tocopherol (37) (41) and δ-tocopherol (42) are all shortened in the same way and excreted as equivalent metabolites.

The proposed degradation pathway (**Fig. 1**) of vitamin K by successive ω- and β-oxidation is supported by the previous unambiguous identification of 5C- and 7C-aglycones (11), the finding of the expected 10C-side chain intermediate (12) and by analogy to the findings of others that the same degradation pathway operates for similar isoprenoid compounds (33) (42) (41).

The increase in 5C- and 7C-aglycone excretion in response to supplementation with K_3 is noteworthy. The generation of MK-4 from K_3 through the addition of a geranyl-geranyl side chain is well described (6) (7), and is the probable source of the increased excretion of the 5C- and 7C-aglycones following K_3 supplementation. It may be possible to use this metabolite assay as an index of the tissue conversion of K_3 to MK-4.

Our ongoing work is directed at evaluating the present assay as a new marker of vitamin K status. An obvious advantage over current serum measurements of vitamin K is that the urinary excretion reflects degradation of all K vitamins whereas most serum assays are only directed at the assay of a single vitamer, K_1. Thus the measurement of urinary excretion might be suitable as a measure of overall vitamin K status. The relative contribution to total urinary excretion from K_1 and MKs remains to be determined. It seems likely that the majority of daily variability stems from

excretion of K_1 since this is by far the major dietary form, and long-chain MKs are thought to have a slower turnover (43). In addition to the prerequisite for normal renal function, a possible disadvantage of urinary measurements as a marker of vitamin K stores is that metabolites of vitamin K are also excreted via the bile so that urinary excretion will not reflect the total elimination of vitamin K from the body.

ACKNOWLEDGMENTS

We warmly thank Dr. Paul Clarke and the staff of the Neonatal Unit at Hope Hospital Salford, UK for their perseverance and diligence in providing us with urine collection from neonates. We thank the adult volunteers who participated in the vitamin K supplement studies. Special thanks are due to Dr Leon Schurgers, Maastricht University who provided us with urine samples pre- and post- supplementation with MK-7. We are also grateful to Mr. David Card for his assistance in the preparation of this manuscript.

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Fig. 1. Structural formulas of phylloquinone (vitamin K_1), menaquinones (vitamin K_2), 5C-aglycone metabolite, 7C-aglycone metabolite, vitamin K γ-lactone and assay internal standard

I Phylloquinone (vitamin K_1)

II Menaquinones (vitamin K_2)

III 2-Methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone (7C-aglycone metabolite)

IV 2-Methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone (5C-aglycone metabolite)

V 2-Methyl-3-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-naphthoquinone (vitamin K γ-lactone)

Fig.2. Flowchart illustrating the analytical stages involved in the determination of the urinary vitamin K aglycone metabolites.

Fig. 3. Hydrodynamic voltammograms for the methylated urinary vitamin K 5C- (○) and 7C-(■) aglycone metabolites and the IS (Δ). The amounts of each compound injected were 16.4 fmol (4.7 pg), 15.7 fmol (4.9 pg) and 956.1 fmol (314 pg)) respectively.

Fig. 4. Representative chromatograms obtained for calibration standards and urinary extracts.

(A). Chromatogram of a calibration standard showing the resolution of the methylated synthetic 5C-aglycone, 7-C aglycone, and the IS. On-column injection amounts were 166fmol (47.5 pg) for the 5C-aglycone, 157fmol (49.0 pg) for the 7C-agylcone and 960fmol (314 pg) for the IS respectively.

(B). Chromatogram showing measurement of aglycone metabolites at physiological urinary concentrations (i.e. from unsupplemented subject). A volume of 0.5 ml urine was extracted and a fraction of 10/40 μl of the purified extract injected. The measured 5C- and 7C-aglycone concentrations were 14.76 nmol/l (4.22 μg/l) and 2.82 nmol/l (0.88 μg/l) respectively.

(C). Chromatogram showing measurement of urinary aglycone metabolites at raised urinary concentrations (i.e. after oral vitamin K supplementation with 50 mg vitamin K_1, Konakion MM). A volume of 0.05 ml urine was extracted and a fraction of 10/100 μl of the purified extract injected. The measured 5C- and 7C-aglycone concentrations were 336.36 nmol/l (96.20 μg/l) and 29.32 nmol/l (9.16 μg/l) respectively

Table 1. Assay reproducibility for the determination of the 5C- and 7C-aglycone metabolites of vitamin K in urine

	5C-aglycone	7C-aglycone
	nmol/l	
Urine sample A		
Inter-assay reproducibility* (n=45)		
Mean ± SEM	14.13 ± 0.21	3.62 ± 0.06
CV%	9.84	12.75
Urine sample B		
Intra-assay reproducibility (n=10)		
Mean ± SEM	26.43 ± 0.49	5.86 ± 0.06
CV%	5.95	2.85

Two separate urine collections from healthy subjects were analysed for the 5C- and 7C-aglycone metabolites of vitamin K to assess inter-assay (A) and intra-assay reproducibility (B) respectively. *Inter-assay reproducibility was assessed over a period of 5 months.

Table 2. Urinary concentrations (nmol/mmol creatinine) of vitamin K metabolites (5C- and 7C-aglycones) in unsupplemented and vitamin K-supplemented healthy adults and neonates.

Subject	Supplement	Dose	Day 1 Pre-dose 5C	Day 1 Pre-dose 7C	Post-dose sampling time	Day 2 Post-dose 5C	Day 2 Post-dose 7C
UNSUPPLEMENTED SUBJECTS*							
			nmol/mmol creatinine			*nmol/mmol creatinine*	
A			2.5	1.1		1.3	0.7
B			1.7	0.5		1.3	0.5
C			0.3	0.1		0.8	0.3
D			0.8	0.2		1.0	0.2
E			0.6	0.1		0.5	<0.1
SUPPLEMENTED SUBJECTS**							
		mg	*nmol/mmol creatinine*		*hours*	*nmol/mmol creatinine*	
F	K_1	2	2.5	0.4	8	11.0	0.6
G	K_1	50	1.7	0.4	0-24	161.7	11.6
H	MK-4	45	1.2	0.2	0-24	118.6	14.5
I	MK-4	45	1.7	0.3	0-24	57.2	15.7
J	MK-4	45	1.1	0.1	0-24	76.6	21.4
K	MK-7	1	0.9	0.1	24	4.2	0.6
L	MK-7	1	1.6	0.4	4	4.8	1.4
M	K_3	20	1.3	0.4	5	5.6	1.8
N	K_3	20	2.5	0.5	6	17.8	5.1
Neonate A	K_1 (IM)	1	0.2	0.2	5	5.4	14.0
Neonate B	K_1 (IM)	1	0.6	0.4	24	12.7	4.4
Neonate C	K_1 (IM)	1	0.2	0.2	48	153.8	32.1

* Unsupplemented healthy adult subjects A–E collected spot urines at 0900 on consecutive days after a 14 hr fast. **Supplemented healthy adult subjects F–N took the indicated vitamin K supplement orally and collected urine pre- and post-supplementation. The pre-supplementation urines for subjects F–L were 24-hour collections and for subjects M–N were spot urines (early AM) immediately prior to taking the vitamin K supplement. The post-supplementation urines were either spot urines collected at the time indicated (subjects F, and K–N) or entire 24-hour collections (subjects G–J). The neonates received 1mg of K_1 intramuscularly (IM) on the first day of life. The pre-supplementation neonatal samples represented the first void spot urines after delivery. The post-supplementation neonatal samples were spot urines collected at the indicated times after IM K_1.

Figure 1

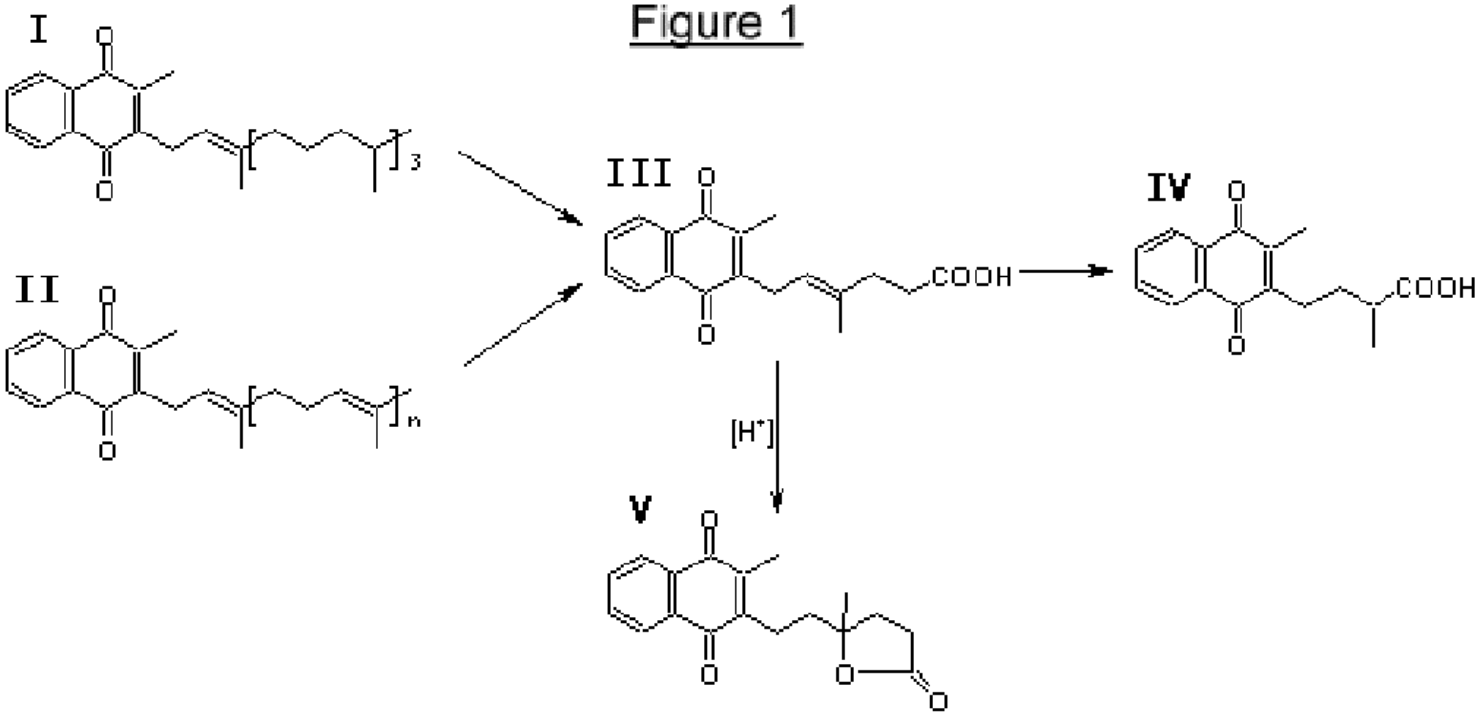

Figure 2

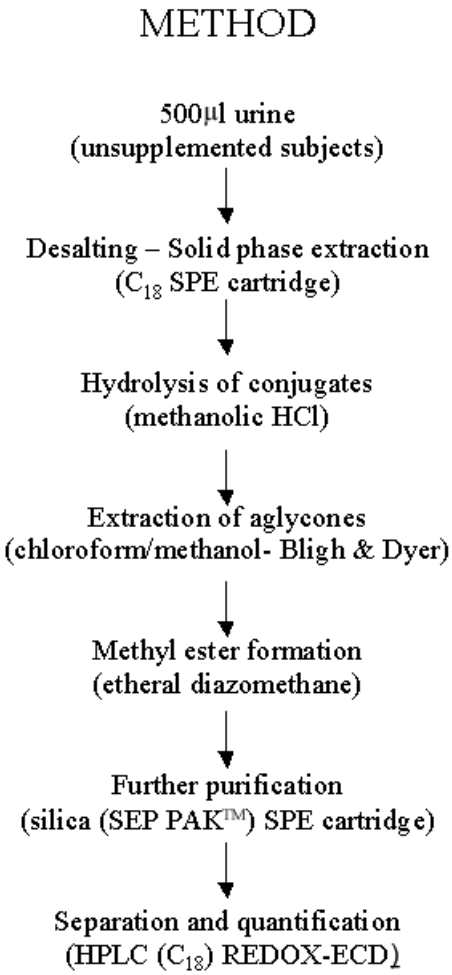
METHOD
500µl urine
(unsupplemented subjects)
Desalting – Solid phase extraction
(C_{18} SPE cartridge)
Hydrolysis of conjugates
(methanolic HCl)
Extraction of aglycones
(chloroform/methanol- Bligh & Dyer)
Methyl ester formation
(etheral diazomethane)
Further purification
(silica (SEP PAK™) SPE cartridge)
Separation and quantification
(HPLC (C_{18}) REDOX-ECD)

Figure 3

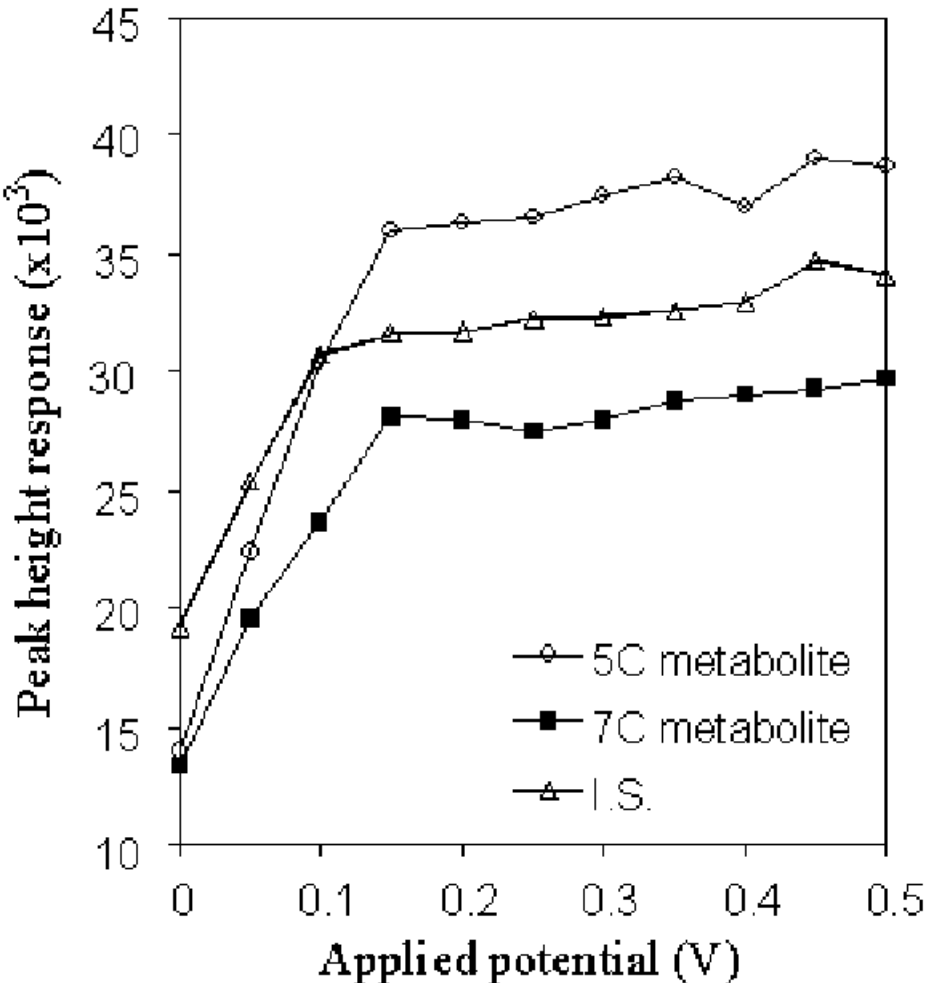
45
40
35
30
25
20
15
10
Peak height response (x10^{-3})
0
0.1
0.2
0.3
0.4
0.5
Applied potential (V)
5C metabolite
7C metabolite
I.S.

Figure 4