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Method for the Determination of Vitamin $K_{1(20)}$ in Human Plasma by Stable Isotope Dilution/Gas Chromatography/Mass Spectrometry

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A method for the quantitative determination of vitamin $K_{1(20)}$ (VK), an essential cofactor in the carboxylation of clotting factors, is presented. The assay is based on gas chromatography/electron impact mass/spectrometry. The preparation of deuterium-labelled vitamin $K_{1(20)}$ for use as an internal standard is described. The method involves extraction of VK from human plasma and its derivatization to the heptafluorobutyryl ester after reduction of one carbonyl group with zinc. The detection limit was found to be 1.0 pg and the limit of quantitation 2.0 pg ml^{-1} plasma. This permits the measurement of vitamin $K_{1(20)}$ even in small quantities of plasma, which is highly desirable in investigations dealing with clotting abnormalities in neonates and infants.

INTRODUCTION

Vitamin $K_{1(20)}$ (VK) is the generic name of 2-methyl-3-eicosa-2-ene-1,4-naphthoquinone, a compound with the physiological activity of preventing haemorrhagic diseases. Natural VK is formed by plants, especially green vegetables, and is the main form of vitamin K in human food. Different types of vitamin K (e.g. vitamin $K_{2(n)}$) are formed by bacteria (*E. coli*) or have to be synthesized, such as vitamin K_3 (menadione).[1]

VK (phylloquinone) is a very lipophilic substance with a molecular mass of 450 and is sensitive to light, temperature and alkali metal hydroxides. Reducing agents convert VK into its chromenol and chromanol,[2] which can be silylated or transformed into other derivatives.[3]

VK is an essential cofactor in the post-translational carboxylation reaction of glutamic acid residues in a number of blood clotting factors and in some other proteins.[1] In these reactions, VK is essential for activation of the inactive precursor prothrombins to (calcium-dependent) active thrombins. Several different methods[4–8] have determined VK concentrations in human plasma samples from 20 pg/ml up to 3 ng/ml. Shearer *et al.*[5] found a VK concentration of about 200 pg ml^{-1} in plasma from healthy mothers at the time of birth, whereas the VK concentrations in cord plasma of their babies was below the detection limit of 20 pg ml^{-1}.

VK deficiency in newborn children, derived from poor placental transfer and the provision of small amounts of VK during breast feeding, may cause bleeding in the first week of life in previously healthy neonates (haemorrhagic disease of the newborn), whereas after the neonatal period, VK deficiency may be associated with serious intracranial bleeding (late haemorrhagic disease). These occurrences during the first 3 months of life justify prophylaxis with VK. The highest efficiency has been obtained with intramuscular administration of a 1 mg dose. In 1990 and 1992, however, Golding and co-workers[9,10] reported an association of childhood cancer with this VK prophylaxis. Since many questions about the efficiency and risks of VK prophylaxis remain unanswered, it is very important to develop a method for the quick and accurate detection of low VK concentrations in small quantities of plasma.

Several methods have been employed to determine physiological plasma levels of VK, most of them based on high-performance liquid chromatographic methods with different detectors.[4–8] The major disadvantages of these methods are time-consuming procedures and/or the use of relative large quantities of plasma. Several workers have described sensitive methods using gas chromatography together with an electron-capture detector[11] or coupled with a mass spectrometer.[12] Based on the work of Gleispach *et al.*,[3] we enhanced the method using gas chromatography/mass spectrometry (GC/MS) by combining mass-selective detection with the benefits of stable isotope dilution methods for internal standardization. As VK is not commercially available in a suitable labelled form, we describe the preparation of deuterium-labelled VK and its use as an internal standard in the GC/MS determination of the vitamin with electron impact ionization.

EXPERIMENTAL

Materials

Vitamin $K_{1(20)}$ was obtained from Hoffmann La Roche (Basle, Switzerland) and 1,4-naphthoquinone, phytol, acetic acid-d_3, trifluoracetic anhydride, pentafluoropropionic anhydride, heptafluorobutyric anhydride and the corresponding fluorinated acids from Aldrich (Vienna,

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Austria). All other solvents and reagents, of analytical grade, were obtained from Merck (Darmstadt, Germany).

Gas chromatography/mass spectrometry

A Fisons Model 8000 gas chromatograph coupled either to a Fisons MD 800 or a Fisons Trio 1000 quadrupole mass spectrometer was used. The column was directly connected to the ion source of the mass spectrometer. The gas chromatograph was equipped with a DB-5MS fused-silica capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness) from Fisons. The splitless Grob injector was kept at 260 °C. Helium was used as the carrier gas. The initial column temperature was 160 °C for 1 min, followed by an increase of 30 °C min^{-1} to 290 °C, an isothermal hold of 4 min, followed by another increase of 30 °C min^{-1} to 310 °C and a final isothermal hold of 2 min. The transfer line between the GC and MS instruments was kept at 308 °C. The ion source temperature was 215 °C. Electron impact (EI) mass spectra were recorded with an electron energy of 70 eV and an emission current of 100 μA.

Synthesis of deuterium-labelled vitamin $K_{1(20)}$ (VK-d_3)

2-Methyl-d_3-1,4-naphthoquinone[13] (menadione-d_3). A 1 ml volume of deuterated glacial acetic acid (CD_3COOH), 2 g of 1,4-naphthoquinone and 1.28 g of silver nitrate ($AgNO_3$) were dissolved at 80 °C in 80 ml of acetonitrile and 40 ml of water. To the stirred solution, 3.8 g of ammonium peroxodisulphate ($(NH_4)_2S_2O_8$), dissolved in 50 ml of water, were added during 15 min. After stirring at 80 °C for 5 h and at room temperature overnight, 200 ml of water were added and the solution was extracted with 2 × 50 ml of dichloromethane. The organic layer was dried over Na_2SO_4, filtered and the solvent distilled off under reduced pressure. The dark-brown oil was purified by recrystallization from light petroleum.

2-Methyl-d_3-3-eicosa-2-ene-1,4-naphthoquinone[14,15] (VK-d_3). A 100 mg amount of 2-methyl-d_3-1,4-naphthoquinone was dissolved at room temperature in 20 ml of light petroleum and a few milligrams of Lindlar catalyst were added. The stirred suspension was hydrogenated for about 10 min with a slow stream of hydrogen, filtered and the solvent evaporated.

The residual oil was dissolved in 1 ml of dry dioxane under a nitrogen atmosphere and 0.1 ml of phytol and 0.1 ml of boron trifluoride–diethylther (50%) were added. The well stoppered vial was heated at 75 °C for 1 h and then stored in the dark at room temperature. After 20 h, 5 ml of diethyl ether were added and the solution was extracted once against water, and then against potassium hydroxide (2% in water, containing 5% sodium hydrosulphite). The etheral solution was dried over sodium sulphate and filtered. A 100 mg amount of silver oxide was added to this stirred solution and after 1 h the oxidizing material was removed by filtration. The resulting etheral solution was reduced to a small volume and the product (VK-d_3) was purified by column chromatography, using a small quantity of silica and benzene–light petroleum (1:1) as eluent. Because of the instability of the product, this purification should be done rapidly and protected from daylight.

Derivatization of VK by reduction with zinc and reaction with *N*-perfluoroacyl anhydrides

N-perfluoroacyl derivatives were prepared by dissolving the VK in a mixture of 1 ml of hexane, 50 μl of the corresponding anhydride (trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA) or heptafluorobutyric anhydride (HFBA)) and 25 μl of the corresponding *N*-perfluoro acid. A 20–30 mg amount of zinc dust (reducing agent) was added and the suspension was stirred at room temperature. After 1 h, 1 ml of water was added, the mixture was stirred for further 5 min, centrifuged and the supernatant was evaporated under a stream of nitrogen. The residue was dissolved in hexane.

Analytical method validation

Calibration graphs were established in the range 4.0–4000 pg per 40 μl of sample. The detection limit was estimated by derivatizing suitable amounts of unlabelled VK and a constant amount of labelled VK in the manner described above and injecting aliquots of 10 μl into the GC/MS system. Inter-assay variations were determined by carrying five identical samples at VK concentrations of 11.0 ng ml^{-1} and 230 pg ml^{-1} plasma through the analytical procedure. Intra-assay variations were estimated by analysing one sample at the same concentrations as above five times by GC/MS.

Sample preparation

A 120 μl portion of the internal standard solution (deuterated VK, 100 pg per 120 μl of methanol) was added to 1 ml of plasma, diluted with 1 ml of water, 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. The *N*-perfluoroacyl derivatives were prepared as described above. After evaporation of the solvent, the sample was dissolved in hexane, transferred to autosampler vials and an aliquot of 10 μl was subjected to GC/MS analysis.

RESULTS AND DISCUSSION

Synthesis of deuterium-labelled Vitamin $K_{1(20)}$ (VK-d_3)

As described previously,[3] VK can be measured by GC/MS with and without derivatization. The sensitivity obtained, however, was not good enough for the detection of VK in biological material. This and the fact that

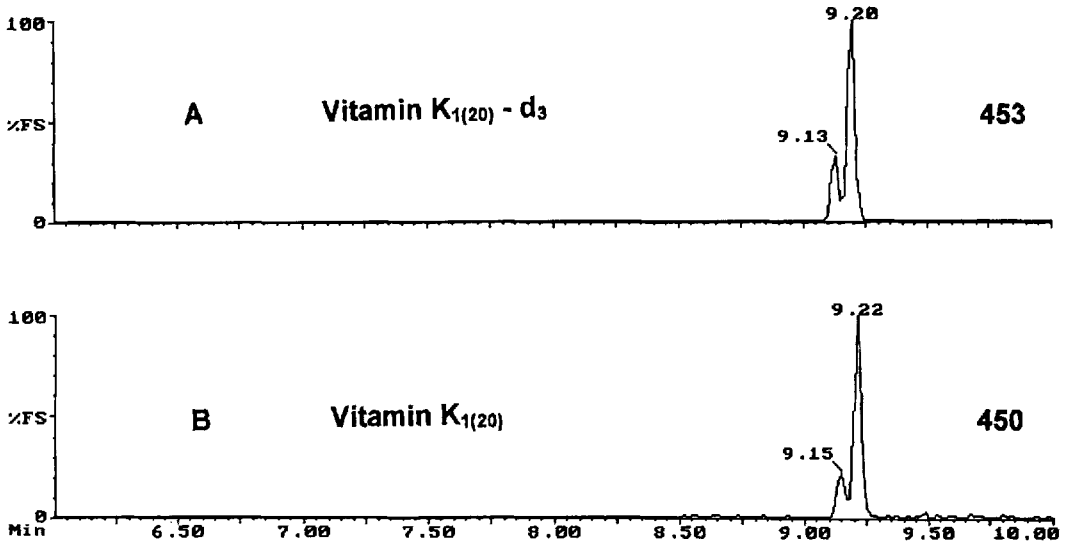

Figure 1. Typical *SIR* mass chromatograms of (B) unlabelled and (A) deuterium-labelled vitamin $K_{1(20)}$ without derivatization. The double peaks correspond to the *cis* (9,13; 9,15) and *trans* (9,20; 9,22) isomers of synthetic VKs.

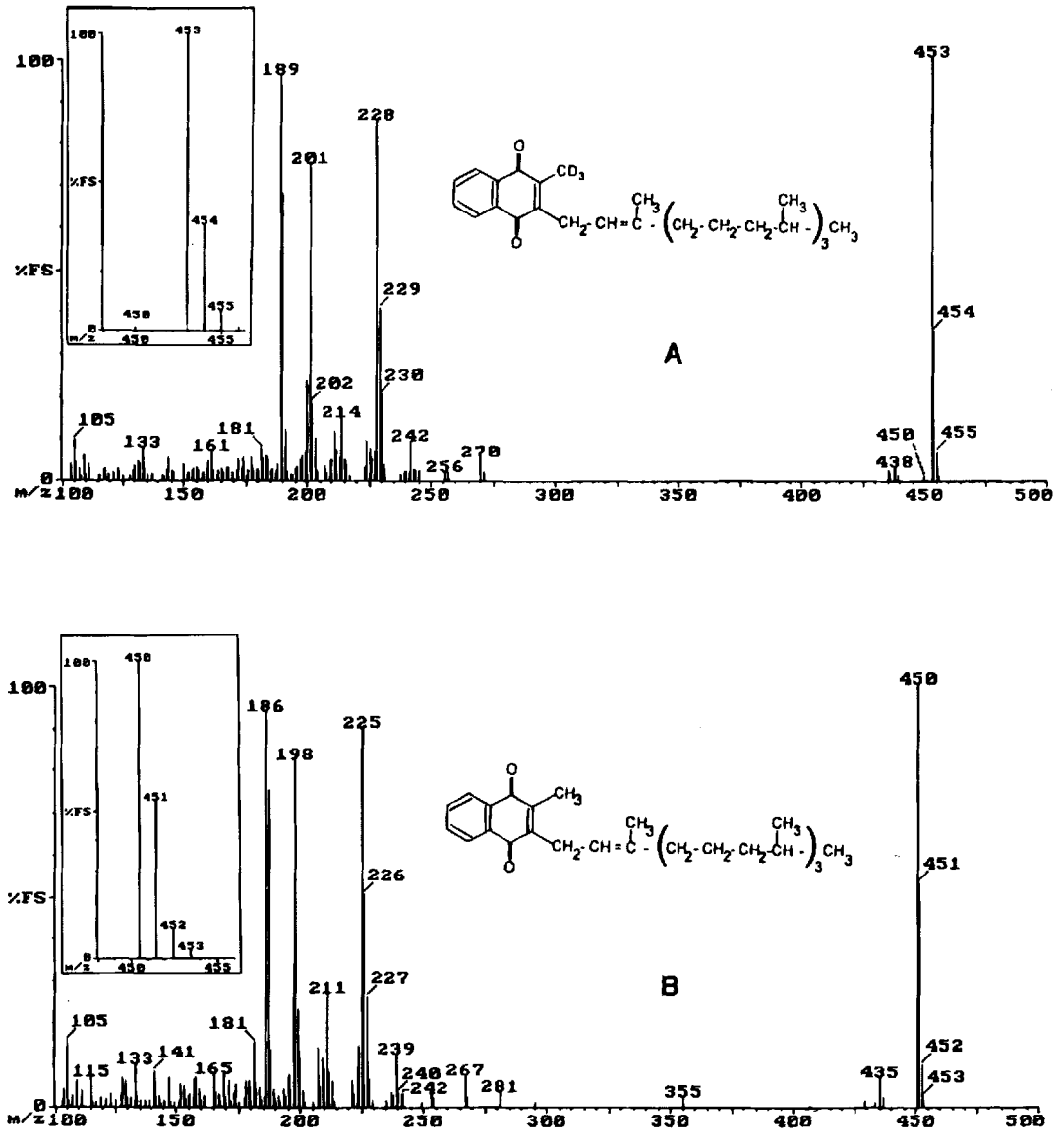

Figure 2. EI mass spectra, molecular structures and isotopic composition of the molecular ion (inset) of (B) unlabelled and (A) deuterium-labelled vitamin $K_{1(20)}$ without derivatization. Details are described in the text.

Table 1. Characteristic fragment ions found in the GC/EI mass spectra of unlabelled and deuterium-labelled vitamin $K_{1(20)}$ and the trifluoroacetyl, pentafluoropropionyl and heptafluorobutyryl derivatives[a]

	m/z			
Compound	Molecular ion M^+	Fragment ion $[M-225]^+$	Fragment ion $[M-265]^+$	Base peak
Vitamin $K_{1(20)}$ (VK)	450	225	(186)[b]	57
Vitamin K-d_3	453	228	(189)[b]	57
Trifluoroacetyl-VK	548	323	283	283
Trifluoroacetyl-VK-d_3	551	326	286	286
Pentafluoropropionyl-VK	598	373	333	333
Pentafluoropropionyl-VK-d_3	601	376	336	336
Heptafluorobutyryl-VK	648	423	383	57
Heptafluorobutyryl-VK-d_3	651	426	386	57

[a] Details are described in the text. Typical EI mass spectra are shown in Figs 2 and 3.
[b] In the cases of underivatized VK, this fragment ion appears at a mass of $[M-264]^+$ instead of $[M-265]^+$, owing to reduction of one carbonyl of the underivatized VK.

no stable isotope-labelled VK was available made it important to synthesize deuterium-labelled VK in our laboratories. The method of preparation is given above and resulted in 500 mg of 2-methyl-d_3-1,4-naphthoquinone and 10 mg of 2-methyl-d_3-3-eicosa-2-ene-1,4-naphthoquinone. The purification step of cooling the final solution to $-20\,^{\circ}C$ and filtering off impurities, as described in the literature,[15] failed because of the small quantities that were used in this preparation.

A typical single-ion-recording (SIR) mass chromatogram of unlabelled and labelled VK without derivatization is shown in Fig. 1 and the corresponding mass spectra together with the molecular structures are shown in Fig. 2. The isotopic composition of the molecular ions (*m/z* 450 and 453) are shown in the insets in Fig. 2. The fact that there is also a small amount of unlabelled VK present in the spectrum of labelled VK is considered to indicate impurities of the deuterium-labelled acetic acid used in the preparation of menadione-d_3. This inconvenience, which is also detectable in the spectra of menadione (data not shown), however, is corrected by calibration graphs.

As can be seen in the SIR mass chromatograms in Fig. 1, the underivatized VK elutes as a double peak with a ratio of about 20:80. This is due to the fact that the phytol, used in the synthesis of VK-d_3, is a mixture of *cis* and *trans* isomers (in relation to the double bond in position 2 of phytol), and can be detected in all samples where artificial VK was used, whereas in biological systems only the *trans* form is active.[16] This double peak is also detectable in silica gel thin-layer chromatography using benzene–light petroleum (1:1) as the elution solvent (B_f 0.58 and 0.54, respectively).

Derivatization

Three different derivatives of vitamin $K_{1(20)}$ were prepared and checked for their potential use in GC/MS assay. Comparison of the chromatograms showed complete derivatization of all perfluoro homologues. The EI mass spectra of the trifluoroacetyl, pentafluoropropionyl and heptafluorobutyryl esters of both labelled and unlabelled VK were compared with those of the underivatized VK. In Table 1 the *m/z* values of the molecular ions, base peaks and other important fragment ions are presented.

Figure 3 shows typical EI mass spectra of the heptafluorobutyryl derivatives of (B) unlabelled and (A) deuterium-labelled VK. In the negative-ion chemical ionization mass spectra of derivatized VK (performed with methane as the moderating gas), however, virtually no molecular ions are detectable and the base peaks (*m/z* 113 or 197) are identified as low-mass fragment ions deriving from the perfluoroacyl group. As shown in Table 1, there is a prominent fragment ion present in all of the EI spectra of the homologous derivatives at values of $[M-265]^+$, except those of the underivatized VK, where the value is $[M-264]^+$. The results indicate that one of the carbonyls of the underivatized VK is hydrogenated during the GC/MS procedure. The losses of 225 and 265 u, detectable in the spectra of all perfluoro homologues and also in those of the underivated VK, is caused by elimination of $C_{16}H_{33}$ and $C_{19}H_{37}$, respectively, from the phytol side-chain of the molecule.[12]

Although identical in ease of preparation and response, the heptafluorobutyryl derivative was chosen because it shows the least interference from the plasma matrix, owing to its high molecular mass. For the same reason, in single-ion recording, we used the mass of the molecular ion instead that of the (up to three times more intense) base peak. In addition, the perfluoro derivatives, in comparison with the underivated VK, are less sensitive to light and temperature.

Sample preparation

The yield of the extraction from diluted plasma (checked by comparing samples of the same amounts of VK with and without extraction) was quantitative at VK concentrations of 11.0 ng ml^{-1} and 100 pg ml^{-1}.

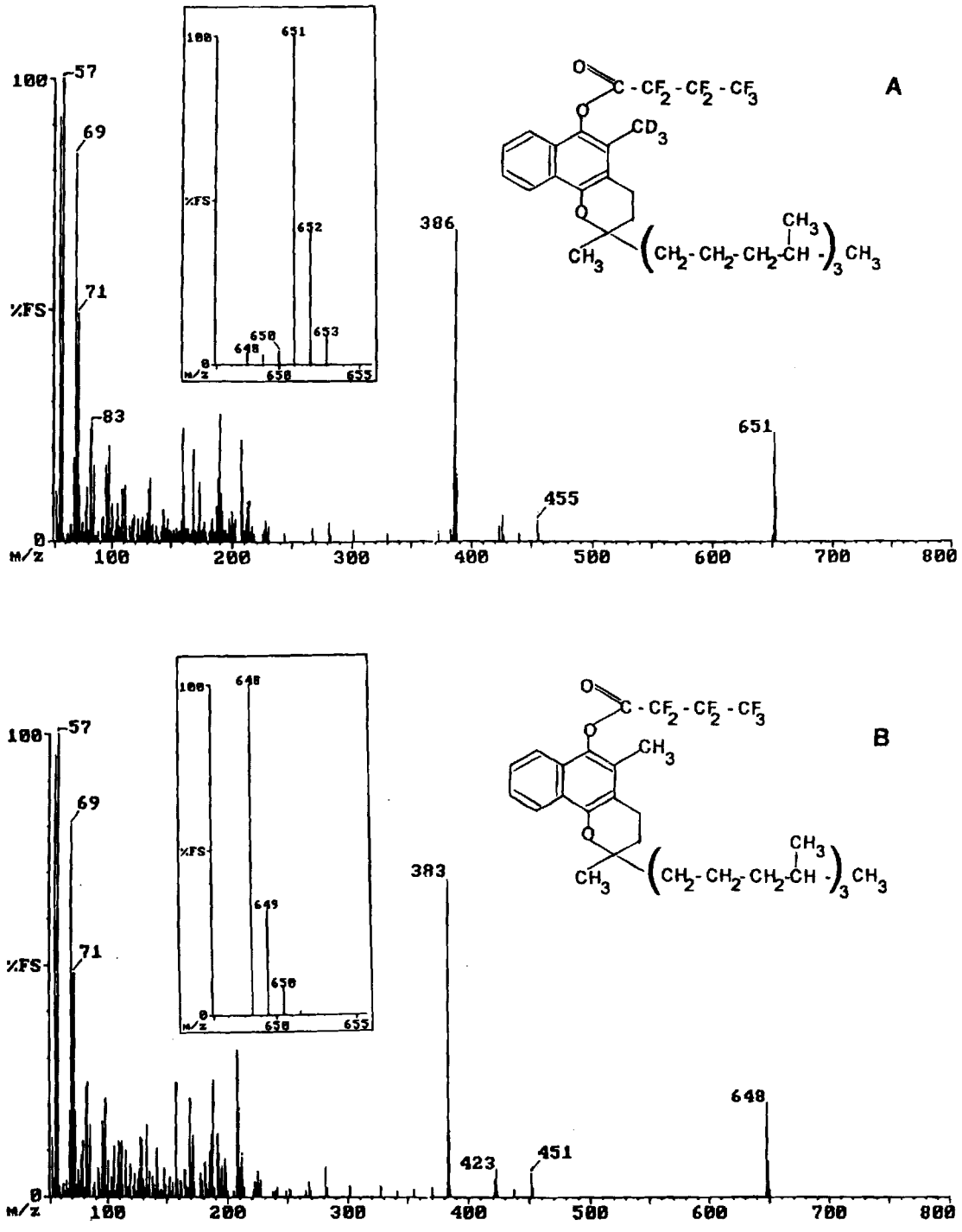

Figure 3. EI mass spectra, molecular structures and isotopic composition of the molecular ion (inset) of (B) unlabelled and (A) deuterium-labelled heptafluorobutyric derivative of vitamin $K_{1(20)}$. Labelling and derivatization were carried out as described in the text.

To reduce the risk of cross-contamination, the machine-washed glass vials were heated for a few minutes at 550 °C.

Solid-phase extraction on Bond Elut C_{18} cartridges showed no advantages over solvent extraction, owing to partial reaction of VK with the column material. Also, the purification of samples after derivatization on Bond Elut C_{18} cartridges and also on small silica gel columns failed, because of hydrolysis of the derivatized VK. A typical single-ion recording mass chromatogram obtained after analysis of VK in plasma from a human volunteer is shown in Fig. 4.

Fifteen different plasma samples from human volunteers were analysed, and VK concentrations between the lower limit of quantitation (2.0 pg ml^{-1}) and 650 pg ml^{-1} were measured, which is in close agreement with the literature.[1,4–8]

Analytical method validation

The calibration graph established was linear within the range 4.0–4000 pg per 40 µl of sample ($r^2 = 0.998$). The limit of detection was found to be 1.0 pg (actually

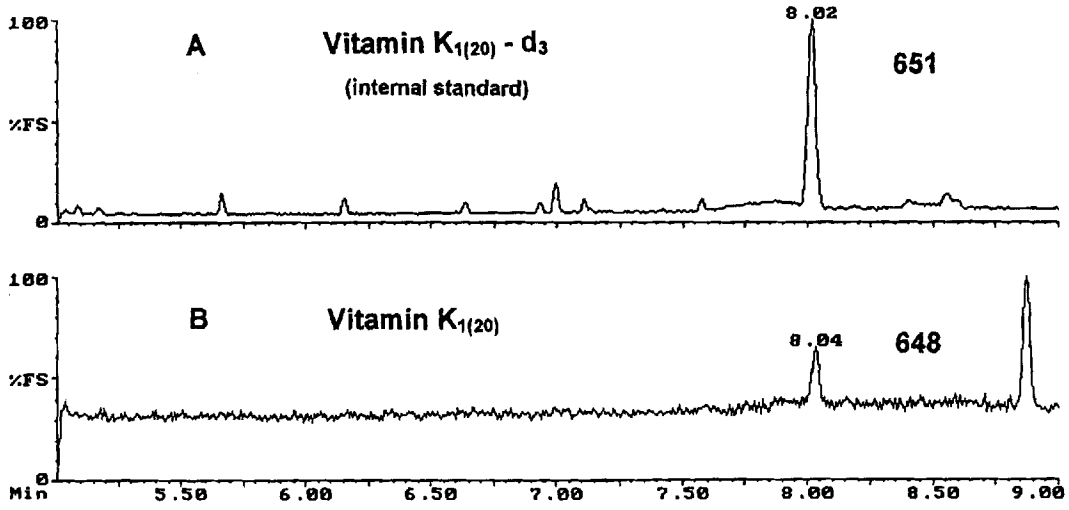

Figure 4. Typical *SIR* chromatogram obtained after analysis of VK in a plasma sample from a human volunteer after reduction with zinc and derivatization with heptafluorobutyric anhydride. The amount analysed corresponds to 0.44 ng ml^{-1} plasma.

injected) at a signal-to-noise ratio of at least 4:1. Inter-assay variations (mean ± SD) were estimated to be 10.96 ± 0.77 ng ml^{-1} and 237.3 ± 6.9 pg ml^{-1} plasma, respectively. Intra-assay variations (mean ± SD) were estimated to be 10.98 ± 0.47 ng ml^{-1} and 233.6 ± 3.1 pg ml^{-1} plasma, respectively.

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