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A novel liquid chromatography-tandem mass spectrometry method for determination of menadione in human plasma after derivatization with 3-mercaptopropionic acid

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ABSTRACT

Menadione (VK_3), an essential fat-soluble naphthoquinone, takes very important physiological and pathological roles, but its detection and quantification is challenging. Herein, a new method was developed for quantification of VK_3 in human plasma by liquid chromatography-tandem mass spectrometry (LC–MS/MS) after derivatization with 3-mercaptopropionic acid via Michael addition reaction. The derivative had been identified by the mass spectra and the derivatization conditions were optimized by considering different parameters. The method was demonstrated with high sensitivity and a low limit of quantification of 0.03 ng mL^{-1} for VK_3, which is about 33-fold better than that for the direct analysis of the underivatized compound. The method also had good precision and reproducibility. It was applied in the determination of basal VK_3 in human plasma and a clinical pharmacokinetic study of menadiol sodium diphosphate. Furthermore, the method for the quantification of VK_3 using LC–MS/MS was reported in this paper for the first time, and it will provide an important strategy for the further research on VK_3 and menadione analogs.

1. Introduction

Vitamin K (VK) is a family of structurally similar and fat-soluble vitamins. All forms of VK share a common naphthoquinone ring, but differ in the position-3 side chain. There are three major types of VK, including phylloquinone (VK_1), menaquinones (VK_2), and menadione (VK_3) (see Fig. 1). VK_1 and VK_2 are the natural compounds. The most common form of VK_2 in humans is menaquinone-4 (MK-4) [1]. Although VK_3 (2-methyl-1,4-naphthoquinone) is considered as a synthetic analog, Davidson et al. [2–4] found that dietary VK_1 can be cleaved to form VK_3 by bacteria in the intestine. It is also an intermediate metabolite in the conversion of VK_1 to MK-4 [5], and plays an important role in blood coagulation as a cofactor for the synthesis of clotting factors in the liver and in bone mineralization [4,6].

Recently, the interest in VK_3 has grown because of its antitumor activity against various human cancer cells [7,8] and other actions [9–11]. It has shown antiproliferative effects against various kinds of cancer cells, including pancreatic [12], hepatic [13], oral cavity [14], breast [15], leukemia [16] and several glioma cell lines [17]. Both *in vivo* and *in vitro* studies showed a synergistic effect when VK_3 was combined with conventional chemotherapeutic agents, such as 5-fluorouracil, mitomycin C, doxorubicin, bleomycin, cisplatin, dacarbazine and so on [3,18]. The development of the VK_3 prodrugs as antihemorrhagic and anticancer agents is promising, and attracts more and more interest of scientists. To evaluate the safety and efficacy of those developing new drugs or prodrugs of VK_3, it is very important to obtain the plasma concentration data of VK_3 converted by those drugs. In addition, an increasing body of work indicates that VK deficiency may be associated with osteoporosis and possibly with hepatocarcinoma and atherosclerosis [9]. The efficacy of VK in the prevention or treatment of these diseases deserves further studies. Therefore, to detect the low endogenous concentrations and elucidate the effects and molecular mechanisms of vitamin Ks in human, it is essential to establish accurate, sensitive, and selective methods for the quantification of vitamin Ks in human plasma. There are many methods reported in the literature for determination of VK_1 [19] and VK_2 [20] in plasma, but there is no highly sensitive and effective method or LC–MS/MS method reported for the determination of VK_3 in human plasma. The reasons mainly include two aspects. On the one hand, there is no ionizable functional group in its chemical structure, so that the mass spectrometric response of it is very poor, and it is relatively difficult to establish a sensitive and robust LC–MS/MS method for its unchanged form. On the other hand, VK_3 is highly unstable in plasma, its plasma concentration level is very low, and meanwhile it decreases rapidly in plasma [21,22].

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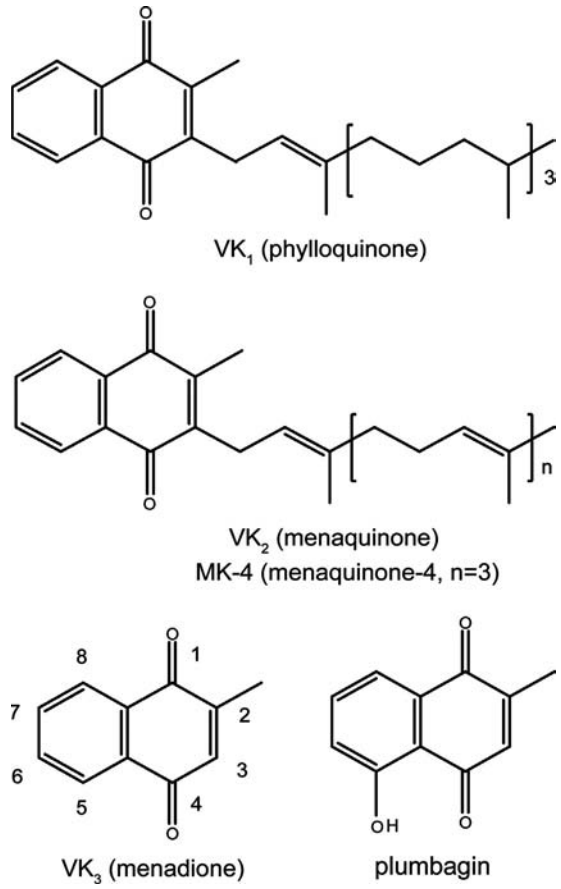

Fig. 1. Chemical structures of vitamin Ks and plumbagin. Note the *n* on vitamin K_2, which represents the number of unsaturated isoprenyl groups.

Various methods [5,6,23–27] have been reported for the determination of VK_3 in various matrices. Among the methods only a few were reported for the determination of VK_3 in plasma. Akman et al. [23] reported a differential pulse polarographic assay for VK_3 in plasma with a low limit of quantification (LLOQ) of 30 ng mL^{-1}. Hu et al. [24] reported the HPLC method for the determination of VK_3 in rabbit plasma with an LLOQ of 10 ng mL^{-1}. Both methods were not sensitive enough for determining the basal VK_3 level in plasma. Recently, Al Rajabi et al. [5] reported an HPLC method using a C_{30} column, post-column zinc reduction and fluorescence detection to measure urinary VK_3 with an LLOQ of 0.3 pmol mL^{-1}. The method offered increased sensitivity, but it only applied to the determination of VK_3 in urine not in plasma. Furthermore, VK_3 is not endogenously fluorescent and requires a post-column zinc reduction for fluorescent detection. To the best of our knowledge, although the LC–MS/MS methods for determination of vitamin K_3-glutathione conjugate in hepatocytes [28] and in liver [29] have been published over the years, no successful methods for the quantitative determination of VK_3 using LC–MS/MS are reported so far.

In recent decades, LC–MS/MS has been proven to be an extremely sensitive and specific technique for biomedical analysis. However, some chemical substances are difficult to detect by the wide-spread LC–MS/MS method due to the lack of ionizable functional group. Introducing ionizable group, by a pre-column derivatization method, into the structure of these poorly ionizable drugs is a useful strategy to improve both detection specificity and sensitivity by mass spectrometry [30–32].

Menadiol sodium diphosphate (MSD) is a prodrug of VK_3. It is converted to its active form VK_3 *in vivo*. The drug is currently undergoing phase II clinical trials in China as an antihemorrhagic agent. In this study, we developed the sensitive LC–MS/MS method to determine VK_3 in human plasma using a simple derivatization reaction of VK_3 with 3-mercaptopropionic acid. The method was successfully applied to the clinical pharmacokinetic study of MSD, and the plasma concentration-time profiles of VK_3 in human after oral administration of MSD were acquired for the first time. The proposed method also provides a new strategy for the detection of menadione analogs.

2. Experimental

2.1. Optimization of the derivatiazation procedures

Derivatization efficiencies were evaluated at various reaction temperatures, time, media, amounts of derivatization reagent and pH levels. The optimum derivatization conditions of VK_3 with MPA were determined by the amounts of the derivatized VK_3.

2.2. Preparation of calibration standard and quality control samples

The primary stock solution of 1.0 mg mL^{-1} VK_3 was prepared by dissolving accurately weighed amounts of the reference substance in methanol. Working solutions of VK_3 were prepared at concentration levels of 1.5, 5.0, 15, 50, 150, 500, 1000, 1750 and 2500 ng mL^{-1} by serial dilution of the primary stock solution with methanol. The working solution of the internal standard (IS, 200 ng mL^{-1}) was prepared by diluting a 1.0 mg mL^{-1} plumbagin stock solution with methanol. All solutions were kept at −20 °C in dark and brought to room temperature before use. The derivatizing reagent was freshly prepared by diluting the 3-mercaptopropionic acid (MPA, 1.22 g mL^{-1}, 20 °C) to the concentration of 122 μg mL^{-1} with methanol.

We prepared VK_3-free blank plasma by exposing plasma from healthy subjects to light for eight hours[33]. The treated plasma contained no detectable VK_3. The calibration standards of VK_3 were prepared by spiking 20 μL of the corresponding working solutions mentioned above into 1.0 mL treated blank plasma to yield the concentrations of 0.03, 0.1, 0.3, 1.0, 3.0, 10, 20, 35 and 50 ng mL^{-1} for VK_3. The quality control (QC) samples were prepared using a different stock solution of VK_3 to obtain the plasma concentrations of 0.08, 0.2, 2.0 and 40 ng mL^{-1}.

All operations were performed under red lamps to avoid any losses due to light sensitivity of VK_3 and plumbagin.

2.3. Sample preparation

2.3.1. Extraction procedure

After being drawn from volunteers, the blood samples were collected into heparinized tubes in ice-water bath, and centrifuged immediately at 4 °C to separate the plasma. An aliquot of 1.0 mL plasma sample was transferred to a glass tube in the ice water bath and 20 μL of the IS working solution (200 ng mL^{-1}) was added and vortex-mixed for 10 s. Then 5 mL ethyl acetate was added to the mixture, vortex-mixed for 3 min, and centrifuged for 8 min at 1700 g. The supernatant was separated and evaporated to dryness under a gentle stream of nitrogen in a water bath of 35 °C.

2.3.2. Derivatization procedure

The residue of the extracted plasma sample was reconstituted with 100 μL MPA methanol solution (122 μg mL^{-1}) and 100 μL methanol-water (1:1, v/v). After being vortex-mixed for 3 min, the mixture was reacted at 70 °C for 2 h, and then centrifuged for 5 min at 17000 g. An aliquot of 5 μL supernatant was then used for the LC–MS/MS analysis.

The whole analysis procedure was carried out in a dark room to avoid light-induced decomposition of the analytes.

2.4. LC–MS/MS conditions and performance parameters

An Agilent 1260 series liquid chromatographic system (Agilent Technologies, USA) was used in the LC–MS/MS system. Chromatographic separation was performed on a Hedera ODS-2 column (5μm, 150 mm × 2.1 mm i.d., Hanbon Science and Technology, China) with a Security Guard C_{18} guard column (5 μm, 4 mm × 2.0 mm i.d., Phenomenex, USA). Isocratic elution employed a mobile phase of 5 mM ammonium acetate buffer solution containing 0.1% formic acid and methanol (30:70, v/v) at a flow rate of 0.4 mL min^{-1}. The column temperature was maintained at 38 °C. The autosampler was maintained at 4 °C and the injection volume was 5 μL. The total run time was 5.0 min.

Tandem mass spectral analysis was carried out on an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, USA) equipped with Turbo Ionspray interface. Selection and optimization of the mass spectrometric parameters were performed by continuous infusion of MPA derivative solutions at a flow rate of 10 μL min^{-1} using a syringe pump (Harvard Apparatus Inc., USA). The instrument was operated with the ion spray voltage set at −4.5 kV and the heater gas temperature set to 600 °C. A nebulizer gas (gas 1) of 40 psi, a heater gas (gas 2) of 50 psi, a curtain gas of 30 psi, and a collision gas of 7 psi were used. Mass-dependent parameters declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were respectively set at −42 V, −13 V, −15 V and −20 V for theVK_3 and IS derivatives. Multiple reaction monitoring (MRM) was employed for data acquisition. The optimized MRM fragmentation transitions were *m/z* 275.0→*m/z* 203.0 for derivatized VK_3 and *m/z* 291.0→*m/z* 219.0 for derivatized IS. The dwell time for each transition was 500 ms. Data acquisition was performed with Analyst 1.5.2 software (Applied Biosystems, USA).

2.5. Method validation

The analytical methodology was validated according to the FDA's guidance for industry on bioanalytical method validation [34,35]. The selectivity, linearity, matrix effects, recovery, precision and accuracy, carryover and stability were assessed, respectively.

2.6. Application to real samples

The validated LC–MS/MS method was applied to determine the plasma concentrations of VK_3 in 30 healthy Chinese subjects enrolled in a clinical pharmacokinetic study of MSD. The study was approved by the Ethics Committee of the First Affiliated Hospital of Lanzhou University and was conducted in accordance with the Declaration of Helsinki. Blood samples (4 mL) were collected into heparinized tubes in the ice water bath at 0 h (before drug administration) and at 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 40 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, 6 h and 7 h after drug administration. Then, the samples were treated according to the procedures described above and instantly frozen at −80 °C until analysis. Time profiles for the plasma concentrations of VK_3 were acquired for each subject.

3. Results and discussion

3.1. Optimization of the derivatization reaction

3.1.1. Selection of the derivatization reagent

VK_3, as a quinone compound, contains an α, β-unsaturated ketone structure, which makes it very prone to react with the nucleophiles [36]. Thus, a suitable reagent could be chosen to attack its α, β-unsaturated ketone structure to introduce a readily ionizable group into VK_3 for mass spectrometry detection.

Until now, various reagents have been used for the nucleophile attack of the carbonyl functional groups in carboxylic acids, aldehydes and ketones [37]. Derivatization of carbonyl compounds with 2,4-dinitrophenylhydrazine (DNPH) forming the corresponding 2,4-DNP hydrazones is one of the most widely used derivative analytical methods [38]. The reactions are simple and rapid, and performed in a water matrix. However, a drawback is that stereoisomeric hydrazone derivatives are formed, which may be hydrolyzed under nonacidic conditions. Moreover, since the hydrazines not only react with aldehydes but also with ketones and small carboxylic acids, this derivatization lacks selectivity. Besides, the addition of thiols to VK_3 have been well documented [22,28,39,40], involving nucleophilic Michael addition to give a conjugate. In this work, different kinds of thiol reagents, including glutathione, D-cysteine and MPA were tested to evaluate their effects on increasing analytical performance and MS sensitivity. Results showed that glutathione and D-cysteine could not

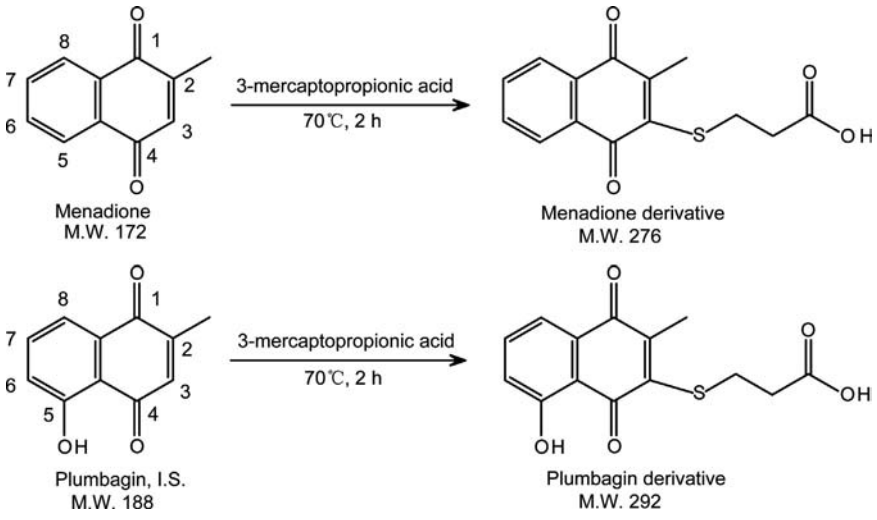

Fig. 2. Derivatization pathways for menadione and plumbagin with 3-mercaptopropionic acid.

meet the specificity requirement for the determination of VK_3, as they are both endogenous substances. MPA was effectively applied as the derivatization reagent in our experiment for the following advantages: (1) easily deprotonated, its carboxyl group is readily ionized to negative ion, and makes it be sensitively detected by negative ESI-MS; (2) simple and rapid derivatization reaction; (3) low cost and high stability of derivative products; and (4) good chromatographic properties of its derivative products. The derivatization pathway of VK_3 with MPA is shown in Fig. 2.

3.1.2. Reaction media

In our study, different reaction media including methanol, acetonitrile and water were tested. The results (see Fig. S1) showed that methanol-water reaction system performed the highest derivatization rate.

3.1.3. Reaction temperature and reaction time

The effect of temperature on the derivatization reaction was examined at 25, 37, 50, 60, 70 and 80 °C reacted for 2 h (Fig. 3A). The amounts of derivatives were also examined at different reaction times of 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4 and 8 h, while maintaining the reaction system temperature at 70 °C (Fig. 3B). The derivatization reaction was almost completed within 2 h at 70 °C, and the ESI-MS response of the products remained unchanged in the reaction time period of 2–8 h. Hence, reaction at 70 °C for 2 h was chosen as the optimum condition for the derivatization.

3.1.4. Amount of the derivatization reagent

To investigate the optimal amount of MPA added in the reaction system, 5 different concentration levels of MPA solutions were prepared by dilution of the MPA (1.22 g mL^{-1}, 20 °C) in methanol to give concentrations of 12.2 mg mL^{-1}, 1.22 mg mL^{-1}, 122 μg mL^{-1}, 12.2 μg mL^{-1} and 1.22 μg mL^{-1}, respectively. 100 μL aliquots of these five MPA reagent solutions were added to the derivatization reaction systems containing the reconstructed extraction residuals of the QCs (0.2, 2.0 and 40 ng mL^{-1} of VK_3 spiked in VK_3-free blank plasma), respectively. Each reaction system was kept at 70 °C in the dark for 2 h. The amounts of reaction products of VK_3 and IS were assessed. The results (see Fig. S2) indicated that the amounts of reaction products of analytes increased dramatically when the MPA concentration increased from 1.22 to 122 μg mL^{-1}, but decreased graduately when the MPA concentration increased from 122 μg mL^{-1} to 12.2 mg mL^{-1}. The reason was that excessive amount of acid may decrease the rate of the reaction. Finally, the optimal addition amount of the derivatization reagent was 100 μL of 122 μg mL^{-1} MPA.

3.1.5. Reaction pH

To examine how pH affects the derivatization reaction, the pH of the water portion added in the reaction system was adjusted with acetic acid and ammonia to pH 5, 6.5, 7, 7.5, 8 and 9. The derivatization reaction was performed at 70 °C for 2 h. The test results showed that neither the derivatization rate nor yield had significant differences at different pH levels of water portion studied. So, there is no need to control pH levels of the reaction system.

3.2. Selection of the internal standard and sample preparation

In this study, plumbagin (PB, 5-Hydroxy-2-methyl-1,4-naphthoquinone, see Fig. 1), a VK_3 analog, was chosen as the IS. PB is chemically similar to VK_3, and mimics VK_3 in any sample preparation steps, such as extraction and derivatization processes. Moreover, the derivative of PB with MPA has similar chromatographic behavior and ionization property to the VK_3s. So PB is a proper IS for this analytical method. Usually the amount of the IS added to the treated sample should be controlled so that the mass response ratio of VK_3 at mid concentration level of the standard curve to the IS was approximately 1:1. The results showed that the derivatives of VK_3 and the IS have approximate responses at the same concentration level. So 20 μL of the IS working solution (200 ng mL^{-1}) was added into 1.0 mL plasma sample, which was close to the mid concentration of VK_3.

VK_3 is stable in methanol solution, but when added to blood or plasma it rapidly disappears[21], since it can react with the free sulfhydryl groups of the endogenous peptide and proteins in plasma at the third carbon [22]. The reaction is very rapid at room temperature, which leads to inaccuracy of the determination. To address the problem of extreme instability of VK_3 in plasma, different extraction procedures like protein precipitation (PPT) and liquid-liquid extraction (LLE) were tested. The test results showed that VK_3 and the IS were stable in the supernatant of LLE, while in the supernatant of PPT were not. So LLE is suitable for the extraction of VK_3 and the IS. In this study, we tried to extract analytes with several organic solvents (ethyl acetate, chloroform, n-hexane, cyclohexane, dichloromethane, benzene and methyl tertiary butyl ether) individually as well as with the various combinations of these solvents. The results showed that ethyl acetate served as a good extraction solvent for VK_3 and the IS. Fortunately, we found that the VK_3 spiked into human plasma was stable in ice-water bath for one hour at least. So, the collected fresh blood samples could be put into the ice-water bath to

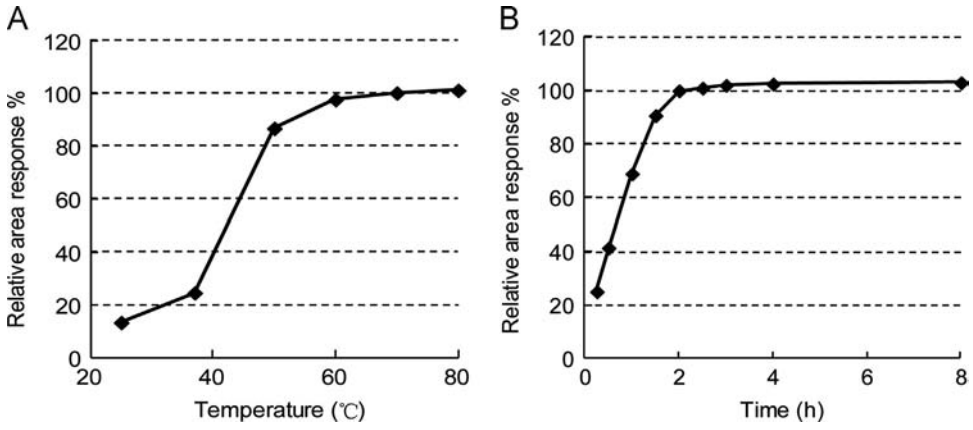

Fig. 3. Influence factors of derivatization: (A) temperature profile of menadione derivative for 2 h (the mass response of the analyte reacted at 70 °C on the *Y* axis represents 100%) and (B) time profile at 70 °C of menadione derivative (the mass response of the analyte reacted for 2 h on Y axis represents 100%).

prevent VK_3 degradation before the plasma separation procedure, and the separated plasma samples also could be kept stable in the ice-water bath for one hour before extraction.

3.3. Optimization of LC–MS/MS conditions

The LC–MS/MS method was initially studied using the ESI or APCI interface in both the positive and negative detection modes. Using ESI, no corresponding signal was observed. Under APCI, VK_3 formed a radical anion $[M]^{\bullet -}$ at *m/z* 172, and cyproterone acetate ($[M\text{-}H]^-$, *m/z* 415.1→ 355.1) was chosen as the IS. Then, the conditions of LC-APCI-MS/MS were optimized. The limit of detection could reach 1.0 ng mL^{-1} and the mass response was very unstable resulting in poor precision and reproducibility. Maybe the radical anion $[M]^{\bullet -}$ is unstable, whose lifetime is limited to within mass spectrometers [41]. Direct determination of menadione in ESI or APCI mode did not meet the requirement of sensitivity.

Ultimately, a chemical derivatization with MPA was adopted to introduce ionizable group into the structure of VK_3. As the VK_3 derivative contains a carboxyl group which is easily deprotonated, the negative ionization mode was selected. ESI was chosen as the ionization source in this experiment because ESI offered better intensity of the analyte compared to APCI. Under the experimental conditions, the base peak of VK_3 derivative obtained was the deprotonated molecule ions $[M\text{–}H]^-$ at *m/z* 275.0. The product ion mass spectrum of $[M\text{-}H]^-$ of the analyte showed major fragments at m/z 203.0 resulting from the cleavage of the S-C bond. The MRM transition of *m/z* 275.0→*m/z* 203.0 offered high signal-to-noise ratio. No interference from endogenous substances was observed. Then the parameters of MS/MS were optimized to obtain the highest intensity of the analyte. Similar to the process employed to quantify VK_3, MRM reaction of the IS at *m/z* 291.0→*m/z* 219.0 was used for quantification. Moreover, the derivatives were identified by the mass spectra. Collision-activated decomposition MS/MS spectra of the VK_3 and IS derivates are shown in Fig. 4.

3.4. Method validation

3.4.1. Linearity and LLOQ

Plotted calibration curves and correlation coefficients >0.997 confirmed that the calibration curves were linear over the concentration ranges of 0.03–50 ng mL^{-1} for VK_3. The LLOQ (signal-to-noise ratio >10) of this method was 0.03 ng mL^{-1} for VK_3. Fig. 5 shows the typical chromatograms of VK_3 and the IS in human plasma.

3.4.2. Precision and accuracy

The intra- and inter-run precision and accuracy were calculated by using ANOVA for the QC samples at four concentration levels (each $n=5$) over three days. The accuracy and precision data are shown in Table 1. The results demonstrated acceptable accuracy and precision of the present method.

3.4.3. Matrix effect and extraction recovery

The absolute matrix effects of six different batches of VK_3-free human plasma were $98.3 \pm 3.8\%$, $96.7 \pm 4.3\%$, and $99.7 \pm 4.2\%$ for VK_3 at concentrations of 0.2, 2.0, and 40 ng mL^{-1}, respectively. The matrix effect of IS was $100.0 \pm 5.0\%$. The RSD values of the relative matrix effects were less than 2.9%. The results indicated that no co-eluting substances influenced the ionization of the analytes. The extraction recoveries of VK_3 were $64.8 \pm 1.7\%$, $65.5 \pm 5.3\%$, and $67.4 \pm 2.6\%$ ($n=5$) at concentrations of 0.2, 2.0, and 40 ng mL^{-1}, respectively. The extraction recovery of the IS was $66.0 \pm 2.9\%$.

3.4.4. Stability

The stability tests of the analytes were designed to cover anticipated conditions for the preservation of the clinical samples. The stability results were summarized in Table 2. Due to the instability of VK_3 in human plasma at room temperature, the post-preparative extract residue samples were prepared and stored at $-80\,^{\circ}C$ until analysis in the clinical study. Besides, it is necessary to protect derivatized samples from light. Otherwise, the signals would be

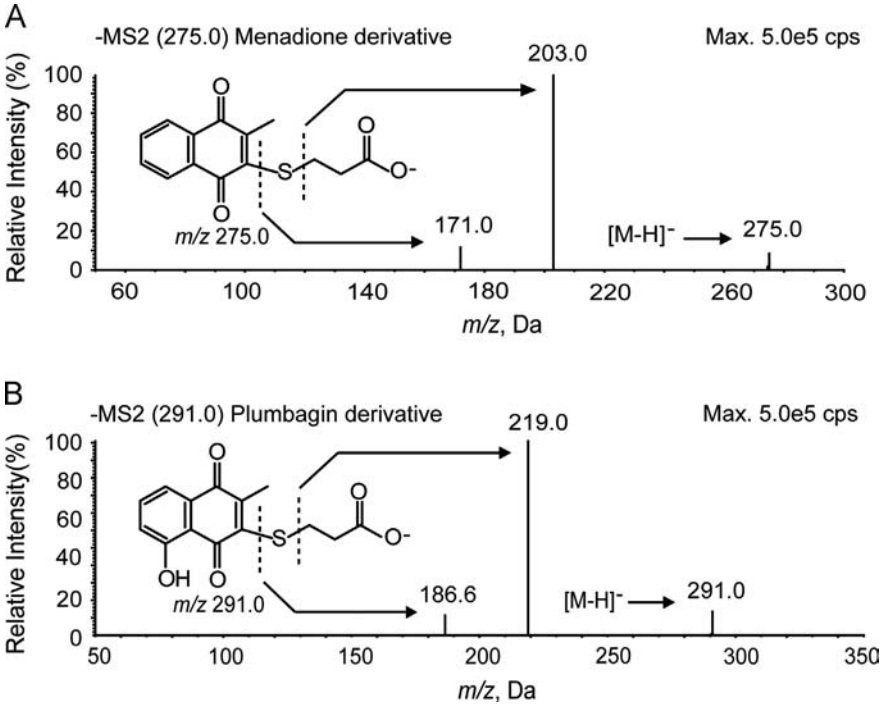

Fig. 4. Collision-activated decomposition MS/MS spectra of menadione derivative (A) and plumbagin derivative (B).

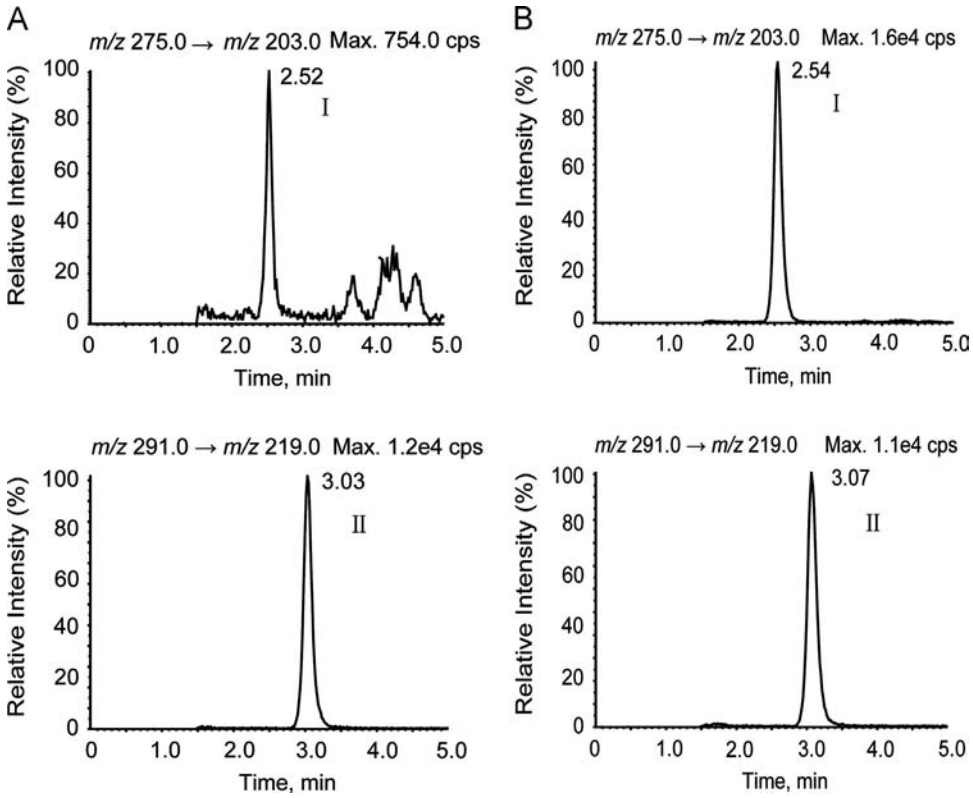

Fig. 5. Typical MRM chromatograms of menadione derivative (I) and plumbagin derivative (IS, II) in human plasma: (A) blank plasma sample spiked with menadione at 0.08 ng mL^{-1} and plumbagin (IS) at 4.0 ng mL^{-1}, and (B) a plasma sample (3.773 ng mL^{-1}) obtained from a subject at 25 min after oral administration of 10 mg Menadiol sodium diphosphate.

Table 1
Precision and accuracy data for the analysis of menadione in human plasma.

Nominal plasma concentration (ng mL^{-1})	Mean measured concentration (ng mL^{-1})	Relative error (%)	Intra-day RSD (%)	Inter-day RSD (%)	*p* Value
0.08200	0.08204	0.05	10.1	6.9	0.6354
0.2050	0.2017	−1.6	4.8	10.0	0.0387
2.050	2.017	−1.6	6.0	11.7	0.0546
41.00	40.63	−0.9	3.2	8.2	0.0122

Table 2
Summary of stability of menadione in human plasma under various storage conditions ($n=3$).

Storage conditions	Nominal plasma concentration (ng mL^{-1})	Mean measured concentration (ng mL^{-1})	Accurracy (%)	Relative error (%)	RSD (%)
Room temperature for 20 min	0.2050	0.1934	94.4	−5.7	9.3
	2.050	2.024	98.7	−1.3	5.5
	41.00	42.28	103.1	3.1	0.3
Ice-water bath for 1 h	0.2050	0.2021	98.6	−1.4	4.3
	2.050	2.076	101.3	1.3	6.0
	41.00	41.84	102.0	2.0	3.3
Post-preparative room temperature for 7.5 h	0.2050	0.2038	99.4	−0.6	4.0
	2.050	2.018	98.4	−1.6	4.6
	41.00	41.42	101.0	1.0	1.8
Auto sampler at 4 °C for 12 h	0.2050	0.1983	96.7	−3.3	4.1
	2.050	2.015	98.3	−1.7	7.3
	41.00	41.27	100.6	0.7	2.2
Post-preparative long-term at −20 °C for 2 days	0.2050	0.1911	93.2	−6.8	5.6
	2.050	2.019	98.4	−1.5	3.7
	41.00	41.12	100.3	0.3	3.2
Post-preparative long-term at −20 °C for 12 days	0.2050	0.2026	98.8	−1.2	5.7
	2.050	1.969	96.1	−4.0	0.5
	41.00	40.18	98.0	−2.0	3.1
Post-preparative long-term at −20 °C for 30 days	0.2050	0.2005	97.8	−2.2	13.6
	2.050	2.102	102.5	2.5	3.5
	41.00	39.67	96.7	−3.2	1.8

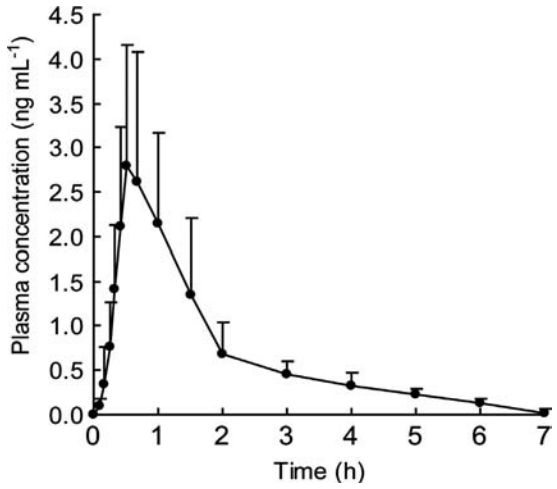

Fig. 6. Mean plasma concentration–time profile of menadione after oral administration of 10 mg Menadiol sodium diphosphate to Chinese healthy subjects ($n=10$).

reduced to 30% of their initial magnitude within 4 h. Thus, the whole processes of this study should be performed away from light.

3.5. Application

The present method was successfully applied to the determination of basal VK_3 level in human plasma, and the clinical pharmacokinetic study in which plasma concentrations of VK_3 in Chinese healthy subjects were determined up to 7 h after oral administration of MSD. The mean plasma concentration-time curves of VK_3 after oral administration of 10 mg MSD in Chinese healthy subjects are presented in Fig. 6. Currently, there is no report on the pharmacokinetic study of VK_3 in healthy subjects. After 10 mg single dose administration of MSD, the pharmacokinetic parameters of VK_3 were obtained. The maximum plasma concentration (C_{max}) was 3.365 ± 1.235 ng mL^{-1}. The time to reach the maximum plasma concentration (T_{max}) was 0.5 ± 0.2 h, and the half-life of drug elimination ($t_{1/2}$) was 2.3 ± 0.7 h. The data obtained from this study is useful for the evaluation of the safety and efficacy of MSD, and that is required for its registration as a new drug. It also provides some references to the subsequent clinical trials of the medicine.

4. Conclusions

A novel derivatization method using MPA as the reaction reagent was developed to improve the assay sensitivity of VK_3 in human plasma during the LC–MS/MS analysis. This method achieved a much lower LLOQ of 0.03 ng mL^{-1}, which was about 33-fold better than that for the direct analysis of the underivatized compound. Compared with existing methods, this method also exhibited high selectivity, good repeatability, short run time, and low-cost. Moreover, the method was successfully applied to the real sample analysis. More importantly, the method for the quantitative determination of VK_3 using LC–MS/MS was reported in this paper provides an important tool in the further research on VK_3 and menadione analogs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.039.

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