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HPLC–APCI–MS for the determination of vitamin K_1 in human plasma: Method and clinical application

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ABSTRACT

A sensitive HPLC–APCI–MS method for the determination of vitamin K_1 (VK-1) in human plasma was established. Target ions at $[M+H]^+$ m/z 451.5 for VK-1 and $[M+H]^+$ m/z 331.4 for the I.S. (teprenone). Calibration curve was linear over the range of 0.3–1000 ng/ml. The lower limit of quantification was 0.3 ng/ml. The intra- and inter-batch variability values were less than 8% and 15%, respectively. The C_{max} was 210.1 ± 86.7 ng/ml while the elimination half-life ($t_{1/2}$) was 8.8 ± 1.7 h and time to the C_{max} was 5.5 ± 0.8 h after administration of soft capsule containing 10 mg VK-1.

1. Introduction

Vitamin K_1, 2-methyl-3-[(2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecenyl]-1,4-naphthalenedione (see Fig. 1A), is an essential cofactor for the post-translational γ-carboxyglutamate (Gla) residues in several blood coagulation factors [1]. Vitamin K_1 may play a variety of health-promoting roles. Administration of vitamin K_1 results in an increase in bone-mineral density and a reduction in bone resorption in humans and rodents [2–6].

The "normal" physiological concentration of serum vitamin K_1 was reported with mean values as low as 100 pg/ml and as high as 4 ng/ml. With the consequent differential influence of previous timings and amounts of food consumption, plasma vitamin K_1 concentration did not vary significantly [7,8]. The pharmacokinetic profile of VK-1 in healthy humans has not been reported so far. As entrusted by Xi'an daheng pharmaceutical Co., Ltd. (Xi'an, China), we investigated the pharmacokinetics of a new formulation of VK-1 soft capsule. In order to investigate the pharmacokinetics of VK-1 in humans, sensitive and accurate assays were needed. Previously, several methods for determination of plasma vitamin K_1 by high-performance liquid chromatography (HPLC) with ultraviolet (UV) [9], fluorescence detection [10,11], GC–MS [12–14] and electrochemical detection [15] were reported, in which the lower limit of quantification (LLOQ) was 2–120 pg/ml. These methods offered reliable and sensitive analysis of VK-1 in human plasma, but the sample preparation procedures of the methods were tedious and time-consuming, which was not suitable for the analysis of a great deal of biological material. Besides, the results of the pilot bioavailability study in our laboratory showed that the human plasma concentration levels of VK-1 on the terminal elimination phase were all above 0.3 ng/ml, so the concentration of 0.3 ng/ml as LLOQ was sufficient to meet the requirement of pharmacokinetics of VK-1. This paper reports a simple and sensitive method to determine VK-1 in human plasma by HPLC–APCI–MS and the pharmacokinetics of VK-1 soft capsule in healthy Chinese volunteers.

2. Experimental

2.1. Chemicals

VK-1 (99.3% purity) was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Teprenone (99.1% purity) was obtained from Eisai China, Inc. (see Fig. 1B). The test formulation was vitamin K_1 soft capsule (each soft capsule containing 10 mg VK-1) provided by Xi'an daheng pharmaceutical Co., Ltd. Methanol was of HPLC grade (Merck, Germany). Isopropanol was of HPLC grade (SK Chemicals, Korea). Ethanol was of analytic-grade purity and purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Cyclohexane was of analytic-grade purity and purchased from Shanghai No. 4 Reagent & H.V. Chemical Co., Ltd. (Shanghai, China). Distilled water was used throughout the study.

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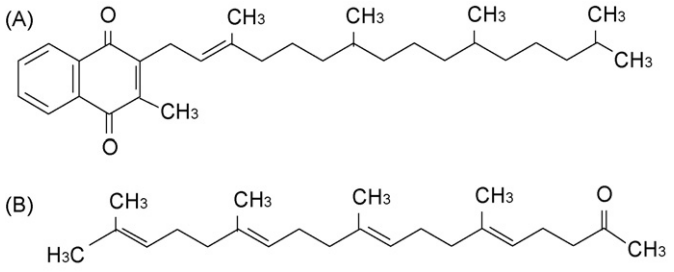

Fig. 1. Chemical structures of vitamin K_1 (A) and teprenone (B).

2.2. Instrumentation and conditions

The HPLC–APCI–MS method was performed using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA) with a Sepax GP-C8 column, 5 μm, 250 mm × 4.6 mm i.d. (Sepax Technologies, Inc., USA). The signal acquisition and peak integration were performed by Agilent's the ChemStation software (10.02 A). The mobile phase was methanol–water containing 20% isopropanol (97:3, v/v), and the column temperature was maintained at 35 °C. A constant mobile phase flow-rate of 1.0 ml/min was employed throughout the analyses. The HPLC–APCI–MS was carried out using nitrogen to assist nebulization. A single quadruple mass spectrometer equipped with an APCI source was set with a drying gas (N_2) flow of 4.5 l/min, nebulizer pressure of 50 psi, drying gas temperature of 300 °C, vaporizer temperature of 350 °C, capillary voltage of 4.0 kV, corona current of 3.0 μA and the positive ion mode. The fragmental voltage was 100 V. HPLC–APCI–MS was performed in the selected-ion monitoring (SIM) mode using the target ions at $[M+H]^+$ *m/z* 451.5 for VK-1 and $[M+H]^+$ *m/z* 331.4 for the I.S. Fig. 2 shows the typical full-scan APCI mass spectrum of VK-1 and the I.S.

2.3. Preparation of stock and working solutions

The stock solution (1 mg/ml) and working solutions (10 μg/ml, 1 μg/ml, 100 ng/ml and 10 ng/ml) of VK-1 were prepared by dissolving an accurately weighed quantity of VK-1 in methanol and serial dilution with methanol. The stock (1 mg/ml) and working solution (1 μg/ml) of the I.S. were prepared in the same way. All the solutions were stored at −20 °C. All the solutions prepared above contained 2 mg/ml alpha-tocopherol. Both VK-1 and teprenone (the internal standard) are the compounds of easy-oxidative degradation. So alpha-tocopherol was added as the antioxidant in the solutions. Teprenone was chosen as the internal standard for the determination of VK-1, because of that VK-1 and teprenone possess the same mechanisms in their degradation way in the solutions, and in their ionization behaviors in the APCI source.

2.4. Sample preparation

Aliquot of 0.5 ml even plasma sample and aliquot of 50 μl I.S. (1 μg/ml) were both placed into a 10 ml glass centrifuge tube and vortex-mixed for 10 s, then to which 2 ml ethanol was added and vortex-mixed for 2 min to precipitate the protein. After protein precipitation, the mixture was extracted with 3 ml cyclohexane by vortex mixing for 3 min, and then centrifuged for another 10 min. The cyclohexane phase was separated and evaporated to dryness under a stream of nitrogen in a water bath of 30 °C. The residue was reconstituted with 120 μl mobile phase, and a 20 μl aliquot was injected into the HPLC–APCI–MS for analysis [16–18].

2.5. Bioanalytical method validation

The selectivity of the assay was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank sample should be tested for no endogenous interferences.

Known concentrations of VK-1 ranging from 0.3 to 1000 ng/ml were added to blank plasma samples. The samples were extracted and chromatographed as outlined above. Calculate the peak area ratio (*f*) of VK-1 (*As*) to that of the internal standard (*Ai*), $f = As/Ai$. VK-1 is an endogenous substance in human plasma, so its concentration level in the blank plasma should be subtracted from the calibration plasma samples before constructing the calibration curves of VK-1. The obtained peak area ratio of the blank plasma was defined as f_{BK}. The adjusted *f* value of the calibration samples was defined as *f'*, $f' = f - f_{BK}$. Calibration curves of VK-1 were constructed by plotting the *f'* values against the amount of VK-1 added to the blank samples. The calibration curve was prepared and assayed along with the quality control (QC) samples. The four QC samples were prepared in 0.5 ml blank plasma at an increase of concentrations of 0.6, 16, 160 and 900 ng/ml separately and stored at −20 °C. The QC samples were assayed along with clinical samples to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown clinical samples analyzed.

Validation samples were prepared and analyzed on three batches to evaluate the accuracy, intra- and inter-batch precisions of the method. The accuracy, intra- and inter-batch precisions of the method were determined by analyzing five replicates of the above-mentioned QC samples along with one calibration curve on each of three batches. Assay precision was calculated using the relative standard deviation (R.S.D.%). The accuracy is the degree of closeness of the determined value to the nominal or known true value

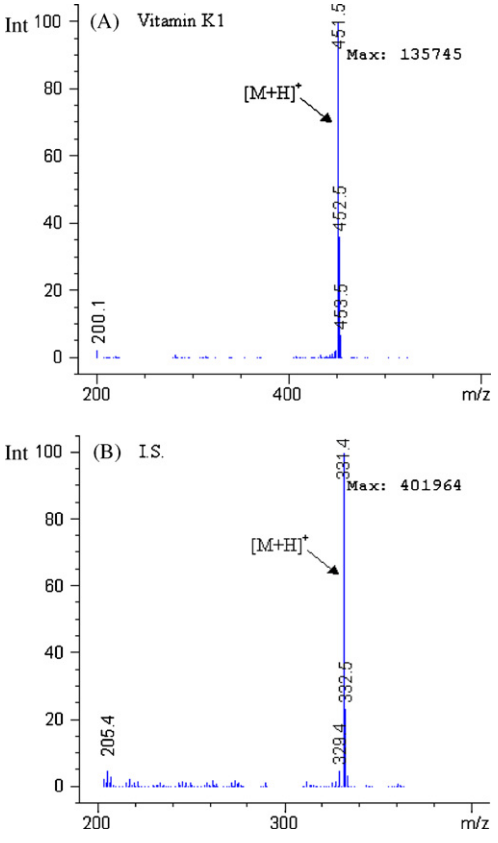

Fig. 2. Mass spectra of the positive ions of vitamin K_1 (A) and I.S. (B) at 70 V.

under prescribed conditions. The accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (R.E.%). It was calculated using the formula:

$$\text{R.E.\%} = \frac{E - T}{T} \times 100.$$

2.6. Clinical study design and pharmacokinetic analysis

The method described above was applied to the pharmacokinetic study in which plasma concentrations of VK-1 in twenty healthy Chinese male volunteers were determined up to 48 h after an oral dose of 10 mg VK-1. The drug was administrated 30 min after the breakfast. The blood was sampled pre-dose and at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 24, 48 h post-dose. The Ethic committee of Xijing Hospital affiliated to the Fourth Military Medical University approved the clinical pharmacokinetic study. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Model-independent pharmacokinetic parameters were calculated for VK-1. The maximum plasma concentration (C_{max}) and the time to it (t_{max}) were noted directly. The elimination rate constant (k_{el}) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_{el}$. The area under the plasma concentration–time curve AUC_{0-48} to the last measurable plasma concentration was calculated by the linear trapezoidal rule.

3. Results and discussion

3.1. Sample preparation

The protein precipitation and liquid–liquid extraction methods were tested in the sample preparation procedure, respectively. The results showed that the extraction efficiency of these two kinds of sample preparation methods was very low. However, higher extraction recovery of the analytes may be achieved by extracting the analytes using the method of liquid–liquid extraction following protein precipitation. Thus, the method of liquid–liquid extraction following protein precipitation was applied to prepare the plasma samples. Several reagents were tested as the protein precipitation and liquid–liquid extractant. The test results showed that the use of ethanol and cyclohexane as the precipitant and extractant respectively gave higher recoveries and less interference of the analytes. Therefore, ethanol and cyclohexane were finally chosen as the reagents in sample preparation procedure.

3.2. Conditions of chromatography

In order to achieve the maximum signal response under APCI+ condition and the shortest analysis time, the percentage of organic phase in the mobile phase was maintained as high as possible while still avoiding the early front peak which contained most of the hydrophilic response-suppressing endogenous interferences. Methanol and acetonitrile were screened for the organic phase. Methanol was selected due to the better selectivity. The aqueous portion was also investigated. The results showed that using 3% pure water as the aqueous portion of the mobile phase could reach good separation for analysts. So, the mobile phase of water–methanol (3:97, v/v) was chosen. But the result was disappointing due to long analytical time (22 min) and deformed peak shape. It is generally accepted that addition of isopropanol into the mobile phase could improve the chromatographic peak shape

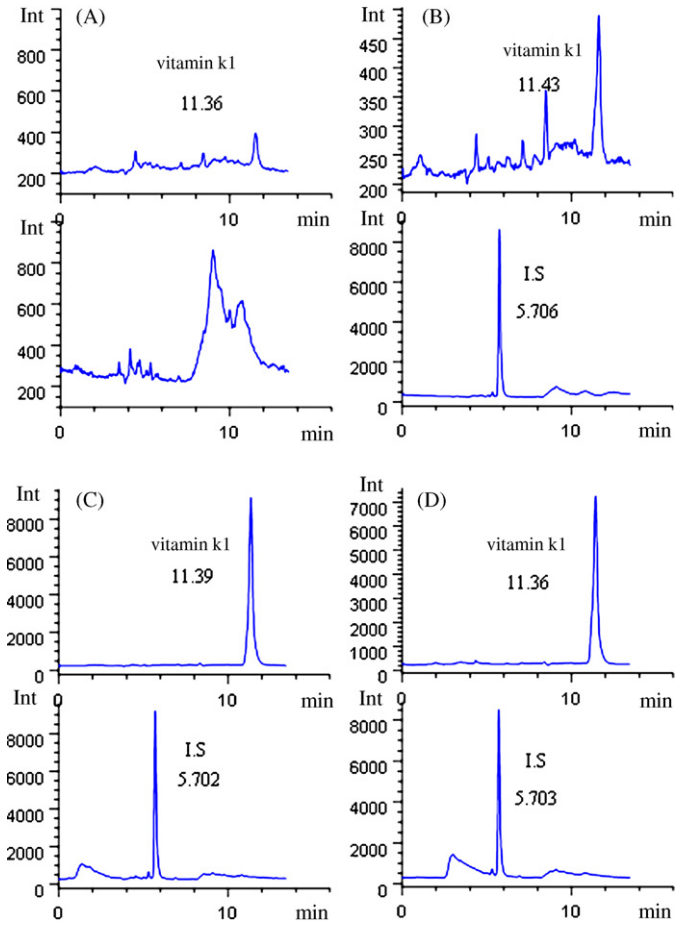

Fig. 3. Typical SIM mass chromatograms of blank sample (A), LLOQ for vitamin K_1 (0.3 ng/ml) and I.S. in plasma (B), plasma spiked with vitamin K_1 (100 ng/ml) and I.S. (C), plasma obtained from a volunteer at 5 h after oral administration 10 mg vitamin K_1 (D).

and shorten the analysis time as well. The different ratios of isopropanol at 10%, 20% and 30% were tested in the mobile phase. The test results showed that the addition of 20% isopropanol into the aqueous portion was satisfactory enough to improve the chromatographic peak shapes and the analysis time (13.5 min). Finally, the acceptable retention and separation of VK-1 was obtained by using an elution system of water containing 20% isopropanol–methanol (3:97, v/v) as the mobile phase. The representative selected-ion chromatograms were shown in Fig. 3. Typical retention times were about 11.3 min for VK-1 and 5.7 min for the I.S.

3.3. Conditions for APCI–MS

The mass spectrometric conditions were optimized to obtain maximum sensitivity. Because VK-1 is a low-polarity and neutral compound, The APCI in positive ion mode was adopted for the LC–MS determination of VK-1. The LC–APCI–MS was performed in the selected ion-monitoring (SIM) mode. In order to select the target ion for monitoring VK-1, the APCI mass spectra obtained in the full scan mode at different fragmentor voltage were investigated. The test results showed that the base peak (the highest ion peak in the mass spectrum, which can be selected as the target ions of the analytes) in the mass spectra of VK-1 obtained at different fragmentor voltage was of the same ion at m/z 451.5, which was the protonated molecule $[M+H]^+$ of VK-1.In order to determine the optimal fragmentor voltage which is set for the ion-source fragmen-

Table 1
Accuracy and precision for the analysis of vitamin K_1 in human plasma (in prestudy validation, three runs, five replicates per run).

Analytes	Added C (ng/ml)	Found C (ng/ml)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	R.E. (%)
Vitamin K_1	0.6384	0.6613	3.1	13.5	3.5
	53.20	52.01	4.5	4.5	−2.2
	532.0	538.1	7.5	7.5	1.1
	957.6	951.3	6.1	6.1	0.7

Note: R.S.D., relative standard deviation; R.E., relative error.

tation, the intensities of VK-1 protonated molecular $[M+H]^+$ m/z 451.5 were compared at fragmentor voltages of 30, 50, 70, 90, 120, 150 V in the SIM mode. The highest sensitivities could be obtained using a 100 V fragmentor voltage. At this fragmentor voltage, the base peak in the mass spectrum of the I.S. was the protonated molecular $[M+H]^+$ m/z 331.4. So, the protonated molecular $[M+H]^+$ at m/z 331.4 was selected as the target ion for the I.S. in the method. The nebulizer pressure can also influence the response of the analytes. The MS response was compared at the nebulizer pressure of 10, 20, 30, 40, 50 psi. The highest response could be obtained using 50 psi as the nebulizer pressure.

3.4. Method validation

The selectivity of the assay was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank plasma sample was tested using the proposed extraction procedure and HPLC–APCI–MS conditions to ensure no interference of analytes and internal standards from plasma.

Five calibration analyses were performed on 5 batches ($r > 0.998$). The mean standard curve for vitamin K_1 was: $f = 0.00015 + 0.01602 \times C$, and $r = 0.999$ over the concentration range of 0.3–1000 ng/ml. The calibration curves, which related the concentrations of the analytes to the area ratio, showed acceptable linearity within the chosen ranges. The back-calculated results showed good day-to-day accuracy and precision.

The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within ±20% and accuracy was within ±20%, and it was established by using five independent samples of standards. The LLOQ of VK-1 were prepared by spiking appropriate amounts of the standard solutions in blank plasma, and then were extracted and assayed. The LLOQ for VK-1 in plasma were 0.3 ng/ml.

Table 2
Matrix effect evaluation of vitamin K_1 and the I.S. in human plasma ($n = 5$).

Samples	Spiked concentration (ng/ml)	Matrix effect (%)
Vitamin K_1	0.6384	110.1
	53.20	108.5
	532.0	107.1
	957.6	102.3
I.S.	1000	105.7

Note: S.D., standard deviation; n, number of replicates.

3.5. Assay precision and accuracy

Table 1 summarizes the intra- and inter-run precision and accuracy for VK-1. The precision was calculated by using one-way-ANOVA. The results in Table 1 demonstrate that the precision and accuracy of this assay are within the acceptable range and the method is accurate and precise.

3.6. Matrix effect

The matrix effect (ME) was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample. It was examined by comparing the peak areas of the analytes and I.S. among three different sets of the samples. In set 1, a control sample was resolved in mobile phase, and the obtained peak areas of analytes were defined as A. In set 2, analytes was resolved in the blank plasma sample's reconstituted solution, and the obtained peak areas of analytes were defined as B. In set 3, analytes was resolved in mobile phase, and the obtained peak areas of analytes were defined as C. ME was calculated by using the formula: ME $(\%) = (B - A)/C \times 100$ or ME $(\%) = B/C \times 100$. Usually, if the analytes are the endogenous substances in human plasma, the former formula is used to evaluate the ME values of the analytes, otherwise, the latter formula should be applied. Because of VK-1 is an endogenous compound existing in human plasma, the former formula, ME $(\%) = (B - A)/C \times 100$, was applied to evaluate the matrix effect of VK-1. The latter formula, ME $(\%) = B/C \times 100$, was used to calculate the matrix effect of the I.S. The matrix effect of the assay was evaluated at the mentioned QC samples above and the I.S. concentration level of 1 μg/ml. The blank plasmas used in this study were from five different batches of healthy human blank plasma. If the ratio <85% or >115%, an exogenous matrix effect is implied. The results in Table 2 showed there was no matrix effect of the analytes observed in this study.

3.7. Extraction recovery

Ethanol and cyclohexane were chosen as the solvents for the higher extraction efficiency to the two target compounds. It can

Table 3
Stability data of vitamin K_1 in human plasma under various storage conditions ($n = 3$).

Analytes	Storage conditions	Added C (ng/ml)	Found C (ng/ml)	Inter-run R.S.D. (%)	R.E. (%)
Vitamin K_1	Room temperature (8 h)	0.6384	0.6460	1.0	1.2
		957.6	894.8	1.1	−6.6
	Three freeze–thaw cycles	0.6384	0.6835	8.6	7.1
		957.6	1013	0.8	5.8
	4 weeks at −20 °C	0.6384	0.6241	10.2	−2.2
		957.6	1063	3.8	7.6

Note: R.S.D., relative standard deviation; R.E., relative error; n, number of replicates.

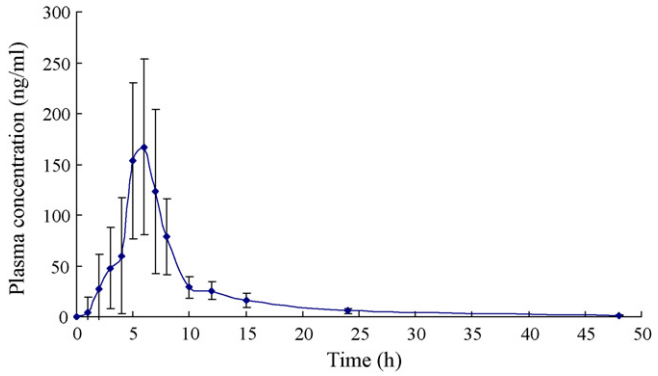

Fig. 4. Mean plasma concentration–time profile in 20 volunteers after single oral administration of 10 mg vitamin K_1 soft capsule.

Table 4
Mean pharmacokinetic parameters of vitamin K_1 for 20 volunteers after single oral administration of the vitamin K_1 soft capsule (mean ± S.D., $n = 20$).

Parameters	Vitamin K_1 capsule
$t_{1/2}$ (h)	5.5 ± 0.8
C_{max} (ng/ml)	210.1 ± 86.7
t_{max} (h)	8.8. ± 1.7
AUC_{0-48} (h ng/ml)	1032 ± 204

Note: S.D., standard deviation; *n*, number of replicates.

not only eliminate the interference of endogenous substances, but also meet the requirement of sensitivity for the method. The recovery of VK-1, determined at the above-mentioned QC samples were 110.1 ± 10.2%, 108.5 ± 4.9%, 107.1 ± 4.9% and 102.3 ± 9.9% ($n = 5$).

3.8. Stability

The stability of VK-1 was studied under a variety of storage and handling conditions. The results (see Table 3) showed that no significant degradation occurred at ambient temperature for 8 h. And there were also no significant degradation occurred during the three freeze–thaw cycles for VK-1 plasma samples. VK-1 in plasma at −20 °C was stable for 4 weeks.

3.9. Application

The method was successfully applied to determine the plasma concentration of VK-1 up to 48 h after an oral administration of the reference and the test formulations of vitamin K_1 capsule to 20 volunteers. The mean plasma concentration–time curve of VK-1 of the test formulation was shown in Fig. 4. The main pharmacokinetic parameters of VK-1 of the test formulation in 20 volunteers were calculated and summarized in Table 4. The relative bioavailability of the test formulation was 99.7 ± 21.2%, based on the test-reference ratio of *AUC*. There were no remarkable differences between the test formulation and the reference formulation in bioavailability.

4. Conclusion

The assay achieved good sensitivity and specificity for the determination of VK-1 in human plasma. No significant interferences caused by the endogenous compounds are observed. This simple and sensitive method is suitable for the pharmacokinetic study of VK-1 in human subjects, and can also be used as a therapeutic drug monitoring method in clinic to check the plasma concentration of VK-1 in the patients.

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