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Short communication

Simple and sensitive determination of menatetrenone and its epoxide metabolite in human plasma

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Abstract

A simple and rapid quantification method was developed for determining menatetrenone and its epoxide metabolite in human plasma. After a simple protein precipitation with methanol, the analytes were chromatographed on a reversed-phase C_{18} column and detected by LC/MS/MS with atmospheric pressure chemical ionization. The coefficient of variation of the assay precision was less than 10.1%, and the accuracy ranged from 98.0 to 106.5%. The limit of detection of menatetrenone and its epoxide was 0.5 and 0.2 ng/ml, respectively. This method was used to simultaneously measure the plasma concentration of menatetrenone and its epoxide metabolite from healthy subjects after a single 30 mg oral dose of menatetrenone.

Keywords: Menatetrenone; Epoxide metabolite; LC/MS/MS

1. Introduction

Osteoporosis characterized by decreased bone density as a result of enhanced bone resorption compared to bone formation may lead to bone fracture. In the bone formation system γ-carboxylation of glutamyl residue of osteocalcin, a calcium binding protein containing γ-carboxyl glutamic acid is essential, and the enzymatic reaction mediated by γ-carboxylase is potentiated by menatetrenone as a cofactor. Consequently, menatetrenone (2-methyl-3-tetraprenyl-1,4-naphthoquinone), a vitamin K analogue has been used for the treatment of osteoporosis [1–3].

In previous literatures, menatetrenone in human plasma has been mainly determined using high-performance liquid chromatography with ultraviolet or fluorescence detection [4,5]. Recently liquid or gas chromatography with a mass spectrometry offers more sensitive assay techniques [5–7]. Vitamin K analogues in plasma have been chromatographed following a liquid–liquid and/or a solid phase extraction [5,6]. Since the sample clean-up procedure is the time-demanding and rate-limiting step in the quantification of analytes in matrices, one makes an effort to simplify the step using a protein precipitation frequently used for fast sample pretreatment and disrupting protein–drug binding [8]. On the other hand, the ionization suppressive effect of endogenous materials in plasma, even after the protein precipitation, is the most common reasons for assay failure in LC/MS/MS.

We develop a simple, sensitive and accurate method for determining menatetrenone and its epoxide metabolite in human plasma using liquid chromatography with a tandem mass spectrometry following a simple protein precipitation with an organic solvent. This method was successfully applied to characterize the pharmacokinetics of menatetrenone in humans.

2. Experimental

2.1. Reagents and chemicals

Menatetrenone and vitamin K_1 were kindly donated by Samil Pharmaceutical Co. Ltd. (Seoul, South Korea). Menatetrenone epoxide was chemically synthesized at a medicinal chemistry laboratory in College of Pharmacy, Catholic University of Daegu. The chemical identity of the products was confirmed by nuclear magnetic resonance spectrometry and high-resolution

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mass spectra. HPLC-grade organic solvent was purchased from Merck Co. (Darmstadt, Germany), and all the other chemicals and solvents were of the highest analytical grade available.

2.2. Preparation of standards and quality controls

Menatetrenone, menatetrenone-epoxide and vitamin K_1 (internal standard, IS) were dissolved in methanol at 1 mg/ml, respectively. The standard solutions were serially diluted with methanol and added at drug-free plasma to obtain the concentrations of 2.5, 5, 10, 25, 50 and 100 ng/ml for menatetrenone and menatetrenone-epoxide. IS was diluted with methanol at 5 ng/ml. Calibration graphs in plasma were derived from the peak area ratios of menatetrenone and menatetrenone-epoxide to IS with a linear regression.

Quality controls were prepared daily in 180 μl of blank human plasma by adding 20 μl of standard solution to evaluate the inter- and intra-day precision and accuracy of this assay method.

2.3. Characterization of the product ions using tandem mass spectrometry

One hundred micrograms per milliliter of menatetrenone, menatetrenone-epoxide and IS solutions were infused into the mass spectrometer separately at a flow rate of 10 μl/min together with methanol at 300 μl/min using three-way cock to characterize the precursor and product ions of each compound. The precursor ions, $[M+H]^+$, and the pattern of fragmentation were monitored using positive ion mode. The major peaks observed in the MS/MS scan were used to quantify each molecule.

2.4. Analytical system

The concentrations of analytes in human plasma were quantified using liquid chromatography–mass spectrometry with a PE SCIEX API 4000™ LC/MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with an atmospheric pressure chemical ionization interface used to generate positive ions $[M+H]^+$. The compounds were separated on a reversed-phase column (XTerra® C_{18}, 2.1 mm × 50 mm internal diameter, 3.5 μm particle size; Waters, USA) with a mobile phase, methanol. The mobile phase was eluted using an HP 1100 series pump (Agilent, Wilmington, DE, USA) at 0.3 ml/min.

The turboion spray interface was operated in the positive ion mode at 5500 V and 350 °C. The operating conditions were optimized by flow injection of a mixture of all analytes and were determined as follows: nebulizing gas flow, 1.04 l/min; auxiliary gas flow, 4.0 l/min; curtain gas flow, 1.44 l/min; orifice voltage, 80 V; ring voltage, 400 V; collision gas (nitrogen) pressure, 3.58×10^{-5} Torr. Quantitation was performed by multiple reaction monitoring (MRM) of the protonated precursor ions and the related product ions for menatetrenone and menatetrenone-epoxide using the internal standard method with peak area ratios. The mass transition used for menatetrenone, menatetrenone-epoxide and IS were *m/z* 445.9 → 187.1 (declustering potential 50 eV, collision energy 32 eV, dwell time 200 ms), *m/z* 461.5 → 311.9 (declustering potential 54 eV, collision energy 21 eV, dwell time 200 ms) and 451.7 → 187.2 (declustering potential 50 eV, collision energy 32 eV, dwell time 200 ms), respectively. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1.4.1, Applied Biosystems, Foster City, CA, USA).

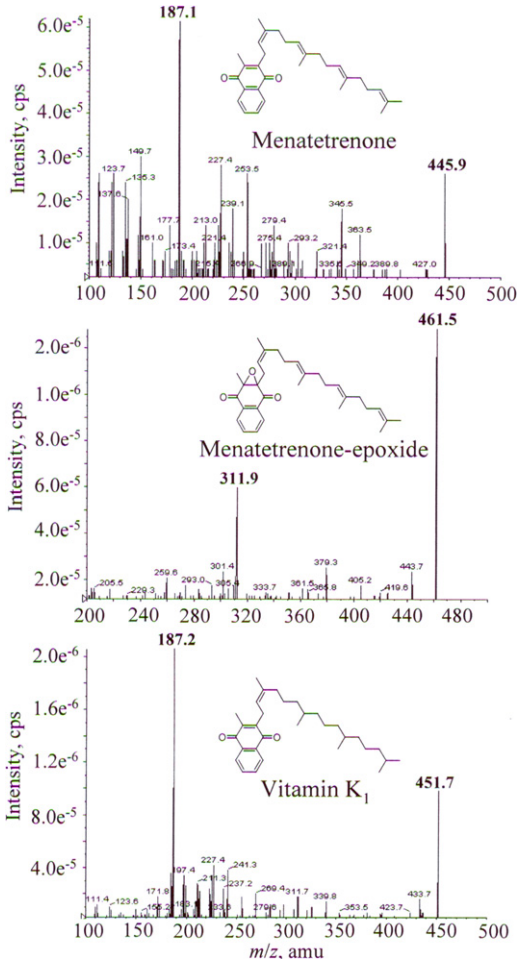

Fig. 1. Mass–mass spectra of menatetrenone (top), menatetrenone-epoxide (middle) and vitamin K_1 (bottom) obtained using atmospheric pressure chemical ionization mode.

2.5. Sample preparation

Eight hundred microliters of internal standard (5 ng/ml) was added to 0.2 ml of plasma, followed by 10 s vigorous vortex-mixing. The mixture was centrifuged at 13,200 rpm for 20 min. Ten microliters of the supernatant was injected onto the analytical column.

2.6. Validation procedure

The validation parameters were selectivity, extraction recovery, precision, and accuracy. Five batches of blank heparinized human plasma were screened to determine the specificity. The extraction recoveries of menatetrenone and menatetrenone-

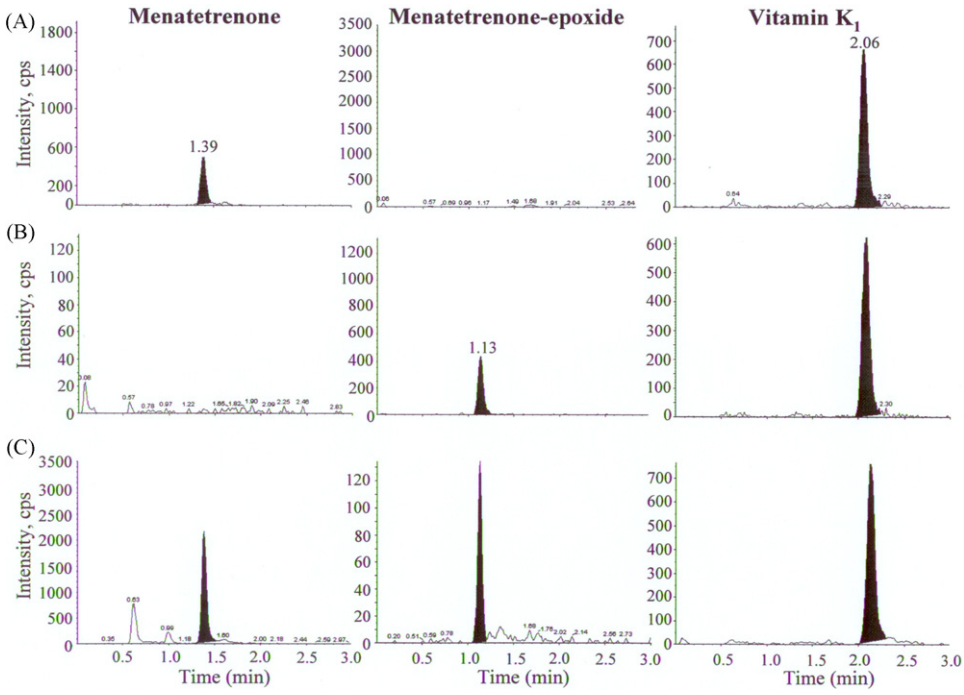

Fig. 2. Chromatograms of menatetrenone, menatetrenone-epoxide with vitamin K_1 (internal standard). A and B: plasma spiked with 5 ng/ml of menatetrenone and menatetrenone-epoxide including 5 ng/ml of internal standard, respectively; C: plasma sample from a volunteer.

epoxide were calculated by comparing the peak area ratio measured for the standard solution with that obtained for plasma extracts after the extraction procedure. The precision and accuracy of the intra- and inter-day assay validation were estimated using the inverse prediction of the concentration of the quality controls from the calibration curves.

2.7. Clinical application

Twenty-four healthy subjects who gave written informed consent took part in this study. Health problems, drug or alcohol abuse, and abnormalities in laboratory screening values were exclusion criteria. This study was approved by the Institutional Review Board of Sun Medical Center (Daejeon, South Korea). After an overnight fast, all the subjects were given a single 30 mg oral dose of menatetrenone capsule. Blood samples (6 ml) were taken before and 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12 h after drug administration and stored at −80 °C until analysis.

3. Results

3.1. Mass spectra

Precursor ions for menatetrenone, menatetrenone-epoxide and vitamin K_1, and their corresponding product ions were determined from spectra obtained during the infusion of standard solutions into a mass spectrometer using an atmospheric pressure chemical ionization source, which operated in positive ionization mode with collision nitrogen gas in Q2 of MS/MS system. Menatetrenone, menatetrenone-epoxide and vitamin K_1 mainly produced protonated molecules at *m/z* 455.9, 461.5 and 451.7, respectively. Their product ions proposed were scanned in Q3 after collision with nitrogen in Q2 at *m/z* 187.1 for menatetrenone and vitamin K_1, and at *m/z* 311.9 for menatetrenone-epoxide. These are the most sensitive product ions for quantification (Fig. 1).

3.2. Determination of menatetrenone and its epoxide

There were no interfering peaks at the elution times for either analyte (menatetrenone, 1.4 min; menatetrenone-epoxide, 1.1 min) or IS (vitamin K_1, 2.1 min), indicating that endogenous compounds did not hinder the measurement of menatetrenone and its epoxide in human plasma. Fig. 2 represents the typical chromatograms for plasma spiked with 50 ng/ml for menatetrenone (A) and menatetrenone-epoxide (B) together with 5 ng/ml for IS. The plasma sample from a volunteer is shown at the bottom (C) of Fig. 2.

3.3. Linearity, precision and accuracy

The calibration curves in plasma provided a reliable response from 2.5 to 100 ng/ml for menatetrenone as well as menatetrenone-epoxide ($r^2 > 0.9999$). The intra- and inter-day precision and accuracy of this method were listed in Table 1. The coefficients of variation of the precision of the intra- and inter-day validation were less than 9.0 and 10.1%, respectively. The accuracy of the method ranged from 98.0 to 106.5%.

3.4. Clinical application

Time profiles of plasma menatetrenone-, and its epoxide-concentration after an oral administration of 30 mg menate-

Table 1
Precision and accuracy of the intra- and inter-day assay ($n = 5$)

Concentration (ng/ml)	Menatetrenone		Menatetrenone-epoxide	
	Intra-day	Inter-day	Intra-day	Inter-day
2.5	103.5 ± 6.7[a] (6.4)[b]	100.1 ± 5.3 (5.3)	99.5 ± 5.6[a] (5.6)[b]	90.1 ± 7.2 (8.0)
5	105.4 ± 6.1 (5.8)	98.2 ± 6.0 (6.1)	101.2 ± 5.2 (5.1)	99.4 ± 10.0 (10.1)
10	100.2 ± 8.0 (8.0)	106.2 ± 4.6 (4.3)	102.2 ± 6.5 (6.4)	102.5 ± 8.6 (8.4)
25	99.0 ± 7.3 (7.4)	105.1 ± 7.8 (7.4)	98.0 ± 5.3 (5.4)	99.5 ± 6.5 (6.5)
50	99.4 ± 6.1 (6.1)	102.4 ± 6.3 (6.1)	103.5 ± 4.1 (4.0)	106.5 ± 8.5 (8.0)
100	100.0 ± 9.0 (9.0)	106.0 ± 7.0 (6.6)	103.0 ± 6.2 (6.0)	99.0 ± 8.1 (8.2)

[a] Accuracy (mean% ± S.D.).
[b] CV, coefficient of variance (%).

trenone was illustrated in Fig. 3, and the pharmacokinetic parameters were listed in Table 2.

4. Discussion

Since menatetrenone, a type of vitamin K occurred in nature has been known to play a potent role for bone formation, its clinical use is now available for the treatment of osteoporosis [1,2]. Determination of the compound in biological samples, e.g., plasma, bone marrow, etc., should be essential for the assessment of a generic product as well as for the relationship between the kinetic behavior and the dynamic response.

So far, a couple of sensitive methods have been introduced for the determination of vitamin K analogues in human specimens using GC/MS or HPLC/MS/MS [5–7]. By the way, the clean-up process of plasma was very complicate in the previous work: after denaturing protein with ethanol, a liquid–liquid extraction was performed with hexane, and followed by a solid-phase extraction, so that it took a lot of time and effort for the pretreatment of samples.

Therefore, a simple and sensitive method has been introduced in the present study. A simple protein precipitation with methanol was employed for the determination of menatetrenone and its epoxide, simultaneously. Acetonitrile was not as good as methanol to get a sensitivity; 20% of zinc sulfate (10% aqueous solution) in methanol showed no improvement of peak intensity despite the better precipitation efficiency of plasma protein. Different volumes of methanol were investigated to find the optimal amount of the organic solvent (300–1000 μl of methanol) to get a sufficient sensitivity, at least a 2.5 ng/ml of the quantitation limit. Finally, an 800 μl of methanol represented the best peak shape and intensity. The mobile phase (100% methanol) used in this report was better than those in the previous work, ethanol/methanol (80:20, v/v) or methanol/0.1% acetic acid aqueous (95:5, v/v) at the present chromatographic condition as well [6,7]. The limit of detection came up to 0.5 and 0.2 ng/ml for menatetrenone and menatetrenone-epoxide at a signal-to-noise (S/N) ratio of 3, respectively.

The use of LC/MS/MS is popular more and more in the hospital to conduct a therapeutic drug monitoring as well as a clinical trial due to its specificity and sensitivity. Additionally, simpler method should be needed to get the plasma concentration of a target drug on time through a faster pretreatment procedure.

In that sense, the present LC/MS/MS method would be worth to provide an efficient way to measure the plasma concentration of menatetrenone and its epoxide metabolite at the same time.

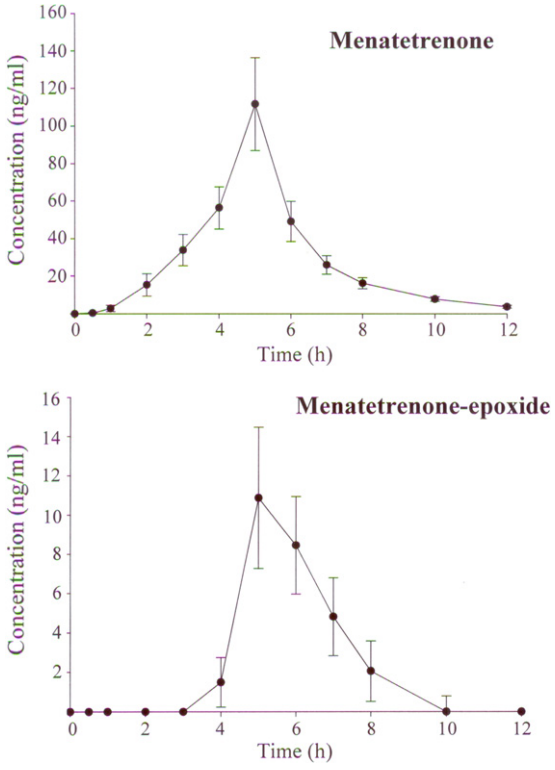

Fig. 3. Time course of the plasma concentrations of menatetrenone (top) and menatetrenone-epoxide (bottom) in healthy subjects after a single 30 mg oral dose of menatetrenone. Each point represents the mean ± S.E. ($n = 24$).

Table 2
Pharmacokinetic parameters after a single oral administration of menatetrenone 30 mg in volunteers (mean ± S.E., $n = 24$)

Parameter	Menatetrenone	Menatetrenone-epoxide
C_{max} (ng/ml)	110.4 ± 20.0	12.8 ± 2.8
T_{max} (h)	4.5 ± 0.2	6.1 ± 0.3
AUC_t (ng h/ml)	296.1 ± 41.2	31.9 ± 6.7
$t_{1/2}$ (h)	1.6 ± 0.1	1.1 ± 0.2

5. Conclusion

In conclusion, the present LC/MS/MS method is very simple, sensitive to determine both menatetrenone and its epoxide metabolite in plasma, and is suitable for the human pharmacokinetic studies of menatetrenone.

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