Simultaneous Determination of Menaquinone-4 and Its Metabolite in Human Osteoblasts by High-Performance Liquid Chromatography/Atmospheric Pressure Chemical Ionization Tandem Mass Spectrometry

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A simple and sensitive method is described for the determination of menaquinone-4 and its metabolite menaquinone-4 epoxide in human periosteal osteoblasts. The method is based on high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry, using deuterated menaquinone-4 as an internal standard. Selected reaction monitoring (SRM) is employed for selectivity and sensitivity. This enables quantification in a short period of time (within 5 min) over the extended ranges 1–1000 ng/ml for menaquinone-4 and 2.5–5000 ng/ml for menaquinone-4 epoxide with acceptable precision and accuracy. The method was used to determine both compounds in human osteoblasts, following incubation with menaquinone-4. It is suggested that menaquinone-4 is metabolized to menaquinone-4 epoxide in bone as well as in liver, indicating the drug has the same role in γ -carboxylation of vitamin K dependent proteins in both tissues. It is expected that this method will contribute to elucidating the pharmacological mechanism of action of menaquinone-4 on bone metabolism.

Keywords Menaquinone-4, vitamin K, mass spectrometry, osteoblast, metabolism, γ -carboxylase

Menaquinone-4 (vitamin K₂) is a potent cofactor for γ -carboxylase, which was originally observed in liver. This enzyme plays an essential role in γ -carboxylation of gla(γ -carboxy glutamic acid)-containing proteins of the bone matrix, such as osteocalcin. In bone, γ -carboxylated osteocalcin is known to be involved in the bone remodeling system^{1–3}, and the pharmacological activity of menaquinone-4 has been demonstrated in human osteoblasts *in vitro*^{4–6} and also in ovariectomized rats, the latter serving as an *in vivo* model for human osteoporosis.⁷ Menaquinone-4 is now available as a medication for the treatment of osteoporosis.⁸

The pharmacokinetic profile of menaquinone-4 in rodents and dogs has already been reported.^{9–14} The specific distribution of menaquinone-4 into bone, the target tissue of the compound, after oral dosing, has also been demonstrated in dogs and ovariectomized rats.^{15–17} The metabolism of menaquinone-4 in bone following distribution has not been elucidated, although the metabolism is thought to be similar in bone and liver. The latter recycles vitamin K *via* its epoxide for the γ -carboxylation of vitamin K dependent proteins such as

blood coagulation factors.

In this study, we developed a simultaneous assay method for menaquinone-4 and its metabolite menaquinone-4 epoxide in human osteoblasts, where bone formation actively takes place, using high-performance liquid chromatography (HPLC)/atmospheric pressure chemical ionization tandem mass spectrometry. The method was used to determine both compounds in human osteoblasts following incubation with menaquinone-4 for 10 d. The metabolic profile of menaquinone-4 in bone is described for the first time.

Experimental

Chemicals and reagents

Menaquinone-4 (2-methyl-3-tetraprenyl-1,4-naphthoquinone) was synthesized by Eisai Chemical Co., Ltd. (Ibaraki, Japan). [²H₇]menaquinone-4 (deuterated menaquinone-4) was synthesized and supplied by Huntingdon Life Sciences (Cambridgeshire, UK). The isotopic purity of deuterated menaquinone-4 was 98.5%. Menaquinone-4 2,3-epoxide was synthesized by Eisai Co., Ltd. (Tokyo, Japan). The structures of these compounds are

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Fig. 1 Chemical structures of menaquinone-4, menaquinone-4 epoxide and [²H₇]menaquinone-4 (deuterated menaquinone-4).

shown in Fig. 1. Solvents for HPLC were of HPLC grade obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) and α -minimum essential medium (α -MEM) were purchased from Irvine Scientific (Santa Ana, CA, USA). Disodium α -glycerophosphate (α -GP) was purchased from Tokyo Kasei Co. (Tokyo, Japan). Warfarin was obtained from Eisai Co., Ltd. (Tokyo, Japan).

Apparatus for LC/APCI/MS/MS

HPLC was performed on an ODS column (L-column ODS, 150 mm $\times$ 1.5 mm i.d., Chemicals Inspection and Institute, Tokyo, Japan) using a Hewlett-Packard HP 1050 series system (Waldbronn, Germany). The mobile phase was EtOH/MeOH (80:20) with a flow rate of 0.15 ml/min.

Mass spectrometric detection was carried out with a Finnigan TSQ 7000 triple-quadrupole instrument (San Jose, CA, USA) using positive ion atmospheric pressure chemical ionization (APCI) as an LC/MS interface. The temperatures of the vaporizer and heated capillary were set at 400°C and 125°C, respectively. The sheath gas pressure and auxiliary gas flow were set at 65 psi and 5 units, respectively. Selected reaction monitoring (SRM) was employed using argon as the collision gas at 2 mTorr, with collision energies of -25 eV for menaquinone-4 and deuterated menaquinone-4 and -12.5 eV for menaquinone-4 epoxide during collision-induced dissociation (CID). Parent to daughter transitions were monitored for m/z 445 to m/z 187 for

menaquinone-4, m/z 461 to m/z 311 for menaquinone-4 epoxide and m/z 452 to m/z 194 for deuterated menaquinone-4. Total scan time was set at 2 s.

Preparation of calibration curve

The stock solutions of menaquinone-4 and menaquinone-4 2,3-epoxide (both 0.5 mg/ml) were prepared in methanol and stored at -20°C. Standard solutions for the calibration curves in each analysis were prepared by sequential dilution of the stock solution with methanol (final concentrations of 1, 2.5, 5, 10, 50, 100, 500, 1000 and 5000 ng/ml) and by mixing with an internal standard, deuterated menaquinone-4 (100 ng/ml as a final concentration). Twenty-microliter aliquots of these samples were subjected to LC/APCI/MS/MS analysis. QC samples (5, 50 and 500 ng/ml in duplicate) were also prepared by adding the appropriate concentrations of standard solutions and deuterated menaquinone-4 (100 ng/ml as a final concentration) to 1 ml blank samples (cell extract). The reliability of the measurement was confirmed using these QC samples.

Quantification method

Calibration curves were constructed by plotting the peak area ratios of the analytes (menaquinone-4 and menaquinone-4 epoxide) to the internal standard (deuterated menaquinone-4) against the concentration of analytes. A weighted ($1/X^2$) linear regression line was fitted over the 1000-fold concentration range. The concentrations of menaquinone-4 and menaquinone-4 epoxide in test samples were calculated from this line and are expressed as ng/ml or ng/well.

Incubation of human osteoblasts with menaquinone-4

Human periosteal osteoblasts were incubated with menaquinone-4 to investigate the metabolism of the compound. The isolation and cell culture methods have been described previously.^{5,18} Incubation media consisted of α -MEM supplemented with 10% FBS containing menaquinone-4 (2.5×10^{-6} M) with or without warfarin (2.5×10^{-5} M). Cells at 18th population doubling levels in 12-well multi-well plates (4 cm²/well) that had reached confluence were incubated in media at 37°C in 5% CO₂/95% air for 10 d in the presence of 2 mM α -GP, as 10 mM β -GP, to accelerate the mineralization process. Menaquinone-4 and warfarin were dissolved in dimethyl sulfoxide (DMSO) and water, respectively. The final concentration of DMSO was less than 0.1%; the same amount of DMSO was added to the control culture. Media were changed every other day.

Sample preparation

The media was removed after incubation, the cells were washed twice with 1.5 ml phosphate buffered saline (Ca²⁺ and Mg²⁺ free) containing 1% DMSO and extracted with 1.5 ml methanol. Deuterated menaquinone-4 (final concentration of 100 ng/ml) was added to the extract and 20 μ l aliquots were subjected to LC/APCI/MS/MS analysis.

Fig. 2 APCI positive mass spectra of menaquinone-4 (a), menaquinone-4 epoxide (b) and deuterated menaquinone-4 (c).

Results and Discussion

LC/MS/MS conditions

First we attempted to raise the intensity of the protonated molecular ions of menaquinone-4 and its metabolite on LC/MS to achieve high sensitivity detection. Figure 2 shows the APCI positive mass spectra for menaquinone-4, menaquinone-4 epoxide and deuterated menaquinone-4. The protonated molecular ions for these compounds were observed as base peak ions under these conditions. A preliminary study revealed that electrospray ionization (ESI) positive mass spectra of menaquinone-4 indicated a mixture of some adduct ions to the molecular ion, including Na adduct ions, and they were not altered in any component of the mobile phase and at any heated capillary temperature examined (data not shown). We also observed that highly aqueous mobile phases, such as 50% aqueous methanol, result in the simple protonated molecular ion (m/z 445) instead

Fig. 3 The effects of vaporizer (a) and heated capillary (b) temperatures on the intensity of the protonated ions of menaquinone-4 and menaquinone-4 epoxide.

of these adduct ions, although menaquinone-4 was not eluted from the HPLC column under such aqueous conditions. The weak intensity of the individual adduct ions was expected to result in low sensitivity on LC/MS/MS analysis. In general, Na adduct ions cannot be fragmented during subsequent CID to obtain an appropriate product ion. We concluded therefore that ESI is not suitable for LC/MS/MS analysis of menaquinone-4 or its metabolite, while APCI, in which any component of the HPLC mobile phase can generate the protonated molecular ions for the parent ions on LC/MS/MS analysis, is suitable as an LC/MS interface.

For the HPLC mobile phase, we found that organic solvents and salts such as ammonium acetate did not affect peak shape or the intensity of menaquinone-4. Ethanol/methanol (80:20) was selected as the mobile phase since it can separate the analytes from each other within a short time (5 min) on HPLC chromatograms.

Figure 3 shows the effects of vaporizer (a) and heated capillary (b) temperatures on the intensity of the protonated molecular ions of menaquinone-4 and menaquinone-4 epoxide. It can be seen that the intensity is quite dependent on these two parameters. Both temperatures also affected the background levels on chromatograms (data not shown). The vaporizer and heated capillary temperatures were set at 400°C and

Fig. 4 Product ion mass spectra of the protonated ions of menaquinone-4 (a), menaquinone-4 epoxide (b) and deuterated menaquinone-4 (c).

125°C, respectively, taking into consideration the signal-to-noise ratio of the peak intensity. The sheath gas pressure and auxiliary gas flow did not greatly affect the sensitivities of these compounds. Under the conditions mentioned above, menaquinone-4 and its metabolite produced the protonated molecule the most effectively with no apparent fragmentation.

We decided to use selected reaction monitoring (SRM) for the determination of these compounds to achieve high selectivity and sensitivity. Figure 4 shows the product ion mass spectra of the protonated ions of menaquinone-4, menaquinone-4 epoxide and deuterated menaquinone-4. Fragmentation of the parent ions is apparent, with m/z 187, 311 and 194 being prominent product ions for the three compounds, respectively. The effects of the collision energy and collision gas pressure on the intensity were investigated (data not shown) in order to obtain a maximum response of the product ions. Based on this result, the energy level was set at -25 eV for

Fig. 5 Typical LC/APCI/MS/MS chromatograms for menaquinone-4, menaquinone-4 epoxide and deuterated menaquinone-4. MK-4: menaquinone-4; MK-4 epoxide: menaquinone-4 epoxide.

menaquinone-4 and deuterated menaquinone-4 and -12.5 eV for menaquinone-4 epoxide. The argon gas pressure was 2 mTorr for both.

Calibration curves

Figure 5 presents typical LC/APCI/MS/MS chromatograms for menaquinone-4, menaquinone-4 epoxide and deuterated menaquinone-4 obtained from the calibration samples. It should be noted that menaquinone-4 and menaquinone-4 epoxide were sufficiently separated from each other in a short period of time (within 5 min) and the respective chromatograms of the compounds did not interfere with each other. No interfering peak appeared at the retention time of the two compounds on the chromatogram of the blank sample. Figure 6 shows typical calibration curves for menaquinone-4 and menaquinone-4 epoxide. Responses in each analysis were linear over the range from 1 to 1000 ng/ml for menaquinone-4 ($r \geq 0.996$) and from 2.5 to 5000 ng/ml for menaquinone-4 epoxide ($r \geq 0.996$), indicating satisfactory concentration ranges with which to determine the compounds in biological samples.

Precision and accuracy

The accuracy and intra- and inter-day precision were determined for the present assay method. The results

Fig. 6 Typical calibration curves for menaquinone-4 (a) and menaquinone-4 epoxide (b). Menaquinone-4: 1–1000 ng/ml ($r=0.9966$); menaquinone-4 epoxide: 2.5–5000 ng/ml ($r=0.9975$). The plots over the short range are shown in the insets.

Table 1 The accuracy and intra- and inter-day precision for menaquinone-4 and menaquinone-4 epoxide in the calibration samples

Nominal concentration/ ng ml ⁻¹	Injection amount/ pg	MK-4			MK-4 epoxide		
		Accuracy, % [n=5]	Intra-day precision (RSD, %) [n=5]	Inter-day precision (RSD, %) [n=3]	Accuracy, % [n=5]	Intra-day precision (RSD, %) [n=5]	Inter-day precision (RSD, %) [n=3]
1	20	-1.00	6.06	5.05			
2.5	50	4.00	9.62	10.89	-0.40	13.65	4.12
5	100	-2.20	4.09	5.60	2.60	6.63	7.82
10	200	2.60	1.36	8.02	-4.40	5.23	1.48
50	1000	3.28	1.37	1.45	0.70	2.48	1.80
100	2000	2.64	2.13	2.06	0.27	3.49	2.37
500	10000	-1.22	1.29	3.00	6.50	1.26	3.79
1000	20000	-7.61	1.25	3.97	3.26	1.47	0.18
5000	100000				-8.64	0.97	3.14

MK-4: menaquinone-4; MK-4 epoxide: menaquinone-4 epoxide.

Table 2 The accuracy and inter-day precision for menaquinone-4 and menaquinone-4 epoxide in QC samples

Nominal concentration/ ng ml ⁻¹	Injection amount/ pg	MK-4		MK-4 epoxide	
		Accuracy, % [n=3]	Precision (RSD, %) [n=3]	Accuracy, % [n=3]	Precision (RSD, %) [n=3]
5	100	1.80	4.13	-3.60	8.92
5	100	-1.00	4.04	-2.40	11.27
50	1000	6.18	3.99	9.44	1.96
50	1000	6.30	2.99	6.82	3.05
500	10000	-6.99	2.29	-0.43	0.73
500	10000	-11.12	2.86	-4.69	1.81

MK-4: menaquinone-4; MK-4 epoxide: menaquinone-4 epoxide.

Table 3 The concentrations of menaquinone-4 and menaquinone-4 epoxide in osteoblasts incubated with menaquinone-4 (2.5×10^{-6} M) with or without warfarin (2.5×10^{-5} M) for 10 d

Incubated compound	Found concentration		Ratio (Epo/MK-4)
	Menaquinone-4 (ng/well)	Menaquinone-4 epoxide (ng/well)	
Menaquinone-4	8.66	12.20	1.41
Menaquinone-4 with warfarin	7.17	19.99	2.79

Epo: menaquinone-4 epoxide; MK-4: menaquinone-4. Each value represents the mean of 4 samples.

are shown in Table 1. The accuracy (mean of 5 replicates) for both menaquinone-4 and menaquinone-4 epoxide was satisfactory (within $\pm 8.64\%$ over the calibration range). The intra- and inter-day precision (5 and 3 replicates, respectively) were also acceptable for both compounds. The RSD values were generally less than 10%, except for the intra-day precision of the lowest menaquinone-4 epoxide concentration, which was 13.65%. Based on these results, we concluded that the quantification limits for menaquinone-4 and menaquinone-4 epoxide were 1 ng/ml (20 pg per injection) and 2.5 ng/ml (50 pg per injection), respectively.

To evaluate the effects of endogenous interferences extracted from osteoblasts on mass chromatograms, QC samples were prepared by adding menaquinone-4 and menaquinone-4 epoxide to the cell extracts at concentrations of 5, 50 and 500 ng/ml. These mixtures were then subjected to LC/MS/MS analysis. The results are presented in Table 2. The accuracy of all six QC samples (2 lots at 3 concentrations) was within $\pm 11.12\%$ (mean of triplicates). This result is acceptable and indicates that interfering peaks on the chromatograms are negligible. The present LC/MS/MS assay method therefore is thought to be sufficiently reliable for measuring menaquinone-4 and menaquinone-4 epoxide in samples extracted from osteoblasts.

Measurement of menaquinone-4 and menaquinone-4 epoxide in osteoblasts

The method has been used to determine menaquinone-4 and menaquinone-4 epoxide in human osteoblasts following incubation with menaquinone-4 (2.5×10^{-6} M) for 10 d with or without warfarin (2.5×10^{-5} M) in order to investigate the metabolism of the compound in bone. The concentrations for all of the control samples, to which no menaquinone-4 was added before incubation, were below the quantification limit for both compounds, confirming that endogenous compounds do not interfere with the measurement of menaquinone-4 and menaquinone-4 epoxide in osteoblasts. It has been reported¹⁻³ that vitamin K (quinone form) was metabolized to its epoxide *via* the hydroquinone form, and then converted to the quinone form by the enzyme vitamin K epoxide reductase in liver. Warfarin is thought to inhibit the enzyme, and the epoxide increased when the liver was exposed to the drug.¹⁻³ In the present study, menaquinone-4 epoxide was detected in cell extracts

Fig. 7 Typical LC/MS/MS chromatograms for menaquinone-4 and menaquinone-4 epoxide extracted from osteoblasts following incubation with menaquinone-4 (2.5×10^{-6} M). MK-4: menaquinone-4; MK-4 epoxide: menaquinone-4 epoxide.

following incubation with menaquinone-4. The concentration of menaquinone-4 epoxide increased upon exposure to warfarin (Fig. 7, Table 3). It is therefore suggested that menaquinone-4 is metabolized to menaquinone-4 epoxide in bone as well as in liver.

In conclusion, an LC/MS/MS method for the simultaneous determination of menaquinone-4 and its metabolite menaquinone-4 epoxide with minimum pretreatment of biological samples and a short analysis time per sample has been established. The sensitivity, accuracy and precision of the method are satisfactory for measuring the compounds in samples extracted from

human osteoblasts following incubation with menaquinone-4. The quantification limit of menaquinone-4 in this method is relatively sensitive compared to a previous HPLC method with fluorescence detection (50 pg per injection)¹⁴ and linear calibration ranges (over 10³-fold range of concentrations) for both compounds are larger compared to previous studies¹⁹⁻²³ in which drugs were determined using LC/APCI/MS/MS with calibration ranges of several 10 or 10²-fold concentrations. Furthermore, we have demonstrated using this method that menaquinone-4 is metabolized to menaquinone-4 epoxide in bone as well as in liver, indicating the drug has the same role in γ -carboxylation of vitamin K dependent proteins in both tissues, even though it has never been reported that vitamin K is metabolized in tissues other than the liver. It is anticipated that this assay method will contribute to clarifying the pharmacological mechanism of action of menaquinone-4 on bone metabolism.

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