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Determination of anticancer drug vitamin K_3 in plasma by high-performance liquid chromatography

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Abstract

Synthetic vitamin K_3 (VK_3, 2-methyl-1,4-naphthoquinone, or menadione) has been found to exhibit antitumor activity against various human cancer cells at relative high dose. Parallel to our study on the mechanism of VK_3 action and for future clinical trials in Taiwan, we developed a simple, sensitive and accurate high-performance liquid chromatographic method for the determination of VK_3 in biological fluids. VK_3 was extracted from the plasma samples with *n*-hexane. The chromatographic separation employed an ODS analytical column (5 μm, 250 × 4.6 mm I.D.) with a mobile phase of methanol–water (70:30, v/v) and UV detection at 265 nm. On completely drying of the extraction solution, *n*-hexane, by a stream of nitrogen, menadione was lost to a great extent. Methanol (70%, 200 μl) was added to the extraction solvent after extraction and centrifugation to prevent the loss of menadione. The absolute recovery was 82.4 ± 7.69% ($n = 7$). The within-day and between-day calibration curves of VK_3 in plasma in the ranges of interest (0.01–10.00 μg/ml; 0.01–5.00 μg/ml) showed good linearity ($r > 0.999$) and acceptable precision. The limit of quantitation of VK_3 was 10 ng/ml in plasma. This method has been successfully applied to a pilot pharmacokinetic study of VK_3 in rabbits receiving an intravenous high-dose bolus injection of 75 mg menadiol sodium diphosphate (Synkayvite). The pharmacokinetic properties of menadione could be described adequately by an open two-compartment model. The mean half-life of menadiol (transformation to menadione) was 2.60 ± 0.12 min. The elimination half-life, volume of distribution and plasma clearance of menadione were 26.3 ± 2.97 min, 25.7 ± 0.78 l, and 0.68 ± 0.10 l/min, respectively.

1. Introduction

Vitamin K_3 (VK_3, 2-methyl-1,4-naphthoquinone, or menadione) is a synthetic quinone derivative of naphthalene. In addition to its anticoagulation function [1], VK_3 has been shown to have a anticancer activity [2]. A greater than 50% decrease in colony formation occurred in 86% of human tumors when tested at 1 μg/ml. The interest in VK_3 grew in part because of its chemical structure, which is similar to that of the commonly anthracyclic chemotherapeutic agents doxirubicin and daunorubicin. In a series of in-vitro and in-vivo animal studies, menadione showed significant antineoplastic activities against both malignant cell lines and a variety of human tumor cells [3]. It was also suggested that menadione prevented the action of certain carcinogens, such as benzo[a]pyrene and quinazo-

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line in rat liver, and reduced the number of benzo[a]pyrene-induced tumors in rats significantly. Potential mechanisms of VK_3 antineoplastic activity include generation of semiquinone [4,5], free radical [6] and superoxide [7] causing glutathione depletion [8], lipid peroxidation [9], and single (double)-strand DNA breaks [10].

There are several methods available for analysis of VK_3, e.g. stopped-flow spectrophotometer [11], gas chromatography [12], reversed-phase [13,14] and normal-phase [15] high-performance liquid chromatography (HPLC), and differential pulse polarographic assay [16]. HPLC methods with ultraviolet (UV) [17,18] and fluorescence detection [13,19] using reversed-phase columns and subsequent post-column derivatization have been developed for animal feeds and premixes. Among these methods, only few were developed for plasma. Akman et al. [16] developed a pulse polarographic assay for VK_3 in human plasma. The limit of quantitation was (10 ng/ml), and the calibration curve is linear from 0.6 to 10 μM (0.1–1.72 μg/ml). This polarographic method had been applied in two patients with advanced colorectal carcinoma treated with a single i.m. dose of menadiol sodium diphosphate (Synkayvite). This method showed good sensitivity; however, to remove bias in the differential pulse polarography method, time-consuming sample preparation was required. K. Kusube et al. [20] reported an HPLC method with fluorescence detection after on-line electrochemical reaction (ECR), which was applied to the post-column electrochemical derivatization of vitamin K analogues (naphthohydroquinone); this method was selective and sensitive. The limit of quantitation was 10 ng/ml in plasma.

This paper reports on an HPLC method for the determination of VK_3 in plasma. Samples were prepared by a simple, inexpensive one-step liquid–liquid extraction process and without post-column derivatization. The procedure is rapid and gives a good limit of quantitation. The integrity of menadione in plasma was validated. Also, the HPLC method has been applied for a pilot pharmacokinetic study in two rabbits receiving a single i.v. bolus of menadiol sodium diphosphate.

2. Experimental

2.1. Chemicals

VK_3 (purity >99%), carbazole (purity >98%) (Merck, Germany), methanol (HPLC grade, Mallinckrodt, KY, USA), *n*-hexane (analytical grade, Riedelde Haen, Germany) and Synkayvite (menadiol sodium diphosphate, Roche Laboratories, NJ, USA) were purchased from their respective suppliers.

2.2. Equipment and chromatographic conditions

HPLC was performed on a combined Waters and Kratos liquid chromatographic system with a Waters Model 510 pump, a Waters Model 715 Ultra WISP autosampler (Waters Assoc., Milford, MA, USA) and an ABI Model 783A UV detector (Kratos, Ramsey, NJ, USA) set at 265 nm and a sensitivity of 0.0005 AUFS. A SIC Chromatocorder 12 integrator (System Instruments, Tokyo, Japan) and a Beckman Ultrasphere ODS HPLC column (5 μm, 250 × 4.6 mm I.D.) (Beckman Instruments, Berkeley, CA, USA) were used. A guard column (10 μm, Bondapak C_{18}) was also used.

VK_3 and carbazole (as internal standard) were isocratically eluded with a mobile phase of methanol–water (70:30, v/v) at a flow-rate of 0.8 ml/min. Peak heights were integrated and recorded by the SIC-12 integrator. The mobile phase was filtered through a 0.45-μm millipore filter and degassed before use.

2.3. Preparation of standards

VK_3 was accurately weighed and dissolved in the mobile phase to prepare a 1 mg/ml stock solution. Calibration standards of VK_3 (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 8, and 10 μg/ml) were prepared by mixing blank plasma (0.8 ml) with VK_3 stock solution (0.1 ml).

Carbazole (35 μg/ml in mobile phase) was prepared as internal standard. The calibration curve in plasma was freshly constructed for each assay run during the animal study.

2.4. Sample preparation

All tubes were wrapped with aluminum paper and processed under dark conditions to avoid exposure to light. All glassware was siliconized with Sigmacote (Sigma). An 100-μl aliquot of internal standard carbazole (35 μg/ml) was added to plasma containing various concentrations of VK_3. The mixture was extracted with 6 ml of *n*-hexane and was mechanically rotated for 45 min. After centrifugation (1080 *g*, 15 min) and freezing at −76°C for 30 min, the upper organic layer was poured into a clean tube and added with 200 μl of 70% methanol before evaporation under nitrogen gas to prevent loss of menadione during evaporation. The samples containing menadione were then analyzed by HPLC as described above.

2.5. Assay validation

To assess the precision and accuracy of the method, within-day ($n = 6$) and between-day ($n = 6$) calibration curves with various concentrations of VK_3 (described in the section 2.3) in plasma were constructed. The precision of the method was expressed as the within-day and between-day correlation coefficient, coefficient of variation (%) and the accuracy was expressed as the mean derivation of all concentrations.

2.6. Pharmacokinetic study

Each of the two New Zealand white rabbits (weighing 3.16 and 3.85 kg, respectively) was injected with a high dose of 75 mg Synkayvite in a 2-ml ampoule by i.v. bolus. Serial blood samples were drawn at 0, 1, 2, 3, 4, 5, 7, 10, 12, 15, 20, 25, 30, 45, 60, 90 and 120 min after injection. Between each sampling time, heparin (100 unit/ml) was injected to prevent blood clotting. Blood samples (1 ml) obtained from the ear-vein were collected, placed into 1.5-ml Eppendorf tubes and kept at −20°C until analysis.

2.7. Pharmacokinetic analysis

The menadione plasma concentration obtained from HPLC was fitted to a three-exponential equation:

$$C_p(t) = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + C \cdot e^{-k_f t}$$

where $C_p(t)$ is the plasma concentration at time t after drug administration, A, B, and C are intercepts on the y-axis for each exponential segment of the curve in units of concentration, with the use of PCNONLIN [21], a non-linear regression computer program. A weighting function of $1/C_p(t)$ was used. The estimates of the initial parameters which were required for non-linear regression were obtained with the use of a linear regression computer program (CSTRIP) [22]. The weighted residual sum of squares, correlations, and residual plots were used to obtain the best estimate of A, B, C and α, β, and k_f. Pharmacokinetic parameters, such as area under the plasma concentration–time curve to time infinity (AUC_∞), transformation and elimination half-lives [$t_{1/2}(k_f)$, $t_{1/2}(\beta)$], the volume distribution (Vd), and total plasma clearance (CL) were calculated for each individual subject according to the standard formula [23].

3. Results and discussion

A simple, sensitive and inexpensive sample extraction procedure is presented here. Representative chromatograms of the HPLC analysis of rabbit plasma samples extracted by *n*-hexane are shown in Fig. 1. The retention times of menadiol, menadione and carbazole were 5.2, 8.7 and 12.5 min, respectively. The highly polar menadiol usually eluted with plasma impurities. The calibration curves were constructed from the peak-height ratio of menadione to carbazole. The limit of quantitation for VK_3 in plasma was

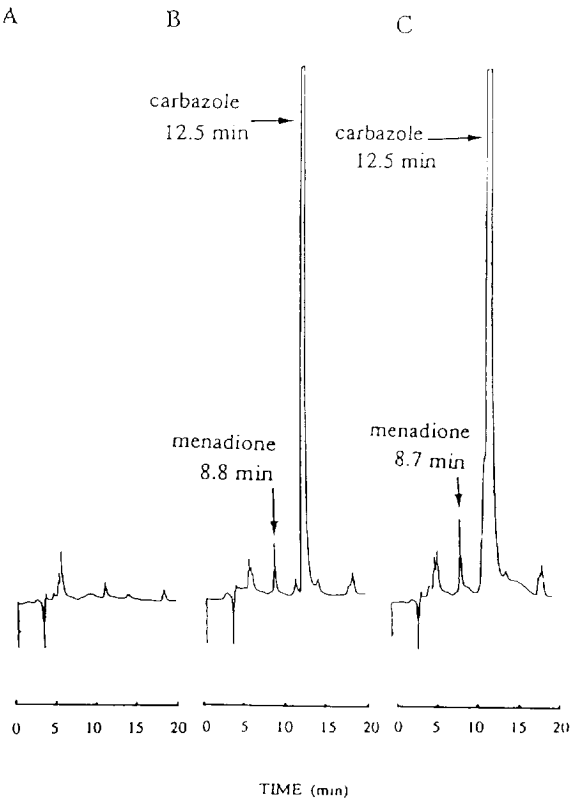

Fig. 1. Representative chromatograms of menadione in rabbit plasma. (A) blank plasma; (B) plasma spiked with 0.05 μg/ml menadione; and (C) plasma sample after administration of 75 mg Synkayvite, the manadione concentration was 0.05 μg/ml.

10 ng/ml. The direct-injection technique is by far the simplest and most rapid method used for treating biological samples prior to their introduction onto an HPLC system. The reasons for choosing the extraction method instead of the direct-injection technique are that protein precipitation [with acetonitrile or methanol–acetone (1:1)] is time-consuming, and also that the samples are further diluted, thereby deteriorating the limit of quantitation. We found that the use of *n*-pentane and *n*-heptane resulted in higher extraction efficiencies for menadione than the use of *n*-hexane. In the present experiment *n*-hexane was selected as extraction solvent, since a cleaner extraction and less noisy baseline were obtained.

The accuracy of the method was confirmed by determining a known concentration of menadione spiked in plasma using calibration curves. To assess the precision and accuracy of the method, within-day ($n = 6$) and between-day ($n = 6$) calibration curves with various concentrations of VK_3 (0.01–10.0 μg/ml and 0.01–5.00 μg/ml) in plasma were constructed (Tables 1 and 2); the curves showed good linearity ($r > 0.999$). Within-day coefficients of variations over the menadione concentration range 0.01–10.0 μg/ml were less than 6.00%. The mean absolute recovery was 82.4 ± 7.69% at a concentration of 0.01–5.00 μg/ml ($n = 7$). If the extraction solvent *n*-hexane was completely dried by a stream of nitrogen, menadione was lost to a great extent. To improve the recovery of menadione, a 200-μl volume of 70% methanol in water was added to the extract to prevent loss of menadione during evaporation. The actual mechanism of this process is unclear. We propose

Table 1
Within-day assay accuracy and precision for the analysis of menadione in plasma ($n = 6$)

Menadione (μg/ml)	Found (μg/ml)	S.D.	Coefficient of variation (%)	ERR (%)
0.01	0.01	0.0001	1.00	0.00
0.05	0.05	0.001	2.00	0.00
0.25	0.26	0.01	3.85	4.00
1.00	0.93	0.05	5.37	−7.00
2.50	2.34	0.09	3.85	−6.40
5.00	4.78	0.15	3.14	−4.40
8.00	7.32	0.36	4.92	−8.50
10.00	10.8	0.30	2.78	8.00

Table 2
Between-day assay accuracy and precision for the analysis of menadione in plasma ($n = 6$)

Menadione (μg/ml)	Found (μg/ml)	S.D.	Coefficient of variation (%)	ERR (%)
0.01	0.01	0.0001	1.00	0.00
0.025	0.026	0.001	3.85	4.00
0.05	0.049	0.002	4.08	−2.00
0.10	0.10	0.007	7.00	0.00
0.25	0.24	0.007	2.92	−4.00
1.00	0.95	0.04	4.21	−5.00
2.50	2.51	0.10	3.98	0.40
5.00	5.22	0.15	2.87	4.40

that methanol prevents adsorption of menadione on the polar surface of the glass.

The HPLC method has been successfully applied to a pharmacokinetic study of VK_3 in rabbits receiving one (75 mg) i.v. bolus of Synkayvite (menadiol sodium diphosphate). The mean age of the two rabbits was 2.1 yr, and the mean body weight was 3.51 kg. The plasma concentration–time curve (mean ± S.D.) of menadione after administration of Synkayvite is shown in Fig. 2. After intravenous administration of Synkayvite, the menadione concentration in plasma quickly increased and decreased in a multi-exponential manner according to a two-compartment model. The corresponding pharmacokinetic parameters for two rabbits are listed in Table 3. In vivo, Synkayvite is very rapidly converted to menadione. At 4 and 7 min after injection, the respective menadione peak concentrations were 2.37 and 2.36 μg/ml for the two

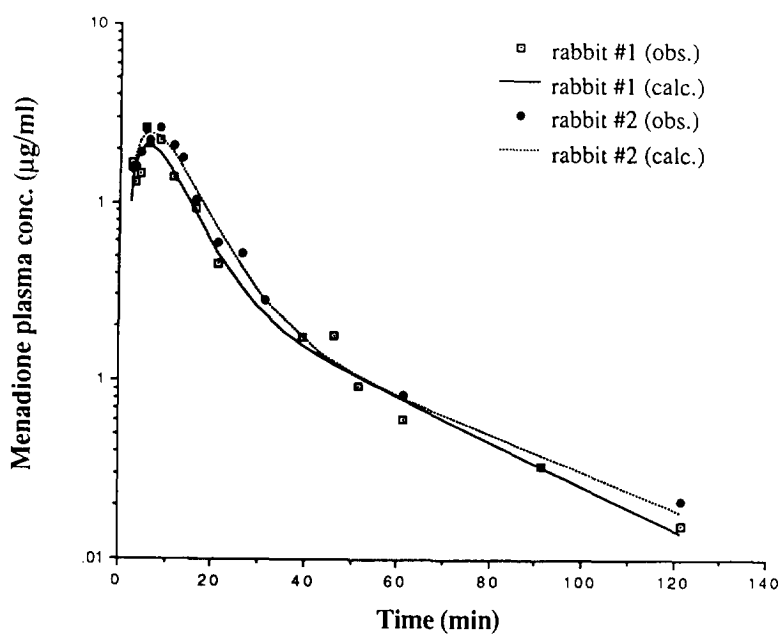

Fig. 2. Concentration–time curve of menadione in plasma after i.v. administration of menadiol sodium diphosphate (75 mg) in each of two rabbits. The symbols represent the observed data, and the lines indicate the calculated data according to the estimated pharmacokinetic parameters.

Table 3
Pharmacokinetic parameters of menadione after i.v. administration of 23.2 and 19.5 mg/kg menadiol sodium diphosphate in rabbits

Pharmacokinetic parameters	Rabbit	
	No. 1	No. 2
Dose (mg/kg)	23.2	19.5
A (μg/ml)	10.5	10.6
B (μg/ml)	0.40	0.32
C (μg/ml)	−10.9	−10.9
α (min^{-1})	0.18	0.15
β (min^{-1})	0.029	0.024
K_f (min^{-1})	0.277	0.258
$t_{1/2}(\alpha)$ (min)	3.87	4.58
$t_{1/2}(\beta)$ (min)	24.2	28.4
$t_{1/2}(k_f)$ (min)	2.51	2.68
Vd/F (l)	26.2	25.1
T_{max} (min)	4.00	7.00
C_{max} (μg/ml)	2.37	2.36
AUC_∞ (μg min/ml)	33.3	40.6
CL/F (l/min)	0.75	0.61

Equation: $C_{(p)} = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-K_f t}$: where A, B, C are intercepts; α, β, and k_f: rate constants for distribution phase, elimination phase, and transformation phase, respectively; $t_{1/2}(\alpha)$: the half life of drug in the distribution phase; $t_{1/2}(\beta)$: the half life of drug in the elimination phase; $T_{1/2}(k_f)$: the half life of drug in the transformation phase; Vd/F: volume distribution; AUC_∞: area under plasma concentration–time curve to time infinity; CL/F: total plasma clearance; F: fraction of menadiol converted to menadione.

rabbits, the half lives of the transformation phase and the elimination phase were 2.60 ± 0.12 and 26.3 ± 2.97 min, respectively. In other words, 90% of the fraction of menadiol eventually converted to menadione was formed within 10 min and this amount was excreted within 2 h after administration. The apparent volume distribution (Vd/F) of menadione was 25.7 ± 0.78 l. The systemic clearance (CL/F) of menadione was 0.68 ± 0.10 l/min. Since the extent of menadiol transformation to menadione was not known, the Vd/F and CL/F were not the actual Vd and CL of menadione, unless menadione was injected intravascular.

There are only few kinetic data reported on menadione or its water-soluble forms. In 1969, Thierry and Suttie [24] used radiolabelled Synkayvite on rats with a dose of 1.6 to 2.0 μg/rat (160 to 210 μCi). Akman and coworkers [16,25], treating two cancer patients with Synkayvite, reported that no serious side-effects were observed and that menadione cleared relatively slowly (lasting for approximately 4 h) from plasma after parenteral administration of 20 mg/m^2 (i.m.). In our study, menadione was more rapidly removed from rabbits, the menadione concentrations in plasma at 120 min after injection being almost undetectable (<0.01 μg/ml). As we know, menadiol sodium diphosphate is metabolized in vivo to menadione [24,26] and the most prominent metabolite in liver is the glucuronide of 2-methyl-1,4-naphthoquinone. However, Will et al. [27] and Martius [28] reported that menadione in animals was only metabolized to small amounts of menaquinone-4 (VK_2). VK_2, which has the alkyl group at position 3, may play an important role in glutamic acid carboxylation reactions [29,30]. In the present study, we did not find significant chromatographic interference with menadione at a dose of 75 mg per rabbit.

We have reported a simple, one-step extraction method without post-column derivatization for the determination of menadione in plasma. This precise and accurate method has been successfully applied to the pharmacokinetic study of high-dose menadione in rabbits.

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