



Comparison of two reducing agents dithiothreitol and tris(3-hydroxypropyl) phosphine for *in vitro* kinetic assay of vitamin K epoxide reductase

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ABSTRACT

Vitamin K epoxide reductase (VKOR) is a target enzyme for anticoagulants, such as warfarin, that are used as medicines or rodenticides. Assessing VKOR activity is required to ensure the proper usage of these drugs. Dithiothreitol (DTT) is a typical disulfide reductant that is used as a substrate for *in vitro* VKOR assays. However, DTT is considered problematic because of its side effects. Tris(3-hydroxypropyl)phosphine (THP) has been found to be a reliable alternative to DTT, as shown by kinetic analyses of the VKOR with them. THP showed significantly lower V_{max} and K_m values than those of DTT; however, there was no significant difference in their V_{max}/K_m and IC_{50} for warfarin.

1. Introduction

Vitamin K epoxide reductase (VKOR) is a membrane protein of the endoplasmic reticulum (ER) that catalyzes the conversion of vitamin K epoxide (VKE) to vitamin K quinone (VK) (Li et al., 2004). This reaction is necessary for the production of proteins dependent on vitamin K, such as blood clotting factors II, VII, IX, and X, via the γ -carboxylation of specific glutamate residues. However, some coumarin derivatives can also act as inhibitors of VKOR and cause delay in blood coagulation. These compounds, such as warfarin, have been widely used as rodenticides since the 1950s (Oldenburg, Marinova, Müller-Reible & Watzka, 2008). However, the increasing emergence of rodenticide-resistant rats has become a serious issue. The first cases of rodenticide resistance were observed in Scotland in 1958; since then, these have been observed worldwide (Buckle & Smith, 2015). Some rats are known to have a mutation in the VKOR gene; as a result, VKOR shows low sensitivity to rodenticides (Rost et al., 2004). For adequate pest control, it is necessary to investigate the sensitivity of each VKOR to different rodenticides by *in vitro* analysis.

For its reaction, VKOR requires electron donation via a shuttle of two disulfide bonds. Four cysteine residues comprising Cys43, Cys51, Cys132, and Cys135 for rat VKORC1 are entirely conserved among

VKOR orthologs (Czogalla et al., 2017). However, the source of their physiological equivalents is still unknown. Dithiothreitol (DTT) has been used as a reductant for VKOR reactions *in vitro* because it is a typical disulfide bond reducer (Whitlon, Sadowski & Suttie, 1978). However, several studies have found that the use of DTT in facilitating VKOR assays is problematic because it can reduce VKE to VK directly and produce 3OH-KO as a byproduct (Hildebrandt, Preusch, Patterson & Suttie, 1984). In addition, some researchers have reported that inhibition tests with DTT are not consistent with *in vivo* sensitivity results (Whitlon et al., 1978). Therefore, a novel assay to evaluate the physiological sensitivity of VKOR is clearly warranted.

A possible alternative for DTT is tris(3-hydroxypropyl) phosphine (THP); it is a water-soluble and air-stable compound that can reduce disulfide bonds (McNulty, Krishnamoorthy, Amoroso & Moser, 2015). Krettler, Bevans, Reinhart, Watzka and Oldenburg (2015) reported that THP does not directly reduce VKE and that it showed a comparable capacity for VKOR activity compared with DTT (Krettler et al., 2015). However, there is limited information about the kinetic application of THP to estimate the enzymatic activity of VKOR and its inhibition test with rodenticide. In this study, we developed a THP-driven kinetic analysis of VKOR and compared it with DTT.

Abbreviations: VKOR, Vitamin K epoxide reductase; DTT, Dithiothreitol; THP, tris(3-hydroxypropyl)phosphine; VKE, vitamin K epoxide; VK, vitamin K quinone; ER, endoplasmic reticulum

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2. Materials and methods

2.1. Chemicals employed

We purchased vitamin K1 from Kanto Chemicals (Tokyo, Japan), and Vitamin K1 epoxide from Sigma-Aldrich (St. Louis, MO). Vitamin K1(D7) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA). From Wako Pure Chemical Industries (Osaka, Japan), we purchased acetic acid, diethyl ether, DTT, ethanol, HEPES, methanol, K_2HPO_4 , KH_2PO_4 , NaOH, and warfarin–sodium. THP was obtained from Santa Cruz Biotechnology (Dallas, TX).

Method validation of quantification by HPLC-MS

	Recovery rate (%)	LOD (nM)	LOQ (nM)	Ionization mode	Precursor product (m/z)	Product (m/z)	Dwell time (ms)	Q1 pre bias (V)	CE	Q3 pre bias (V)
Vitamin K1	83.89 ± 1.62	1.40nM	4.24nM	-	450.2	185.0	100.0	12.0	35.0	18.0
Vitamin K1 epoxide	77.89 ± 1.49	5.21nM	15.8nM	-	465.2	210.25	100.0	13.0	31.0	20.0
Vitamin K1 d7	83.49 ± 1.64	3.04nM	9.21nM	-	456.2	245.0	100.0	12.0	34.0	24.0

2.2. Preparation of liver microsome from rat liver

Four male 9-week-old Sprague-Dawley rats were introduced from Japan SLC, INC. (Shizuoka, Japan). They were housed under a 12/12 h light/dark cycle at 20 °C–23 °C. Rats were provided *ad libitum* access to food (CE-2, CLEA, Tokyo, Japan) and water. After 10 days of acclimatization, rats were sacrificed by isoflurane overdose, and their liver was removed for analysis. Liver microsomes were prepared according to the method described by Omura & Sato, 1964. The protein concentration of these microsomes was measured using the method described by Lowry, Rosebrough, Farr and Randall (1951).

All animal care and experimental procedures were performed in accordance with the Guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International and were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Approved number; 18-0150).

2.3. VKOR activity and inhibition test

A reaction mixture was prepared in a HEPES buffer (pH 7.4, 0.1 M), with a total volume of 100 μ L. It contained 1.0 mg protein/mL of microsomes, and 5, 10, 25, 50, 100, 300, and 750 μ M of VKO. Samples were pre-incubated for 5 min, and reactions were started by the addition of either DTT or THP (1 mM, final concentration). The reactions were continued for 20 min at 37 °C and were terminated by the addition of 1 mL of iced diethyl ether. For the inhibition tests, we used mixtures containing 1.0 mg protein/mL of microsomes, 50 μ M of VKO and 0, 0.01, 0.05, 0.1, 0.25, 0.5, and 1 μ M of warfarin–sodium. Direct reduction of VKO by reductants was also measured by a reaction mixture without microsomes and subtracted from the value developed by including microsomes. The appropriate concentrations of VKO and warfarin to use were decided by preliminary experiments. Each of these experiments was performed in duplicate.

VK and VKO were extracted from the reaction mixture using the liquid–liquid method, and vitamin K1(D7) was added as an internal standard (100 nM, final concentration). Liquid–liquid extraction was performed with 5 mL of diethyl ether, and the organic layer was acquired and evaporated under a gentle stream of N_2 gas. The residue was dissolved in 1 mL of methanol.

2.4. Vitamin K detection

The concentrations of these compounds were determined by high-performance liquid chromatography (HPLC) (Shimadzu 20 series;

Shimadzu, Kyoto, Japan) coupled with atmosphere pressure chemical ionization triple quadrupole mass spectrometry (APCI/MS/MS, LC-8040; Shimadzu) equipped with a C18 column (Inertsil ODS-3, 2.1×150 mm, 5.0 μ m), from GL Science, Tokyo, Japan. The HPLC process was undertaken according to the method described by Suhara, Kamao, Tsugawa and Okano (2004). Collision energies and other MS parameters were optimized and are shown in Table S-1. The recovery rate of extraction, limit of detection, and limit of quantification calculated using the standard curve ($n = 6$) are also presented in Table S-1.

2.5. Data analysis

VKOR activity values were fitted by nonlinear regression with the Michaelis–Menten equation or inhibition curve. Estimations of apparent K_m , V_{max} , and IC_{50} values were obtained using the GraphPad Prism 5 system (GraphPad Software Inc., San Diego, CA). Data were analyzed using a Student's *t*-test with a significance level of $p = 0.05$ using JMP 14 (SAS Institute, Cary, NC).

3. Results

In preliminary experiments, the protein concentration and incubation time for reactions were confirmed to retain linearity using the method described above. Hence, 1 mM of reductant and 20-min incubation periods were used throughout the study. We performed kinetic assays of VKOR to estimate V_{max} and K_m at the VKO concentration levels of 5, 10, 25, 50, 100, 300, and 750 μ M (Fig. 1A, Table 1). Both reductants showed good regression to the Michaelis–Menten curve and reached a plateau at 750 μ M of VKO. However, DTT showed greater activity than THP, which resulted in the significantly higher V_{max} and K_m shown in Table 1. However, there were no significant differences in V_{max}/K_m (enzyme efficiency) between the two reductants. An inhibition test was performed with a representative VKOR inhibitor, warfarin at a concentration of 50 μ M of VKO (Fig. 1B and C). Both of the reductants generated typical sigmoid curves, although DTT showed higher basal activity (Fig. 1B). When adjusted to the ratio by its native activity (without warfarin) as shown in Fig. 1C, they generated regression curves that were completely the same, and their IC_{50} values showed no significant differences (Table 1).

4. Discussion

Intra/Inner-species differences in VKOR are an important factor in determining sensitivity to an anticoagulant. For example, avian species showed different IC_{50} values compared with rodents (Watanabe, Saengtienchai, Tanaka, Ikenaka & Ishizuka, 2010), and single nucleotide polymorphisms of VKOR in humans can affect the appropriate dosage of warfarin for the treatment of thrombosis. An accurate analysis of VKOR is required from the point of view of comparative pharmacology and toxicology. In this study, THP, a new disulfide reductant, was applied to a kinetic analysis of VKOR as an alternative to a DTT-driven assay, and it showed almost the same V_{max}/K_m and IC_{50} values as those resulting from using DTT. The ratio V_{max}/K_m is well known as an indicator of physiological enzyme efficiency, and IC_{50} is a metric for the potency of a substance in inhibiting a

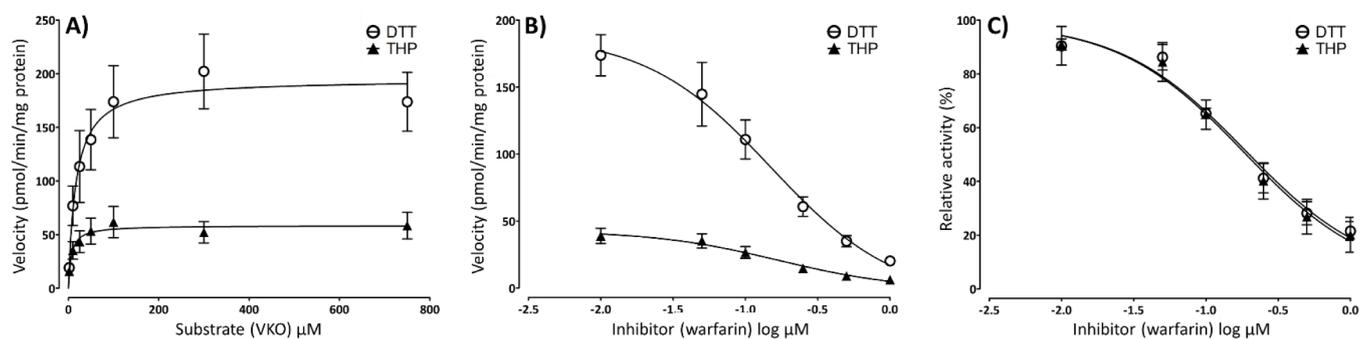


Fig. 1. Michaelis–Menten plots of VKOR activity

(A) VKOR activity *versus* various concentrations of VKO. (B) VKOR activity *versus* various concentrations of inhibitor, warfarin. (C) inhibition plotting fitted by VKOR activity without warfarin. Data were fitted to nonlinear regression curves by GraphPad Prism 5. All kinetic values calculated from these figures are shown in Table 1. The plots show the mean \pm SE. $N = 4$ for each analysis.

Table 1

Kinetic parameters of VKOR.

	DTT	THP
V_{max} (pmol/min/mg protein)	195.2 \pm 18.1*	58.1 \pm 5.5
K_m (μ M)	17.0 \pm 7.0*	5.6 \pm 3.2
V_{max}/K_m (mL/min/mg protein)	11.5 \pm 4.4	10.3 \pm 2.9
IC_{50} (μ M)	0.162 \pm 0.027	0.189 \pm 0.028

Kinetic parameters of VKOR-dependent activities of rat liver microsomes were estimated with THP and DTT. V_{max} , maximum response velocity; K_m , substrate concentration at $V_{max}/2$; V_{max}/K_m , enzyme efficiency. The values are given as means \pm SE ($n = 4$). * indicates a significant difference (Student's t -test, $p = 0.05$).

biological function. Based on the results we have observed in this study, there seem to be few differences between the two reductants in estimating physiological enzyme efficiency although THP lowered the direct reduction of VKO.

It is noteworthy that Fregin et al. reported that their inhibition test with HEK293 cells co-expressed VKOR and that clotting factor IX demonstrated relatively natural sensitivity to the rodenticide (Fregin et al., 2013). The benefit of a recombinant cell assay is that it uses an endogenous reductant, and it shows a good correlation with its phenotype *in vivo*. On comparing the IC_{50} values across these studies, we found that the IC_{50} of the cell-based assay was 24.7 nM, whereas our values were 162 and 189 nM. Interestingly, the classical DTT-driven assay also showed a dramatically different value than ours, namely 3034.8 nM (Rost et al., 2004). This result may be due to differences in methodology because our study used 1 mM of DTT whereas they used 5 mM. In addition, our sample was a liver microsome, but they used recombinant HEK 293 cells expressed VKOR.

Further study with numerous VKOR variants is needed to assess the reliability of a THP-assay. Although a cell-based assay is suitable to calculate IC_{50} , there are some benefits to using a liver microsome, in that it can reflect the natural expression level of VKOR, and it is possible to perform enzyme kinetic analysis with it. Therefore, alternative methods of performing *in vitro* assays using liver microsomes are still needed.

One difference between THP and DTT is the significantly lower V_{max} produced by THP. This outcome is likely not due to the saturation of the reaction since the linearity of the reaction at a high concentration of VKO with incubation time was confirmed. The possible reason for a higher V_{max} produced by DTT is that it can bypass the first disulfide bond and directly reduce the second disulfide bond close to VKO, which lies within the lumen of the ER (Rost et al., 2005). DTT can pass through the ER membrane because it is an amphiphilic compound (logP: 0.18) and is known to be soluble in both water and organic solvents, such as ether. However, it is difficult for THP to access the

second disulfide bond in the lumen of the ER because it is a hydrophilic compound whose predicted logP by Molinspiration is -1.35 . Indeed, VKOR solubilized in detergent showed higher activity with THP (Krettler et al., 2015), supporting this hypothesis. The lower V_{max} produced by THP indicates that the reducing ability of THP is 'more physiological' than DTT. This point should be investigated using a recombinant VKOR that lacks the first disulfide bond to examine the superiority of THP. To develop a greater understanding of VKOR, the identification of its physiological reductant is also required.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Supplementary materials

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