QUANTIFICATION OF THE VITAMIN K_3 CONCENTRATION

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ABSTRACT

Recent study revealed that menaqunone-4 (MK-4) may be a preferred form among vitamin K (VK) homologues and it would play an important role in the body. And MK-4 would be converted from VK_3 in our body. Thereby if the serum VK_3 concentration can be detected, the relationship between some diseases and VK_3 can be clarified. However, no studies have ever examined the serum VK_3 concentration. The conventional method to detect the VK is the fluorescence detection way. This method utilizes the VK fluorescence after its reduction to the hydroquinone form. However, this hydroquinone form is easily oxidized and converted into quinone form in a short time. Therefore, the relationship between VK_3 concentration and their fluorescence amount has not ever been clarified. Consequently, the purpose of this study was to clarify the relationship between the VK_3 concentration and amount of fluorescence. First, the experimental system was constructed for the fluorescence measurement. The system includes the inverted microscope and the photomultiplier. Preliminary study was performed to evaluate the feasibility of the experimental system. The result showed a slight different fluorescence amount with large scale of photon count levels by the different levels of VK_3 concentration. However, as a further problem, the positional gap of the measurement point and the external light intrusion in the experimental system was revealed. Against these problems, we adopted in our experimental system the special stage to fix the glass capillary, and the low-pass filter for cutting the external light. And we performed the additional experiment with VK_3 concentration of 0% as the negative control study. Result, the output value becomes 200,000 to 300,000 counts/50ms, stability was increased. Further study should be performed under variety of VK_3 concentrations.

1. INTRODUCTION

There are two different kinds of natural vitamin K (VK) homologues, vitamin K1 and vitamin K_2 (MK-n). On the other hand, vitamin K_3 (VK_3) lacks the side chain part of vitamin K homologues. Recent study revealed that menaqunone-4 (MK-4) may be a preferred form among VK homologues and would play an important role in the body[1)]. There is also consistent evidence that the MK-4 is produced by conversion from dietary VK homologues[2)] and then accumulated in various tissues with a high concentration[3)4)]. Some epidemiological studies have been investigated through measurement of serum vitamin K_1 or K_2 concentration[5)]. We assumed that the VK3 would be the precursor of the MK-4 in the body. And if the serum VK_3 concentration can be detected, the relationship between some diseases and VK_3 would be clarified. However, VK_3 is thought to have only a few picomole in the body, because measurement is difficult, the study has not yet been conducted. This background information prompted us to develop a sensitive and stable method to detect the serum VK_3. The conventional method to detect the VK is the fluorescence method[6)]. This method utilizes the fluorescence of VK after reduction of the quione form to the hydroquinone form. However, this hydroquinone form is easily oxidized and converted into quinone form in a short time, in addition, its molecules are too small to detect the accurate fluorescence. Therefore, the relationship between VK_3 concentration and the fluorescence amount has not been established yet. Consequently, the purpose of this study was to clarify the relationship between the VK_3 concentration and amount of fluorescence.

2. METHOD

2.1 Design of the Experimental system

We have built an experimental system that allows the

fluorescence measurement of hydroquinone, which is the reduced form of VK_3.

The system contains the photomultiplier (Hamamatsu Photonics, H10682-210, following PTM) with the time resolution of 20 nanoseconds and the measurement sensitivity peak of 400 nanometers, and inverted microscope (OLYMPUS, IX71) with fluorescence mirror unit (OLYMPUS, U-MWU2), and mercury lamp as a light source. The prepared materials for the experiment were the methanol (Wako, reagent grade) and Vitamin K_3 (Wako, Wako special grade), which was then reduced at the Suhara laboratory in Shibaura Institute of Technology. In the measurement of VK_3, because the fluorescent light of hydroquinone is assumed to be weak, we adopted the inverted microscope with the connection through the photomultiplier to the Personal computer.

Negative control study was conducted using the blackout curtain, so external light does not enter into the photomultiplier with the light from the PC also blocked. The excitation filter 330 ~ 385 [nm] and the absorption filter 420 [nm] were both mounted on the microscope. In addition, the 60 times objective lens (OLYMPUS, LUCPLFLN60XPH), and 0.5 times lens between the photon sensor and the microscope, were used. As the system summary, a schematic diagram of the experimental system is shown in Fig.1.

Since we have successfully built the experimental system, we performed the preliminary test to validate the feasibility of prototyped experimental system, like followings.

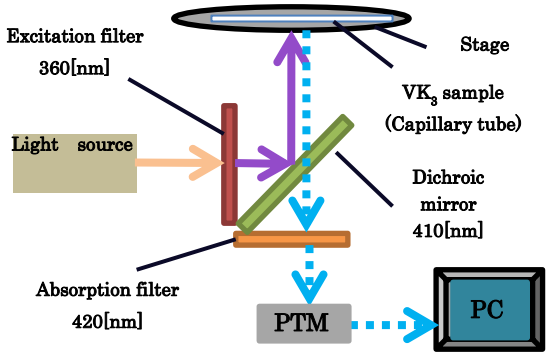


Fig. 1 VK_3 experimental system overview diagram for the amount of fluorescence measurement

2.2 Experimental procedure

At first, the reduced VK_3 was dissolved in methanol, and VK_3 solution having a concentration of 5.81×10^{10} [pmol/L] and 5.81×10^{7} [pmol/L] were prepared respectively. The prepared solution was inserted into the glass capillary tube and placed on the microscope stage. These procedure was performed under the blind condition inside the blackout curtain so that the sample was not exposed to the light. Using the 60 times objective lens, the fluorescent light measurement was carried with the measurement time of 10 seconds and sampling interval of 50 [ms] into the photomultiplier.

2.3 Experimental result

The results of the VK_3's fluorescence measurement test is shown in Fig.2. The figure shows the time series detected photons of fluorescent light of the each VK_3 solution.

The VK_3 solution with the 5.81×10^{7} [pmol/L] and 5.81×10^{10} [pmol/L] showed the photon count average of 1.74×10^{6} [counts/50msec] and 1.79×10^{6} [counts/50msec], respectively. And then we conducted an additional experiment using only methanol without any VK_3, what is the negative control, and it resulted still some level of light with variation among 1.6×10^{3} ~ 1.6×10^{6} [counts/50msec].

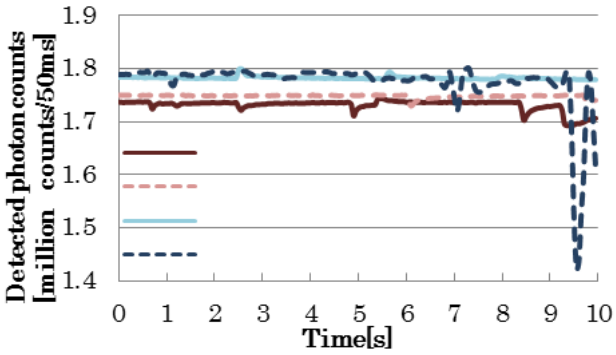


Fig. 2 Fluoresent Light Measurement

2.4 Discussion & Short summary

Our preliminary test successfully showed the slight difference in fluorescent amount according to VK_3 concentration level as shown in Fig.2. However, the time series detected fluorescent light was not constant and it was followed by fluctuation of approximate 100,000 ~ 300,000 [counts/50msec]. Following two aspects was considered as the possible reason. One thing is that the capillary tube is not fixed for the experiment, therefore the capillary position can be changeable each experiment. The possible second reason would be light leakage problem. In order to solve these possible problems, the further development was performed for the better reproducibility.

3. FURTHER SYSTEM IMPROVEMENT AND EXPERIMENT

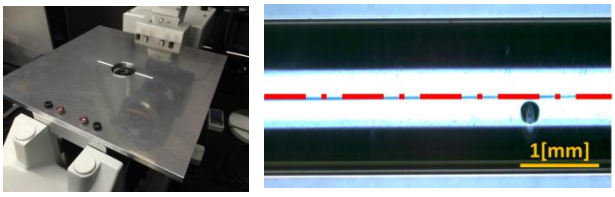
3.1 Improvement of Experimental setup

We prototyped the special stage which can fix the glass capillary in order to improve the reproducibility of the measurement. The prototyped stage is shown in Fig.3. In order to fix the capillary, the groove with width of 2 [mm] was made at the center position, and the capillary position and the objective lens were aligned with those center axis using the thickness tape. Consequently, the center of the capillary to become definitive for the same position, and the experimental reproducibility of was improved.

Furthermore, the new low-pass filter of 430 [nm] to C-mount connection was also adopted to prevent the intrusion of light leakage and external light. This made it possible to suppress increase of the external light, and it

became possible to input the limited fluorescence wavelength light of 410 ~ 430 [nm] into the photomultiplier.

In addition, for the further stability of the PTM, the measurement duration was extended to be 100 seconds from 10 seconds.



(a) (b)

Fig. 3 Special microscopic stage to fix the glass-capillary (a) and observed view of capillary (b)

The further experiment was performed to understand negative control condition. The test procedure is shown in the followings. At first, the ion-exchanged water and methanol was prepared. And those fluids were inserted into the glass capillary and acrylic capillary, respectively.

3.2 Result of the negative control study

Fig.4 shows the result of the negative control studies using the glass or acrylic capillary filled with nothing (cavity), water, and the methanol, respectively. The left and right vertical axis shows the time series detected photon of fluorescent light using the acrylic and glass capillary, respectively. The detected photon count resulted in 200000 ~ 300000 [counts/50msec] for the glass-capillary and 1770000 ~ 1790000 [counts/50msec] for the acrylic capillary, respectively.

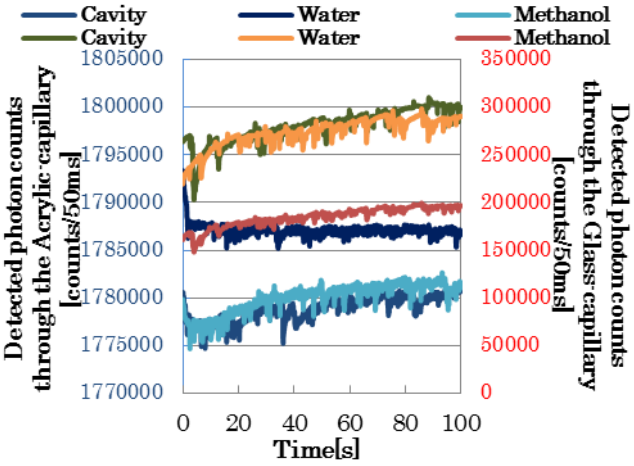


Fig. 4 Result of the negative control study using the capillary made by acrylic and glass material; Duration of the measurement was 100 seconds.

Additionally, Fig.5 shows the time series mean detected photon counts for 100 seconds, in which each plot with standard deviation was derived through the averaging the acquired data during 10 seconds.

Furthermore Fig.6 shows the coefficient of variation of each plot data acquired in Fig.5. This figure represents the relative fluctuation degree of data against the averaging value.

From these graphs, it became to be obvious that the measured data commonly varied for the short time period of 10 seconds right after starting measurement. And the data became to be stable after 40 seconds. Furthermore, the detected photon level increased with time elapsing as shown in Fig.5.

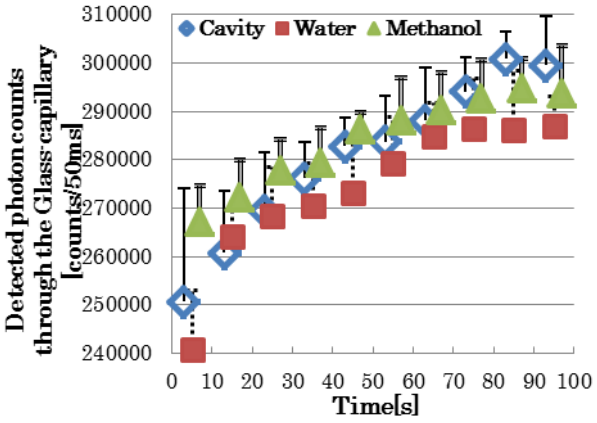


Fig.5 The result of negative control study using the glass-capillary; Each plot with its standard deviation was derived through the averaging the measured data for 10 seconds.

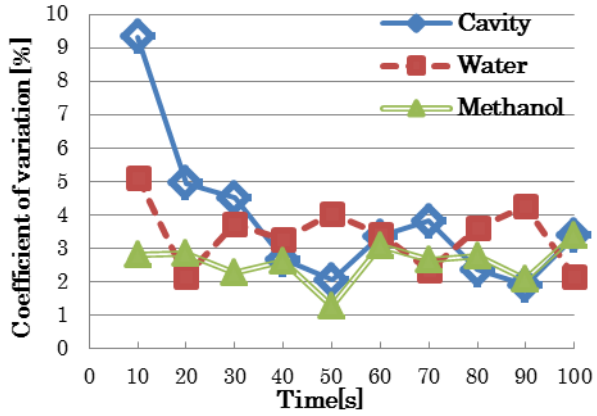


Fig. 6 The coefficient of variation of the measured photon count as the negative control study corresponding to the acquired data in Figure5; This figure should represents the stability of photon measurement.

3.4 Discussion

As shown in Fig.4, the data of the acrylic capillary output varied between 1.77 ~ 1.79 million [counts/50msec]. Therefore, little change was given with the comparison to the data using the previous system improvements. This possible reason is the possible fluorescent characteristics of acrylic material used in the capillary part, and the acrylic material's fluorescence was allowed to be detected into the fluorescence wavelength range between 410 ~ 430 [nm], which is possible peak fluorescence of VK_3.

On the other hand, the data acquired by glass capillary became much less levels like 0.2 ~ 0.3 million [counts/50msec] with the decrease amount of 1.5 million

[counts/50msec]. From this fact, the majority of fluorescent light of the glass material was possibly filtered by adopting new filter. In addition, the beginning of measurement seemed to be unstable, and it became to be relative more stable after 40 seconds, furthermore, measured photon count showed tendency to rise as the function of time elapsing in our experiment with the time duration of 100 seconds. Therefore, the longtime measurement would be also less reliable. Therefore, data acquisition between 60 ~ 70 seconds after starting measurement would be adequate way for this measurement because the coefficient of variation was low level of approximate 2.67 % and the average value did not excessively increase at this range. Further our study will perform additional experiments with variety of controlled VK_3 concentration levels.

4. CONCLUSION

We prototyped the experimental system which can be applied for the VK_3 quantification within the solution, and further improved it. The preliminary study showed slight different fluorescent light amount under the different VK_3 concentration. However, negative control test still showed some level of fluorescent light detection which should be cancelled. With our current test system condition, the data acquisition between 60 ~ 70 seconds after starting measurement would be most adequate measurement way.

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