# The Effect of Sample Storage Temperature on the Phenytoin Level in Serum

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## ABSTRACT

**Objective:** To study the effect of temperature on phenytoin concentration in serum and to determine the sample were kept at 2-8°C and 35°C.

**Methods:** Serum samples of patients, who had been treated with phenytoin. had their phenytoin level determined on the first day. The sample was stored for seven days and analyzed on days three, five and seven of the study and divided into three storage concentrations from 2.5-10, 10.1-20 and 20.1-30 µg/ml compared using 50 samples per group.

**Results:** Phenytoin level was found to have increased over time to three days at a temperature of 35°C and five days at 2-8°C. It increased in the level of statistical significance at $p<0.05$. This study's data were all well correlated at $r^2 = 0.9981$-$0.9999$.

**Conclusion:** Collected samples for analysis of phenytoin level must be stored at 2-8°C and stored for at least five days, to prevent degradation of protein which cannot bind to the drug. The phenytoin levels in serum increased to make the diagnosis of dosage mistakes.

**Keywords:** Phenytoin, stability, storage

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## INTRODUCTION

Phenytoin (5, 5-diphenylhydantion, PHT) or dilantin is an anticonvulsant drug widely used in the treatment of acute seizures and epilepsy, and less commonly used for other conditions such as bipolar disorder.[1] PHT levels in the blood must be maintained within a narrow therapeutic range, because low levels may cause the patient to suffer seizures or precipitate into status epilepticus, whereas high levels may result in toxicity with adverse effects.[2,3]

Maintaining a therapeutic level of PHT in the blood can be a challenge for several reasons. Hepatic enzymes metabolize PHT with different speed among people, under the influence of age, genetic factors, coexisting disorders and organ function. Some patients are very sensitive to small changes in bioavailability and even small increases in dose can cause large increases in blood concentrations, enhancing the severity of side effects and causing PHT toxicity.[4]

PHT in blood is approximately 90% bound to proteins and 10% free;[5,6] thus, a low amount of protein in the blood causes an excess of active drug. Furthermore, PHT often interacts with other drugs, affecting the safety and effectiveness of other medications.[7] Therapeutic drug monitoring in a pediatric sample is necessary because of the

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very different responses in terms of drug availability.[8,9]

The stability of this drug in serum under various conditions of shipment and storage is an important consideration in therapeutic drug monitoring. Kleion V, et al, found that when the PHT concentration in the same sample was determined repeatedly over time, it steadily decreased. When samples were stored at 4°C, it declined by about 20% in 48 days, but when stored at room temperature, it had all disappeared in four weeks.[10] However, Wilensky AJ, found no change in serum PHT concentration during three months of storage at 4°C.[11]

In regions of Thailand, blood is often collected in red-top (no additive) tubes to separate serum from cells. Samples may not be placed on ice immediately before packaging and shipping for antiepileptic drug analysis. Furthermore, because of clinical settings in tropical climates, samples may be exposed to high temperatures for an undisclosed amount of time. Currently, PHT stability data under these conditions are not available. The objective of the present study was to assess the short-term stability of Phenytoin in human serum as a function of storage time and temperature.

## MATERIALS AND METHODS

The serum samples examined in the study were obtained from 150 patients. The samples were divided into 3 groups: the first low concentrations 2.5-10 µg/ml for 50 samples, the second medium concentrations 10.1-20.0 µg/ml for 50 samples and the third high concentrations 20.1-30.0 µg/ml for 50 samples. All were receiving PHT as their treatment for epilepsy. Serum levels of PHT were determined by a fluorescence polarization immunoassay (AxSYM, Abbott Laboratories, Chicago, LLL, U.S.A). An AxSYM instrument (Abbott Labs) equipped with Revision 2.0 software using AxSYM line diluent (0.1M phosphate buffer, pH 7.4, with 0.1% of a proprietary antimicrobial agent) was used for identification of PHT samples. Controls were prepared from analytically pure PHT at concentrations of 7.5, 13.5, and 25.0 µg/mL (low, medium, and high controls, respectively) in diluent containing 0.1% sodium azide. Polarization measurements were reported as millipolarization (mP) units. A standard curve was generated using calibrators prepared gravimetrically from analytically pure PHT at assigned concentrations of 0, 2.5, 5, 10, 20, and 40 µg/mL (A, B, C, D, E, and F calibrators, respectively).

On the first day, a sample of PHT was evaluated under routine laboratory conditions (25+3°C). Then each sample was separated to store at low temperature (2-8°C) and heigh temperature (35°C). Samples from each temperature were subsequently reevaluated on days 3, 5 and 7.

### Statistical analysis

Data were analyzed using paired t-test for comparison of different storage times and temperatures, for which we used the graphical procedure outlined by Bland and Altman.[12]

## RESULTS

To study the effect of storage temperatures on phenytoin levels in serums, 150 tested serums were classified into 3 groups (50) according to their PHT levels which were low, medium and height concentration. The PHT levels tested on days 3, 5 and 7 were from two different storage temperatures.

The effect of temperature on the drug concentration in the low, medium and high level concentrations of 2.5-10,10.1-20.0 and 20.1-30.0 µg/mL, respectively, were studied. We stored at a temperature of 35°C and stored in a refrigerator 2-8°C. Analysis was done on days 3, 5 and 7 and results were compared with the results of the first day. In this example, 50 samples are shown in Table 1.

The results of the analysis showed the relationship between samples collected at the temperatures 35°C and 2-8°C on days 3, 5 and 7, which were compared with the first day of analysis. The results of the drug on days 3, 5 and 7 were tested with a paired t-test and compared with the ones stored at different temperatures are which were found to be statistically significantly different at $p < 0.05$. In order to determine the concordance

**TABLE 1.** Shows the results of the drug phenytoin on days 1, 3, 5 and 7 at concentrations Low, Medium and Height of samples in the refrigerator temperature is 35°C and 2-8°C.

| | Low | | | | | | | Medium | | | | | | | Height | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | day 3 | | day 5 | | day 7 | | | day 3 | | day 5 | | day 7 | | | day 3 | | day 5 | | day 7 | |
| | first day | 35°C | 2-8°C | 35°C | 2-8°C | 35°C | 2-8°C | first day | 35°C | 2-8°C | 35°C | 2-8°C | 35°C | 2-8°C | first day | 35°C | 2-8°C | 35°C | 2-8°C | 35°C | 2-8°C |
| Min | 2.74 | 2.92 | 2.70 | 3.00 | 2.71 | 3.01 | 2.91 | 10.51 | 10.60 | 10.46 | 10.92 | 10.50 | 13.00 | 10.77 | 20.13 | 20.10 | 19.97 | 20.10 | 19.85 | 20.83 | 20.00 |
| Max | 9.78 | 10.07 | 9.73 | 10.24 | 9.75 | 12.15 | 9.84 | 19.81 | 20.12 | 19.76 | 21.12 | 19.82 | 22.14 | 20.06 | 30.00 | 30.14 | 30.00 | 31.14 | 30.00 | 32.00 | 30.20 |
| Mean | 6.23 | 6.42 | 6.24 | 6.91 | 6.25 | 7.92 | 6.31 | 14.88 | 15.09 | 14.89 | 15.65 | 14.90 | 16.74 | 15.04 | 24.56 | 24.82 | 24.57 | 25.34 | 24.59 | 26.28 | 24.68 |
| SD | 2.10 | 2.12 | 2.10 | 2.16 | 2.11 | 2.34 | 2.11 | 2.80 | 2.86 | 2.80 | 2.89 | 2.81 | 2.92 | 2.81 | 3.12 | 3.11 | 3.11 | 3.24 | 3.14 | 3.30 | 3.13 |
| *P*-value | | 0.0000 | 0.3580 | 0.0000 | 0.1050 | 0.0000 | 0.0000 | | 0.0000 | 0.1440 | 0.0000 | 0.0530 | 0.0000 | 0.0000 | | 0.0000 | 0.2740 | 0.0000 | 0.0610 | 0.0000 | 0.0000 |
| r2 | | 0.9961 | 0.9992 | 0.9756 | 0.9986 | 0.9317 | 0.9986 | | 0.9972 | 0.9996 | 0.9690 | 0.9920 | 0.9818 | 0.9993 | | 0.9934 | 0.9992 | 0.9744 | 0.9986 | 0.9331 | 0.9983 |

between the PHT level of day 1 and the PHT level of days 3, day 5, and day 7, the Bland Altman method of analysis was used. The data which were analyzed using Bland-Altman, were consistent on days 3, 5 and 7 compared with the first day. On the X- axis the mean of the PHT level on day 1 and the PHT leveld on days 3, 5, and 7 were plotted and on the Y- axis the differences between the PHT level of day 1 and the PHT levels of days 3, 5, and 7 were plotted. In this plot a line of mean bias and the lines of 95% limits of agreements were present. Bias is the average of differences between the PHT level of day 1 and the PHT levels of days 3, 5, and 7. The majority of the PHT levels were falling within the lines of limits of agreement i.e. ± 2 SD. There were some values were falling outside of ± 2 SD, as shown in Fig 1 and 2.

## DISCUSSION

This study, investigated the effects of temperature on stored samples of PHT in serum, which clearly demonstrated that a increase in concentration must be considered for a storage period of 3 days (at 35°C). Hence the high temperature is not an appropriate condition to store the tested serum caused yielding to abnormally finish. Our results for serum sample represent the need for more increase of initial PHT concentration on the first day in a serum sample. The serum samples, containing PHT at 2-8°C, can be stored up to five days. Interesting results were observed while measuring the remaining amount of PHT in serum sample stored at 2-8°C.

In an in vitro study of PHT binding to plasma proteins, Lunde et al,[13] found that the unbound plasma fraction of PHT determined by the ultrafiltration method, was considerably greater at 37°C than at room temperature. Higher binding under current routine laboratory condition (i.e. room temperature) may be accompanied with changes in the association constant and/or the total concentration of binding sites. Allison et al,[14] showed significant differences in PHT-serum protein binding affinity as a function of temperature. It appears that the differences between different conditions of temperature, determined by

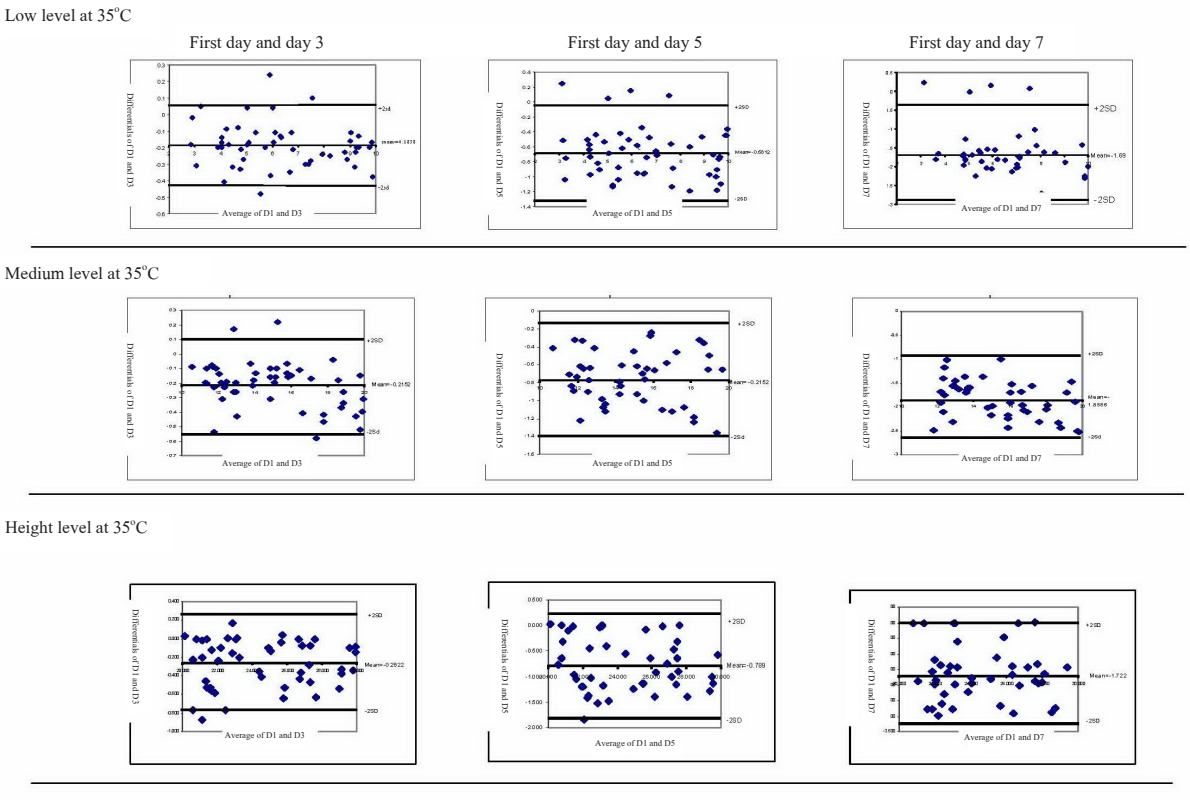


**Fig 1.** Bland-Altman plot showing consistency between Phenytoin serum level analysis of samples at a temperature of 35°C on day 1 to day 3, 5 and 7.

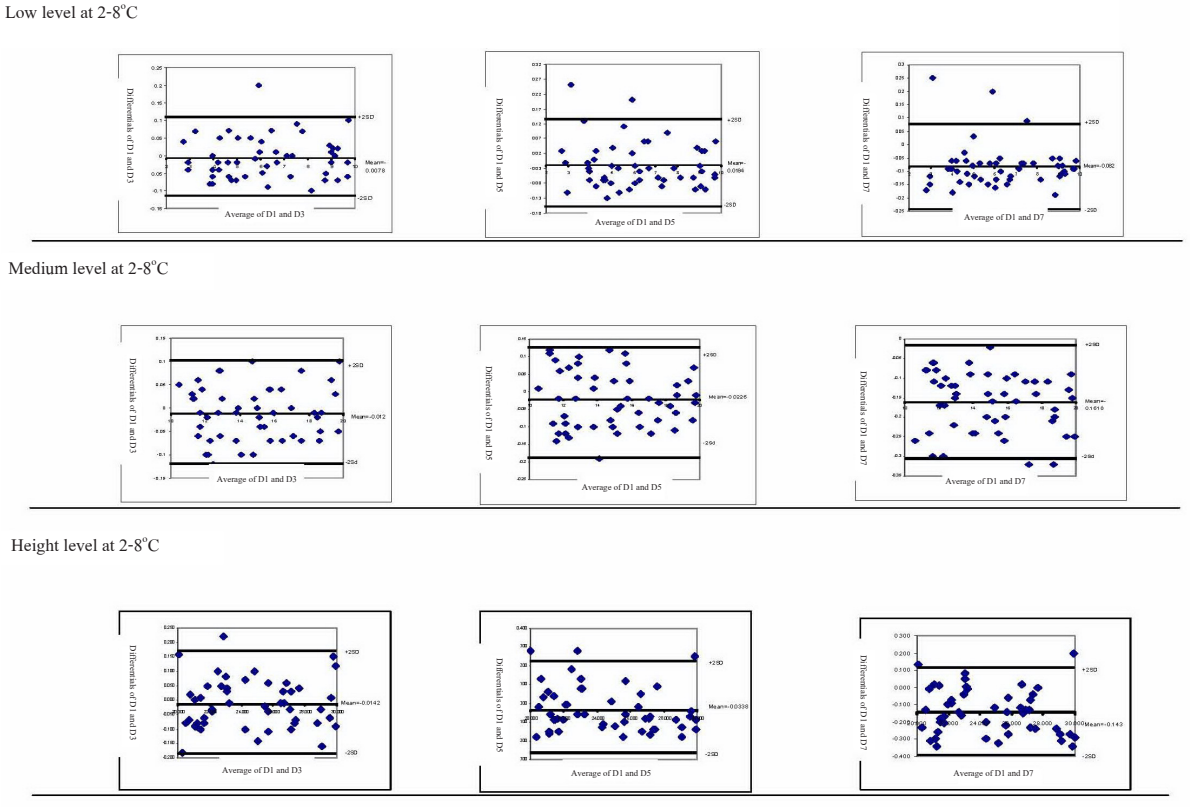


**Fig 2.** Bland-Altman plot showing consistency between Phenytoin serum level analysis of samples at a temperature of 2-8°C on day 1 to day 3, 5 and 7.

ultrafiltration method in the binding affinity of PHT to serum proteins are relatively larger than those in binding capacity, and therefore, effects of temperature on binding kinetics may be greater in binding affinity than in binding capacity.

The results of our study, found that the levels of PHT increased when stored at 35°C and kept long. It is possible that protein degradation occurred.

Figs 1 and 2, show the study comparing the consistency of data with the Bland-Altman plot which found some examples that were inconsistent with the sample group. Albumin may be caused by low level or degeneration, which affects the drug as well.

This study found that the PHT level increased, so the drug is not going to break down. Therefore, the drug level did not decrease and tends to increase when stored for a long time at high temperature. The mechanism of protein degradation should be studied further.

## CONCLUSION

The present investigation covered an observation period of one week, showed that for 2-8°C and 35°C, the time interval between sampling and analysis strongly influences the quantitative determination of phenytoin. The present study also demonstrated that 2-8°C seems to be the best storage temperature with samplesanalysed within 5 days.

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