CHAPTER III

METHODOLOGY

This study was designated as an experimental research and descriptive analysis by the Naresuan University Council on Ethical Certification on 10 February 2009. Sample collection and handling followed universal precautions and biohazard controls.

Material and equipment

1. Chemical agents
   - 1.1 Phenolphthalein
   - 1.2 Potassium Hydroxide
   - 1.3 Distilled Water
   - 1.4 Zinc Dust
   - 1.5 95 % Ethanol
   - 1.6 Hydrogen Peroxide
   - 1.7 Copper plate
   - 1.8 Sodium Hydroxide
   - 1.9 Nickel plate
2. Equipment
   - 2.1 Hot Plate (FINETECH Series SM-10 made in Korea)
   - 2.2 Reflux apparatus
   - 2.3 Auto pipette size 10 µl. and 100 – 1000 µl.(Brand model)
   - 2.4 Transfer pipette made in (Germany)
   - 2.5 Tube 12 x 75 cm.
   - 2.6 Amber bottles volume 1000 ml
   - 2.7 Slides
   - 2.8 Whatman No. 1 filter paper (made in England)
   - 2.9 Aluminum Foil
   - 2.10 Hematology analyzer (Sysmex Company made in Japan)

2.11 Analytical balance (Metter Toledo, Switzerland)

2.12 Reduced phenolphthalein commercial test kit (WARD'S Natural Science)

Population / Sample Collection

A sample collection protocol was provided to each participating hospital and public health center. Each glucose meter and hematology automatic analyzer designated for this study in 20 community hospitals and 100 primary care units of Uttaradit and Pichit Province, Thailand was visually inspected for blood contamination on the outside surface.

Sensitivity of reagent

The sensitivity of the modified reduced phenolphthalein reagent was determined by testing the blood at a dilution between $1:10\text{-}^{1}$ to $1:10^{6}$

Precision of reagent

The precision of the reduced phenolphthalein method was determined by testing a minimum concentration of blood hemoglobin for positive sensitivity. The precision of reduced phenolphthalein is a percentage of reproducibility or repeatability where the repeated measurements are conducted under unchanged conditions and show the same results.

Stability of reagent

A blood sample for a stability test of modified reduced phenolphthalein is the minimum concentration of blood hemoglobin that gave a positive result when tested using a reduced phenolphthalein reagent, tested every month and recorded. Stability of the reduced phenolphthalein is the period of time and remains positive about the heat hemoglobin concentration minimum.

Specificity of reagent

A sample for specificity testing, using substances tested, includes free hemoglobin such as tomato juice, melon juice, detergent, 70%alcohol, sodium, dichoro-S-triazinetrione, red color and cabbage. Whole blood is the positive control and 0.9 % normal saline is the negative control. Specificity measures the proportion of negative results which are correctly identified.

Blood sample degradation

Use of the blood concentration minimum positive during the experiment determines sensitivity by selecting the two lowest concentrations. Quality control includes positive control and negative control. Whole blood is positive control and 0.9 % normal saline is negative control. The blood dilution 10 ul was dropped on a slide and kept at room temperatures for 20 sets, and tested daily for 20 days.

The experiments were divided into 4 sub-studies.

Study 1: Reduced phenolphthalein: Principle, interpretation, and preparations

Principle and interpretation

Blood detection using reduced phenolphthalein

The method for detection of blood contamination by modified reduced phenolphthalein methods is as follows: filter paper Whatman No 1 was dropped with reduced phenolphthalein solution 1-2 drop; then 1-2 drops 3 % hydrogen peroxide, and color changes on the filter paper were observed for 15 seconds. For a positive result the filter paper changes from colorless to pink and for a negative result the filter paper does not change. The positive control and negative control are whole blood and 0.9 % normal saline.

Reduced phenolphthalein preparation

The original Kastle-Meyer method and the modified method were shown in table

Table 1 modified method for reduced phenolphthalein preparations

Methods	Chemical compositions					Heat	Ratio
	Phenolphthalein	Potassium Hydroxide	Sodium Hydroxide	Reducing	Distilled Water		
Original method	2.0 g	20.0 g	-	Zinc dust 20.0 g	100 ml	Reflux 2-3 hr.,*	1:10:50
Method 1	2.0 g	10.0 g	-	Zinc dust 5.0 g	250ml	Boil 2-3 hr.*	1:5:125
Method 2	1.0 g	10.0 g	-	Zinc dust 20.0 g	50 ml	Boil 1 hr*	1:10:50
Method 3	1.0 g	-	10.0 g	Zinc dust 5.0 g	250 ml	Reflux 2-3 hr*	1:10:250
Method 4	2.0 g	20.0 g	-	Zinc dust 20.0	50 ml	Reflux 45 min*	1:10::25**
Method 5	1.0 g	10.0 g	-	Copper 10 .0 g.	250 ml	Boil 2-3 hr*	1:10:250
Method 6	1.0 g	10.0 g	_	Nickel 10.0 g	250 ml	Reflux 2-3 hr*	1:10:250

* Until the solution changes to colorless ** Add Ethanol

Method 1 and 3 prepared by the application from Applied Science BTEC method.

Method 4 prepared by the increasing ratio of phenolphthalein

Study 2: Validations of modified reduced phenolphthalein testing

Sensitivity, precision, specificity, stability, sample degradation, price, and, procedures for reduced phenolphthalein testing were compared among six modified methods and the original method.

1. Sensitivity of modified reduced phenolphthalein reagent:

The EDTA whole blood was diluted in the ratio of1:10^{-1} to 1:10^{-6}. Each dilution at 10 ul was tested with reduced phenolphthalein reagent using a triplicate determination. The positive result was cut off in 2/3 of each dilution. [25, 26]

2. Precision of modified reduced phenolphthalein reagent:

The precision is determined by testing the minimum concentration of blood hemoglobin which responded positively to the sensitivity testing. Twenty measurements each were done within day and between days. The precision was evaluated by standard deviation in the same tested group. [21]

3. Specificity of modified reduced phenolphthalein reagent:

The modified reagent was tested with the sample such as tomato juice, melon juice, detergent (LIPON-F), 70%alcohol, sodium chloride (POSE-AID) and cabbage. The test result should be negative. [25, 26]

4. Stability study of modified reduced phenolphthalein reagent

4.1 Keep at 2- 8 °C in opaque bottles

The modified reduced phenolphthalein was prepared and kept in comber billhead in refrigerate temp 2-8 °C. Finding the maximum day of storing reduced phenolphthalein reagent were prepared by modify form research work at other situations and test result as the first day with dilution was minimum of blood.

5. Degradation of blood sample

The 10 ul whole blood sample 10 was kept on a slide, dried for 20 minutes, then placed in a box and kept at room temperature. The sample was tested every 24 hours for 20 days. [21]

6. Comparison studies of modified reduced phenolphthalein and commercial kit (original method)

The two reagents was tested in 20 samples of fresh blood and old blood (aged 20 days) and results compared using the parameters of sensitivity, precision, specificity, reagent degradation, and sample degradation.

Study 3: Surveys of the user's satisfaction to the new test kit

The new test kits together with the questionnaires were sent to the target laboratory for trials and evaluated the product for 50 laboratories.

Study 4: Survey of blood contamination on glucose meters and hematology analyzers

1. The modified reduced phenolphthalein was used to survey blood contamination in 150 hospitals and 100 Point of Care Unit (PCU) in Uttaradit and Pichit Province, Thailand. After using the reagent, the user must be answering the questionnaire.

2. Blood contamination detection area

The modified reduced phenolphthalein was used to survey blood contamination on glucose meters at 5 positions: the front, side, back, meter storage area and "insert test strip" area. On hematology analyzers the test was done at 4 positions: the keyboard touch screen, start switch, printer, and front panel.

Data analysis

1. The efficiency of the reduced phenolphthalein with respect to sensitivity, precision, specificity, sample degradation, and stability was evaluated using the percentages as calculated according to the following chart.

$$\text{Sensitivity (\%)} = \frac{\text{number of True Positives} \times 100}{\text{Number of True Positives} + \text{number of False Negatives}}$$

$$\text{Specificity (\%)} = \frac{\text{number of True Negatives} \times 100}{\text{Number of True Negative} + \text{number of False Positives}}$$

2. For comparisons of the efficiency of the reduced phenolphthalein (according to sensitivity, precision, specificity) between the modified reduced phenolphthalein and a commercial test kit, the data was analyzed using the Chi-square.

3. Product satisfaction was evaluated using the percentage, mean and standard deviation.

4. Blood contamination on glucose meters and hematology analyzers was evaluated using percentages as presented in the table