CHAPTER 3

METHODOLOGY

3.1 Study design

An analytical cross-sectional study was used in this study to investigate micronutrients status in HIV/AIDS patients who were treated with HAART and to compare micronutrients status between HIV/AIDS patients and healthy group.

3.1.1 Sample size, definition and calculation

3.1.1.1 Study population

The target population of this study was HIV-infected adult males and females who attended at the Maharaj Nakorn Chiang Mai Hospital and were treated with GPO-vir antiretroviral drug between March-September 2005. The healthy subjects were selected from the Blood Bank unit of hospital and from the staffs at the Research Institute for Health Sciences, Chiang Mai University.

3.1.1.2 Sample size

124 HIV/AIDS subjects were treated with GPO-vir antiretroviral drug.

The sample size of HIV/AIDS subjects in this study was calculated by Yamane formula [84] as follow;

$$n = \frac{N}{1 + N(e)^2}$$

$$n = \frac{448}{1 + 448(0.10)^2}$$

$$n = 81.75$$

$$n \cong 82 \text{ subjects}$$

Where n is the sample size, N is the population size and e is sample error (10%). 124 HIV-infected subjects were selected proportional of the place where HIV-infected subjects admitted, regarding incomplete data.

HIV/AIDS patients were recruited from NAPHA project, social security fund and group of self payment and number of the subjects in each group was calculated as shown in Diagram 1.

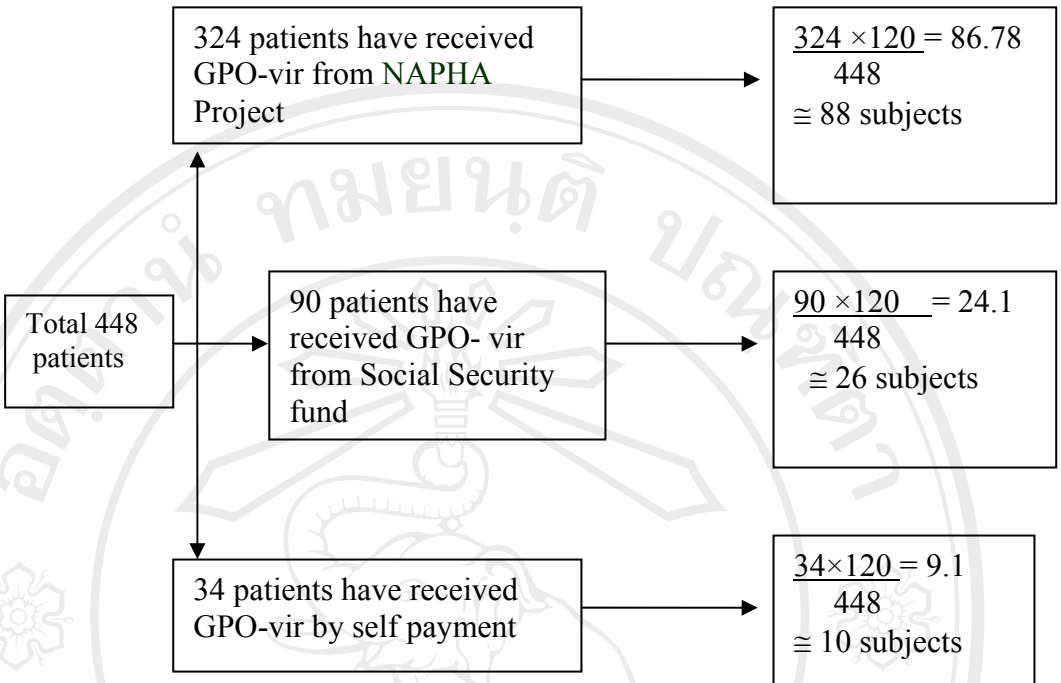

Diagram 1

Each group of subjects was selected by using CD4+ T cell counts < and ≥ 200 cell/mm^3 as follow diagram 2.

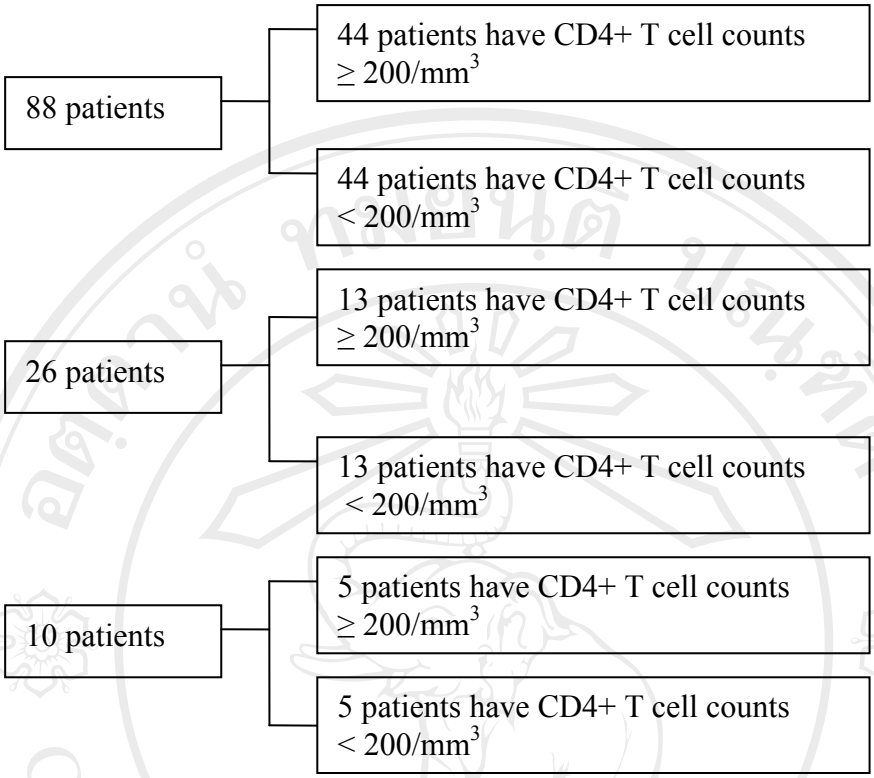

Diagram 2

3.1.2 Subjects inclusion criteria

- Adult males and females (non – pregnancy)
- No clinical diagnosis of liver disease
- No pyrexia (>38 °C) who were receiving treatment for acute opportunistic infection
- Not on therapy for tuberculosis
- No vitamin and mineral supplement

3.1.3 Healthy subjects

A total of 61 healthy subjects were recruited in this study. There were 51 subjects from Blood Bank unit of Maharaj Nakorn Chiangmai Hospital and 10 subjects from Research Institute for Health Sciences by matching sex and age with HIV/AIDS subjects (Ratio 1:2).

3.2 Materials and Methods

3.2.1 Subject characteristics and food frequency dietary assessment

All study subjects were interviewed using questionnaire. The questionnaire composed of two parts:

Part I : Characteristics data.

Part II : Food consumption behavior.

3.2.1.1 Content validity and reliability

The questionnaires were evaluated content validity by Dr. Sakda Pruenglampoo and Mrs.Somluck Nimsakul (MCN), researchers, Research Institute for Health Sciences, Chiang Mai University.

The questionnaire, part of food consumption behavior, was pre-tested in outside target area by interviewing 24 HIV/AIDS patients who were receiving GPO-vir treatment in Sanphatong Hospital, Chiang Mai. The questionnaires

were analyzed for reliability using Cronbach's Alpha coefficient by SPSS program. The reliability result was 0.87. The details were showed as Appendix B to D.

3.2.1.2 Frequency dietary assessment

Food frequency questionnaires (FFQ) consisted of 5 food sources of vitamin A, E, B12, zinc and selenium. The food source of vitamin A, vitamin E, vitamin B12, zinc and selenium contained 26, 5, 10, 17 and 9 items, respectively. Frequency of food intake was obtained from 1 week recall. The subjects were interviewed to describe food intake of each item from each food source. The frequencies of food intake were divided into 5 categories as follow;

0 times/week = 0 score

1-5 times/week = 1 score

6-10 times/week = 2 scores

11-15 times/weeks = 3 scores

16-21 times/weeks = 4 scores

The total scores of each food sources were calculated as percentage of total scores.

3.2.2 Serum collection

Ten mL of whole blood were collected into vacuum tube wrapped with aluminum foil. The blood samples were promptly transported to the laboratory and left for 30 minute at room temperature for allowing the blood to clot. Sera were separated by centrifugation at 3,000 rpm for 10 minutes at 4°C and divided into 1.5 mL of micro centrifuge tubes as followed; 1.2 mL for zinc, 1.2 mL for selenium, 0.5 mL for vitamin B12 and 0.6 mL for vitamin A and vitamin E. All specimens were stored at - 20 °C prior to analysis.

Micronutrients status: Vitamin A, E, B12, zinc and selenium deficiency were defined as [80]:

Vitamin A < 0.3 μg/mL	Zinc < 0.75 μg/mL
Vitamin E < 5 μg/mL	Selenium < 85 μg/L
Vitamin B12 < 200 pg/mL	

3.2.3 Laboratory methods

3.2.3.1 Determination of vitamin A and vitamin E in serum by high performance liquid chromatography (HPLC)

The procedure of validation method was performed in Appendix G [85].

3.2.3.1.1 Quality control of serum vitamin A analysis

(1) Accuracy

The accuracy of the method was perform by recovery study and expressed as % recovery**.** The recoveries data from this method were ranged from 91 to 97 %. These results were recommended to be performed at the 80 to 110% of label claim as The AOAC manual for the Peer Verified Methods program [86] with estimated analyte concentration at ppm (part per million) level. Detail of results showed as Appendix G, article 5.3.

(2) Precision

The precision was determined through the repeated analysis of the pool serum, both intra and inter assay precision. The coefficients of variation (%CV) were calculated by replicating extraction and analysis of the control serum pools. Intra-assay variation was determined regularly by analyzing the pool serum ten times on the same day. For inter-assay variation, two injections of the pool serum control per day were carried out. The results show in Table 3.1.

Table 3.1 Inter-assay and intra-assay of serum retinol concentration in pool serum

	Intra-assay (n = 10)		Inter-assay (n = 13)	
	Mean ± SD (µg/mL)	% CV	Mean ± SD (µg/mL)	% CV
Pool serum	0.580 ± 0.030	4.87	0.607 ± 0.033	5.44

The precisions of this analysis method were recommended to be performed at the 5-11% of label claim as The AOAC manual for the Peer Verified Methods program [86] with estimated for biological samples at 1-100 ppm unit. Detail of results showed as Appendix G, article 5.4.

3.2.3.1.2 Quality control of serum vitamin E analysis

(1) Accuracy

The recoveries data from this method were a range 97-101 %. These results were recommended to be performed at the 80-110% of label claim as The AOAC manual for the Peer Verified Methods program [86] with estimated concentration at ppm (part per million) unit. Detail of results showed as Appendix G, article 8.3.

(2) Precision

The precision was determined through the repeated analysis of the pool serum, both intra and inter assay precision. The coefficients of variation (%CV) were calculated by replicating extraction and analysis of the control serum pools. Intra-assay variation was determined regularly by analyzing the pool serum ten times on the same day. For inter-assay variation, two injections of the pool serum control in each assay. The results show in Table 3.2.

Table 3.2 Inter-assay and intra-assay of serum α-tocopherol concentration in pool serum

	Intra-assay (n = 10)		Inter-assay (n = 17)	
	Mean ± SD (μg/mL)	% CV	Mean ± SD (μg/mL)	% CV
Pool serum	9.12 ± 0.43	4.73	8.97 ± 0.49	5.49

The precisions of this analysis method were recommended to be performed at the 5-11% of label claim as The AOAC manual for the Peer Verified Methods program [86] with estimated for biological samples at 1-100 ppm unit. Detail of results showed as Appendix G, article 8.4.

3.2.3.2 Determination of vitamin B12 in serum by Elecsys® vitamin B12 immunoassay

The procedure of the method was showed in Appendix I [87].

3.2.3.2.1 Quality control of serum vitamin B12 analysis

The quality control of this method employed to monitor accuracy and precision by PreciControl Universal 1 lot 165476 control material and the mean was 1,010 pg/mL (range 707-1,313 pg/mL) and PreciControl Universal 2 lot 165474 control material and the mean was 492 pg/mL (range 300-684 pg/mL). The precision was determined through the repeated analysis of PreciControl Universal 1 and 2, both intra and inter assay and calculating the %CV. Intra-assay variation was determined regularly by analyzing each the controls ten times on the same day. For inter-assay variation, the PreciControl Universal 1 and 2 were analyzed in each assay. The results show in Table 3.3.

Table 3.3 Inter-assay and intra-assay of serum vitamin B12 concentration in PreciControl Universal control material

	Intra-assay (n = 10)		Inter-assay (n = 6)	
	Mean ± SD	% CV	Mean ± SD	% CV
PreciControl Universal 1	1,021.9 ± 12.84	1.25	985.6 ± 68.0	6.90
PreciControl Universal 2	494.32 ± 12.88	2.60	491.4 ± 65.15	13.25

(Detail of results showed in Appendix I, article 3)

3.2.3.3 Determination of zinc in serum by flame-atomic absorption spectroscopy

The procedure of the method was performed in Appendix K.

3.2.3.3.1 Quality control of serum zinc analysis

Accuracy and precision of this method was monitor by using Lyphochek® Assay Chemistry Control, the mean was 0.73 µg/mL, range 0.58-0.87 µg/mL. The precision was determined through the repeated analysis of Lyphochek® Assay Chemistry Control, both intra and inter assay and calculating the %CV. Intra-assay variation was determined regularly by analyzing the Lyphochek® Assay Chemistry Control twenty times on the same day. For inter-assay variation, duplicates extraction of

the Lyphochek® Assay Chemistry Control in each assay were made with a total of fifteen assays (5 days). The results show in Table 3.4.

Table 3.4 Inter-assay and intra-assay of serum zinc concentration in Lyphochek® Assay Chemistry Control

	Intra-assay (n = 20)		Inter-assay (n = 15)	
	Mean ± SD	% CV	Mean ± SD	% CV
Lyphochek® Assay Chemistry Control	0.82 ± 0.03	3.66	0.86 ± 0.03	3.26

The precisions of this analysis method were recommended to be performed at the 5 % for zinc using atomic absorption spectroscopy.

(Detail of results showed as Appendix K, article 5)

3.2.3.4 Determination of selenium in serum by graphite-AAS

The procedure of the method was performed in Appendix L.

3.2.3.4.1 Quality analyze of serum selenium by GF-AAS

Accuracy and precision of this method was monitor by using Seronorm™ Trace elements serum, mean was 81 µg/mL (range 78-84 µg/mL). The precision was determined through the inter assay precision of Seronorm™ Trace elements and calculating the %CV. Inter-assay variation, injection of the

Seronorm™ Trace elements in each assay were made with a total of twenty - one assays (7 days), every tenth sample was analyzed to inserted with the control serum. The result shows in Table 3.5.

Table 3.5 Inter-assay of serum selenium concentration in Seronorm™ Trace elements serum

	Inter-assay (n = 21)	
	Mean ± SD	% CV
Seronorm™ Trace elements serum	80.41 ± 1.86	2.31

(Detail of results showed in Appendix L, article 5)

3.3 Statistical Analysis

The data were analyzed by using SPSS statistical program for window (version 11.0) and were presented as mean ± SD for continuous variable and percentages for discrete variable. Subjects were grouped by HIV status (HIV-infected and healthy subjects), stage of HIV disease as indicated by CD4+ T cell counts (< 200 and ≥ 200 cells/mm^3). Mean concentrations of each micronutrient (micronutrients consist of vitamin A, vitamin E, vitamin B12, zinc and selenium) was compared by using student's t-tests for normal distribution data and Mann-Whitney rank sum test was used for data without normal distribution. Proportions of category data were compared by using the Chi-square test.