III.MATERIALS AND METHODS

A. Materials

[1.] Serum base materials :

Bovine sera were obtained from the slaughterhouse, Chiang Mai Municipality. Human sera were obtained from Clinical Chemistry Laboratory, Central Laboratory Division, Maharaj Nakorn Chiang Mai Hospital.

[2.] Instruments :

- Adjustable automatic pipette, DLALAB p10, p100, p1000 (DLALaboratory, Switzerlands)
- Analytical balance, Mettler H10 (Mettler Instrument, Switzerlands)
- Centrifuge (Kokusan, Japan)
- Clinical chemistry autoanalyzer (Synchron CX5, Beckman, USA)
- Desiccator
- Incubator, SL Shel Lab Model 2005 (Sheldon Manufacturing Inc., USA)
- Lioalfa-10 (Telstar, Spain)
- Magnetic stirrer (Thermolene, USA)
- pH Meter, Model 3560 (Darmstadt, Beckman, USA)
- Electrophoretic Set Apparatus (Biorad Laboratory, USA)
- Power supply (Darmstadt, Beckman, USA)
- Toploading balance
- UV-Visible Double Beam Spectrophotometer UV-160A (Shimadzu Co., Japan)
- Vortex mixer, Scientific Industries (New York, USA)
- Water bath (Hetobirkerod, Denmark)

[3.] Chemicals and Reagents :

Chemicals and reagents used were the analytical grade unless otherwise stated.

- Acetic acid (Merck, Darmstadt, Germany)
- Acetone (Merck, Darmstadt, Germany)

- Acrylamide (Sigma Chemical Co., USA, No. A-8887)
- Albumin, Bovine, Fraction V (Sigma Chemical Co., USA, No. A-4503)
- Ammonium persulfate crystal : APS (J.T. Baker Inc., USA)
- Ammonium sulfate (Merck, Darmstadt, Germany)
- Brilliant blue R-250 (Biorad Chemical Company, USA)
- Bromophenol blue
- Calcium chloride anhydrous (Sigma Chemical Co., USA, No. C-4901)
- Dextran (Sigma Chemical Co., USA, No. D-4133)
- Dextran (Sigma Chemical Co., USA, No. D-9260)
- Diethylaminoethyl Sephacel : DEAE-Sephacel (Sigma Chemical Co., USA, No. I-6505)
- Dithiothreitol (Sigma Chemical Co., USA, No. D-9779)
- Ethanol (Merck, Darmstadt, Germany)
- Ethylenediaminetetraacetic acid : EDTA (Fluka Chemie AG, CH-9470)
- Glycerol (Biomedicals Inc., USA)
- Glacial acetic acid (Merck, Darmstadt, Germany)
- Glycine (Sigma Chemical Co., USA, No. G-7126)
- Heparin lithium salt (Sigma Chemical Co., USA, No. H-0878)
- Hydrochloric acid (Merck, Darmstadt, Germany)
- Lactate dehydrogenase (ICN Biochemicals,USA,No.1511532)
- D-Mannitol (Sigma Chemical Co., USA, No. M-4125)
- 2-mercaptoethanol (Sigma Chemical Co., USA, No. M-6250)
- Methanol (Merck, Darmstadt, Germany)
- N-Butanol (Merck, Darmstadt, Germany)
- N,N'-Methylene-bis-acrylamide (Sigma Chemical Co., USA, No. M-7256)
- N,N,N',N'-Tetramethyl-ethylene diamine : TEMED (Sigma Chemical Co., USA, No. T-8133)
- Saccharose (Merck, Darmstadt, Germany)
- Sodium chloride (Merck, Darmstadt, Germany)
- Sodium dodecyl sulfate (Sigma Chemical Co., USA, No. L-5750)
- Sodium hydroxide (Merck, Darmstadt, Germany)
- Trehalose (Sigma Chemical Co., USA, No. T-5251)
- Tris [hydroxymethyl] aminomethane (Sigma Chemical Co., USA, No. T-1503)

B. Methods

Part I. Preparation of materials used to fortify batches of control serum.

Enzyme concentrates

[1.] Alkaline phosphatase, ALP (Browning, et al., 1986)

The 20 g of pig kidney was homogenized using 2 mL of distilled water per g tissue, then n-butanol is added 1 mL per g tissue and stirred for about one hour. After centrifugation the aqueous layer is fractionated with acetone at $4^{O}C$, the precipitate formed between 33% and 50% (v/v) acetone is retained, dissolved in 0.01 mol/L tris-HCl buffer, pH 7.7 and dialysed overnight against the same buffer. The average yield is 52 units per g tissue and the ALP:AST ratio is greater than 57:1 and ALT activity is undetectable.

Every enzyme are distributed in small vials and stored at $-20^{O}C$ until required.

[2.] Alanine aminotransferase, ALT (Browning, et al., 1986)

The 50 g of pig heart was homogenized in 0.05 mol/L tris-HCl buffer, pH 7.0 containing 10 mmol/L 2-mercaptoethanol and 10 mmol/L EDTA; 3 mL buffer being used per g of tissue. After centrifugation, the supernatant is placed in a water-bath at $60^{O}C$, brought to $52^{O}C$, then rapidly cooled in an ice-bath. The heat-treated sample is fractionated with ammonium sulfate at $4^{O}C$. The precipitate formed when a further 16 g ammonium sulfate per 100 mL are added contains most of the ALT activity and should be separated, dissolved in and dialysed overnight against 0.015 mmol/L tris-HCl buffer, pH 7.25 containing 10 mmol/L 2-mercaptoethanol and 2 mmol/L EDTA. The supernatant from this latter step is retained for AST preparation. The average yield is 14 units per g tissue and the ALT:AST ratio is greater than 4.5:1. The ALP activity in this preparation is negligible.

[3.] Aspartate aminotransferase, AST (Browning, et al., 1986)

A further 14 g ammonium sulfate per 100 mL were added to the supernatant from the ALT preparation. The precipitate formed contains

most of the AST activity. This precipitate is dissolved in and dialysed against 0.015 mol/L tris-HCl buffer to remove ammonium sulfate and is then suitable for addition to quality control material. The average yield is 25 units per g tissue and the AST:ALT ratio is around 25:1.

[4.] Lactate dehydrogenase, LDH (Pridgar, et al., 1984)

LDH was purified from bovine serum by using DEAE-Sephacel chromatography. 70 mL bovine serum was applied to top of a 1x20 cm DEAE-Sephacel column, which had been equilibrated with 20 mmol/L tris-HCl (pH 7.4) containing, per liter, 1 mmol of EDTA, and 2 mmol of dithiothreitol buffer. After that, 50, 100 and 140 mmol/L NaCl in 20 mmol/L tris-HCl (pH 7.4) containing, per liter, 1 mmol of EDTA, and 2 mmol of dithiothreitol buffer was added accordingly to give final elute LDH-3, LDH-2, and LDH-1 from the column. Four milliliters fraction was collected and measured the absorbance at 280 nm for protein determination using a Shimadzu UV-160A spectrophotometer. After the fractions were pooled, LDH activity was measured and concentrated for further used.

Cholesterol concentrate (Terlingen, et al., 1985)

Mix 400 mL of 25 mmol/L calcium chloride solution with 8 mL of 20 g/L lithium heparin solution. Add 80 mL of human serum at room temperature and mix. A precipitate forms immediately. After at least 5 minutes, centrifuge the solution at 1500 x g for 20-30 minutes. Discard the clean supernate. The precipitate containing the beta-lipoproteins dissolves readily in human serum; the cholesterol concentration obtained depends on the volume of serum used to dissolved the precipitate.

Part II. Selection of a suitable excipient for used as stabilizer.

[1.] The effect of excipient on concentrations of proteins, enzymes and lipid components in control serum.

A bovine pool serum previously assayed for total protein (TP), albumin, AST, ALT, ALP, CK, LDH, cholesterol, Tg, HDL-C and LDL-C was aliquot into eight 100 mL pools. The excipient, either trehalose (T), saccharose (S) or mannitol (M) was added to each pool to make a final concentrations of 5 and 10%. The dextran (D) was added in the other to separated pool to make a final concentrations of 1 and 5% respectively. All pools were mixed and assayed for constituents, which described above by using a Beckman CX-5 Autoanalyzer. Results were compared with the bovine serum without adding of excipient. The schematic of the preparation and evaluation of bovine serum pools was shown in Figure 1.

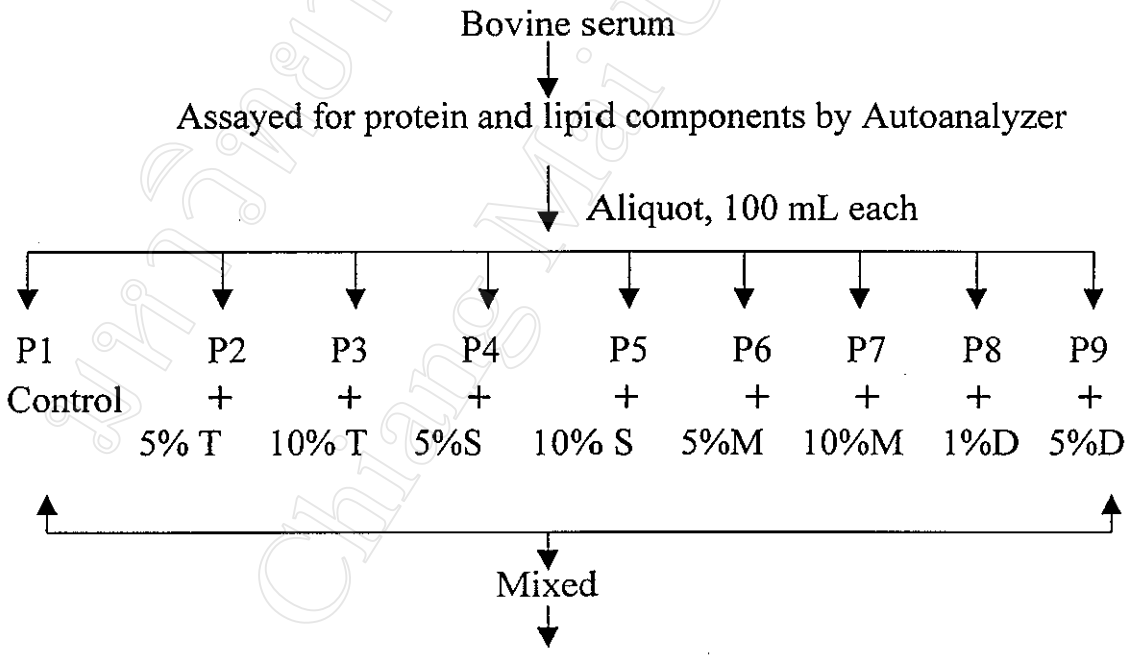

Figure 1. Schematic of the preparation and evaluation of bovine serum pools containing various concentration of each kind of excipient. T, S, M and D are trehalose, saccharose, mannitol and dextran, respectively. P = pooled serum.

[2.] The effect of lyophilization on protein and lipid components, enzyme activities and turbidity of the bovine base matrix control serum containing excipient(s).

[2.1.] Comparisons of the effect of excipient(s) on the concentrations of analytes in lyophilized control sera.

For selecting a suitable excipient, which would be added in control serum, four saccharide excipients were compared for stabilizing effect on protein and lipid components in prepared control sera.

Comparison between saccharose and mannitol.

Eight identical pools of bovine serum (100 mL/each pool) were added with various concentrations of 3.4, 6.2, 8.5 and 17% saccharose (or 100, 180, 250 and 500 mmol/L) and 1.8, 3.3, 4.6 and 9.2% mannitol (or 100, 180, 250 and 500 mmol/L), respectively (Figure 2). The solutions were mixed and then aliquot into 5 mL vials and lyophilized in a Lioalfa-10 for 24-hour cycle. The lyophilized bovine sera were reconstituted with distilled water and analysed for protein and lipid compositions by a Beckman CX-5 Autoanalyzer. Results of each analytes in each pool containing excipient were compared with the control without adding of any excipients.

Comparison of saccharose, trehalose and dextran.

The experiment was carried out the same way as described above but three different excipients in different concentrations were added in six identical pools of bovine serum. As shown in Figure 3, saccharose was used at 1, 5 and 8.5%, trehalose at 1 and 5% and dextran at 1%, respectively. All results obtained after reconstitution were compared with bovine serum without adding of any excipients.

Effect of combinations of excipients on protein and lipid components in lyophilized control serum.

Control sera containing a mixture of disaccharide and dextran were prepared to determine the stability during lyophilized process and storage effect of the excipients. The diagram of preparation was shown in Figure 4. All pools were mixed thoroughly, aliquot 5 mL into vials and lyophilization in a Lioalfa-10 for 24-hour cycle. The lyophilized control

sera containing two different excipients were reconstituted with 5 mL distilled water prior to analysis for protein and lipid constituents using a Beckman CX-5 Autoanalyzer.

[2.2.] The effect of lyophilization on turbidity of the bovine base matrix control sera containing excipient(s).

The effect of lyophilization on turbidity of reconstituted sera were also determined. The clarity of reconstituted control sera were determined by reading the absorbance at 620 nm (Kanluan, et al., 1992) in a Shimudzu UV-160A Spectrophotometer, compared with the liquid control before lyophilization.

The excipient and it's concentration in the prepared control sera were evaluated. Any one which added in and gave the most clarified pool in a reconstituted control serum and that have no effect on analytical concentrations of proteins and lipids, as compared with the previous concentrations analyzed in liquid control, would be selected. The effect of excipients (mostly disaccharides) on glucose determination were also investigated as well as the other components which significant for help diagnosis of diseases. Results were compared and the suitable excipient which gave the analytical results closed to the propose values was selected to use for preparation of two level control sera.

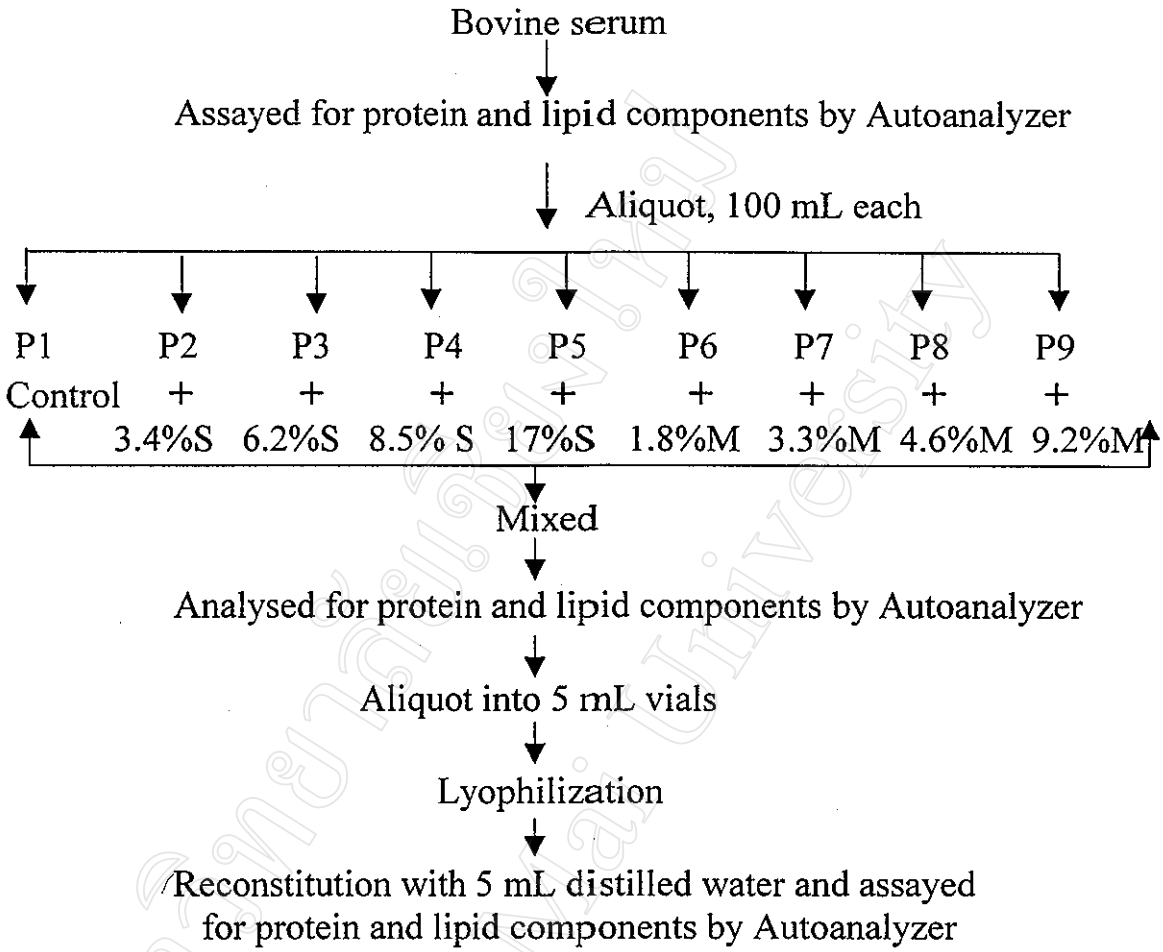

Figure 2. Preparation of bovine serum pools containing various concentrations of each kind of excipient. S and M are saccharose and mannitol, respectively. P = pooled serum.

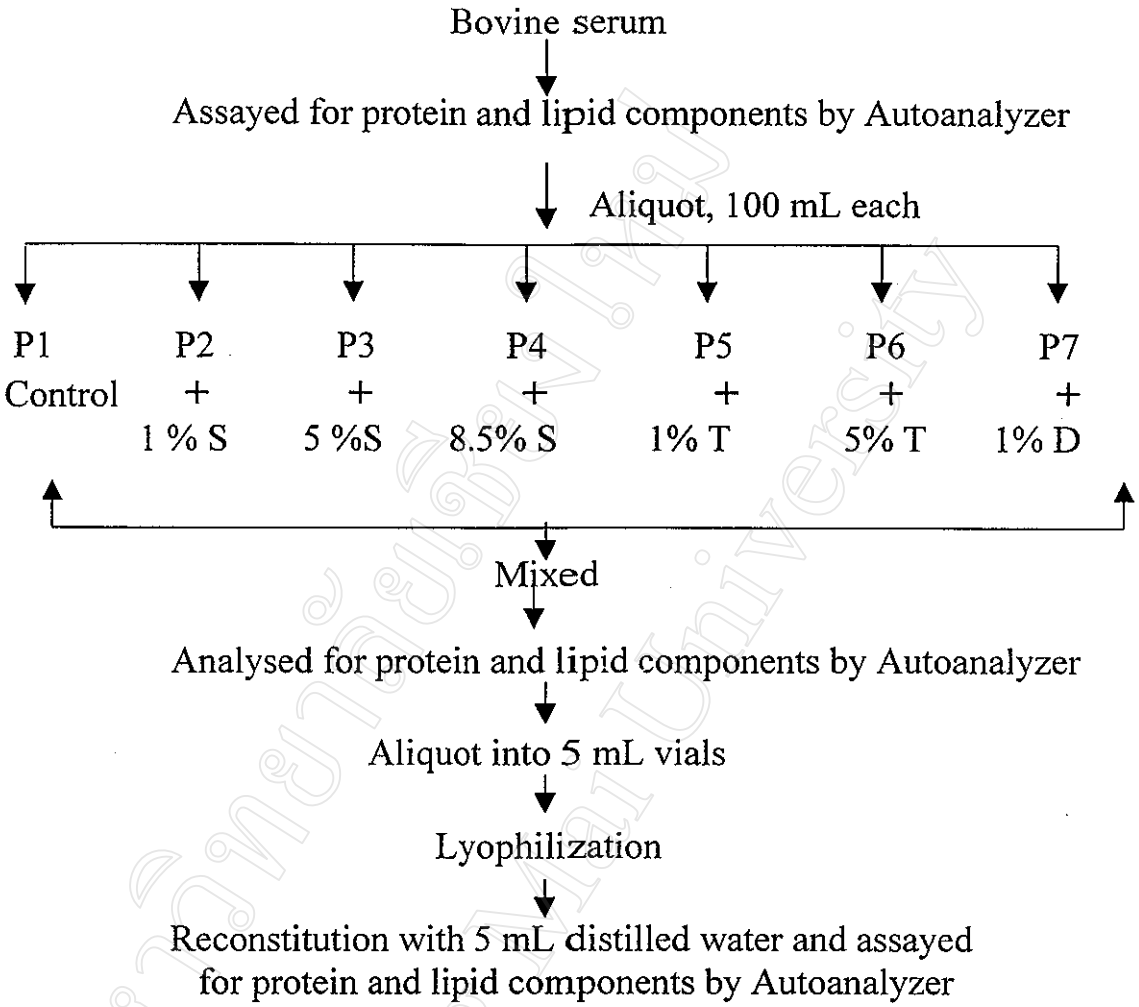

Figure 3. Preparation of bovine serum pools containing various concentrations of each kind of excipient. S, T and D are saccharose, trehalose and dextran, respectively. P = pooled serum.

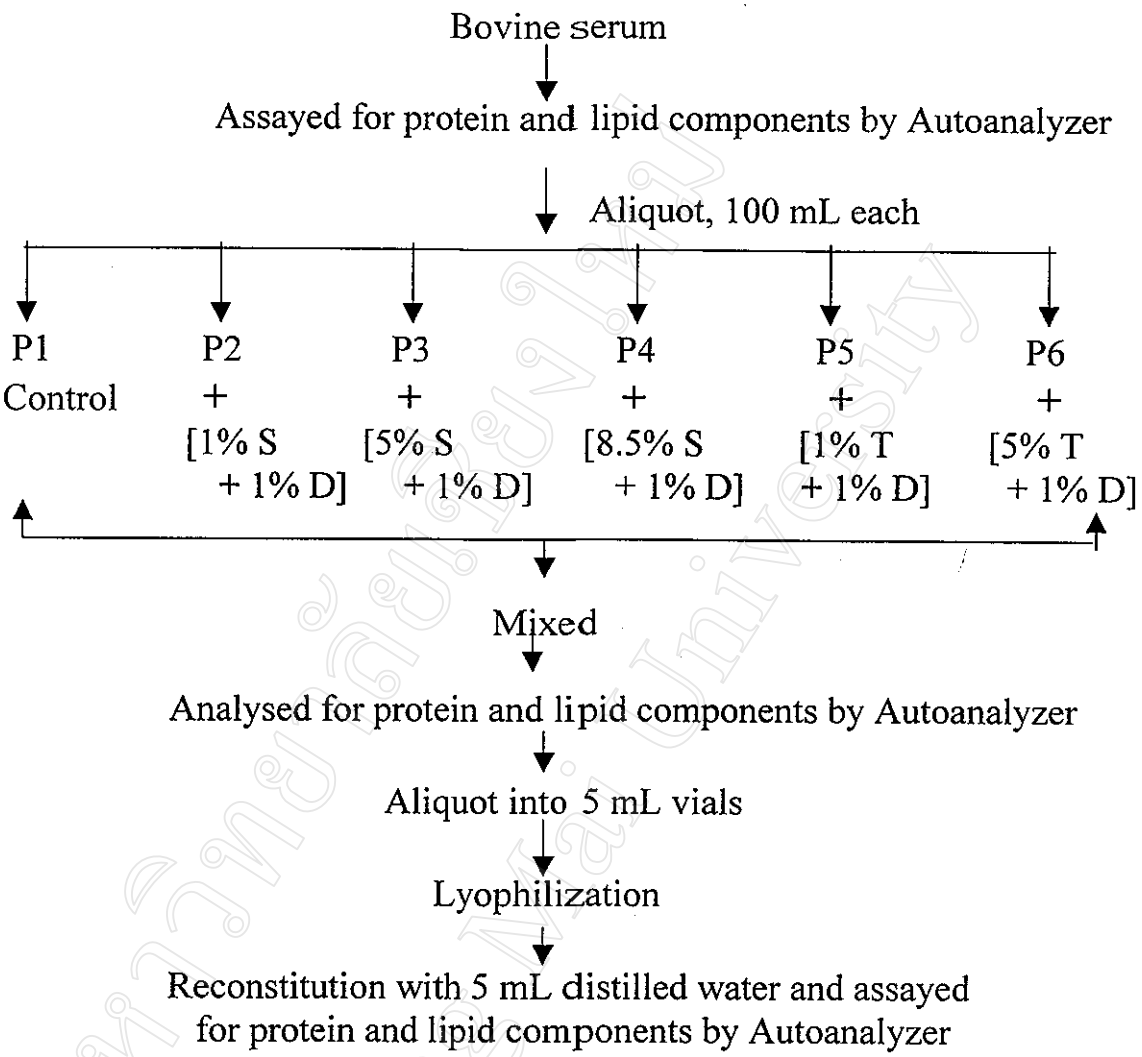

Figure 4. Preparation of bovine serum pools containing various combinations of excipients. S, T and D are saccharose, trehalose and dextran, respectively. P = pooled serum.

[3.] The effect of excipients on the cholesterol rich control sera containing selected excipient(s).

[3.1.] The effect of excipient(s) on cholesterol and other component concentrations in control serum.

A bovine pool serum having cholesterol and lipid profile concentration as shown in Table 2 was used as a matrix for preparation of a cholesterol rich control serum. Method of preparation was to add 12 mg/mL concentrated cholesterol extract (See Method Part I) into the base pooled serum. After mixing, this control serum, which then assayed for protein and lipid profile concentration and turbidity was added with a selected excipient or combinations (See Figure 5) and aliquot into 5 mL vials and lyophilization. The concentrations of proteins and lipids and clarity of the reconstituted control serum containing excipients were examined comparatively with the controls i.e. the liquid control pool before lyophilization and the reconstituted control serum without adding of excipients.

[3.2.] The effect of excipient(s) on turbidity of reconstituted control serum.

The effect of various concentrations of saccharose and 1% dextran on turbidity of control serum specimens were determined by reading the absorbance at 620 nm using a Shimadzu UV-160A Spectrophotometer.

Table 2. Concentrations of protein and lipid components before and after adding cholesterol concentrate.

Test	Bovine serum before adding chol*	Bovine serum after adding chol*
TP (g/dL)	5.7	5.5
Albumin (g/dL)	3.6	4.3
AST (U/L)	50	81
ALT (U/L)	33	42
ALP (U/L)	68	59
CK (U/L)	153	164
LDH (U/L)	344	364
Cholesterol (mg/dL)	138	238
Tg (mg/dL)	61	350
HDL-C (mg/dL)	28	25
LDL-C (mg/dL)	98	144

* Chol = Cholesterol concentrate

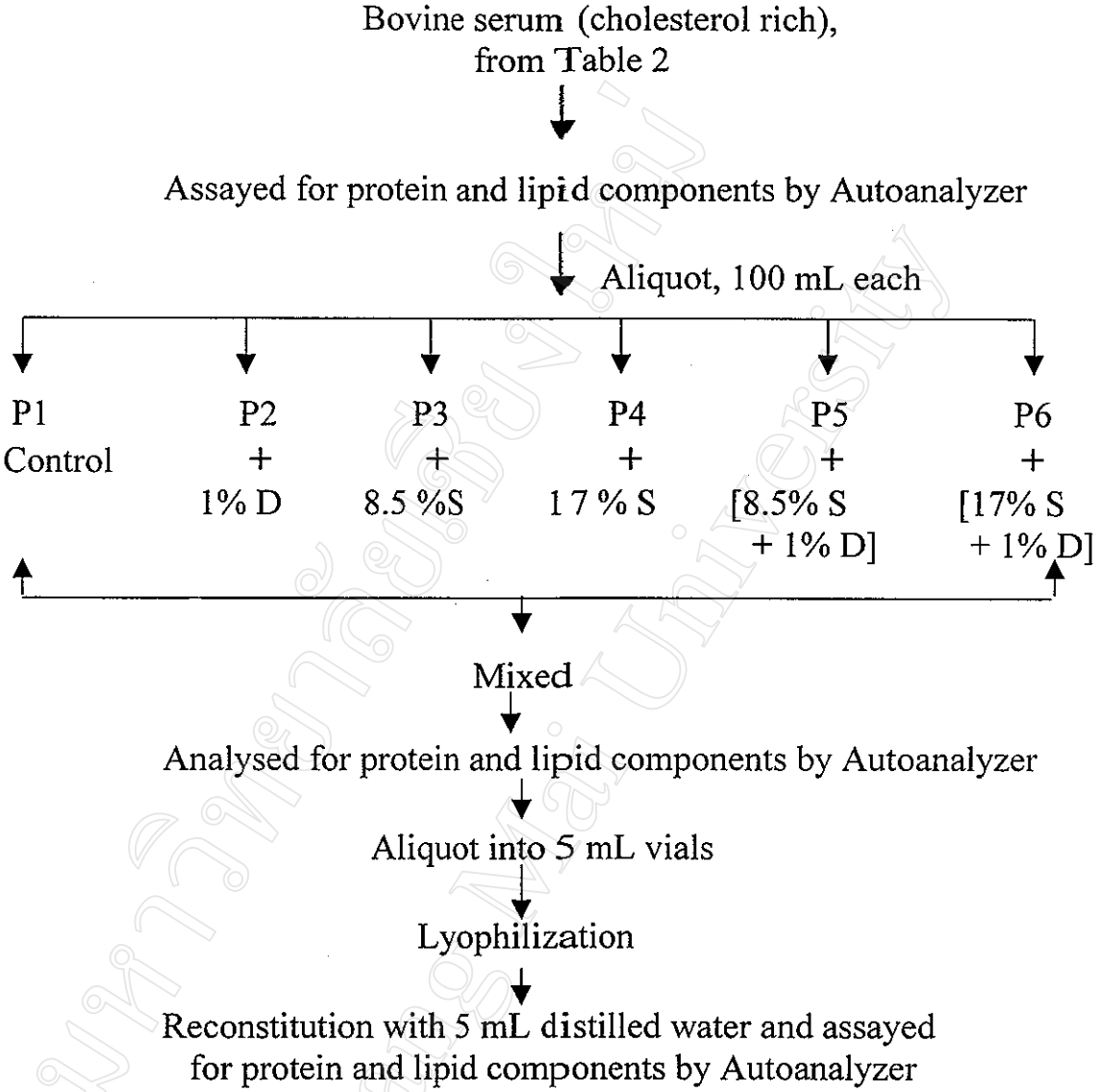

Figure 5. Preparation of cholesterol rich bovine serum pools containing various combinations of excipients; S and D are saccharose and dextran, respectively. P = pooled serum.

[4.] The effect of excipient on stability of protein and lipoprotein in control sera by electrophoretic technique.

To investigate the stabilizing effect of excipient on protein and lipoprotein in control sera during lyophilized process. Total protein and lipoproteins were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel using the procedure which was summarized as follows.

1. Preparation of polyacrylamide gel for Hoefer® mivi-VE electrophoresis.

The separating gel concentration 7.5% was filled into gel sandwich until desired level, the gel was then allowed to polymerize 30-60 minutes at room temperature or until interface appeared. The stacking gel concentration 3.75% was filled on top of separating gel. After that, inserted comb into stacking gel and allowed gel to polymerize 30-60 minutes.

2. Sample preparation.

Total protein electrophoresis; reconstituted lyophilized control serum was loaded in a native form.

Lipoprotein electrophoresis; a 250 μL of reconstituted lyophilized control serum was mixed with 25 μL of dextran sulfate lipoprotein precipitating solution. The mixture was allowed to stand for 2-3 minutes, centrifuged at 2,500 rpm for 10 minutes and the supernate was discard. The precipitate was washed with 100 μL distilled water and dissolved with 20 μL distilled water.

For loading, 20 μL of sample was mixed with 50 μL of sample buffer containing tracking dye (see Appendix I). After the electrophoresis working buffer was added to the tank, the sample solution was gently loaded on the stacking gel surface using a submerged technique.

3.Electrophoresis.

Gels were run at a constant voltage of 200 V for 45 minutes. The position of the tracking dye which monitor the progress of the run was check after 5 minutes. Electrophoresis was carried out until the tracking dye reached the bottom of the gel.

4. After electrophoresis.

The gel was removed and stained with Brilliant blue R-250 for 9 hours. Destaining the gel was performed by immerging the gel in the destaining solution I for 15 minutes, following by the destaining solution II until the background of the gel was clear.

Part III. Preparation of the two level control sera.

Bovine sera were collected for used as a starting material (excluded lipemic, hemolyzed and icteric sera). Excess human sera from routine clinical chemistry laboratory at Maharaj Nakorn Chiang Mai hospital were also screened by the above criteria and collected for used as a reference material. The sera were pooled and kept frozen at $-20^{o}C$ until used.

For preparation, frozen pooled sera were thawed, assayed for protein and lipid components before adjusting the concentrations of analytes by adding various protein, enzymes and cholesterol standards until the target values in two levels (normal and abnormal control sera) were obtained. Then the selected excipient was added into both pools, mixed and aliquot 5 mL into vials. The prepared control sera were lyophilized in a Lioalfa-10 (Telstar, Spain). Reconstitution of the lyophilized sera were made by addition of 5 mL distilled water. Methods for preparations of two level bovine control sera were summarized in Table 3 and 4.

Table 5 and 6 showed the methods for preparations of two level human base control sera which used as reference material in evaluation of the lyophilized preparation of bovine base control sera.

Table 3. Preparation of bovine control serum (level I*).

Analyte	Conc/ 100mL	Conc/ 500 mL	Target/ 100mL	Target/ 620 mL	STD added in	Conc of STD used	Quantity of STD added
TP	6.8	34	6.4	39.68	NA**		
Albumin	4.1	20.5	4.2	26.04	5.54 g	solid	5.54 g
AST	5	25	3.5	21.7	- U	500 U/mL	NA
ALT	3	15	2.5	15.5	0.5 U	200 U/mL	0.0025 ml
ALP	9	45	9	55.8	10.8 U	76 U/mL	0.142 ml
CK	23	115	20	124	9.0 U	500 U/mL	0.018 ml
LDH	31	155	25	155	- U	1000 U/mL	NA
TC	186	930	150	930	- mg	10 U/mL	NA
Tg	105	525					NA
HDL-C	57.8	289					NA
LDL-C	106.7	533.5					NA

*After adding in the standards, the volume of control serum was adjusted to 620 mL with distilled water. The selected excipient was added and the liquid control serum was thoroughly mixed and aliquot into vials before lyophilization.

** NA = Not added.

Table 4. Preparation of bovine control serum (level II*).

Analyte	Conc/ 100mL	Conc/ 800mL	Target/ 100mL	Target/ 1000 mL	STD needed to add in	Conc of STD used	Quantity of STD added
TP	4.9	39.2	5.5	55	NA**		
Albumin	3	24	3.2	32	8 g	Solid	8 g
AST	9	72	12	120	48 U	500 U/mL	0.096 ml
ALT	3	24	6	60	36 U	200 U/mL	0.180 ml
ALP	18	144	20	200	56 U	76 U/mL	0.736 ml
CK	57	456	50	500	44 U	500 U/mL	0.088 ml
LDH	57	456	70	700	244 U	1000 U/mL	0.244 ml
TC	196	1568	200	2000	432 mg	10 mg/mL	43.2 ml
Tg	81	648					NA
HDL-C	102	816					NA
LDL-C	78	624					NA

*After adding in the standards, the volume of control serum was adjusted to 1,000 mL with distilled water. The selected excipient was added and the liquid control serum was thoroughly mixed and aliquot into vials before lyophilization.

** NA = Not added.

Table 5. Preparation of human control serum (level I*).

Analyte	Conc/ 100mL	Conc/ 220mL	Target/ 100mL	Target/ 270mL	STD added in	Conc of STD	Quantity of STD added
TP	6.9	15.18	6.4	17.28	NA**		
Albumin	4.2	9.24	4.2	11.34	2.1 g	solid	2.1 g
AST	4.8	10.56	3.5	9.45	- U	500U/ml	NA
ALT	2.7	5.94	2.5	6.75	0.81 U	200U/ml	0.004 ml
ALP	8.4	18.48	9	24.3	5.82 U	76 U/ml	0.077 ml
CK	20	44	20	54	10.0 U	500U/ml	0.020 ml
LDH	26	57.2	25	67.5	10.3 U	1000U/ml	0.010 ml
TC	186	409.2	150	405	- mg	10mg/ml	NA
Tg	112	246.4					NA
HDL-C	53	116.6					NA
LDL-C	110.6	243.32					NA

*After adding in the standards, the volume of control serum was adjusted to 270 mL with distilled water. The selected excipient was added and the liquid control serum was thoroughly mixed and aliquot into vials before lyophilization.

** NA = Not added.

Table 6. Preparation of human control serum (level II*).

Analyte	Conc/ 100mL	Conc/ 400mL	Target/ 100mL	Target/ 500mL	STD added in	Conc of STD used	Quantity of STD added
TP	5.7	22.8	5.5	27.5	NA**		
Albumin	3.1	12.4	3.2	16	3.6 g	Solid	3.6 g
AST	8	32	12	60	28 U	500U/ml	0.056 ml
ALT	3	12	6	30	18 U	200U/ml	0.090 ml
ALP	19	76	20	100	24 U	76 U/ml	0.316 ml
CK	58	232	50	250	18 U	500U/ml	0.036 ml
LDH	58	232	70	350	118 U	1000U/ml	0.118 ml
TC	195	780	200	1000	220 mg	10 mg/ml	22 ml
Tg	100	400					NA
HDL-C	90	360					NA
LDL-C	85	340					NA

*After adding in the standards, the volume of control serum was adjusted to 500 mL with distilled water. The selected excipient was added and the liquid control serum was thoroughly mixed and aliquot into vials before lyophilization.
**NA = Not added.

Part IV. Optimization of lyophilization process.

The method of a Lioalfa-10 used for lyophilizing control sera without excipient was summarized in Table 7 (Nimsung, et al., 1996-1999)

Table 7. Parameter for lyophilizing control serum.

Operation	Parameter setting
Automatic Operation 1. Prefreeze	Chamber temperature -40°C Shelf temperature -40°C Product temperature -25°C Time 4.5 hours
2. Primary drying	Pump maximum 3.0×10^{-1}mB minimum 3.5×10^{-2}mB Shelf heating +25 °C Time 10 hours
3. Secondary drying	Shelf heating +25 °C Time 4.5 hours
4. Last segment Manual operation	Manual - Vial stoppering - Air entry - Condenser defrosting

In this study, the operations were optimized for lyophilizing control sera containing excipients. The optimized process was carried out by varying the freezing temperatures, freezing times, pump and primary and secondary drying times, the optimized conditions were shown in Table 8.

Table 8. Optimization of lyophilized process for control serum containing excipient preparations.

Operation	Varying ranges
Freezing temperature	-25 ^{O}C ,-38^{O}C to -45^{O}C
Freezing time	5 to 7 hours
Pump	maximum 3.5 to 6.5 x 10^{-1}mB minimum 1.7 to 7.6 x 10^{-2}mB
Primary drying time	9 to 18 hours
Secondary drying time	2.5 to 4.5 hours

Part V. Assessment of lyophilized control serum containing an excipient.

[1.] Determination of optical clarity.

Clarity of control serum was verified by measuring the turbidity of the reconstituted sera pool which using liquid serum before lyophilization as a control. Turbidity of the reconstituted control serum was determined by reading the absorbance at 620 nm (Kanluan, et al., 1992) against distilled water. Absorbance of reconstituted lyophilized serum with and without an excipient were also compared.

[2.] Determination of the analyte concentrations in control serum.

TP, Albumin, AST, ALT, ALP, CK, LDH, TC, Tg, HDL-C and LDL-C were determined using Automated Chemistry Analyzer. Principle of methods employed for all assays in this study were summarized in Table 9 (Beckman instruction manual, 1996).

Table 9. Principle of methods used for analyzing chemical components in control serum (Beckman instruction manual, 1996).

Constituents	Units	Principle
TP	g/dL	Biuret method
Albumin	g/dL	Bromcresol green
AST	U/L	L-aspartate ketoglutarate
ALT	U/L	L-alanine ketoglutarate
ALP	U/L	p-nitrophenyl phosphate
CK	U/L	N-acetyl cysteine (NAC)
LDH	U/L	Lactate →Pyruvate
TC	mg/dL	Cholesterol oxidase
Tg	mg/dL	Glycerophosphate oxidase (GPO)
HDL-C	mg/dL	Dextran sulfate precipitation
LDL-C	mg/dL	Indirect determination*

* LDL-C = TC – (Tg/5 + HDL-C)

[3.] Method for estimating the assign values of constituents in control serum containing excipient.

Five vials of level I and level II control sera, prepared by adding excipient as indicated in the method part III, were reconstituted with 5 mL of 10 and 30 mmol/L bicarbonate diluent, respectively. After mixing and standing for at least 30 minutes, control sera from five vials of each level were assayed for interest components by using a Beckman CX-5 Autoanalyzer. Results obtained were calculated and plotted as the optimal condition variance (OCV) of each test in each level on the Lavey-Jennings charts (OCV graph).

The protein and lipid components in reconstituted control sera from five vials of each level were analysed further in the seven different days (Routine condition variance, RCV). Data were calculated and plotted on the Lavey-Jennings charts prepared for the routine precision determination.

Results obtained were interpreted by the criteria based on the OCV/RCV principle of WHO (Whitehead, 1976), firstly the % coefficient of variation (%CV) of the OCV of each method would be in the acceptable limits of the Beckman CX-5 Autoanalyzer. Secondly the %CV of RCV must not be exceeded than twice of the %CV of OCV.

To determine the assign value of each constituent analysed by each instrument, the acceptable limit was then calculated by adding and subtracting 2 standard deviations of each analysis to its' mean. The

calculated range ($\bar{X}$-2SD to $\bar{X}$+2SD) obtained is the assign value of each test for the method used in that analytical instrument.

Part VI. The study of physical characteristics of lyophilized control serum.

[1.] Determination of moisture content in lyophilized control serum.

The moisture content of lyophilized serum was determined by measuring the loss of weight of a sample when heated in an oven at 80°C, until constant weight is obtained. The initial moisture content is expressed as a percentage of the initial weight, i.e. %moisture content as loss in weight/initial weight multiply by 100 (Shotton and Ridgway, 1974).

[2.] Determination of pH in reconstituted lyophilized control serum.

The lyophilized control serum was reconstituted with 5 mL of either distilled water or bicarbonate solution (10 mmol/L for level I and 30 mmol/L for level II) and the pH was measured by using a Beckman pH meter, Model 3560. The pH meter was calibrated with pH standard solution before used.

[3.] Determination of the suitable diluent for reconstituting control serum containing an excipient.

The prepared control sera were reconstituted with 5 mL distilled water and 10 to 30 mmol/L bicarbonate solution. After standing for at least 30 minutes. The protein and lipid components were analysed by the Beckman CX-5 Autoanalyzer.

Part VII. The study of the effect of excipient on storage of lyophilized control serum.

Two level control sera containing excipients were determined for shelf life by accelerated temperature testing (Rakwatin, 1995). The accelerated test used for determining the decomposition of components may be achieved by increasing the temperature of keeping the product approximately 10-15°C higher than the storage temperature specified for

the usual storage condition. Therefore, the temperature at 45°C is the accelerated temperature for keeping the lyophilized products at room temperature and 20°C is the accelerated temperature for keeping the lyophilized products at 4°C. Two level control sera containing selected excipient were determined for shelf life at 4°C and room temperature by incubating both control sera at accelerated temperature, 20°C (for storage at 4°C) and 45°C (for storage at room temperature). The concentration of chemical components after reconstitution with 5 mL distilled water were determined at 0, 7, 15, 30, 45 and 60 days, respectively.

The interpretation was made by observing the kinetic rate of decomposition of components, which a total of 10% deterioration (or 90% remaining concentration or activity) is usually allowed to accept for its stability.

The common international guideline for prediction of shelf life by accelerated temperature testing are specified as below (Rakwatin, 1995).

1. If the components are stable at 45°C for 4 months, it is predicted that the components should be stable for at least 4 years, at 25°C.
2. If the components are stable at 20°C for 6 months, it is predicted that the components should be stable for at least 4 years, at 4°C.

Part VIII. Feature and cost of lyophilized preparation.

Five mL of control serum containing a selected excipient was filled into vials and lyophilized. Cost of preparation per vial was calculated by compiling all raw material costs together and divided by the numbers of vials. The detail of calculation was shown in Appendix VII.

Part IX. Comparison of methods for constituent analysis in control serum containing excipient.

Two level control sera prepared by the Method in Part III and previously evaluated for protein and lipid components in our laboratory were sent out to evaluate by three external clinical chemistry laboratories. Results obtained from the methods used by the other laboratories, manual method and dry chemistry in principle were compared with our results.

Part X. Statistical analysis.

All data were shown as the average of either duplicate or triplicate (mean $\pm$ SD) test. Comparison between set of results was made using

student *t*-test in the Microsoft Excel 2000 program on a Microsoft Windows 98 in a personal computer. A value of $p<0.05$ was considered significant.