IV.RESULTS

Part I. Preparation of analytes used to add in the control serum.

Enzymes and cholesterol concentrates which prepared for adding in pools of control sera in order to raise concentration of enzymes and cholesterol to their target values of each level were shown in Table 10. Very low specific activity of ALT was obtained, thus the commercial ALT was used in the preparations. Profile of LDH eluted was shown in Appendix II. Cholesterol concentration extracted from human sera was approximately 10 mg/mL.

Table 10. Yield and activity of enzymes extracted from various sources.

Analytes	Starting material	Total Protein (g/L)	Activity, or concentration	Specific activity (U/g)
AST	Heart, 50 g	486	2,567 U/L	5.28
ALT	Heart, 50 g	486	149 U/L	0.306
ALP	Kidney, 20 g	135	2,341 U/L	17.34
LDH	Bovine serum, 70 mL	60	322 U/L	5.36

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

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

The effect of four excipients on concentrations of protein and lipid components was shown in Table 11. Four excipients which were trehalose, saccharose and mannitol (5 and 10% in identical pool of bovine serum) and dextran (1 and 5% in identical pool of bovine serum) in two different concentrations used in this experiment have no effect on the interest analytes

except for the increasing in ALT activity and decreasing in AST activity. (Adding of these excipients may change the pH of the prepared control sera which further affected on the expression of enzyme activity)

Table 11. The effect of four excipients on concentrations of protein and lipid components in the prepared control serum.

Test*/Sample	Control	5%T	10%T	5%S	10%S	5%M	10%M	1%D	5%D
Total protein	9.8	10.4	9.5	9.8	9.6	10.2	9.9	8.4	10.2
Albumin	5.2	4.8	4.8	4.8	4.8	4.9	4.9	5.0	4.9
AST	198	148	137	149	141	140	126	151	161
ALT	36	51	45	51	42	42	39	45	51
ALP	121	114	110	113	108	112	110	112	112
CK	244	243	232	244	227	218	237	233	232
LDH	710	674	648	701	649	665	655	699	692
Cholesterol	241	234	223	230	222	225	219	235	229
Tg	ND	ND	ND	ND	ND	ND	ND	ND	ND
HDL-C	53.9	50.6	60.5	50.6	78.1	49.5	64.9	50.6	44.0
LDL-C	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Unit of total protein, albumin were g/dL; enzymes , U/L and cholesterol and lipoprotein, mg/dL respectively. T=trehalose, S=Saccharose, M=Mannitol and D=Dextran, ND = Not determined

[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.

Comparison between saccharose and mannitol.

The effect of lyophilization on concentrations of protein and lipid components in lyophilized control sera containing various concentrations of saccharose and mannitol was shown in Table 12 and 13, respectively. The concentrations of components in reconstituted control sera adding with each concentration of excipient were compared with those in liquid serum containing the corresponding sugar and with the control serum without adding of an excipient.

Table 12 demonstrated that after lyophilization, all analytes excepted for AST activity in reconstituted control serum were significantly increased ($p<0.05$) as compared with the activity in control serum before lyophilization. After adding various concentrations of saccharose, AST and ALT activities in reconstituted control sera were increased significantly ($p<0.05$) from their original control sera with and without saccharose additions. CK activities in reconstituted control sera adding various concentrations of saccharose were maintained the same when compared with the activity in original control serum without adding of saccharose.

Addition of various concentrations of mannitol in the prepared control sera caused the significant changes ($p<0.05$) in AST and ALT activities (Table 13). AST and ALT activities in original control sera were increased significantly ($p<0.05$) after adding of mannitol. The activity of AST in control serum containing various concentrations of saccharose before and after lyophilization were not different. The expression of ALT activity in reconstituted control sera containing different concentrations of mannitol were significantly lower than that of the corresponding control sera before lyophilization ($p<0.05$). Mannitol caused similar effect of ALT and LDH activities in the prepared control serum.

Comparison of saccharose, trehalose and dextran.

Table 14 showed the effect of saccharose, trehalose and dextran on protein and lipid components in control sera. In good agreement with Table 12 and 13, it was found that the concentration of all components in the reconstituted lyophilized control sera without addition of sugar were decreased. Saccharose and trehalose especially for 5% in control sera help stabilization of some components such as cholesterol and CK. It was noticed that activities of AST and ALT were remained more or less the same as the activity in the liquid control serum without adding of excipient.

Adding 1% concentration of dextran in control serum had no effect on protein, enzymes and lipid components in the prepared control sera. The concentrations of analytes in reconstituted control serum containing 1% dextran were not different from the original liquid control serum without excipient which used as a control of the experiment.

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

It was found that combination of saccharose and dextran (5%S+1%D) caused less effect on concentrations of protein, enzymes and lipid components in lyophilized control sera. Trehalose combined with dextran was not as good as the combination with saccharose. However the result at 8.5% saccharose combined with 1% dextran in lyophilized control serum showed good agreement with the result obtained with 5% saccharose and 1% dextran combination (Table 15).

Table 12. The effect of saccharose on protein and lipid components in liquid and reconstituted control sera.*

Analyte	Cont liq	Cont rec	3.4% S liq	3.4% S rec	6.2% S liq	6.2%S rec	8.5%S liq	8.5% S rec	17%S liq	17%S rec
TP	6.4 ± 0.06	5.9 ± 0.21	6.2 ± 0.06	5.8 ± 0.21	6.1 ± 0.06	5.7 ± 0.15	6.0 ± 0.06	5.2 ± 0.1	5.8 ± 0.0	5.3 ± 0.2
Albumin	3.6 ± 0.06	3.6 ± 0.15	4.0 ± 0.26	3.5 ± 0.15	4.0 ± 0.23	3.6 ± 0.15	4.0 ± 0.15	3.3 ± 0.1	3.8 ± 0.2	3.3 ± 0.1
AST	87 ± 16.2	181 ± 36.4**	122 ± 15.5	193 ± 21.6**	119 ± 22.50	189 ± 7.57**	122 ± 29.9	199 ± 11**	137 ± 16	205 ± 28**
ALT	37 ± 8.9	41 ± 5.1	64 ± 3.21**	44 ± 3.79	64 ± 1.00**	49 ± 4.04	65 ± 2.5**	47 ± 5.6	64 ± 5.1**	51 ± 4.9
ALP	62 ± 1.0	53 ± 3.5	74 ± 6.43	55 ± 3.21	73 ± 6.24	56 ± 4.36	71 ± 5.5	51 ± 2.0	68 ± 5.0	51 ± 2.9
CK	1266 ± 76.5	759 ± 46.4	787 ± 113	1262 ± 55.3	793 ± 117.3	1274 ± 93.5	830 ± 112	1194 ± 90	867 ± 160	1200 ± 34
LDH	459 ± 113	318 ± 64.4	471 ± 17.3	321 ± 59.81	440 ± 15.50	343 ± 75.7	409 ± 25.8	306 ± 97	392 ± 35	431 ± 180
TC	145 ± 2.0	129 ± 3.0	136 ± 3.79	127 ± 2.00	135 ± 2.52	125 ± 0.58	133 ± 2.5	115 ± 2.5	127 ± 1.2	114 ± 1.2
Tg	89 ± 1.7	51 ± 7.4	83 ± 1.00	52 ± 4.16	84 ± 1.53	53 ± 3.61	83 ± 1.5	50 ± 0.6	83 ± 2.5	45 ± 4.4
HDL-C	48 ± 2.5	30 ± 1.5	39 ± 2.08	31 ± 2.08	40 ± 3.06	31 ± 2.08	39 ± 3.6	29 ± 2.5	41 ± 1.2	27 ± 2.1
LDL-C	79 ± 3.6	88 ± 2.8	81 ± 3.71	86 ± 4.04	78 ± 5.49	83 ± 3.12	77 ± 4.9	77 ± 2.4	70 ± 1.2	78 ± 3.8

Cont = Control or serum without saccharose
S = Saccharose, liq = serum before lyophilization and rec = reconstituted lyophilized control serum.
* Data expressed as $\bar{X}$ + SD of the triplicate analyses.
** $p < 0.05$

Table 13. The effect of mannitol on protein and lipid components in liquid and reconstituted control sera.*

Analyte	Cont liq	Cont rec	1.8% M liq	1.8% M rec	3.3% M liq	3.3%M rec	4.6% M liq	4.6%M rec	9.2% M liq	9.2% M rec
TP	6.4 ± 0.00	5.9 ± 4.73	6.7 ± 0.15	6.4 ± 0.12	7.2 ± 0.12	6.8 ± 0.23	7.5 ± 0.1	7.2 ± 0.2	7.8 ± 0.2	7.4 ± 0.2
Albumin	3.9 ± 0.02	3.6 ± 0.37	4.2 ± 0.12	3.6 ± 0.23	4.1 ± 0.21	3.5 ± 0.1	4.2 ± 0.2	3.5 ± 0.2	4.0 ± 0.1	3.4 ± 0.2
AST	87 ± 0.1	181 ± 0.1**	169 ± 6.81**	174 ± 29.9**	162 ± 11.36**	173 ± 32.3**	171 ± 8.1**	208 ± 43**	153 ± 15**	153 ± 130**
ALT	37 ± 0.1	41 ± 0.0	83 ± 6.03**	49 ± 2.65	79 ± 17.67**	49 ± 5.57	85 ± 6.2**	58 ± 0.7	82 ± 25**	46 ± 2.1
ALP	62 ± 1.0	53 ± 0.6	60 ± 3.21	55 ± 0.58	60 ± 1	56 ± 1.53	60 ± 2.1	52 ± 0.6	60 ± 3.2	50 ± 2
CK	1266 ± 1.0	759 ± 6.4	1396 ± 277	1291 ± 63.1	1333 ± 225.1	1303 ± 43.5	1304 ± 233	1135 ± 47	1355 ± 147	919 ± 47
LDH	459 ± 113	318 ± 64	785 ± 59	322 ± 142**	705 ± 101.9	309 ± 129**	715 ± 33	383 ± 192**	554 ± 128	955 ± 30**
TC	145 ± 5.2	129 ± 12.5	136 ± 4.7	127 ± 2.52	138 ± 2.52	124 ± 1.53	136 ± 3.8	126 ± 0	134 ± 5.6	121 ± 3.1
Tg	89 ± 1.3	51 ± 2.7	77 ± 2.65	50 ± 6.08	81 ± 1	50 ± 5.86	79 ± 0	54 ± 0.6	76 ± 1.7	51 ± 0.6
HDL-C	48 ± 0.1	30 ± 0.0	36 ± 2.52	30 ± 2.65	35 ± 1.53	30 ± 2.08	38 ± 2	30 ± 2.7	43 ± 1.5	29 ± 4.2
LDL-C	79 ± 0.1	88 ± 0.8	85 ± 6.15	87 ± 1.42	86 ± 3.03	84 ± 3.87	83 ± 4	85 ± 2.5	75 ± 6.7	82 ± 5.4

Cont = Control or serum without mannitol
M= mannitol, liq = serum before lyophilization and rec = reconstituted lyophilized control serum.
* Data expressed as $\bar{X}$ + SD of the triplicate analyses.
** $p < 0.05$

Table 14. The effect of saccharose, trehalose and dextran on protein and lipid components in liquid and reconstituted control sera.*

Analyte	Cont liq	Con t rec	1% S liq	1% S rec	5% S liq	5% S rec	8.5% S liq	8.5% S rec
TP	7.0 ± 0.2	6.5 ± 0.3	6.9 ± 0.1	6.6 ± 0.1	6.8 ± 0.1	6.3 ± 0.2	6.8 ± 0.1	6.3 ± 0.4
Albumin	4.6 ± 0.3	4.3 ± 0.2	4.5 ± 0.3	4.4 ± 0.2	4.4 ± 0.3	4.2 ± 0.2	4.4 ± 0.3	4.1 ± 0.2
AST	44 ± 3.5	35 ± 2.1	43 ± 1.0	42 ± 3.4	43 ± 2.9	41 ± 3.1	43 ± 1.7	42 ± 2.1
ALT	22 ± 2.1	16 ± 4.4	22 ± 1.7	24 ± 1.7	23 ± 1.5	22 ± 1.5	23 ± 1.2	23 ± 1.5
ALP	102 ± 4.9	76 ± 11	101 ± 1.5	82 ± 6.0	98 ± 2.5	85 ± 2.1	97 ± 4.2	85 ± 2.1
CK	160 ± 15	80 ± 13	151 ± 8	119 ± 4.0	150 ± 12	134 ± 11	151 ± 11	139 ± 4.0
LDH	443 ± 31	422 ± 35	446 ± 11	444 ± 41.	458 ± 8.1	453 ± 21	469 ± 8.7	447 ± 29
TC	185 ± 9.7	156 ± 1.5	179 ± 3.1	159 ± 3.5	176 ± 5.5	158 ± 3.5	177 ± 7.5	157 ± 6.7
Tg	78 ± 2.5	74 ± 1.5	79 ± 0.6	75 ± 0.5	76 ± 2.1	73 ± 1.5	71 ± 1.2	71 ± 3.1
HDL-C	44 ± 0.6	35 ± 2.5	43 ± 2.1	35 ± 3.7	43 ± 1.5	35 ± 5.8	40 ± 3.1	35 ± 1.2
LDL-C	125 ± 9.4	106 ± 4.1	120 ± 4.9	110 ± 2.1	118 ± 6.6	108 ± 3.7	123 ± 4.5	108 ± 6.8

Cont = Control or serum without excipient
S= Saccharose, T=trehalose, D=Dextran, liq = serum before lyophilization and rec = reconstituted lyophilized control serum.
* Data expressed as $\bar{X}$ + SD of the triplicate analyses.

Table 14. The effect of saccharose, trehalose and dextran on protein and lipid components in liquid and reconstituted control sera. (continue)*

Analyte	Cont liq	Con t rec	1% T liq	1% T rec	5% T liq	5% T rec	1% D liq	1% D rec
TP	7.0 ± 0.2	6.5 ± 0.3	6.9 ± 0.1	6.6 ± 0.3	6.8 ± 0.1	6.5 ± 0.5	6.8 ± 0.1	6.6 ± 0.57
Albumin	4.6 ± 0.3	4.3 ± 0.2	4.5 ± 0.3	4.4 ± 0.2	4.4 ± 0.2	4.3 ± 0.1	4.5 ± 0.2	4.5 ± 0.1
AST	44 ± 3.5	35 ± 2.1	42 ± 1.7	29 ± 2.8	43 ± 0.6	41 ± 4.0	43 ± 2.0	41 ± 6.43
ALT	22 ± 2.1	16 ± 4.4	22 ± 3.1	19 ± 5.1	23 ± 1.2	20 ± 4.0	23 ± 1.5	18 ± 2.65
ALP	102 ± 4.9	76 ± 11	101 ± 1.7	82 ± 8.5	100 ± 3.5	89 ± 7.1	98 ± 2.5	83 ± 10.6
CK	160 ± 15	80 ± 13	156 ± 5	108 ± 3.8	150 ± 3.8	129 ± 7.1	250 ± 171	105 ± 4.16
LDH	443 ± 31	422 ± 35	454 ± 22	435 ± 38	472 ± 7.1	458 ± 23	352 ± 167	417 ± 27.1
TC	185 + 9.7	156 + 1.5	180 ± 5.5	159 ± 2.9	176 ± 4.4	158 ± 5.9	179 ± 2.3	147 ± 10.02
Tg	78 ± 2.5	74 ± 1.5	78 ± 1	76 ± 0.6	78 ± 2	73 ± 2.3	78 ± 0.5	76 ± 3.06
HDL-C	44 ± 0.6	35 ± 2.5	43 ± 1.7	34 ± 1.2	42 ± 1	35 ± 2.1	43 ± 1	35 ± 1
LDL-C	125 ± 9.4	106 ± 4.1	121 ± 6.3	110 ± 3.5	118 ± 3.5	109 ± 6.8	121 ± 2.4	97 ± 10.2

Cont = Control or serum without excipient
S= Saccharose, T=trehalose, D=Dextran, liq = serum before lyophilization and rec = reconstituted lyophilized control serum
* Data expressed as $\bar{X}$ + SD of the triplicate analyses.

Table 15. The effect of combined excipients on protein and lipid components in liquid and reconstituted control sera.*

Test	1%S+1%D liq	1%S+1%D rec	5%S+ 1%D liq	5%S+ 1%D rec	1%T+1%D liq	1%T+1%D rec	5%T+1%D liq	5%T+1%D rec	8.5%S+ 1%D,liq	8.5%S+ 1%D,rec
TP	6.8 ± 0.1	6.4 ± 0.3	6.7 ± 0.1	6.3 ± 0.3	6.8 ± 0.1	6.5 ± 0.4	6.8 ± 0.1	6.3 ± 0.4	6.8 ± 0.1	6.2 ± 0.31
Alb	4.5 ± 0.2	4.4 ± 0.17	4.4 ± 0.3	4.3 ± 0.2	4.4 ± 0.3	4.4 ± 0.1	4.4 ± 0.3	4.2 ± 0.2	4.4 ± 0.25	4.2 ± 0.15
AST	43 ± 1.5	40 ± 1.53	43 ± 1.0	40 ± 1.2	43 ± 2.1	59 ± 34	43 ± 3.5	41 ± 2.1	43 ± 1.53	42 ± 0.58
ALT	23 ± 2.0	22 ± 1.0	24 ± 1.2	21 ± 1.2	23 ± 1.5	21 ± 3.5	23 ± 1.2	22 ± 2.0	23 ± 0.58	23 ± 0.58
ALP	98 ± 2.7	82 ± 7.77	95 ± 2.7	85 ± 3.1	100 ± 2.0	83 ± 7.5	99 ± 4.6	88 ± 6.8	98 ± 4.51	86 ± 3.21
CK	152 ± 7.8	110 ± 11.5	151 ± 4.0	134 ± 4.2	152 ± 14	112 ± 7.1	122 ± 46	117 ± 16	156 ± 7.0	89 ± 1.41
LDH	448 ± 2.3	437 ± 36	454 ± 11	438 ± 16	448 ± 18	431 ± 20	465 ± 8.0	452 ± 8.7	476 ± 8.19	449 ± 17.5
TC	178 ± 2.1	146 ± 2.65	173 ± 3.6	152 ± 3.0	177 ± 4.4	147 ± 5.5	176 ± 6.8	149 ± 4.7	176 ± 5.51	157 ± 2.31
Tg	78 ± 1.0	75 ± 1.0	75 ± 1.5	74 ± 2.0	76 ± 1.5	76 ± 0.5	75 ± 2.7	70 ± 0.6	75 ± 2.52	70 ± 2.08
HDL-C	42 ± 1.7	35 ± 2.0	40 ± 0.6	36 ± 3.6	42 ± 2.7	35 ± 1.5	41 ± 1.0	32 ± 1.5	39 ± 1.0	27 ± 6.11
LDL-C	120 ± 1.7	96 ± 4.52	118 ± 2.8	101 ± 6.1	120 ± 1.8	97 ± 5.6	120 ± 5.7	103 ± 6.3	122 ± 4.23	116 ± 7.06

Cont = Control or serum without excipient

S= Saccharose, T=trehalose, D=Dextran, liq = serum before lyophilization and rec = reconstituted lyophilized control serum.

* Data expressed as $\bar{X}$ + SD of the triplicate analyses.

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

Turbidity of control specimens containing various kinds of excipients were also examined. The turbid effect was evaluated by the increase in absorbance of serum specimen reading at 620 nm in a Shimadzu UV-160A Spectrophotometer. Figure 6 showed the absorbances at 620 nm of control sera added with various concentrations of saccharose and mannitol. The more concentration of excipient addition, the lower absorbances were obtained.

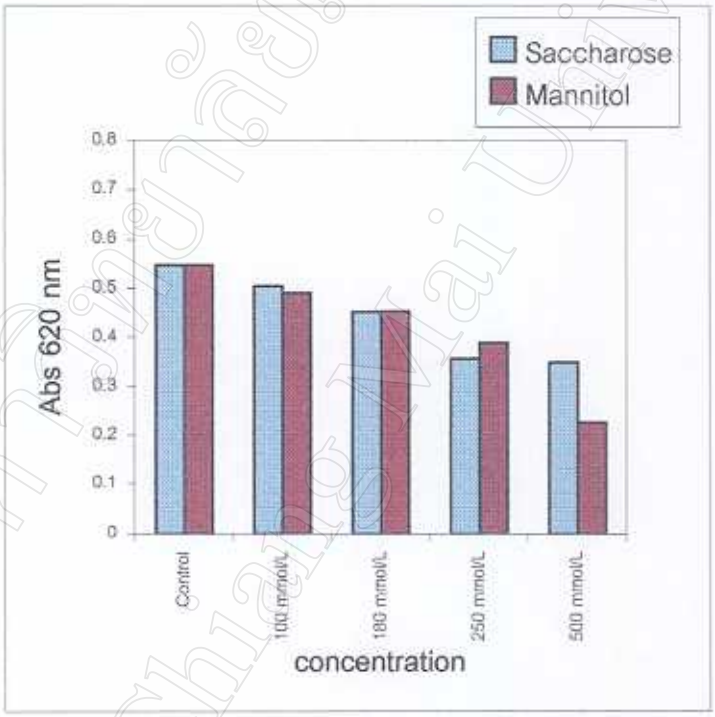


Figure 6. Absorbance at 620 nm of control sera containing various concentrations of saccharose and mannitol.
The concentrations in g% of excipients were converted to the SI unit for comparison.

Figure 7 showed the comparison of absorbance at 620 nm of liquid control serum with and without adding of excipients with the corresponding reconstituted control serum. The absorbance at 620 nm was increased in reconstituted control serum. Lyophilized control serum which adding in 8.5% saccharose had the most clarified matrix when compared with the other control serum pools containing various kinds of excipients.

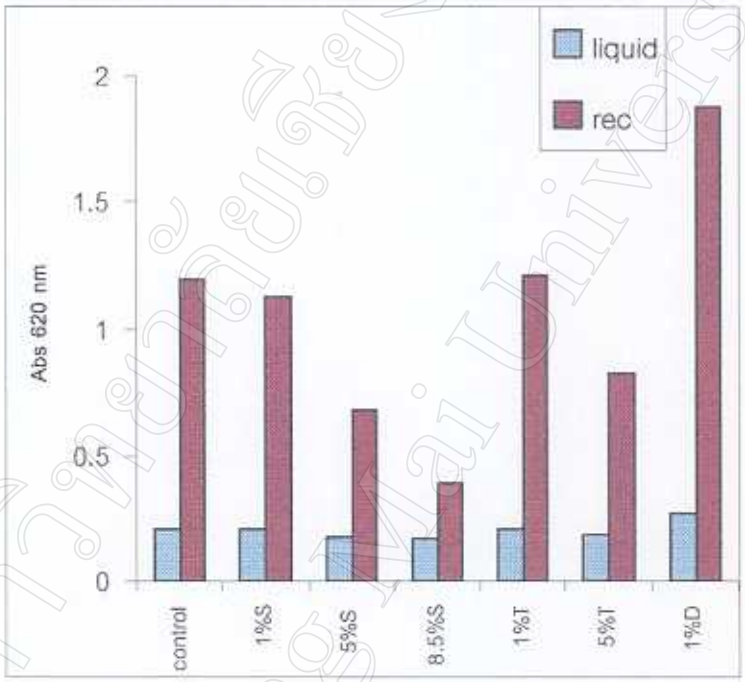


Figure 7. Comparison of the absorbance at 620 nm of the control sera containing various concentrations of excipient.
control=control sera without excipient, S=saccharose, T=trehalose, D=Dextran, liquid=liquid serum and rec= reconstituted control serum.

The absorbance at 620 nm of control sera containing combinations of disaccharide and dextran was shown in Figure 8. The lowest absorbance of reconstituted control serum was found to be the combination of 8.5% saccharose with 1% dextran in the lyophilized control serum.

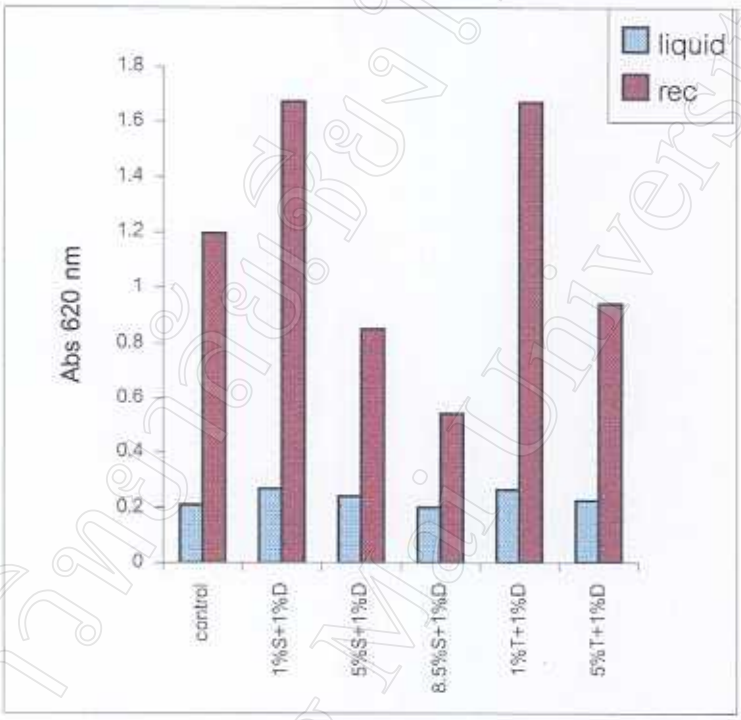


Figure 8. Absorbance at 620 nm of control sera containing combination of two different excipients. S=saccharose, T=trehalose, D=dextran, liquid=liquid control serum, recon=reconstituted control serum.

[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.

It is known that lyophilized control serum containing high level of cholesterol is turbid after reconstitution with distilled water or diluent buffer. Therefore, addition of excipient may increase clarity of the control serum.

Table 16 demonstrated the effect of dextran and saccharose or their combinations on protein and lipoprotein components in lyophilized control sera. The concentrations or activities of constituents in lyophilized control sera containing 1% dextran showed very good agreement with lyophilized control sera without adding excipient.

Table 16. The effect of dextran and saccharose on the cholesterol rich lyophilized control serum*.

TEST	Rich Chol	1%D	8.5% S	17% S	8.5% S +1%D	17% S +1%D
TP	5.3 ± 0.06	5.4 ± 0.06	5.3 ± 0.3	4.7 ± 0.14	5.1 ± 0.25	3.4 ± 0.42
Alb	4.7 ± 0.06	4.9 ± 0	4.0 ± 0.21	3.4 ± 0.07	4.3 ± 0.44	2.8 ± 0.64
AST	79 ± 6.1	78 ± 8.7	72 ± 4	61 ± 1.4	84 ± 11	55 ± 12.7
ALT	35 ± 3.5	36 ± 2.8	36 ± 2.1	34 ± 0.7	37 ± 2.1	19 ± 9.9
ALP	55 ± 1	58 ± 1	55 ± 2	54 ± 0	40 ± 2.5	28 ± 4.9
CK	97 ± 3.8	118 ± 1.5	141 ± 1.5	148 ± 5.7	132 ± 4.5	91 ± 9.9
LDH	320 ± 4.9	322 ± 13.9	331 ± 17	302 ± 19.1	323 ± 25.1	113 ± 22.6
TC	201 ± 3.5	197 ± 4	214 ± 12.2	188 ± 4.2	226 ± 8.5	131 ± 7.1
Tg	459 ± 8.5	454 ± 16.3	444 ± 14.2	333 ± 83.4	405 ± 23.6	268 ± 27.6
HDL-C	19 ± 5.8	23 ± 3	28 ± 8.1	89 ± 26.2	93 ± 96	65 ± 71.4
LDL-C	90 ± 10.6	83 ± 5.9	97 ± 4.7	33 ± 38.6	52 ± 84.6	23 ± 73

Rich chol=reconstituted control without adding excipient, D=dextran, S=saccharose.

* Data expressed as $\bar{X} \pm$ SD of the triplicate analyses.

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

Figure 9 showed the turbidity of the cholesterol rich control sera. After lyophilization, the reconstituted control sera with and without adding excipients were turbid. The most clarified pool was obtained after adding 17% saccharose in control serum. Combination of saccharose and dextran which added in the control sera caused more turbid effect on both original liquid and reconstituted control sera than that obtained from adding an excipient alone.

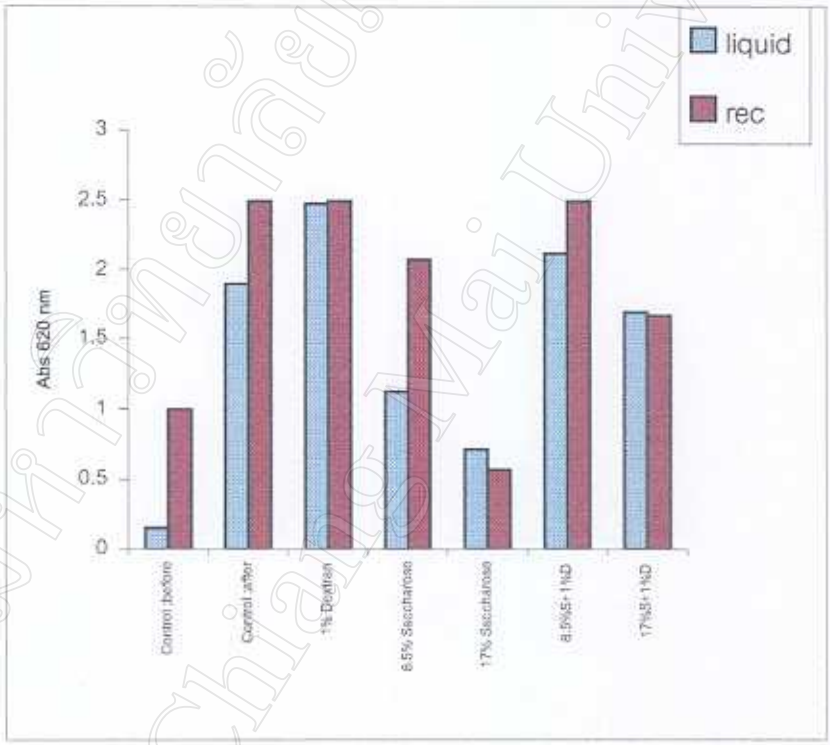


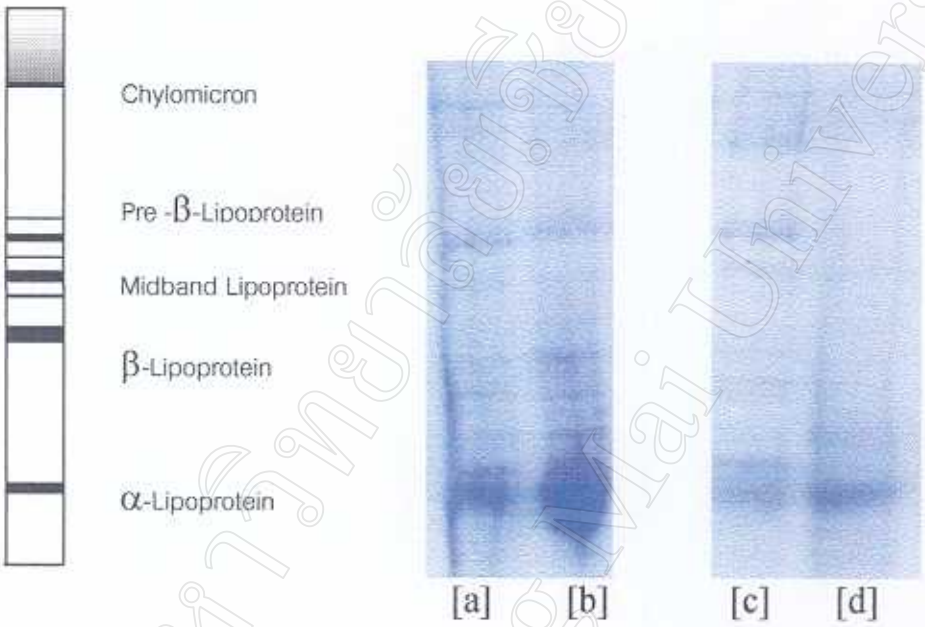
Figure 9. The turbidity of the cholesterol rich control sera with and without excipient(s). S=saccharose, D=dextran, liquid=liquid control serum, rec=reconstituted control. serum, before=before lyophilization, after=after lyophilization.

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

Figure 10 demonstrated the SDS-PAGE of total protein in reconstituted bovine sera comparing with it's corresponded liquid control specimen. Decreased alpha-globulin level (decreased intensity of the stain) was shown in all specimens of reconstituted control sera. The results suggested that there was the denaturation of several enzymes which migrated in this region. There was no changed of albumin in lyophilized control sera. Bovine serum showed the similar pattern when compared with the pattern of human serum used as a reference material.

Figure 10. SDS-PAGE of total protein in reconstituted control sera.
Lane 1 = liquid bovine serum control, level I
Lane 2 = reconstituted bovine serum control, level I
Lane 3 = liquid bovine serum control, level II
Lane 4 = reconstituted bovine serum control, level II
Lane 5 = albumin control, Sigma A-4503 (remainder mostly globulin)
Lane 6 = liquid human serum control, level I
Lane 7 = reconstituted human serum control, level I
Lane 8 = liquid human serum control, level II
Lane 9 = reconstituted human serum control, level II
Lane 10=albumin control, Sigma A-4503 (remainder mostly globulin)

Comparison of lipoprotein patterns of liquid control serum and reconstituted control serum were shown in Figure 11. The color intensity of α- and β- lipoprotein was decreased in all reconstituted specimens compared with the liquid form. The result suggested that the deterioration of lipoprotein occurred during lyophilized process.



Scheme of a lipoprotein electrophoretic pattern on polyacrylamide gel (Kaplan and Pesce, 1984)

Figure 11. SDS-PAGE of lipoprotein in reconstituted control sera.
Lane a = reconstituted bovine serum control
Lane b = liquid bovine serum control
Lane c = reconstituted human serum control
Lane d = liquid human serum control

Protein patterns obtained from SDS-PAGE of the reconstituted control serum containing 8.5% saccharose was shown in Figure 12. There was some change in color intensity in α-globulin region observed in the reconstituted specimen after lyophilization in the presence of 8.5% saccharose and reconstituted control serum (lane 4 and lane 2). Reconstituted human serum (reference matrix) containing 8.5% saccharose exhibited the same results as the bovine serum control (lane 5 and lane 3).

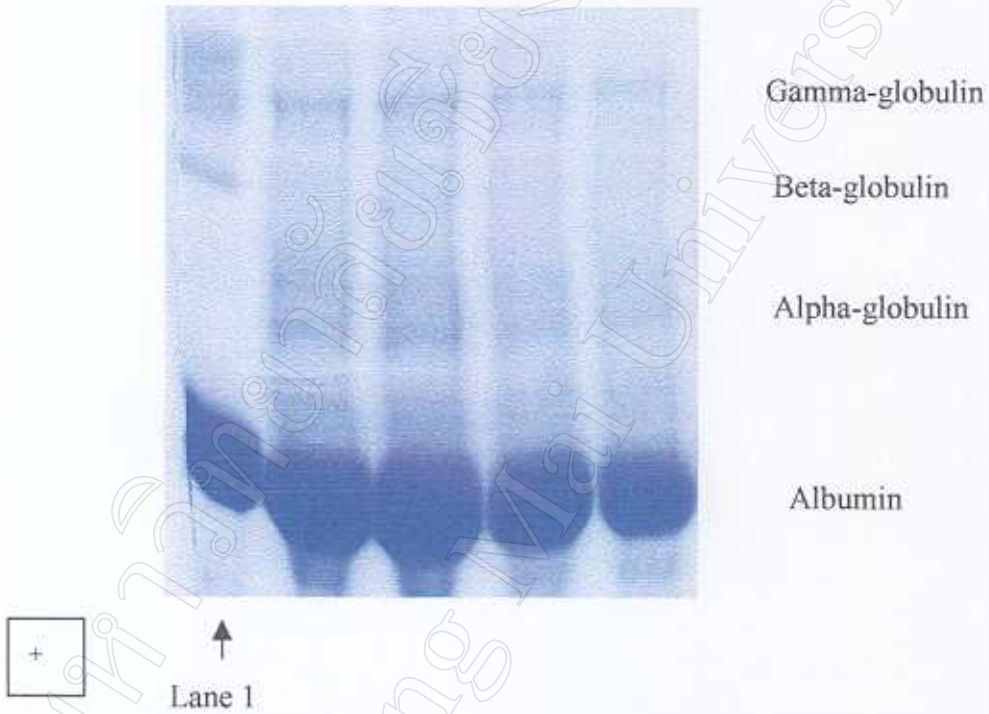


Figure 12. SDS-PAGE of total protein in reconstituted control sera.
Lane 1 = albumin control, Sigma A-4503 (remainder mostly globulin)
Lane 2 = reconstituted bovine control serum
Lane 3 = reconstituted human control serum
Lane 4 = reconstituted bovine control serum with 8.5%saccharose
Lane 5 = reconstituted human control serum with 8.5%saccharose

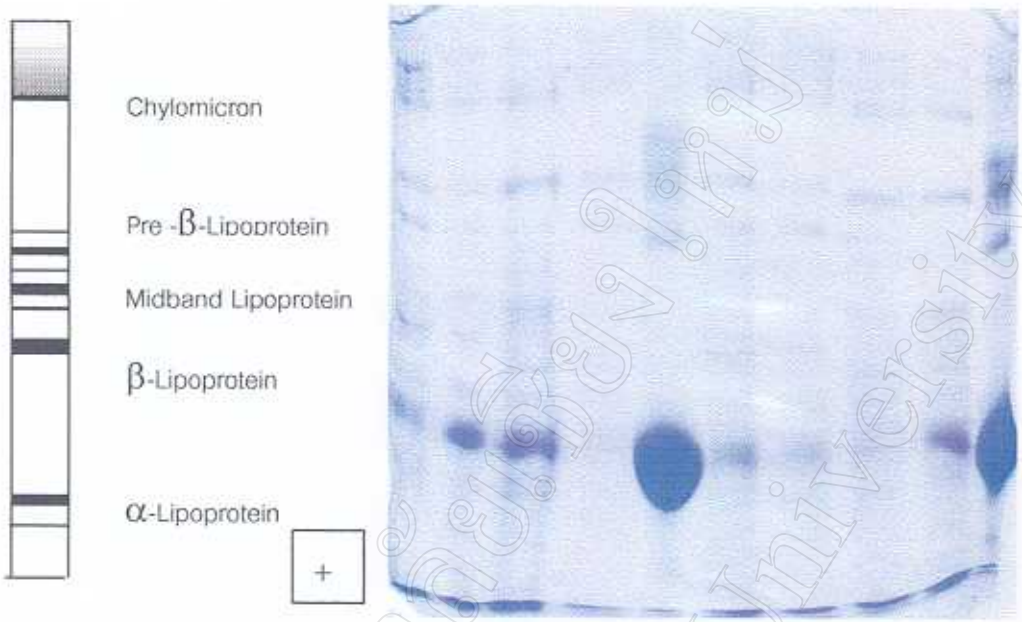
Table 17 showed the percentage of electrophoretic fractionation of protein pattern in Figure 12. It was shown that the percentages of α-globulin fractions in both bovine and human reconstituted control sera containing 8.5% saccharose were increased from those without adding of 8.5% saccharose. These results confirmed the stabilizing effect of 8.5% saccharose on protein (enzyme) concentration in the lyophilized control serum.

Table 17. The percentage of electrophoretic fractionation.

Condition	Albumin	α-globulin	β-globulin	γ-globulin
reconstituted bovine control	71.5	17.0	3.5	6.4
reconstituted human control	73.8	15.4	3.0	5.9
reconstituted bovine control with 8.5% saccharose	68.6	21.2	3.0	6.3
reconstituted human control with 8.5% saccharose	72.9	17.1	1.7	7.1

Figure 13 showed the lipoprotein patterns of the prepared liquid control sera (lane 1 and lane 3), liquid control sera containing 8.5% saccharose (lane 2 and lane 4) and the two samples which corresponded to the previous two liquid control specimens i.e. the lyophilized control sera (lane 6 and lane 8) and lyophilized control sera containing 8.5% saccharose (lane 7 and lane 9). The loss of pre-beta lipoprotein fraction in the lyophilized control serum (without saccharose) was observed, while there was some loss in specimens which added 8.5% saccharose.

In conclusion, saccharose was shown to be the suitable excipient to maintain stability of protein and lipoprotein components in control serum during lyophilized process. At the final concentration of 8.5% saccharose in control serum, the most clarified matrix of reconstituted control serum was obtained.



Scheme of a lipoprotein electrophoretic pattern on polyacrylamide gel (Kaplan and Pesce, 1984)

Figure 13. SDS-PAGE of lipoprotein in reconstituted control sera with 8.5% saccharose compared with the corresponding liquid forms.

Lane 1 = liquid bovine serum control, level I
Lane 2 = liquid bovine serum control, level I with 8.5% saccharose
Lane 3 = liquid bovine serum control, level II
Lane 4 = liquid bovine serum control, level II with 8.5% saccharose
Lane 5 = albumin control
Lane 6 = reconstituted bovine serum control, level I
Lane 7 = reconstituted bovine serum control, level I with 8.5% saccharose
Lane 8 = reconstituted bovine serum control, level II
Lane 9 = reconstituted bovine serum control, level II with 8.5% saccharose
Lane 10 = albumin control, Sigma A-4503 (remainder mostly globulin)

Part III. Results for the preparation of two level lyophilized control sera containing of 8.5% (250 mmol/L) saccharose.

Two level control sera were added with 8.5% saccharose, aliquot and lyophilized by a Lioalfa-10. Results of the analyte concentrations shown in Table 18 were compared with the target values and the values obtained from human reference matrix. Products of this preparation were sent out to the external laboratories for evaluation.

Table 18 showed the concentrations of protein, lipid components and enzyme activities in two levels of the bovine control sera. Results were compared with the reference human control sera. All results of analyses except for some enzymes were agreed with the established target values of both levels. It was demonstrated that ALP and CK activity in level II of both bovine and human control sera were markedly lower than the target values, therefore standard enzymes may be needed to supplement in both abnormal pools.

Table 18. Concentrations of protein and lipid components and enzyme activities in two level lyophilized bovine control sera containing 8.5% saccharose.

Analyte	Level I			Level II		
	Target*	Bovine	Human**	Target*	Bovine	Human**
TP, g/dL	6.4	6.0	6.1	5.5	5.1	5.5
Albumin, g/dL	4.2	3.9	4.0	3.2	3.3	3.2
AST, U/L	35	42	47	120	54	92
ALT, U/L	25	26	26	60	15	34
ALP, U/L	90	75	74	200	169	164
CK, U/L	200	226	217	500	391	391
LDH, U/L	250	214	207	700	750	705
TC, mg/dL	150	137	144	200	181	184
Tg, mg/dL	***	80	74	***	77	96
HDL-C, mg/dL	***	34	33	***	77	69
LDL-C, mg/dL	***	87	89	***	89	96

* Target = target values established by using the medical decision of levels found in serum of health and diseases.

** Reference matrix

*** Depended on cholesterol concentration.

Part IV. Optimization of parameter for lyophilization process.

The method of lyophilization was optimized and the appropriated parameters of the Lioalfa-10 for lyophilization of two level control sera in the presence of 8.5% saccharose were summarized in Table 19.

Table 19. Optimal parameter of the Lioalfa-10 for lyophilization of two level control sera containing 8.5% saccharose.

Operation	Parameter
Freezing temperature	-45 ^{O}C
Freezing time	6 hours
Pump	Segment 1;maximum 6.5×10^{-1}mB minimum 3.5×10^{-2}mB
	Segment 2;maximum 3.5×10^{-1}mB minimum 7.5×10^{-2}mB
Shelf temperature	25 ^{O}C
Primary drying time	12 hours
Secondary drying time	3 hours

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

Two level lyophilized control sera containing 8.5% saccharose were prepared and evaluated for optical clarity. The absorbance at 620 nm of reconstituted control sera were shown in Table 20 comparing with the absorbance of liquid serum before lyophilization. It was found that the absorbance of reconstituted control sera in the presence of 8.5% saccharose were reduced from those of the corresponding liquid sera.

Table 21 showed the comparison of turbidity and cholesterol level between those obtained in our laboratory and commercially reconstituted control serum. Data demonstrated that at higher levels of cholesterol concentrations in both bovine and human matrix control sera, the absorbances at 620 nm of our preparations were lower than that obtained from the commercial control.

Table 20. Clarity of reconstituted control sera.

Specimen	Absorbance at 620 nm	
	Before lyophilization	After lyophilization
Bovine control, level I	1.744	1.040
Bovine control, level II	1.998	1.277
Human control, level I*	1.776	1.085
Human control, level II*	2.179	1.516

* Reference matrix.

Table 21. Comparison of turbidity and cholesterol concentration of our preparation and commercially reconstituted control serum.

Control serum	Source	Absorbance 620 nm	TC (mg/dL)
Wellcomtrol*	Bovine	0.434	122
Humatol*	Bovine	1.224	156
Lyophil*	Bovine	1.977	113
Bovine control, level I	Bovine	1.040	137
Bovine control, level II	Bovine	1.277	181
Human control, level I	Human	1.085	144
Human control, level II	Human	1.516	184

* commercial control serum

The calculated assign values of constituents in control serum containing 8.5% saccharose were shown in Table 22 and 23. Patterns of distribution of OCV and RCV values were demonstrated in Appendix III. Three commercial quality control specimens used for determining the precision of the analyses were Decision 1, 2 and 3. The distribution of RCV values were demonstrated on the RCV-K quality control graph in Appendix IV.

Table 22. Assign values of analytes in lyophilized control serum Level I.

Analyte	OCV (n=5)			RCV (n=7)			Expected range
	$\bar{X}$	SD	%CV	$\bar{X}$	SD	%CV	
TP (g/dL)	6.6	0.08	1.21	6.4	0.24	3.75	6.0-6.9
Alb (g/dL)	3.4	0.05	1.47	3.4	0.05	1.47	3.3-3.5
AST (U/L)	32	13.7	42.7	5	3.64	72.8	0-13
ALT (U/L)	12	1.64	13.7	10	1.07	10.7	8-12
ALP (U/L)	85	1.10	1.29	89	2.41	2.71	84-94
CK (U/L)	226	3.36	1.48	223	16.8	7.5	190-257
LDH (U/L)	212	5.64	2.67	209	10.2	4.86	189-230
TC (mg/dL)	312	13.3	10.1	137	5.86	4.28	125-149
Tg (mg/dL)	79	3.27	4.14	81	3.25	4.01	75-88
HDL-C (mg/dL)	44	10.2	23.1	42	6.72	16.0	29-56
LDL-C (mg/dL)	72	12.4	17.2	79	8.38	10.6	62-95

Table 23. Assign values of analytes in lyophilized control serum Level II.

Analyte	OCV (n=5)			RCV (n=7)			Expected range
	$\bar{X}$	SD	%CV	$\bar{X}$	SD	%CV	
TP (g/dL)	4.8	0.38	7.92	4.8	0.21	4.38	4.4-5.3
Alb (g/dL)	2.7	0.05	1.85	2.8	0.05	1.79	2.7-2.9
AST (U/L)	96	2.17	2.26	84	19.6	23.3	45-124
ALT (U/L)	30	1.41	4.70	22	8.45	38.4	5-39
ALP (U/L)	182	1.92	1.05	185	3.08	1.66	179-191
CK (U/L)	464	3.49	0.75	429	8.94	2.08	411-447
LDH (U/L)	718	5.55	0.77	711	37.0	5.20	637-785
TC (mg/dL)	176	6.38	3.63	177	5.77	3.26	165-189
Tg (mg/dL)	81	3.70	4.57	76	2.99	3.93	70-82
HDL-C (mg/dL)	106	14.0	13.2	92	3.95	4.29	84-99
LDL-C (mg/dL)	54	20.1	37.3	70	4.85	6.93	60-80

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

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

The moisture content in two level lyophilized control sera were ranged from 0.85-2.05 % (See Appendix V).

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

pH of the reconstituted control sera in the presence of 8.5% saccharose dissolved in 5 mL of distilled water were in the range of 6.4-6.8. When these control sera were reconstituted with 10 and 30 mmol/L bicarbonate diluent for level I and II, respectively, the pH of the reconstituted control sera changed and were varied from 8.02-8.25.

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

Lyophilized process caused some change of pH when reconstitution of lyophilized specimens with distilled water. AST and ALT were two components in these lyophilized sera containing 8.5% saccharose, which were the most affected by lyophilization (Table 24). Reconstitution with 10 mmol/L and 30 mmol/L bicarbonate buffer for level I and II lyophilized control serum changed the pH of specimens which then the AST and ALT activity were expressed (Table 24). The bicarbonate diluent in the concentration of 10 and 30 mmol/L were introduced to use as the diluents of two level control sera because they are the diluent buffer previously used to maintain the total carbondioxide concentrations in the bovine matrix control sera (Nimsung, et al., 1996-1999).

Table 24. Effect of diluent on protein, enzymes and lipid components in reconstituted control sera with 8.5% saccharose.

Analyte	Distilled water		Bicarbonate diluent*	
	Level I	Level II	Level I	Level II
TP, g/dL	6	5.1	6.7	5.2
Alb, g/dL	3.9	3.4	3.3	2.7
AST, U/L	4	98	32	95
ALT, U/L	7	44	13	30
ALP, U/L	80	178	84	184
CK, U/L	200	307	224	462
LDH, U/L	198	725	209	727
TC, mg/dL	132	186	108	175
Tg, mg/dL	75	81	80	80
HDLC, mg/dL	37	61	35	118
LDL-C,mg/dL	80	109	57	41

* 10 mmol/L for Level I and 30 mmol/L for Level II.

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

Stability of lyophilized control prepared from a bovine pool serum was shown in Table 25-30 (For details see Appendix VI).

Stability of lyophilized control sera containing 8.5% saccharose were evaluated using the criteria as stated in Method Part VII. In Table 25, it was shown that seven analytes including AST and ALT in 8.5% saccharose added in the level I control sera kept at $4^{O}C$ (control temperature) were unstable. Almost of analytes in lyophilized control sera containing 8.5% saccharose kept at $20^{O}C$ (Table 26) and $45^{O}C$ (Table 27) were stable excepted for AST, ALT and LDL-C. The lower activity of AST and ALT caused by changing of the pH of reconstituted control sera which further affected on expression of enzyme activity. The decrease in LDL-C concentration related to the over estimation of HDL-C concentrations (% remaining concentration was greater than 100). However, the prediction for shelf life of level I bovine control serum

could not be evaluated because of the errors in analyses or the actual instability of the analytes in that control sera kept at $4^{o}C$.

The stability of protein, enzyme and lipid components in level II lyophilized bovine control sera were shown in Table 28, 29 and 30. All components in control sera kept at $4^{o}C$ (Table 28) were stable (% remaining concentrations or activities were not less than 90, see criteria in Method Part VII). The stability of analytes in lyophilized control sera kept at $20^{o}C$ was at least 60 days (Table 29). Therefore, it can predict the shelf life at $4^{o}C$ of the level II lyophilized control products which would be at least one and a half year. The predicted shelf life of these lyophilized control serum, if kept at room temperature, was not exceed 6 months. This could be explained by the loss of ALP activity after 15 days of accelerated temperature testing at $45^{o}C$ (Table 30). For interpretation, if only one component in the lyophilized control serum lost its concentration or activity during storage, it means that the shelf life of the whole product was ended up. However, the long-termed study of stability or shelf life is needed to confirm these results.

Table 25. Stability of level I bovine control serum containing 8.5% saccharose kept at 4^0C.

TEST	Storage duration (days)	%remaining concentration	%remaining activity
TP	Stable (60)	108.1	-
Albumin	Stable (60)	94.02	-
AST	7	-	71.30
ALT	7	-	76.90
ALP	60	-	71.62
CK	Stable (60)	-	196.7
LDH	60	-	81.02
TC	60	68.97	-
Tg	60	69.76	-
HDL-C	Stable (60)	220.0	-
LDL-C	60	38.21	-

Table 26. Stability of level I bovine control serum containing 8.5% saccharose kept at 20^0C.

TEST	Storage duration (days)	%remaining concentration	%remaining activity
TP	Stable (60)	112.3	-
Albumin	Stable (60)	101.9	-
AST	7	-	76.90
ALT	7	-	61.50
ALP	Stable (60)	-	126.5
CK	Stable (60)	-	123.4
LDH	Stable (60)	-	104.5
TC	Stable (60)	101.9	-
Tg	Stable (60)	101.8	-
HDL-C	Stable (60)	230.4	-
LDL-C	30	56.46	-

Table 27. Stability of level I bovine control serum containing 8.5% saccharose kept at 45^0C.

TEST	Storage duration (days)	%remaining concentration	%remaining activity
TP	Stable (60)	113.0	-
Albumin	Stable (60)	98.10	-
AST	7	-	73.10
ALT	7	-	52.30
ALP	Stable (60)	-	126.0
CK	Stable (60)	-	98.00
LDH	Stable (60)	-	110.0
TC	Stable (60)	115.0	-
Tg	Stable (60)	101.0	-
HDL-C	Stable (60)	304.0	-
LDL-C	60	51.20	-

Table 28. Stability of level II bovine control serum containing 8.5% saccharose kept at 4^0C.

TEST	Storage duration (days)	%remaining concentration	%remaining activity
TP	Stable (60)	106.6	-
Albumin	Stable (60)	115.1	-
AST	Stable (60)	-	189.9
ALT	Stable (60)	-	96.30
ALP	Stable (60)	-	97.13
CK	Stable (60)	-	175.6
LDH	Stable (60)	-	132.9
TC	Stable (60)	107.4	-
Tg	Stable (60)	99.09	-
HDL-C	Stable (60)	141.9	-
LDL-C	Stable (60)	92.78	-

Table 29. Stability of level II bovine control serum containing 8.5% saccharose kept at 20^0C.

TEST	Storage duration (days)	%remaining concentration	%remaining activity
TP	Stable (60)	105.4	-
Albumin	Stable (60)	113.5	-
AST	Stable (60)	-	159.5
ALT	Stable (60)	-	98.15
ALP	Stable (60)	-	92.72
CK	Stable (60)	-	341.2
LDH	Stable (60)	-	130.5
TC	Stable (60)	102.4	-
Tg	Stable (60)	99.07	-
HDL-C	Stable (60)	126.0	-
LDL-C	Stable (60)	92.77	-

Table 30. Stability of level II bovine control serum containing 8.5% saccharose kept at 45^0C.

TEST	Storage duration (days)	%remaining concentration	%remaining activity
TP	Stable (60)	104.6	-
Albumin	Stable (60)	109.6	-
AST	Stable (60)	-	136.7
ALT	30	-	81.50
ALP	15	-	82.90
CK	Stable (60)	-	306.5
LDH	Stable (60)	-	130.8
TC	Stable (60)	104.5	-
Tg	Stable (60)	94.43	-
HDL-C	Stable (60)	129.0	-
LDL-C	Stable (60)	96.34	-

Part VIII. Feature and cost of lyophilized preparation.

The feature of lyophilized bovine control containing 8.5% saccharose in a 5 mL vial sealed with aluminium cap was cake-liked structure. Color of the control material was pale yellow to deep yellow.

The total cost was calculated by adding all expenses including whole blood which used as raw material, chemical used for fortification of batches of control serum, the degeneration of equipment especially for the Lioalfa-10, packaging and analytical expenses. The average cost was shown in Table 31, which was approximately 71 Baht/vial. Figure 14 showed the lyophilized bovine control serum containing 8.5% saccharose in an amber vial sealed with aluminium cap.

Figure 14. The feature of lyophilized bovine control containing 8.5% saccharose.

Table 31. The total cost of lyophilized control serum containing excipient of two level batches.

EXPENSES (Baht)	Level I 4L	Level II 4L
Raw material - (Whole blood)	500.-	1,000.*-
Chemicals added	5,230.-**	9,365.91**
Vials with aluminium seal (800 vials)	16,000.-	16,000.-
Expenses used for analyzing chemical compositions - (16 Tests x 40 Baht, replicate test) - (No. of instrument x 5, according to producer)	19,200.-	19,200.-
Expense for Lyophilizer degeneration (200,000 Baht/year), produced 4 times/month Average cost for equipment degeneration/Batch	4,166.-	4,166.-
Expense for salary of workers (average 10%/ Batch)	4,509.60.-	4,973.19
Packaging (25 Baht/Box); packed of 10 vials/box	2,000.-	2,000.-
Total expenses	51,605.60	56,705.10
Average/vial	64.50	70.88

* Separated serum was concentrated before preparation

** See Appendix VII

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

Results of protein and lipid in two level lyophilized control sera obtained from three external clinical chemistry laboratories, using different analytical methods *i.e.* manual method, dry chemistry and normal autoanalyzer were shown in Table 32 compared with that obtained in our laboratory.

From analytical results, it was found that LDH activity in both levels of the prepared control sera assayed by manual and dry chemistry methods were markedly higher than that obtained in our laboratory. This

can be explained by the difference in method of analysis (Beckman instruction manual vs Operation manual of various method on different instruments). Concentrations of other analytes or enzyme activity of the different methods obtained from different laboratory were slightly varied from the results of our laboratory. Their variations were caused by the differences in analytical reagents, the principle of method of each instrument and the precision of analysis of each laboratory.

Table 32. Concentration of the constituents in control serum in the presence of 8.5% saccharose.

Constituents		Our laboratory*	Other laboratory*[1]	Manual method*[2]	Dry chemistry*[3]
Total protein (g/dL)	L.I	6.7	6.6	6.0	6.5
	L.II	5.2	5.9	5.4	5.8
Albumin (g/dL)	L. I	3.3	4.0	4.0	3.1
	L.II	2.7	3.1	3.6	2.6
AST (U/L)	L.I	32	35	96	58
	L.II	95	98	142	135
ALT (U/L)	L.I	13	49	40	52
	L.II	30	61	52	78
ALP (U/L)	L.I	84	72	94	108
	L.II	184	150	138	195
CK (U/L)	L.I	224	116	145	126
	L.II	462	265	313	222
LDH (U/L)	L.I	209	140	812	717
	L.II	727	588	2743	2535
Cholesterol (mg/dL)	L.I	108	144	143	134
	L.II	175	188	199	208
Tg (mg/dL)	L.I	80	96	108	142
	L.II	80	85	89	138
HDL-C (mg/dL)	L.I	35	39	48	-
	L.II	118	82	102	-
LDL-C (mg/dL)	L.I	57	74	101	-
	L.II	41	78	97	-

L.I = Level I and L.II = Level II.

* Beckman CX-5 Autoanalyzer.

*[1] Maharaj Nakorn Chiang Mai Hospital, Merck Mega Autoanalyzer.

*[2] Lanna Medical Laboratory, Chiang Mai.

*[3] Rachawate Hospital, Chiang Mai, Kodak Ektachem.