5. RESULTS

5.1 Precision of methods

Within-run and between-run precision of all tests were evaluated. The within-run coefficient of variation (%CV) of serum glucose, fructosamine, BUN and creatinine were 1.9, 6.4, 8.9, and 4.4% and between-run CV were 2.1, 7.8, 9.2 and 7.6% respectively as shown in Table 1 and Figures 1 and 2

Pooled urine sample both high and normal levels of microalbumin and NAG were tested and found that the within-run and between-run CV in high and normal levels for microalbumin were 1.4, 3.3 and 4.7, 7.8 and for NAG were 1.9, 6.7 and 4.0, 11.7% as shown in Table 2, Figure 3 and Table 3, Figure 4, respectively.

5.2 Linearity study of MA and NAG determination methods

Various concentrations of human serum albumin were tested with the method described in 4.3.1 and found that the method obeys Beer's law for at least 100 mg/dl as shown in Figure 5.

Standard NAG was diluted into various concentrations and tested. The linearity of the test found was upto at least 62.54 U/l as shown in Figure 6.

5.3 Accuracy studies

It was found that the average standard NAG recovered were 112.8%, ranged between 109.3 and 115.0% as shown in Table 5.

The recovery of standard microalbumin were averaged 105.0%, ranged between 99.0 and 109.5% as shown in Table 6.

5.4 Stability of BPB and CNP-NAG reagents

BPB reagent was prepared and evaluated using albumin standards, 20 and 100 mg/dl, every 10 days. The results showed that keeping at 4 °C, the reagent gave very closed values for at least 60 days as shown in Figure 7.

CNP-NAG reagent was prepared and used for determination of urinary NAG (20 and 55 U/l) every 5 days. It was found that CNP-NAG substrate could be kept at 4 °C for 10 days. On day 15 the NAG activity began to decrease significantly especially with 20 U/l urine sample, as shown in Figure 8.

5.5 Correlation of the tests

A. Correlation coefficient (r) of microalbumin in urine and NAG activity was $r = 0.48$, $p < 0.05$ in diabetics and increased to 0.78, $p < 0.05$ in patients with diabetic nephropathy, as shown in Figures 9 and 10.

B. Correlation coefficient (r) of MA, NAG activity and HbA_{1C} in all diabetics were 0.39 and 0.14, respectively, as shown in Figures 11 and 12, in patients with diabetic nephropathy (n=14) the correlation coefficient (r) of MA, NAG with HbA_{1C} was more correlated ($r=0.49$, 0.45 $p<0.05$, respectively.) as shown in Figures 13 and 14.

C. Correlation of MA, NAG with fructosamine in diabetics (n=220) were least correlated ($r=0.17$, 0.14 $p<0.05$, respectively), as shown in Figures 15 and 16. In DN (n=14), the correlation of MA, NAG with fructosamine were higher ($r = 0.43$, 0.38 $p<0.05$, respectively), as shown in Figures 17 and 18.

D. Correlation coefficient (r) of HbA_{1C} and fructosamine as glycemic control. Fructosamine and HbA_{1C} was highly correlated ($r = 0.7$, $p < 0.05$) as shown in Figure 19.

5.6 Cut-off predictive levels of NAG and MA.

The predictive threshold of MA and NAG activity in diabetes were compared with healthy control (n=100) and diabetics (n= 220). Using the cut-off levels of MA and NAG activity at mean + 3SD (MA=2.97 mg./gm creat., NAG=32.40 U/gm. creat.). The NAG activity of healthy control were all normal and only 3 had higher MA than the cut-off level, as shown in Figures 20 and 21. We also used ROC curve to estimate the cut-off levels of MA and NAG activity for diabetics in which 3.49 mg/gm creat. and 37.2 U/gm creat. were found respectively, as shown in Figures 22 and 23.

5.7 Clinical values of urinary MA and NAG determinations

Sensitivity and specificity of urinary MA and NAG activity tests for diabetic nephropathy were evaluated. Urinary microalbumin alone has %sensitivity and specificity of 100% and 89.7% while using NAG activity alone, they were 75.0 and 81.9% respectively. Combination of both tests, the sensitivity and specificity were 100% and 98.3% as shown in Table 7.

5.8 Positive and negative predictive values of MA and NAG activity

The positive predictive values (%) MA, NAG activity and MA in combination with NAG activity were 33.8, 18.1 and 69.9% at 5%

prevalence, for 10% prevalence the values were 51.8, 31.5 and 71.4 % and for 15% prevalence , they were 63.5, 42.5 and 79.8 %, respectively.

The negative predictive values (%) of MA and MA in combination with NAG activity at 5, 10, 15% prevalences were 100% and for NAG activity at 5, 10, 15% prevalences were 95.0, 96.8 and 98.5 %, respectively, as shown in Table 8.

5.9 Reference values

The mean ± 3SD reference values for urinary NAG activity, MA and serum fructosamine in 100 healthy adults were 18.06 ± 4.78 U/gm creat., 1.44 ± 0.51 mg/gm creat. and 1.45 ± 0.56 mmol/l and the reference ranges were 3.72-32.4 U/gm creat, 0.09-2.97 mg/gm creat. and 0.00-3.13 mmol/l, respectively as shown in Table 4.

Table 1. Precision of methods used for serum glucose, fructosamine and BUN, creatinine determinations. (n=10)

	Within-run			Between-run		
	Mean	SD	%CV	Mean	SD	%CV
Glucose (mg/dl)	91.2	1.7	1.9	90.3	1.9	2.1
BUN (mg/dl)	20.2	1.3	6.4	21.9	1.7	7.8
Creatinine (mg/dl)	1.5	0.11	8.9	1.4	0.13	9.2
Fructosamine (mmol/l)	1.8	0.08	4.4	1.7	0.13	7.6

Table 2. Precision of the NAG activity (U/gm creat.) determination using CNP-NAG as substrate. (n =10)

	Within-run			Between-run		
	Mean	SD	%CV	Mean	SD	%CV
High level control	54.2	1.02	1.88	55.0	2.20	4.00
Normal level control	21.4	1.44	6.73	20.4	2.60	11.70

Table 3. Precision of the urinary microalbumin determination with bromphenol blue (BPB) method. (n=10)

	Within-run			Between-run		
	Mean	SD	%CV	Mean	SD	%CV
High level control	92.0	1.3	1.4	92.0	4.3	4.7
Normal level control	15.1	0.5	3.3	15.3	1.2	7.8

Table 4. Reference ranges of urinary NAG activity, MA in urine and serum fructosamine in 100 healthy adults.

	NAG activity (U/gm. creat.)	MA (mg/gm creat.)	Fructosamine (mmol /l)
Mean	18.06	1.44	1.45
SD	4.78	0.51	0.56
Mean ± 2SD	8.50 - 27.62	0.42 - 2.46	0.33 - 2.57
Mean ± 3SD	3.72 - 32.40	0.09 - 2.97	0.00 - 3.13

Table 5. Analytical recovery (%) of standard NAG (64.52 U/l) activity added into urine sample.(Average of 4 determinations)

urine (µl)	NAG in urine(U/l)	Std.NAG added (U/l)	Total NAG (U/l)	NAG found (U/l)	% Recovery of standard
500	20	-	-	-	-
400	16	12.8	28.8	30.6	114.1
300	12	25.7	37.7	40.1	109.3
200	8	38.6	46.6	52.4	115.0
				Mean	112.8

Table 6. Analytical recovery (%) of standard MA (100 mg/dl) added into urine sample. (Average of 4 determinations)

urine (µl)	MA in urine(mg/dl)	Std. MA added (mg/dl)	Total MA (mg/dl)	MA found (mg/dl)	% recovery of standard
500	15.6	-	-	-	-
400	12.4	20.0	32.4	32.2	99.0
300	9.3	40.0	49.3	51.9	106.5
200	6.2	60.0	66.2	69.1	104.8
100	3.1	80.0	83.1	90.7	109.5
				Mean	105.0

Table 7. Sensitivity, specificity of urinary MA (cut-off 2.97 mg/gm creat) and NAG activity (cut-off 32.40 U/gm creat.).

	MA (%)	NAG (%)	NAG & MA (%)
Sensitivity	100.0	75.0	100.0
Specificity	89.7	81.9	95.5
False positive	9.5	16.8	3.6
False negative	0.0	1.8	0.0

Table 8. Positive and negative predictive values of urinary MA (cut- ff 2.97 mg/gm creat.) and NAG activity (cut-off 32.40 U/gm creat.) in diabetics at different prevalences.

Prevalence (%)	Positive predictive value (%)			Negative predictive value (%)		
	5.0	10.0	15.0	5.0	10.0	15.0
MA	33.8	51.8	63.5	100.0	100.0	100.0
NAG activity	18.1	31.5	42.5	95.0	96.8	98.5
MA & NAG activity	69.9	71.4	79.8	100.0	100.0	100.0

Table 9. Sensitivity, specificity of urinary MA (cut-off 3.48 mg/gm creat.) and NAG activity (cut-off 37.20 U/gm creat.) in diabetics (using ROC curve for cut-off level determination).

	MA (%)	NAG (%)	NAG & MA (%)
Sensitivity	94.1	75.0	100.0
Specificity	93.1	90.7	98.3
False positive	6.4	8.6	1.4
False negative	0.5	2.2	0.0

Table 10. Positive and negative predictive values of urinary MA (cut-off 3.48 mg/gm creat), NAG activity (cut-off 37.20 U/gm creat.) in diabetics at different prevalences. (using ROC curve for cut-off level determination).

	Positive predictive value (%)			Negative predictive value (%)		
Prevalence (%)	5.0	10.0	15.0	5.0	10.0	15.0
MA	41.6	46.1	70.1	99.7	99.2	98.9
NAG activity	29.2	45.1	57.5	98.3	96.3	94.3
MA & NAG activity	72.5	87.0	89.8	100.0	100.0	100.0

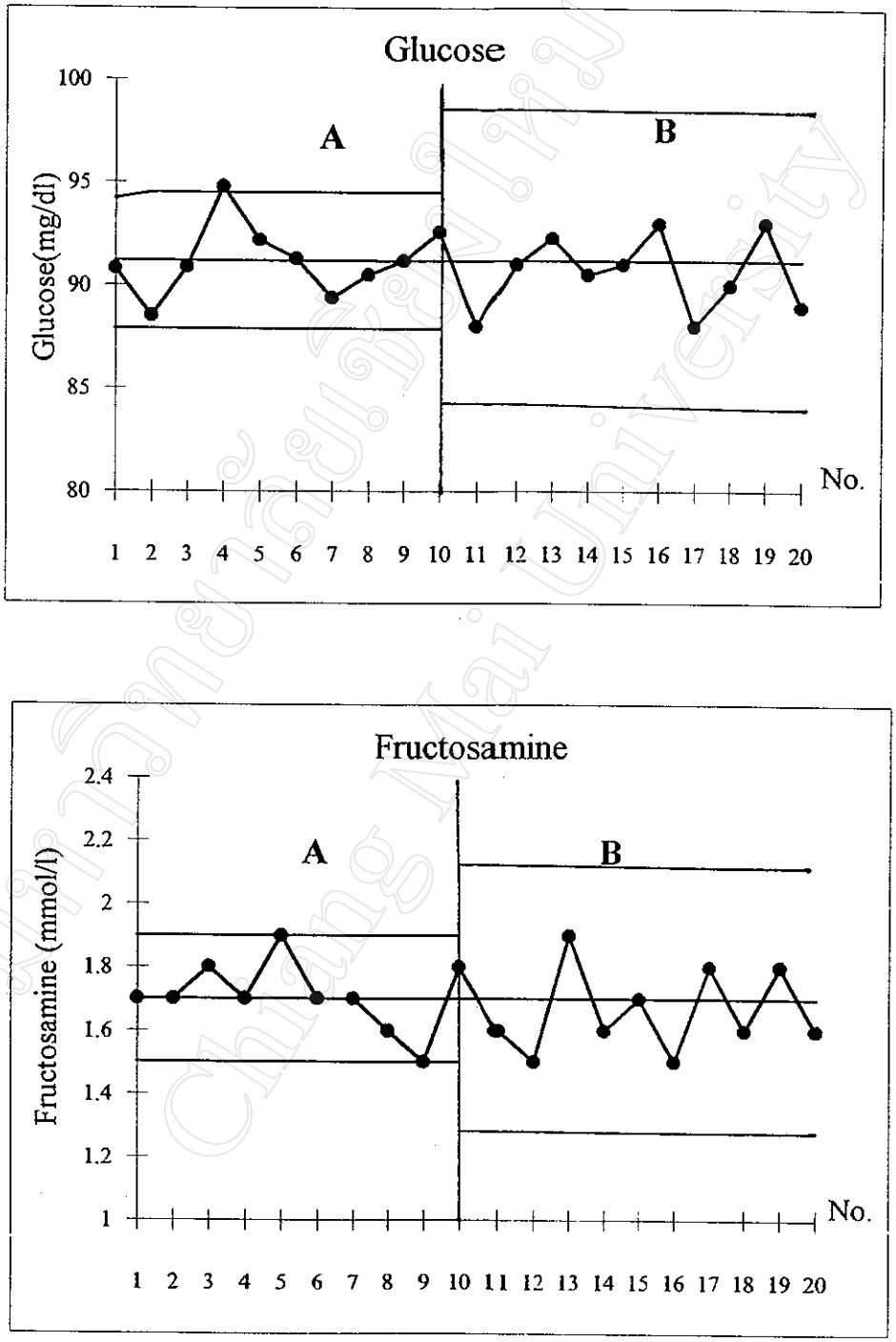
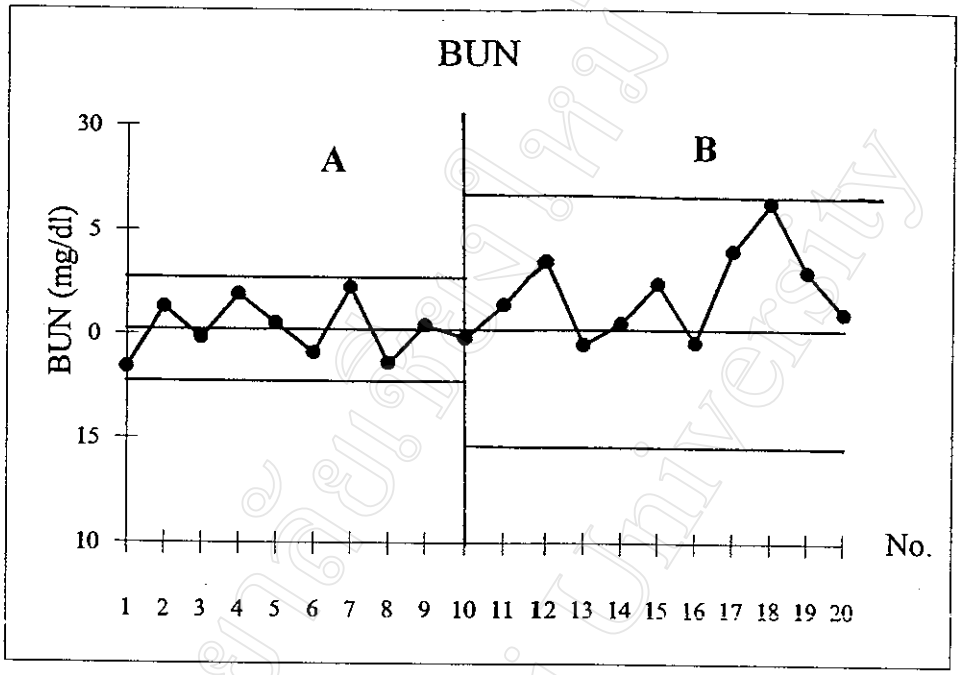


Figure 1. Distribution of glucose and fructosamine in within-run (A) and between-run (B) precision studies.



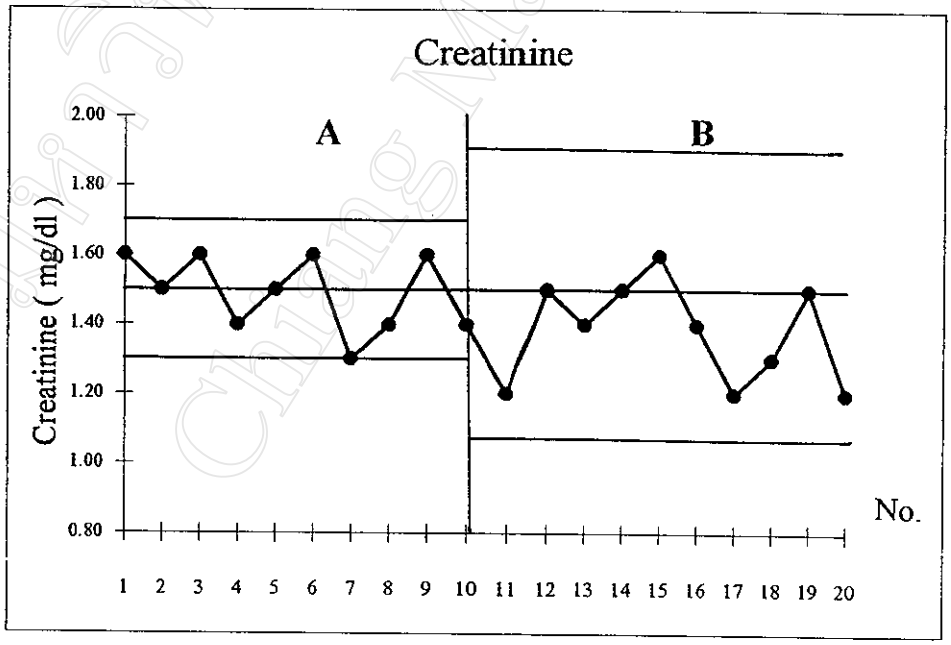
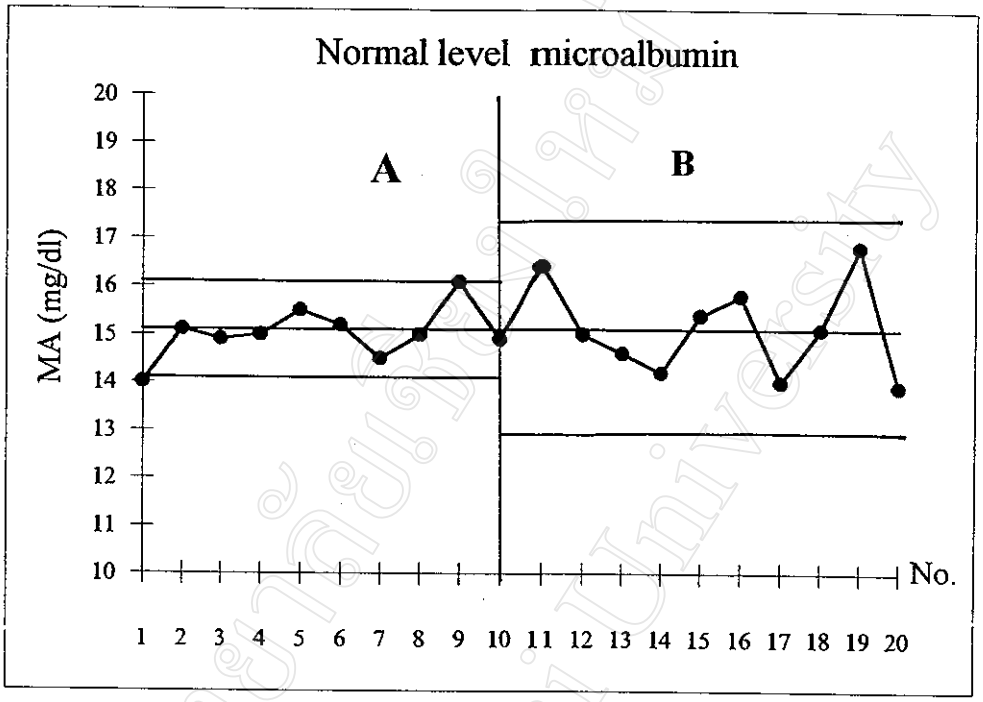


Figure 2. Distribution of BUN and creatinine in within-run (A) and between-run (B) precision studies.



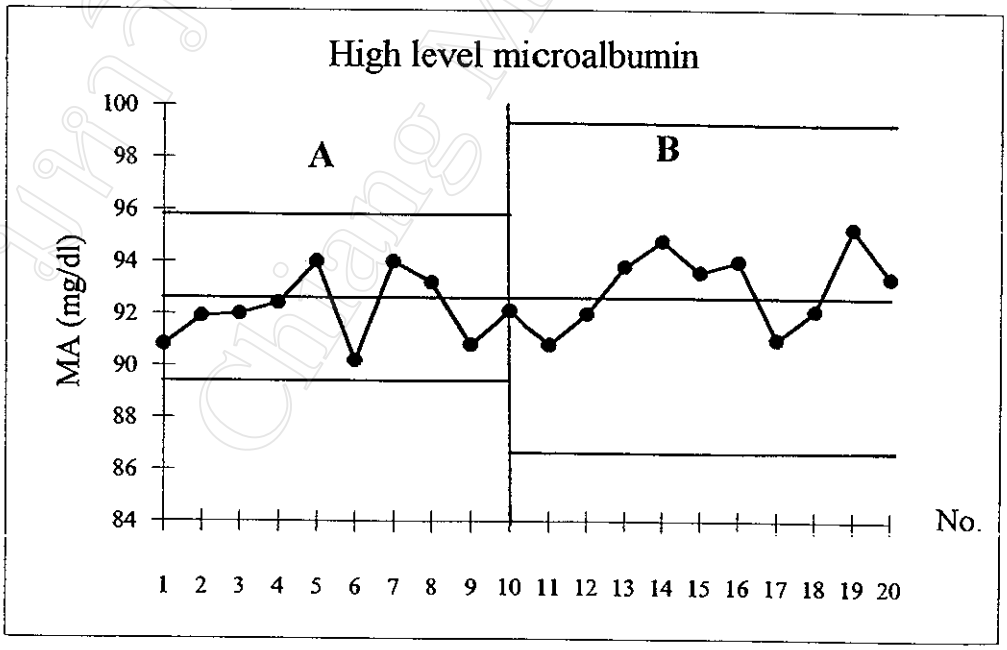


Figure 3. Distribution of normal and high levels MA in within-run (A) and between-run (B) precision studies.

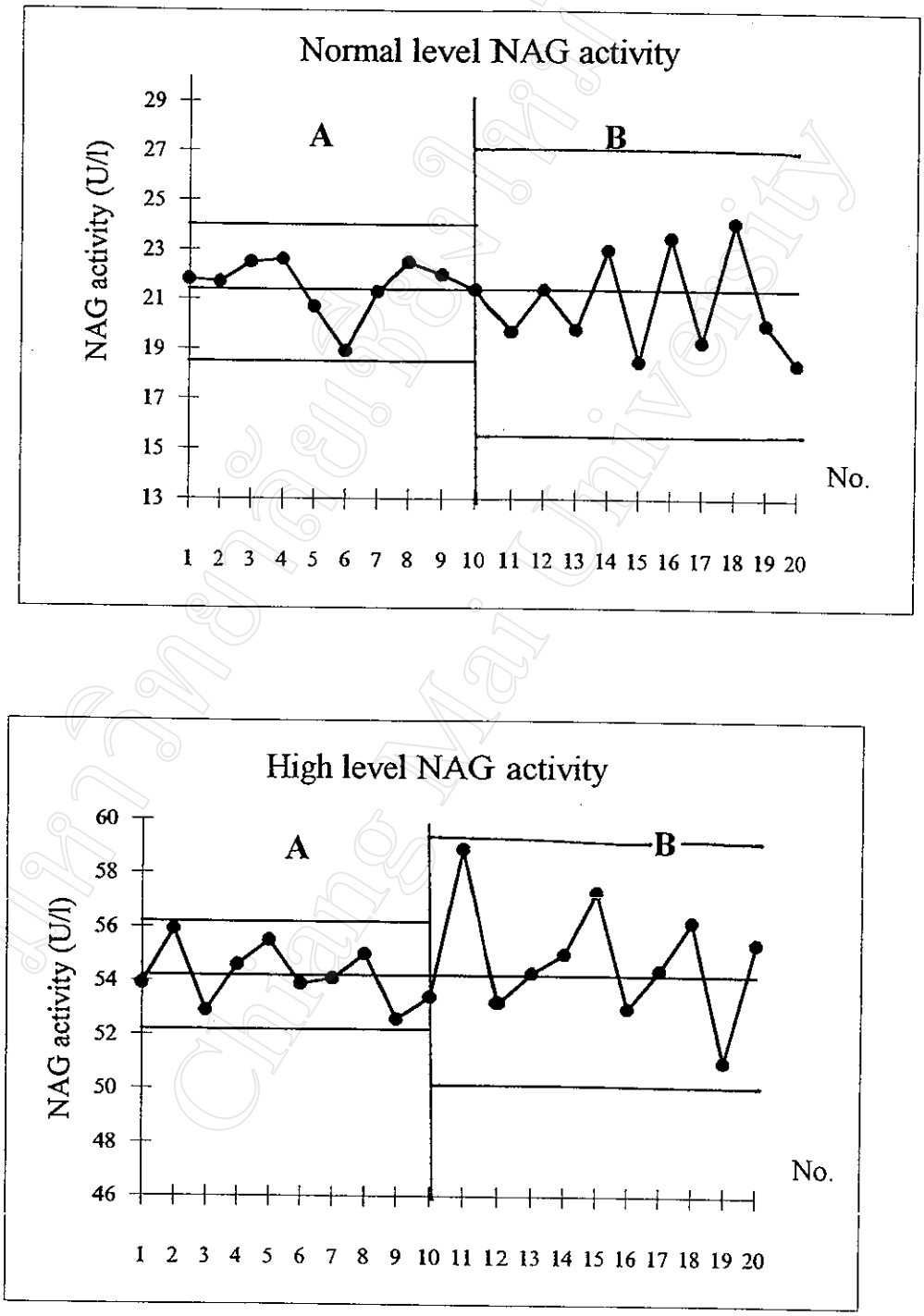


Figure 4. Distribution of normal and high levels of NAG activity in within-run (A) and between-run (B) precision studies.

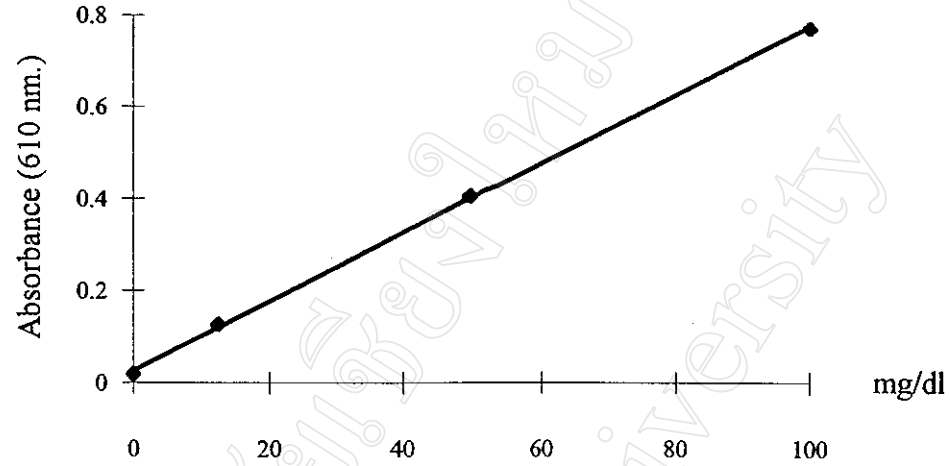


Figure 5. Calibration curve of standard human albumin.

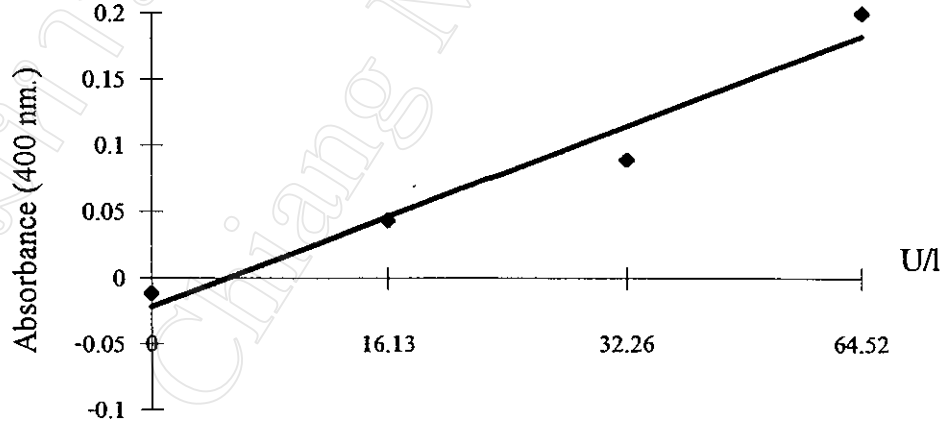


Figure 6. Calibration curve of standard NAG.

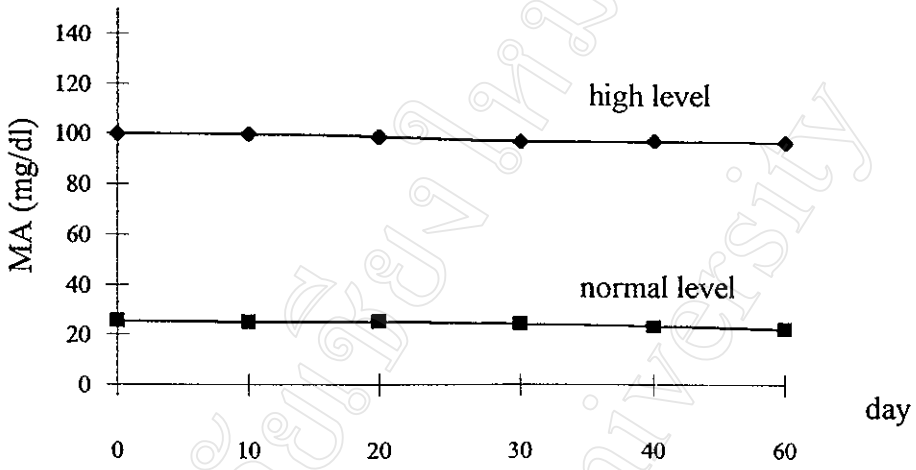


Figure 7. Stability of bromphenol blue reagent.(n=4)

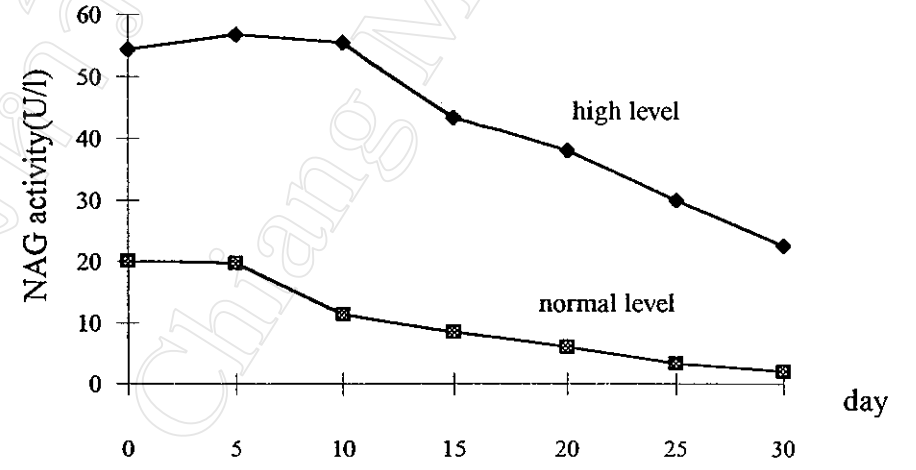


Figure 8. Stability of CNP-NAG reagent (n=4)

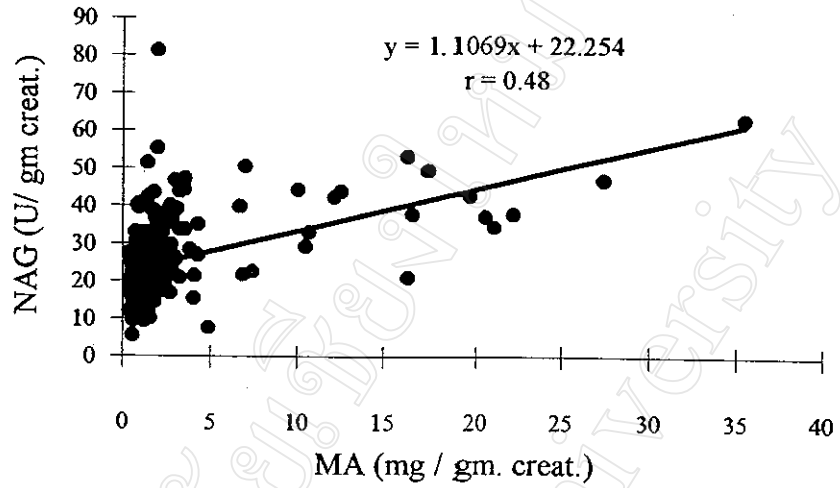


Figure 9. Correlation of MA and NAG activity in diabetics. (n=220)

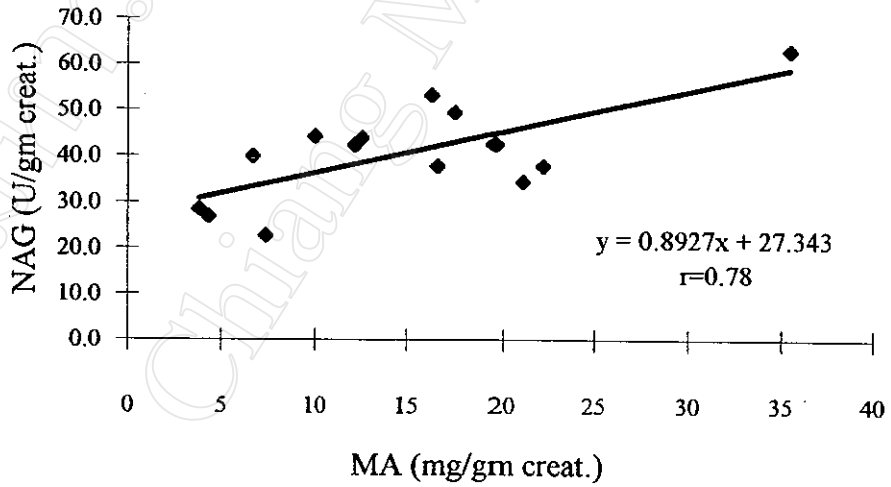


Figure 10. Correlation of MA and NAG activity in patients with diabetic nephropathy.(n=14)

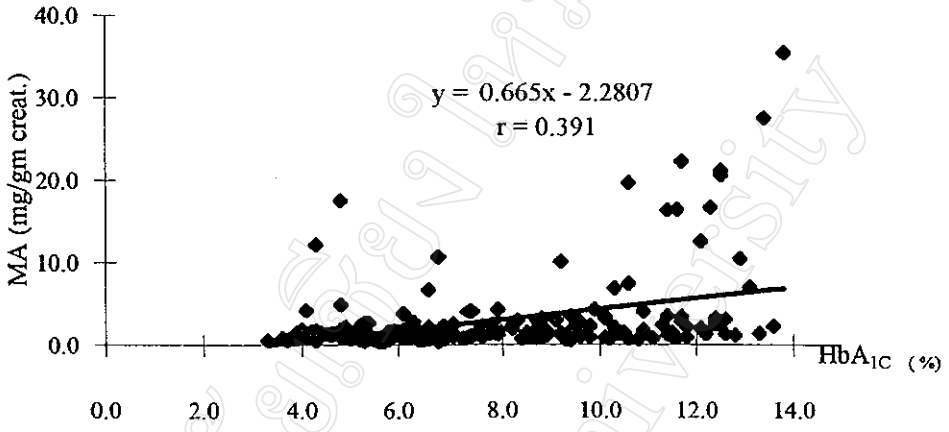


Figure 11. Correlation of MA and HbA_{1C} in diabetics. (n=220)

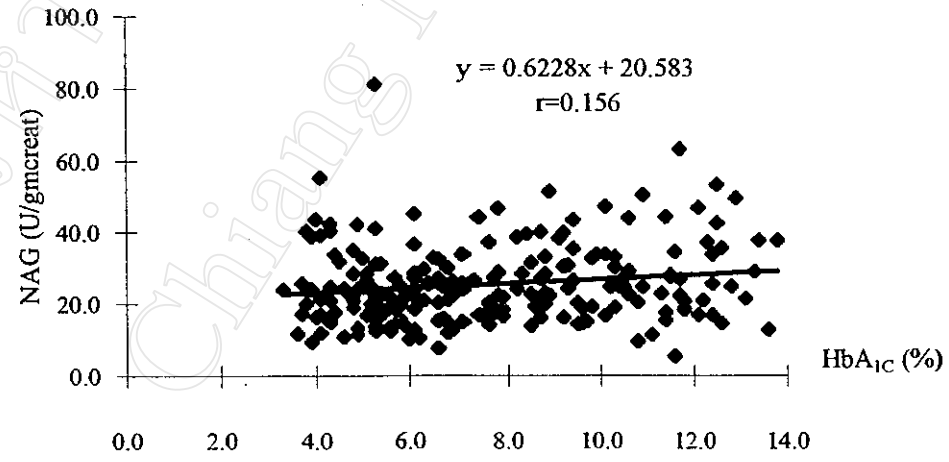


Figure 12. Correlation of NAG activity and HbA_{1C} in diabetics. (n=220)

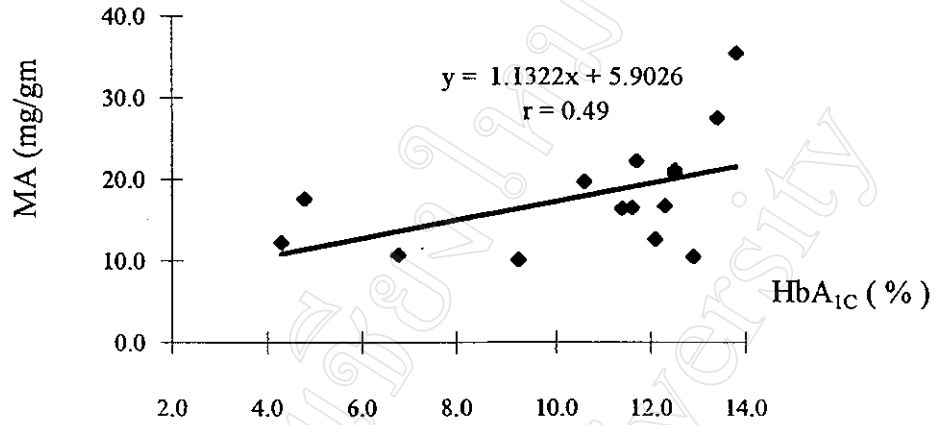


Figure 13. Correlation of MA and HbA_{1C} in patients with diabetic nephropathy. (n=14)

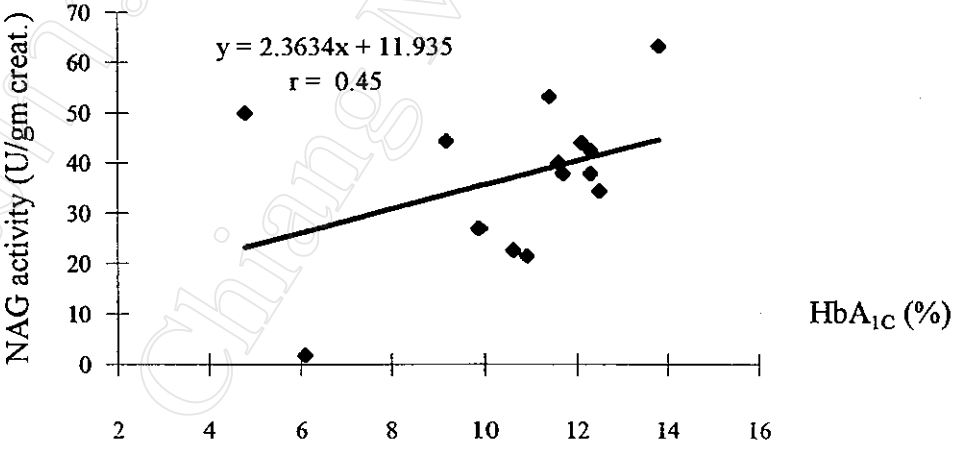


Figure 14. Correlation of NAG activity and HbA_{1C} in patients with diabetic nephropathy. (n=14)

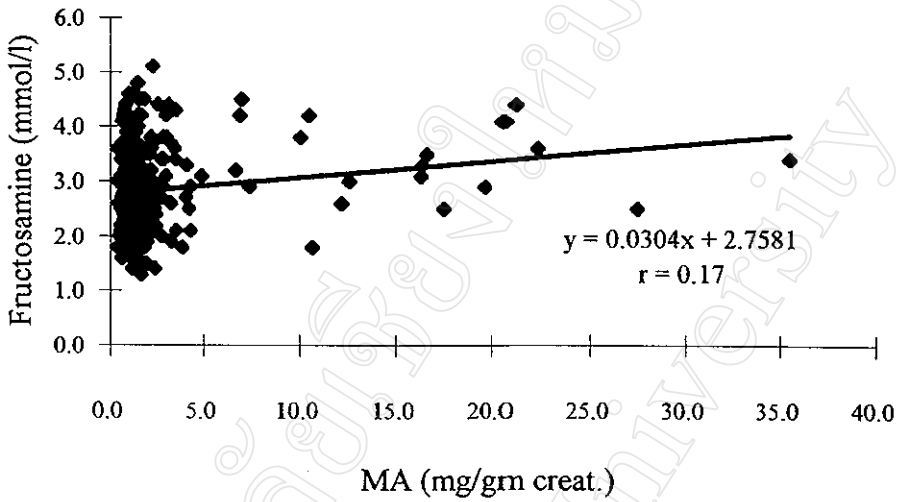


Figure 15. Correlation of MA and fructosamine in diabetics. (n=220)

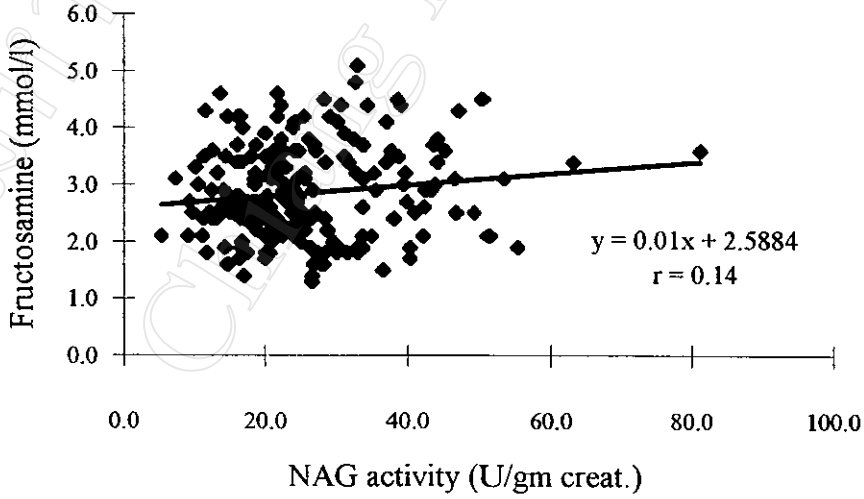


Figure 16. Correlation of NAG activity and fructosamine in diabetics. (n=220)

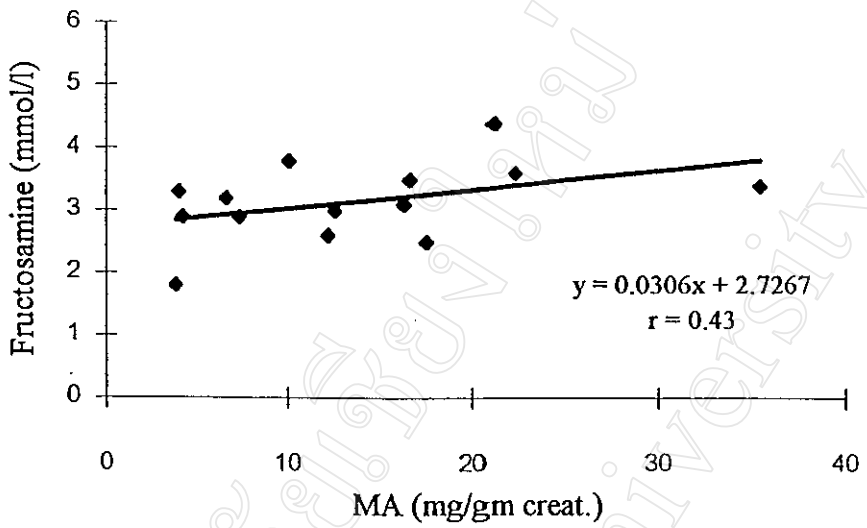


Figure 17. Correlation of MA and fructosamine in patients with diabetic nephropathy. (n=14)

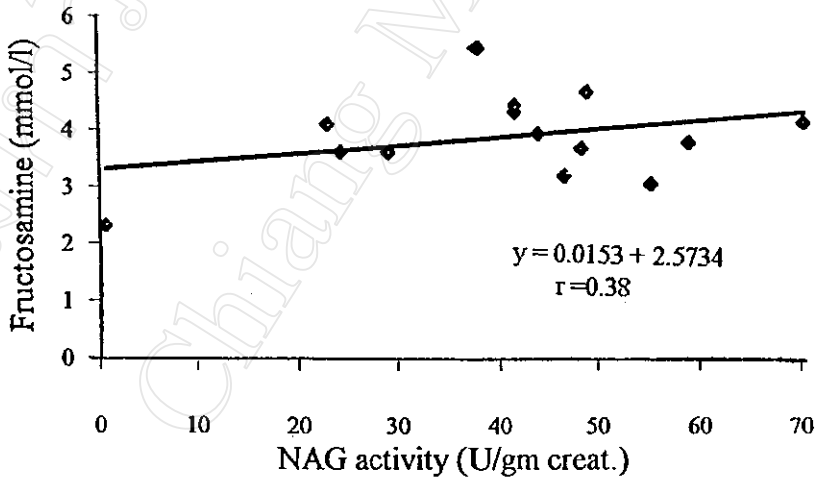


Figure 18. Correlation of NAG activity and fructosamine in patients with diabetic nephropathy (n=14)

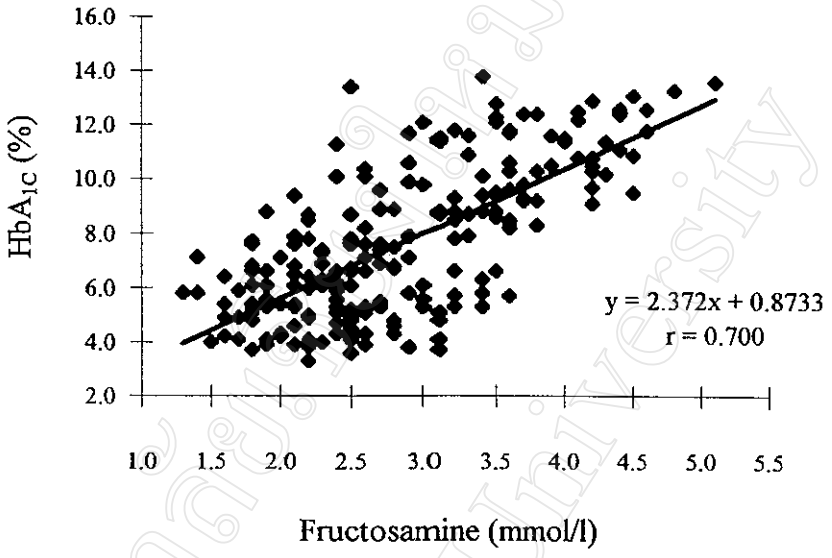


Figure 19. Correlation of fructosamine and HbA_{1C} in diabetics. (n=220)

Figure 20. Distribution of NAG activity in normal control group (n=100) and diabetics (n=220).

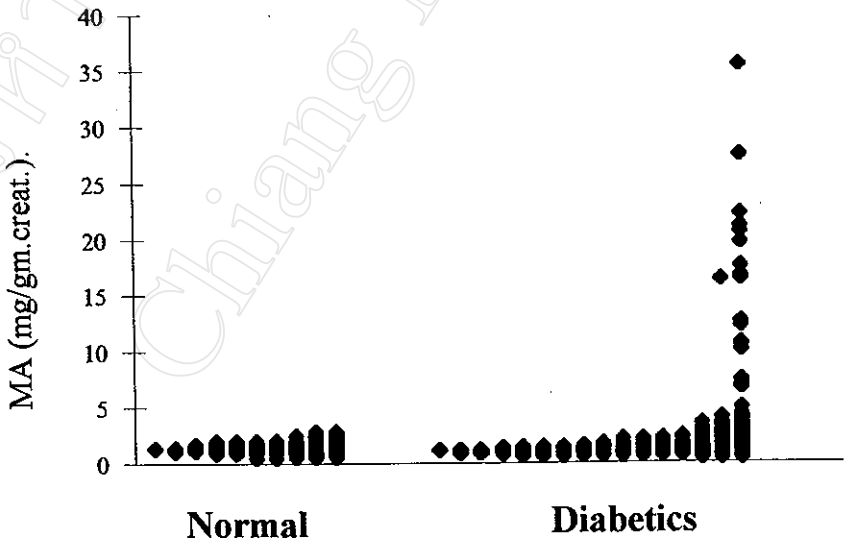


Figure 21. Distribution of MA in normal control group (n =100) and diabetics (n= 220).

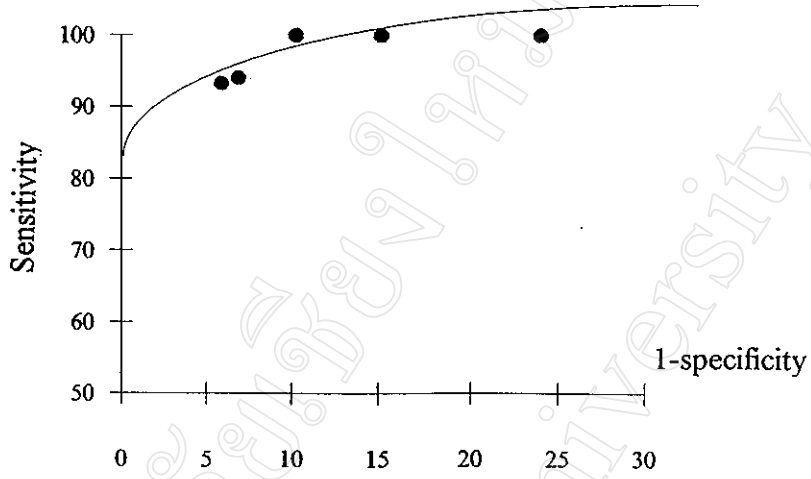


Figure 22. Receiver operating characteristics curve (ROC curve) of MA level in diabetics. (n=220)

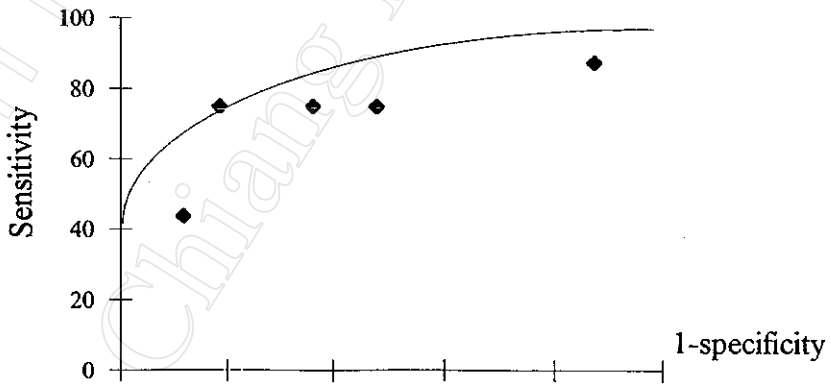


Figure 23. Receiver operating characteristics curve (ROC curve) of NAG activity in diabetics.(n=220)