

## DESIGNING ELISA METHOD FOR MEASURING THE LEVEL OF ANTI-STREPTOKINASE ANTIBODY

Saffari Mahmood<sup>1</sup>, Sameii Azam<sup>2</sup>, Motavalli Mohammadali<sup>3</sup>, Abtahi Hamid<sup>4</sup>

### ABSTRACT

**Objectives:** Streptokinase, a protein produced by group C streptococci, is prescribed as a thrombolysis treatment for the patients who suffer from acute myocardial infarction. Antibodies that are produced to neutralize streptokinase are challenges in treatment of MI patient particularly those who experience the attack for the second time. The purpose of this study was to introduce a simple enzyme immunoassay for determination of anti-streptokinase antibodies in plasma of patients.

**Methodology:** For designing the standard method of Indirect Immunoassay or ELISA, commercially available reagents have been used for the assay, which are calibrated with serums of healthy individuals as control group and patients with acute myocardial as experimental group. Level of anti-streptokinase antibodies of treated patients with streptokinase and healthy voluntaries were assayed after 0, 1 and 6 months and results were analysed with statistical methods.

**Results:** Sensitivity and specificity of this method was 88 and 95 percent respectively and its repeatability was CV, 8%. The mean of streptokinase antibody in the control group which was selected from the healthy population was 0.78 Au/ml (SEM,  $\pm 0.04$ ), where as in the 45 patients of experimental group treated with streptokinase with no prior history of receiving this medication it was 0.95 Au/ml (SEM,  $\pm 0.04$ ). The level of streptokinase measured after time interval of one and six month in both healthy and the experimental group was 0.74 Au/ml (SEM,  $\pm 0.04$ ) and 0.74 Au/ml (SEM,  $\pm 0.04$ ); 6.6 Au/mg (SEM,  $\pm 1.74$ ) and 7.63 (SEM,  $\pm 1.46$ ), respectively.

**Conclusion:** At the first stage, a significant correlation ( $r = 0.92$ ,  $P < 0.0001$ ) between the measured antibody was observed, whereas, a considerable change after receiving the first dose of medicine in the experimental group was detected (seroconversion, 0.89), and this change was even more pronounced following the administration of the second dose. These findings indicate the need for accessibility of a quick and efficient method for assessing the level of anti-streptokinase antibody prior to the prescription of this medicine, particularly after the second or subsequent doses.

**KEYWORDS:** Streptococcus group C, Streptokinase, Thrombolytic treatment.

Pak J Med Sci April - June 2009 (Part-II) Vol. 25 No. 3 474-478

### How to cite this article:

Mahmood S, Azam S, Mohammadali M, Hamid A. Designing ELISA method for measuring the level of anti-streptokinase antibody. Pak J Med Sci 2009;25(3): 474-478.

---

### Correspondence

Saffari Mahmood, Ph.D,  
Email: saffarimahmood@yahoo.com

- \* Received for Publication: August 21, 2008
- \* Revision Received: January 28, 2009
- \* 2<sup>nd</sup> Revision Received: April 23, 2009
- \* Final Revision Accepted: May 11, 2009

## INTRODUCTION

The use of fibrinolytic system as a choice for the treatment of thrombolysis started in 1965<sup>1,2</sup> and then new generations were developed.<sup>3</sup> This method is still in use in the treatment of patient who suffer from myocardial infarction or stroke as the selective method<sup>1,2,4</sup> and for the

treatment of loculated pleural effusion<sup>2</sup> and is a good alternative for surgical procedure in femoropopliteal.<sup>5</sup> The medicines that are used as the activating of fibrinolytic system in all cases lead to the formation of protease serine known as plasmin through a cascade mechanism.<sup>6</sup> This newly formed matter has the capability of dissolving clot and removing blockage.<sup>1,3,7</sup>

One of the methods most often used is the treatment of thrombolysis is through the use of streptokinase and related medicines immediately following MI which leads to the vasodilation of coronary arteries of the lesion area.<sup>2,8</sup> The application of these medicines as an effective measure in patients who suffer a heart attack is still the first choice. In various studies, the superiority of this treatment compared to other choices for fibrinolysis including activating plasminogen with tissue originality has been demonstrated.<sup>1,8,9</sup> It is known as an ideal medicine, but on the other hand, neutralizing streptokinase antibody that is produced in these patients especially following the consumption of this medicine can act as the nonphysiologic controlling factor causing disturbance in adhesion to plasminogen and further limiting streptokinase function<sup>7,8,10,11</sup>

Additionally, streptokinase is a naturally occurring product from the bacteria streptococci. Because streptokinase is a bacterial product and an antigen, it seems that many individuals who have had previous streptococcal infection (e.g. strep throats) have anti-streptokinase antibodies in their blood. These antibodies neutralize streptokinase when it is administered as a drug.<sup>12,13</sup> Since the application of streptokinase as the method of managing these types of patients require knowledge about the presence of these antibodies in the circulation of recipient of the medicine,<sup>10,14</sup> this research was designed to introduce a method with high sensitivity, precision, and repeatability plus high specificity to measure the level of streptokinase antibodies in patients who are the subject of this treatment. The method is an immunologic immunoassay which is based on the reaction of antigen and antibody<sup>15,16</sup> and promise to be very useful.

## METHODOLOGY

*Samples:* The sample for this research included two groups of individuals; 45 patients in the first group served as the experimental group with the age range of 30 to 79 years that were candidate for streptokinase administration. The second group consisted of 55 healthy volunteers (control group) with the age range 20 to 69 years. These groups voluntarily participated in the project and following the completion of informed consent form and application containing demographic information they underwent the treatment. All the subjects in these groups gave 10 ml of venous blood on three different stages; beginning, one and six months after the initiation of the project. Plasmas were prepared from samples anticoagulated with 1:10 volume of a 0.109 M solution of trisodium citrate, pH 6.0 and were kept at -20°C.

*Enzyme immunoassay:* For the purpose of measuring the level of streptokinase antibody in the serum of subjects, a method of measuring antibody through enzyme marking or ELISA was designed.<sup>10,16</sup> The microtitre plates were incubated overnight with 100µl of streptokinase solution for coating (Heber Biotec Company, Germany, 500 IU/ml, prepared in PBS; 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2 ) for 1 h at 37°C.

Afterwards microtitre plates were washed by buffer ( 1000ml PBS supplemented with 0.5 ml tween 20, pH 7.4 ) and incubated with PBS+ ( PBS supplemented with 0.35 M NaCl , 0.002 M EDTA , 1.5% gelatin , 1ml/l tween 20) for 1 h at room temperature to block remaining binding sites. Thereafter non-bound material was removed and the microplates were washed three times with buffer, the amount of 100µl of various concentration of serum was added, incubated at 37°C for five minutes, and washed by buffer three times. The amount of bound anti- streptokinase antibody was determined by incubation with 100µl HRP (Horse radish peroxidase) - conjugated anti-human IgG diluted 1:20000 (DAKO,Denmark) in PBS+ at room temperature for five minutes. After washing again, 100µl of chromogen substrate containing TMB reagent (Biogene company)

was added to the wells, and incubation done at room temperature for five minutes. The reaction was stopped by adding 100 µl of 1 N sulfuric acid and the absorbance read at 450 nm using a microplate reader.

*Anti-streptokinase antibodies assay:* For the purpose of obtaining semiquantitatively arbitrary unit of the level of streptokinase antibody, one internal standard curve was plotted. For this purpose, the mean of mixing the prepared serums of the 2<sup>nd</sup> and 3<sup>d</sup> stage from the experimental group was obtained<sup>16,17</sup> and the designed test was applied on various concentrations of serums. The OD level of these serums at 1:204800 concentrations was set as blank OD. Therefore at 1:102400, more than one arbitrary unit of antibody was available.<sup>15-17</sup> Also, in order to prevent background discoloration reactions,<sup>8</sup> one mixture of control group serums was also prepared and the test was applied at various concentrations. This serum at the 1/800 concentration was leveled to OD equal to blank. As a result, the 1/400 concentration contained one Au/ml antibody. This concentration was used as the threshold concentration. All the serums at 1/400 concentration, with OD equal to blank were classified as negative and those with equivalent at concentration of 1/800 and more were labeled as one, two, three, ... Au/ml of antibody, respectively<sup>10,15,17</sup> According to this criterion of internal standard prepared for the mixture (stored), the serums of the experimental group at the

2<sup>nd</sup> and 3<sup>rd</sup> stage contained 8 Au/ml (8 units) of antibody (1:800;1, 1:3200;2, 1:6400;3, 1:204800;8 AU/ml). The results were calculated using a standard dilution curve of reference plasma, which was plotted against the absorbance on a double-logarithmic scale. Through this procedure, the level of antibody in all samples shown by plotting the standard curve measuring the antibody in Au/ml against OD was assessed.

## RESULTS

In this study, the antibody measured as the neutralizing antibody is defined as the anti-streptokinase. The mean value of this antibody in serum sample of control group at the first stage was 0.78 Au/ml (SEM, ±0.04) and in the experimental group, before the injection of streptokinase, it was 0.95 (SEM, ±0.04). The results of independent t-test showed that there was no significant difference between the levels of neutralizing antibody ( $p < 0.05$ ). In addition, in 24 out of 55 individuals in the control group (44%), the measurable level of streptokinase neutralizing antibody between one to three units was observed. This marker for 22 out of 45 individuals in the experimental group was 49%. At the second stage, the mean value of antibody in the control group was 0.74 Au/ml (SEM, ±0.04) and in 23 out of 51 individuals (45%), the value of neutralizing antibody reached one unit or more. The results of t-test showed that there was a significant difference

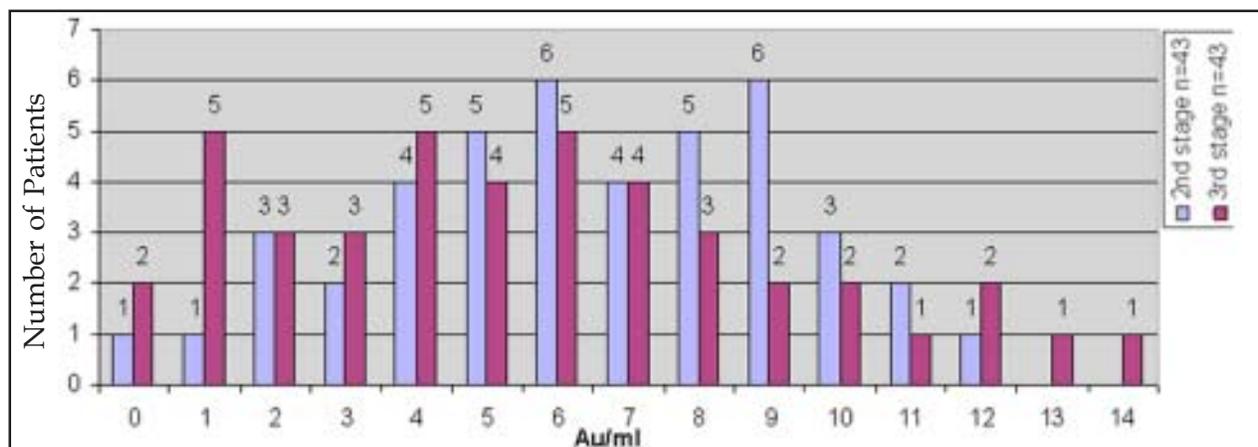


Fig-1: distribution of anti-streptokinase antibodies in experimental group at the 2nd & 3rd stage. The mean value of antibody in these stages was 7 times increased comparing to the time prior to the prescription of streptokinase.

between the two mean values ( $p < 0.001$ ). The mean value of antibody in this stage for the experimental group (after one month) was 6.6 Au/ml (SEM,  $\pm 1.74$ ), a 7 times increase comparing to the time prior to the prescription of streptokinase (Fig-1). In addition, the maximum level of antibody observed was 12 Au/ml. At the third stage, the mean value of antibody in the control group was 0.74 Au/ml (SEM,  $\pm 0.04$ ) and in 25 out 55 individuals (46%), the level of streptokinase antibody was between 1 to 3 units. At this stage, the mean value of antibody in the experimental group was 7.63 Au/ml (SEM,  $\pm 1.46$ ).

## DISCUSSION

The results of analysis of data indicated that at the first stage of assessment, anti-streptokinase antibodies were observed in 44% of the individuals in the control group and 49% of the subjects in the experimental group. Overall, the mean of anti-streptokinase antibodies was 46%. These results can be considered as the touch markers of streptokinase compared to other studies that have reported the value of 61<sup>10</sup> and 57%.<sup>15</sup> However, in the present study, due to the correction and elimination of background and using the concentration of 1:400 as the arbitrary base unit, this percentage is lower than the similar studies reported earlier.

The repetitions of the test at the second stage one month later showed that the measured marker in the control group had high correlation with the first stage ( $p < 0.001$ ). This is an indication of consistency and accuracy of the designed test. For the purpose of evaluating the accuracy, precision, and repeatability of the designed test, statistical analysis was used on two samples of control (N 28) group. The results indicated a co efficiency of variation equal to 8% (CV 8%). Considering the fact that most references report the antibody threshold equal to 2 Au/ml or more,<sup>10,17</sup> the predicted threshold has positive predictive value of 88% (PPV 88%) and negative predictive value of 90% (NPV 90%). The mean value of serum antibody in the subjects in the control group at the second stage was 6.6 Au/ml. Anti-streptokinase antibodies increases approximately seven times as the first stage. In some studies, an increase of 20 to 200 times for antibody streptokinase antibody titre following 3 to 5 days after contact with streptokinase has been reported.<sup>14,18</sup> Also, in other studies, a follow up of one month or more had shown an increase in antibody titre of 5 to 10 times in healthy population.<sup>10,19</sup>

It should be noted that in this study, due to the elimination of background, the possibility of anti-streptokinase antibody was controlled. In some studies, it has been shown that anti-streptolysin antibody (ASO) is an antibody

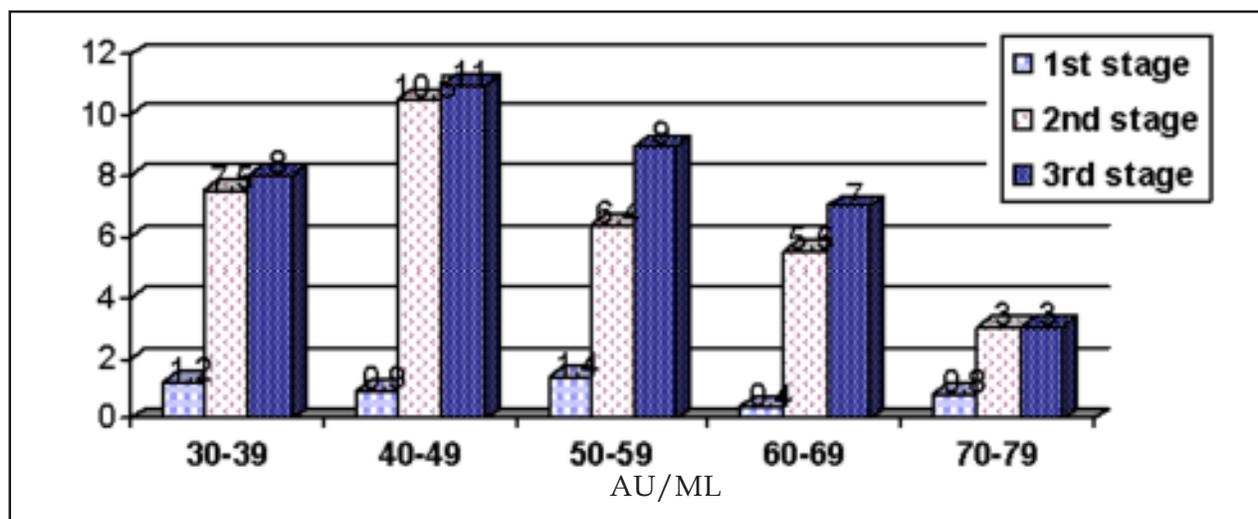


Fig-2: The mean value of antibody for different age groups at the 3 stages. The level of antibody after streptokinase treatment is much more in the middle age groups.

which is different from the one that neutralizes streptokinase due to some of its specific properties.<sup>10,15</sup> Streptokinase is neutralized by its antibody and has no relation with ASO.<sup>14</sup> The repetition of the experiment at the third stage, i.e. after six month following the first contact with the streptokinase for the control group, also indicated the consistency of marker at the second and third stages, but the marker in the experimental group showed negligible increase (Fig-1).

The presence of antibody at a considerable quantitative value in the circulation of the patient can be an important variable for the physician to treat the patient with streptokinase medication. The high antibody level is important in neutralizing streptokinase effect, particularly when any effect is detected,<sup>7,8,12</sup> and in such cases prescribing streptokinase will not lead to an appropriate treatment response.<sup>20</sup> It should be mentioned that all of these cases are not due to the presence of neutralizing antibodies,<sup>3,21</sup> and other inhibitors for the streptokinase have been reported.<sup>7</sup> Prescription of streptokinase for the patients who show high values of anti-streptokinase antibody can lead to the possible occurrence of reactions with mechanisms of the type III reactions.<sup>11</sup>

Fig-2 reveals that the mean values of antibody for various age groups are different. The implication of such a finding is that the seroconversion for anti-streptokinase antibody is present for all the age groups, but this phenomenon is more pronounced for the middle age groups.<sup>3</sup>

#### ACKNOWLEDGEMENTS

We are grateful to Dr. Abdare Isfahani from cardiology department, Dr. Abdoli from internal medicine department, Dr. Sayyah for statistical analysis and others for their valuable contribution in this study.

#### REFERENCES

1. Grager CB, Calf RM, Topal EJ. Thrombolytic therapy for acute myocardial infarction. *Drug* 1992;44(3):293-325.
2. Tanel L, Teet P. Effect of intrapleural streptokinase administration on antistreptokinase antibody level in patients with loculated pleural effusions. *Chest* 2003;123:432-5.
3. Longstaff C, Williams S, Thelwell C. Fibrin binding and the regulation of plasminogen activators during thrombolytic therapy. *Cardiovasc Hematol Agents Med Chem* 2008;6(3):212-23.
4. Robbins KC. Therapy, biochemical mechanism. *Semin Thromb Haemostas* 1991;(17):1-6.
5. Mularczyk T, Kostewicz W. Fibrinolytic intra-arterial therapy in treatment of arterial occlusion in femoropopliteal segment. *Int Angiol* 2008;27(4):313-8.
6. James P, McRedmond, Patrik H, Brian W, Desmond J, Fitzgerald. Streptokinase-induced platelet activation involves antistreptokinase antibodies and cleavage of protease-activated receptor-1. *Blood* 2000;95(4):1301-8.
7. Boxrud PD, Fay WP, Bock PE. Streptokinase binds to human plasmin with high affinity perturbs the plasmin active site and induces expression of a substrate recognition exosite for plasminogen. *J Biol Chem* 2000;275(19):14579-89.
8. White HD, Cross DB, Williams BF, Norris RM. Safety and efficacy of repeat thrombolytic treatment after acute myocardial infarction. *Br Heart J* 1990;64(3):177-81.
9. Gemmill JD, Hogg KJ, Dunn FG, Rae AP, Hillis WS. Predosing antibodies levels and efficacy of thrombolytic drugs containing streptokinase. *Br Heart J* 1994;(72):222-5.
10. Lynch M, Pentecost BL, Litter WA, Stockley RA. The distribution of antibodies to streptokinase. *Postgrad Med J* 1996;72(847):290-2.
11. Schellekens H. How to predict and prevent the immunogenicity of therapeutic proteins. *Biotechnol Annu Rev* 2008;14:191-202.
12. Blackwell N, Hollins A, Gilmore G, Norton R. Antistreptokinase antibodies: Implication for thrombolysis in a region with endemic streptococcal infection. *J Clin Pathol* 2005;58:1005-7.
13. Boon va, Munro JL, Kan GW, Burnell J, Speare R. Seroreactivity against streptococcal DRS (distantly related to SIC) protein is a predictor for ESRF. *Clin Vaccine Immunol* 2008;6.
14. Juhin P, Bortrom PA, Torpa, Bredberg A. Streptokinase antibodies inhibit reperfusion during thrombolytic therapy with streptokinase in acute myocardial infarction. *J Cardiol* 1999;245(5):483-8.
15. Alexander T, Krishnaswami S, Khanduri U. Anti-streptokinase levels in Indian patients. *Int J Cardiol* 1991;32(3):361-4.
16. Bom J, Victor J, Brugemann J. Rapid enzyme immunoassay of anti-streptokinase antibodies in human plasma. *Chemica Acta* 1993;(218):121-9.
17. Soada H, Pakyad P, Sattari M, Seyed N. Assessment of antistreptokinase antibody in patients with heart disease and normal subjects. *I J I* 2004;1(1):63-70.
18. Lynch M, Litter WA, Pentecost BL, Stockley RA. Immunoglobulin response in intravenous streptokinase in acute myocardial infarction. *Br Heart J* 1991;66(2):139-142.
19. Bernham M. Hypersensitivity and streptokinase (letter) *Lancet* 1990;(3):33520.
20. Sezer M, Oflaz H. Intracoronary streptokinase after primary percutaneous coronary intervention. *N Engl J Med* 2007;356(18):1823-34.