DEVELOPMENT OF NEPHELOMETRIC α_1-AT METHOD AND ITS PERFORMANCE CHARACTERISTICS

Asist. Prof. Mustafa ÇALAPOĞLU
Department of Biochemistry, Medical School of Karadeniz Technical University, Trabzon, Turkey, calapoglu@hotmail.com

Prof. Dr. Orhan DEĞER
Department of Medical Biology, Medical School of Karadeniz Technical University, Trabzon, Turkey

Dr. Ahmet ALVER
Department of Biochemistry, Medical School of Karadeniz Technical University, Trabzon, Turkey

Asist. Prof. Nilüfer Şahin ÇALAPOĞLU
Department of Medical Biology, Medical School of Karadeniz Technical University, Trabzon, Turkey

Asist. Prof. Ersan KALAY
Department of Medical Biology, Medical School of Karadeniz Technical University, Trabzon, Turkey

Asist. Prof. C. Kurtuluş BURUK
Department of Microbiology, Medical School of Karadeniz Technical University, Trabzon, Turkey

INTRODUCTION

α_1-AT, a glycoprotein of molecular mass of 52 kDa, is a major plasma serine protease inhibitor (serpin). α_1-AT plays a central role as a protease inhibitor in controlling tissue degradation. As a major protease inhibitor in human plasma, α_1-AT can complex with a broad spectrum of proteases including elastase, trypsin, chymotrypsin, thrombin, and bacterial leukocyte elastase. α_1-AT is an acute phase reactant whose plasma concentration can rise by 3 to 4-fold above normal during inflammation, infection and malignant disease. α_1-AT deficiency, one of the most common hereditary disorders, mainly affects the liver and lung [1,2]. The concentrations of α_1-AT in plasma can be measured either by functional assays or by immunochemical methods. Functional assays evaluate inhibition of trypsin or elastase. Immunologic methods include radial immunodiffusion, electroimmunoassay and automated nephelometric methods. The

results of immunologic methods frequently vary considerably between laboratories because of the differences in commercial standards supplied. Imunonephelometry is the most commonly used methods for determination plasma α_1-AT levels in various pathological conditions, and is the stage 1 test for diagnosis of α_1-AT deficiency [3].

Many laboratories rely on dedicated nephelometer and commercial reagent kits for evaluation of serum proteins. However with growing emphasis on more-precise methods, laboratories are forced to seek additional rapid, automated and precise methods efficiencies by capitalizing on the use of existing laboratories whenever possible.

We report that a nephelometric method was developed with the purified IgG antibodies that can be used to directly measure concentrations of α_1-AT in serum. The assay is based on commercially available reagents and has potential usefulness in studies of the role of α_1-AT in disease.

MATERIALS and METHODS

Reagents

Tween-20, coomassie brilliant blue R-250, 2-mercaptoethanol (C_2H_6OS), acrylamide (C_3H_5NO), ammonium persulfate, ammonium sulfate $(NH_4)_2SO_4$, N,N'-methylene bis-acrylamide, N,N,N',N'-tetramethylendiamine (TEMED), bovine serum albumin (BSA), Cat. No: A 7030, DEAE sepharose CL-6B anion exchanger, Cat. No: DCL-6B-100, Freund's adjuvant complete, Cat. No: F-5881, Freund's adjuvant incomplete, Cat. No: F-5506, human α-1-antitrypsin, Cat. No: A 6150, rabbit anti-human α-1-antitrypsin, Cat no: A 0409, anti-human IgG (Fc spesific), Cat no: A 9544, sigma 104 phospatase substrate purchased from Sigma Cehemical Co, St Louis, USA. N reaction buffer, Code No: OUMS 65, N diluent, Code No: OUMT 65, rabbit anti-human α_1-antitrypsin, Lot 15418920, N/T protein control SL/M, Code No: OQIO 15, N protein standart SL, Code No: OQIM 13 obtained from Dade Behring SA, France.

Subjects

We studied 80 healthy adults (50 men and 30 women), ranging in age from 22 to 44 years with no significant medical histories or any pathological conditions affecting α_1-AT levels. After an overnight fast, serum samples were separated from the retracted clot as soon as possiple and stored aseptically at –70 °C until analysis.

Polyclonal Antibody Production and Purificatin of the IgG Fraction

Human α_1-AT were used as antigen, and polyclonal antibodies aganist this antigen were raised intramuscularly in New Zealand white rabbits. Two rabbits were immunized with this antigen, and antiserum from the rabbit that had the highest antibody titre was used in the experiment described in present paper.

1/1 complete Freund's adjuvant and 1mg/mL purified antigen in phospate-buffered saline (PBS) were emulsified, and 0,5 mL emulsion was injected into each thigh muscle of hind legs. 4 week later, 1 mg antigen emulsified in complete Freund's adjuvant (1/1) were injected to rabbits as described above. Booster immunization 2 weeks after initial boost was repeated. Ten days after second booster immunization the rabbits were bleeded from marginal vein of the ear. Blood was collected in 50 mL plastic centrifuge tube. The blood was allowed to clot normally for 15 min at room temperature [4]. Blood was centrifuged for 25 min at 10000 g at 4^{o}C and pellet was discarded. The IgG fraction of the antiserum was precipitated by $(NH_4)_2SO_4$ to produce 45% final saturation and was stirred at 4^{o}C overnight. Subsequently, it was centrifuged at 4000 g for 20 min at 4^{o}C and resultant supernatant was discarded. Precipitate was disolved in PBS (0.14 M NaCl, 2.7 M KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) to give 50% of the original volume and was dialysed against PBS (0.07 M, pH 6.3) at 4^{o}C for 24 h with two buffer changes. The antiserum was applied to DEAE-sepharose CL-6B column, and column was washed the ion exchanger with 2 column volumes of PBS. The eluate was collected, and monitored for the absorbance of the eluate at 280 nm [5-7].

ELISA (Enzyme Linked Immunosorbent Assay)

Polyvinily chloride microtiter plates were coated with 20 μL/mL α_1-AT as antigen in carbonate/bicarbonate buffer (0.2 M, pH 9.6) overnight at 4 ^{o}C and washed with TBS (25mM tris, 150 mM NaCl, pH 7.5) three times. Subsequently, the remaining binding sites were blocked by TBS containing 3% BSA during a 30-min incubation at room temperature. Sera were incubated for 1 hour at various dilutions in a solution of %3 BSA and 0.05 Tween 20 in TBS and washed with TBS containing 0.05% Tween 20 three times. Goat IgG directed aganist rabbit IgG conjugated to alkaline phosphatase at a 2000-fold dilution in the BSA-tween 20 solution was incubated for 1 h at room temberature to detect the bound antibodies to the coated antigen and washed with TBS containing 0.05% Tween 20 three times. p-nitrophenyl phosphate in 1.5 M of 2-amino 2-methyl 1-propanol, pH 10.3 was used as a substrate. The absorbance was read after 15 min at 405 nm aganist the signal from nonimmun rabbit serum as a blank.

Electrophoresis

Serum protein electrophoresis was performed Titan gel Serum Protein Kit (cat No: 3041, Helena Laboratories) according to the manufacturer's instructions.

SDS-PAGE was performed on 10% acrylamid gel according to Laemmli [8]. Samples loaded after addition of β-mercaptoethanol (10% v/v) and boiled for 6 min. Samples were stained by Coomassie Birilliant Blue R-250.

RID (Radial Immunodiffusion)

Antisera we produce and commercial antisera were assayed by RID [9]. The RID plates, standarts and samples were equilibrated to room temperature and 5 μL aliquots of samples were dispensed into the wells. The plates were incubated for 18 h at room tempe-

rature and diameter of the precipitin ring was measured. The antibody concentrations for each samples were determined from a standard curve plotted on a semilog graphic paper.

Development of Nephelometric α_1-AT Method

Immunonephelometric assay method was performed by Behring Nephelometer 100 analyzer (BNA) system. Fixed-time method to determine α_1-AT in serum was used. An initial measurement was done 10 second after preparing the reaction mixture. A second value was measured after 6 minutes. Multipoint calibration to calculate concentrations were chosen. Reference curve was automatically constructed by the analyzer from multiple dilution of the N protein standart SL and nephelometer was calibrated by purified polyclonal IgG antibodies. The reference curve was automatically determined using the logit-log-function and linear regression analysis. The deviations of the measured points from the calculated curve was constructed as 5%.

Original nephelometric method (1st method, Behring BN 100) was optimized by 20 μL sample volume and 40 μL antiserum volume. Developing of nephelometric method with purified IgG antibodies (2nd method) was optimized by 20 μL sample volume and 40 μL antiserum volume. Method developed with purified commercial polyclonal antibodies (3rd method) was optimized by 10 μL sample volume and 100 μL antiserum volume.

RESULTS

Antisera

Protein quantitation and antibody titer were used to produce standart curve of nephelometric methods. Protein quantitations were determined by the preimmun serum, and purification steps of antiserum according to Lowry using bovine serum albumin as a standart [10]. Results of protein quantitations were shown in **Table 1**. Presence or absence of antibodies was controlled by ELISA. Specificitiy for α_1-AT of antisera were determined by RID **(Table 1)**.

Table 1: Result of the protein levels and antibody titer in antisera

Sera	Protein concentrations (mg/mL)	ELISA (OD) n:4	RID (mg/mL)
Preimmun serum	68.00	0.050	-
Immun antiserum	69.00	0.180	1.310
Commercial nephelometric antiserum	42.10	0.162	1.158
Antiserum purified with ammoniumsulfate	22.32	0.181	1.652
Antiserum purified with ammoniumsulfate and ion-exchange chromatography	8.235	0.830	2.730
Commercial purified antiserum	10.38	0.425	2.010

Purity of IgG fractions that precipitated with ammoniumsulfate and obtained by ion-exchange chromatography, was controlled by protein electrophoresis **(Figure 1)** and SDS-PAGE **(Figure 2).**

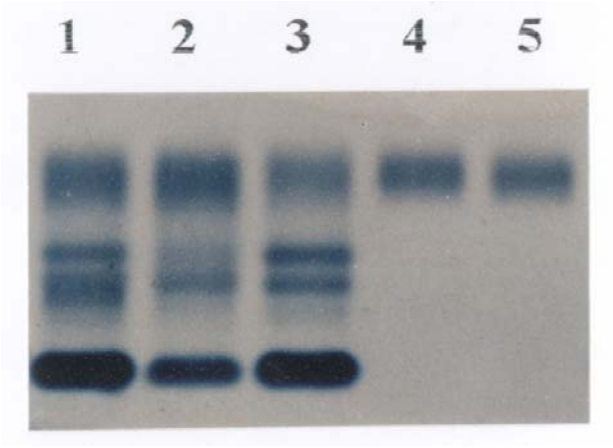

Fig 1: Electrophoretic pattern of antisera. Protein electrophoresis of the immune antiserum (line 1), antiserum purified by precipitation of IgG with ammoniumsulfate (line 2), commercial nephelometric antiserum (line 3), antiserum purified with precipitation of IgG with ammoniumsulfate and by ion-exchange chromatography (line 4), and commercial antiserum (line 5). Electrophoretograms obtained from immunized antiserum, five bands (albumin,α_1, α_2, β, γ) are seen.

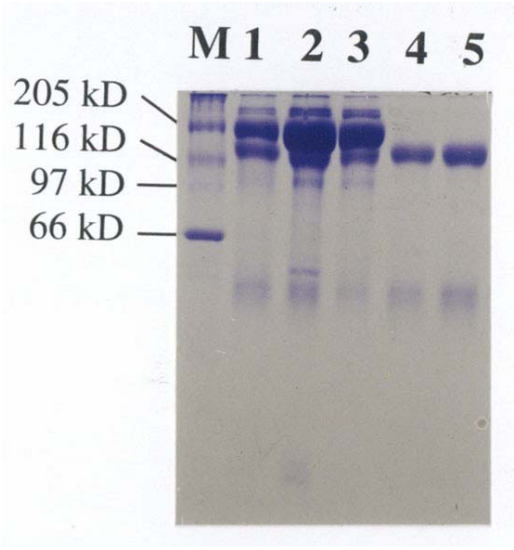

Fig 2: SDS-PAGE of the antisera. Protein molecular weight marker (line M). Antiserum purified with precipitation of IgG with ammoniumsulfate (line 1), immune antiserum (line 2), nephelometric antiserum (line 3), antiserum purified with precipitation of IgG with ammoniumsulfate and by ion-exchange chromatography (line 4), and commercial antiserum (line 5).

Sensitivity and Detection Limits

The sensitivity of the assay was established by the lower limit of the reference curve. Therefore, sensitivity depends upon the concentrations of the α_1-AT in the standart. The assay ranges of the α_1-AT reagent varies from 30 to 960 mg/dL for a sample dilution of 1/20. Typical limit of detection for α_1-AT on the Behring Nephelometer 100 is 4.9 mg/dL when using samples dilution of 1/5.

The standart serum dilutions ranged from 1/5 to 1/160. Dilutions were prepared by the analyzer in sample cups in the standart serum casette using N diluent. The number of dilution per standart was programmed for purified IgG antiserum reagent and commercial purified IgG antiserum. Standart dilutions, therotical concentrations, calculated concentrations by Behring BN 100 software and delta deviations are shown in **Table 2**.

Table 2: Parameter of the Assay sensitivity and detection limit

Theoretical diluted standart concentrations (150 mg/mL)		Calculated concentrations			Delta concentrations (%)			Lower limits of detection
		Methods			Methods			
		1.	2.	3.	1.	2.	3.	
30.0000	(1/5)	30.19	30.67	30.36	0.6	2.2	1.2	4,9 mg/dL (1:5 sample dilution)
15.0000	(1/10)	14.79	14.38	14.72	-1.4	-4.1	-1.8	
7.5000	(1/20)	7.597	7.673	7.488	1.3	2.3	-0.2	
3.7500	(1/40)	3.725	3.801	3.859	-0.7	1.4	2.9	
1.8750	(1/80)	1.877	1.808	1.802	0.1	-3.6	-3.9	
0.9375	(1/160)	0.938	0.962	0.960	0.1	2.6	2.3	

Method 1: The assay method of commercial nephelometric α_1-AT

Method 2: The assay method of nephelometric α_1-AT that was developed from prufied IgG fraction in our laboratory

Method 3: The assay method of nephelometric α_1-AT that was developed from commercial prufied polyclonal IgG fraction

Linearity

Serum pool was diluted with N diluent. Linearity results were shown in **Table 3**.

The assays were found to be linear from 5.9 mg/dL to 190 mg/mL. The linearty of high levels of α_1-AT can be obtained by predilution of sample.

Table 3: The linearty of the assays as determined by measuring the dilution recoveries by diluting serum pool with N diluent, and by calculating expected /observed percentages (%R)

Dilu-tions	Serum pool (190 ± 10 mg/dL)	Method 1			Method 2			Method 3		
		Mea-sured	R%	Bias	Mea-sured	R%	Bias	Mea-sured	R%	Bias
1/1	190.0	198.5	104.47	8.5	186.2	98	-3.8	194.1	102	4.1
1/2	95.0	103.0	108.4	8.0	100.4	105.68	5.4	99.6	104.8	4.6
1/4	47.5	49.2	103.58	1.7	45.7	96.21	-1.8	46.8	98.52	-0.7
1/8	23.8	25.3	106.3	1.5	26.6	111.7	2.8	25.0	105.04	1.2
1/16	11.9	13.2	110.9	1.3	13.1	110.8	1.2	11.0	92.43	-0.9
1/32	5.94	6.9	116.16	0.96	6.6	111.1	0.66	6.0	101.01	0.06
1/64	2.97	<5.9			<5.9			<5.9		
Mean (± SD)			108.30± 4.67	3.66± 3.57		105.58± 6.93	0.74± 3.26		100.63±4. 70	1.39± 2.41

Comparison of Methods

Serum samples were assayed with the purified IgG antiserum, commercial N antiserum and commercial purified IgG antiserum. Correlation of the result were shown in the **Table 4**.

Table 4: Comparison of the methods

Methods	Number of sera	Linear Regression	Coefficient of correlations
1	20	y(1) = 0.828x (2) + 33.688	0.939
2	20	y(2) = 0.999x (3) - 1.470	0.977
3	20	y(1) = 0.892x (3) + 23.073	0.965

Accuracy and Precision

Coefficent of variations (CV) were found with the commercial N antiserum, purified IgG antiserum and commercial purified IgG antiserum on the Behring Nephelometer 100 Analyzer **(Table 5)**.

Table 5: Coefficient of variation (CV) with the antisera to human α_1-AT on the Behring Nephelometer 100

		Within-run				Between-run			
Methods	N/T protein control serum (mg/dL)	n	$\bar{X}$	SD	%CV	n	$\bar{X}$	SD	%CV
1	149mg/dL	5	155.24	3.84	2.46	4	162.55	6.00	3.58
2	149mg/dL	5	148.52	4.18	2.82	4	143.8	4.55	3.57
3	149mg/dL	5	149.54	1.48	1.00	4	150.13	1.60	1.07

Normal Ranges of Methods

A Behring Nephelometer 100 analyzer system was used in the evaluation of the nephelometrric α_1-AT methods and to determinate the normal ranges of methods. Comparison of normal ranges of immunonephelometric methods are shown in **Table 6**. 80 normal healthy individuals with no pregnancy and medication were used to asigned the value of confidence intervals. No statistical difference was found among methods used (by ANOVA test).

Table 6: Comparison of normal ranges of immunonephelometric α_1-AT methods in serum

Methods	No of subjects	Confidence intervals, mean, ±SD, and ranges (mg/dL)
1	80	138 ± 23.07 (132.87 - 143.13)
2	80	133 ± 26.15 (127.13 - 138.82)
3	80	134 ± 25.26 (128.38 - 139.62)

DISCUSSION

α_1-AT can be quantified by immunochemical methods, immunoturbidimetry and immunonephelometry, the most commonly used methods at present. Early methods, including RID, RIA, ELISA and functional assays were generally slower, and too time consuming to perform in a routine biochemistry department. Therefore, the development of rapid, automated, and more-precise methods for determinating α_1-AT to evaluate the diagnostic potential of this analyte in the clinical stuations has been important. Although there are commercially available α_1-AT methods, it is necessary to have as many as possiple to make this assay both analytically and financially competitive for routine use. Here, we have evaluated an immunonephelometric method for the immunological quantitation of serum α_1-AT. The assay based on a purified polyclonal anti α_1-AT antibodies produced by rabbits against human protein in our laboratory.

A great variety of experimental animals have been exploited in the production of antibody. Our studies require only the production of immune sera containing polyclonal antibody. In this case, almost any animals on a valid protocol may be used. We used two animals/immunogen. This ensured aganist the failure of one of the animals owing to mishap or biological variability [11].

Addition of appropriate amounts of ammonium sulfate causes precipitation of IgG from all mammals, and can be used for serum. Although such IgG is usually contamined with other proteins, the ease of this precipitation procedures coupled with the high yield of IgG has led to its wide use in producing enriched IgG preperations. Ammonium sulfate precipitation is the most widely used and adaptable procedure, yielding a 40% pure preperation [5].

IgG may be purified from serum by a simple one-step ion-exchange chromatography procedure. The method is widely used and works on the principle that IgG has a higher or more basic isoelectric point than most serum proteins. The high capacity of ion-exchange columns allows for large-scale purification of IgG from serum. DEAE Sepharose CL-6B is useful for this purpose.

When is necessary to determine the presence of specific antibody in a solution, ELISA is one of the simplest methods to use. ELISA can easily and reliably detect small quantities (of the order of 1μg/mL) of antibody in immune serum [12]. Times, volumes, and concentrations of reagents were adjusted in order to give good, reproducible results. Hovewer, general sceheme outlined is likely to give a reliable yes-or-no result on the first attempt.

Nephelometric method that developed with purified polyclonal IgG antibodies in our laboratory, was found to produce a good linearity through the operating range, and when compared the other nephelometric methods showed a high degree correlation. Results obtained on the commercial nephelometric method were found to be more positively biased when compared with developed with purified polyclonal IgG fraction.

Rapid determination of α_1-AT concentration is important to permit early diagnosis in two clinical situations. (i) Failure to inactivate proteolytic enzymes by α_1-AT released from dead bacteria or leukocytes in the lung may result in an accumulation of enzymes, and proteolytic destruction of pulmonary tissue with the development of severe panacinaremphyzema. (ii) Congenital deficiency of α_1-AT is a well-established cause of liver disease in infancy and childhood, producing sign of obstructive jaundice in infants, 5-30% of cases are throught to be due to α_1-AT deficiency, progression to hepatic decompensantion, ascites and hepatic failure sometimes taking place within mounts [13].

Because of their speed and ease, nephelometry are widely used to measurement of the α_1-AT. Detection limits of approximately 10 μg/mL can be attained with nephelometric method. RID methods can detect down to a minimum of 10 to 20 μg/mL [14]. RIA methods are very sensitive and can measure levels as low as nanograms per mililiter level. In our method, detection limit was set up for 1/5 sample dilution and

found to be 49 μg/mL. Determination of detection limit of this method was taken into consideration of routine clinical usage.

Nephelometric method developed in our laboratory is sensitive and specific, with acceptable precision, and correlated with the commercial nephelometric method and the other method which is developed with commercial purified IgG antibody in our laboratory. These nephelometric methods gave us good results within and between batch precision with 1% to 3.58%.

In conclusion, α_1-AT antibody we developed may be used for a robust, fully automated, and rapid method, essential for the quick turn around necessity in a routine hospital laboratory. The described nephelometric method is suitable for analysis of single sample or for large-scale screening. A full age-related, sex-related reference range can be determined with this method and compared with others. Additionally, this method might be important in the examination of heterozygotes and homozygotes who have subnormal levels of α_1-AT.

Acknowledgements

This study was supported by research fund of Karadeniz Technical University (project no: 96.114.001.12).

REFERENCES

1. COX D.W. **α_1-AT deficinecy**. In: The Scriver CR, Deaudet AL, Sly WS, Walle D, editors. Metabolic and molecular bases of inherited disease. London: Mc Graw-Hill Companies., 1995. p. 4125-4148.
2. MICHAEL, R.N. **Molecular basis, clinical consequences and diagnosis of α_1-AT deficiency**. Ann Clin Biochem 1997; 34:230-46.
3. CAMPBELL E.J. **Alpha1-antitrypsin deficiency: Incidance and detection program**. Respiratory Medicine 2000; 94:(Suppl. C):18-21.
4. AUSABEL F., BRENT R., KINGSTON R., MOORE D., SEIDMAN J.G., SMİTH J., STRUHL K. **Preparation of Polyclonal Antisera**. Canada: Greene Puplishing Associates and John Wiley and Sons, 1992. p. 37-41
5. PAGE M., THORPE R. **Purification of IgG by precipitation with sodium sulfate or ammonium sulfate**. In: Walker JM, editor. The Protein Protocols Handbook. Tatowa, New Jersey: Humana Press Inc., 1996. p. 721-2.
6. DEUTSCHER M.P. **Guide to protein purification**. In: Abelson JN, Simon MI, Editors. Methods in enzymology. California: Academic Press., 1990 p. 57-9, 72-7.
7. PAGE M., THORPE R. **Purification of using DEAE-sepharose chormatography**. In: Walker JM, editor. The protein protocols handbook. Tatowa, New Jersey: Humana Press Inc., 1996. p. 725-6.
8. HAMES B.D. **One-dimensional polyacryamic gel electrophoresis**. In: Hames

BD, Rickwood D, editors. Gel electrophoresis of proteins. A practical approach. New York: Oxford University Press., 1990. p.1-59.

9. BAILEY G.S. **Single radial immunodiffusion**. In: Walker JM, editor. The protein protocols handbook. Tatowa, New Jersey: Humana Press Inc., 1996. p.753-5.
10. HENRY R.J, CANNON D.C, WINKELMAN J.W, editors. **Clinical chemistry: Principles and techniques**. New York: Harper & Row., 1974.p. 424-8.
11. CARTER J.M. **Production and characterization of antibodies aganist peptides**. In: Walker JM, editor. The protein protocols handbook. Tatowa, New Jersey: Humana Press Inc., 1996.p. 711-6.
12. MICHALSKI J.P., McCOMPS C.C., SHETH S., McCARTHY M., de SAHAZO R.A. **Modified double antibady sandwich enzyme-linked ımmunosorbent assay for measurement of α_1-AT in biologic fluids**. Journal of Immunological Methods 1985; 83: 101-12.
13. GEMMELL R.S. **Determination of plasma alpha-1-antitrypsin by enzyme-linked immunosorbent assay**. Medical Laboratory Sciences 1986; 43:135-9.
14. JACKSON G. **Immunglobulin quantitation**. In: PESCE A.J., KABLAN L.A., editors. Methods in clinical chemistry. USA: The C.V. Mosby Company., 1987 p. 735-41.