OVINE ANTIVENOM FOR *VIPERA AMMODYTES*

CRISTIAN CĂPITĂNESCU[a], CLAUDIU ŞTEFAN TURCULEŢ[b], ZIZI NICULESCU[c], ADRIANA CĂPITĂNESCU,[a]
VICTOR STRÂMBU[b], GHEORGHE MANOLE[d], BOGDAN ŞTOLICĂ[e], CĂTĂLIN CÎRSTOVEANU[f]
and EMANUEL GALATESCU[g]

[a] S.C. Ecodet Activ S.R.L., Fizicienilor Street, No. 22, Bucharest, district 3, 032066, Romania; adriana_capitanescu@yahoo.com
[b] " Carol Davila" University of Medicine and Pharmacy, Dionisie Lupu Street, No. 37, Bucharest, district 2, 020021, Romania
[c] "Ovidius" University of Constanţa. Department of Internal Medicine, Ilarie Verona Street, No. 7, Constanţa, 900684, Romania
[d] Colentina Hospital, Stefan cel Mare Blvd, No. 19-21, Bucharest, district 2, 020125, Romania
[e] Institute of Public Health Bucharest, Dr. Leonte Street, No.1-3, Bucharest, district 5, 050463, Romania
[f] "Maria Sklodowska Curie" Hospital, Constantin Brancoveanu Blvd., No. 20, Bucharest, district 4, 041451, Romania
[g] National Medicinal Science Academy, Stefan cel Mare Blvd, No. 19-21, Bucharest, district 2, 020125, Romania

Received November 3, 2008

This study describes in detail the procedure for producing in sheep antivenom serum for *Vipera ammodytes*. This procedure includes also the reason and criteria for selection of animals intended to be used for antivenom production. The most significant aspect of the immunization process is represented by splitting this into three distinct phases. First of this phases requires adsorbtion of the venom on the alginate in order to gradually release into the animal body.
It was tested also a simple method for purification and concentration of the immunoglobulin using caprylic acid.
The antivenom efficacy of the crude serum and immunoglobulinic concentrate was investigated based on the inhibitory action of the antivenom on the venom enzymes, particularly L-aminoacid-oxidase. Due to precision of instrumental techniques, the antienzymatic antivenom potency assay seems to become a very suitable strategy for better pharmaceutical and biochemical characterization of antivenoms.
Based on the less time consumption and resources reasonability, the method proposed in this study is very appropriate for monitoring the immunoglobulin concentration during antivenom production.

Key words: Antivenom; Vipera; L-aminoacidoxidase; Spectrophotometric.

Antivenoms are the most efficient pharmaceutical preparations in therapy of bites from venomous snakes[1–3]. They are obtained from serum of animals hyperimmunized with venom of one or more species[4–6] and look like concentrate solutions of antibodies or lyophilized antibodies[7, 8].

Antivenom manufacturing was tested for different species of snakes and mammalians, including rabbits, ghosts, sheep and horses. From chronological point of view, the main process represents hyperimmunization of animals. This is performed by injection of the animal with a well established quantity of venom of interest species. The quantities of venom are established so that to not jeopardize the animal health or life. Immunization can be done versus the venom of a single specie, when a mono-potent antivenom is obtained or versus the venom of many species, when a multi-potent antivenoms can be obtained. Generally, multi-potent antivenoms are obtained by sequential immunization of the test animals with each type of venom.

Hyperimmunization involves several aspects on: the venom to be injected (fresh liquid or solid), route of administration (subcutaneous or intramuscular, with or without adjuvant), administration timetable.

At present, animals hyperimmunization to produce antivenom is performed almost exclusively by using solutions of different concentrations of venom in solid state (crystallized or lyophilized – the venom is very stable and can be characterized in this form) dissolved in 0.9% sodium chloride. Venom administration can be done intramuscular or subcutaneously.

Until recently, it was considered that horse is the most suitable animal to be used for producing therapeutic antivenoms, based on the following reasons:

– it is a resistant mammifer, with a big blood volume, which could produce a big amount of antibodies;

– has a predictable and docile behaviour, being suitable for hyperimmunization and serum harvesting;

– has a wide geographical area.

Recently, the orientation for using sheeps as animals for producing antivenoms appeared, mainly due to some advantages versus using of horses, namely:

– lower risk of adverse reactions;

– higher flexibility of immunization procedures;

– using of lower quantities of venom for immunization.

The serum harvesting and processing is made after a convenient concentration of antibodies in the blood of producing animals is attained. After separation of figurate elements from plasma, this undergoes further processing, as well for antibodies concentration as for decreasing of immunogenic potential of plasma itself. The most used methods are based on precipitation with salts and solvents, steric exclusion chromatography or affinity chromatography, ultrafiltration or digestion with proteolitic enzymes.

Most antivenoms were in liquid presentation, but some of them are lyophilized antivenoms.

In order to express the therapeutic capacity of antivenom, it is necessary its analytical characterization, mainly to assess the antivenomous potential. This is based on biochemical properties of antivenomous serum and almost exclusively on antigene-antibody reaction, respectively on the study of reaction between venom and antivenom. The effects of this interaction can be followed into different media („in vitro" or „in vivo"), using variable supports or methods and measurement instruments more or less specific. The parameter that describes quantitatively the interaction venom-antivenom and express antivenom efficacy is effective average dose ED_{50}[1,9]. Different categorizations of the methods for determination of antivenom efficacy can be made based on different criteria.

EXPERIMENTAL PART

1. Antivenom producing

Animals selection

Even if sheep has a lower blood volume than horse and sometimes is not so docile, for this experiment, indigen sheeps were used as animals producer of antivenom for *Vipera ammodytes*.

These choice were justified by the following:

– the possibility to obtain antivenom with reduced anaphylactic shock potential;

– decreased costs for maintaining and food of animals;

– decreased costs for immunization (due to smaller quantities of venom to be used).

The sheeps which became producers of antivenom were selected based on the following criteria: health, sex, age.

So, healthy animals, without ethyopathologic antecedent were selected. The animals were tested sanitary-veterinary, maintained into quarantine for 60 days, and case forms for each individual were prepared.

Two males were selected, based on the reason that physiological variations related to reproduction are more reduced than in case of females of the same species.

Individuals of very closed age were selected to allow the results to be compared. The age was of 2 years +/– 3 months.

The animals selected according to the above presented criteria were inoculated in accordance with a timetable which will be detailed presented. During immunization process, no antibiotics were administered.

Hyperimmunization

It was considered that subcutaneous route of administration is the most suitable method of inoculation for ovines. Performing of hyperimmunization process is conditioned by achieving of the phases to be further described.

Primary immunization

Two main phases were performed to achieve the primary immunization.

1st phase

Principle

Increasing doses of venom dissolved into adjuvant (sodium alginate) solution prepared as described below were injected subcutaneously.

Reagents

Doses of 18 mg crystallized venom into each 1.8 ml tubes.

1.25% (w/v) sodium alginate solution.

Method

A single dose of venom was injected subcutaneously every week.

1.8 ml solution of alginate is added with a single use syringe into the tube corresponding to the working week.

Dissolution is performed slowly, in 25–30 minutes, by slow rotations of the tube around its longitudinal axe, to avoid foaming. It is not shaken because of foaming.

The volume of venom specified into the table below is injected subcutaneously.

Table 1

Timetable of the 1st phase of primary immunization

Week number	1	2	3	4	5	6	7	8
Tube number	1	2	3	4	5	6	7	8
Volume of venom solution injected (mL)	0.1	0.2	0.3	0.4	0.5	0.6	1	1.5
Quantity of venom used (mg)	1	2	3	4	5	6	10	15

The venom dissolution should not be performed earlier then one hour before sample injection.

The venom solution remained unused in the tube after injection of the specified volume is discharged (it will not be used for future inoculation).

Preparation of alginate solution

The alginate is a polysaccharide extracted from sea algae. It is a fine, light brown powder. It is soluble in water, but in presence of calcium ions turns into gel.

It will be avoided the contact of alginate solutions with vessel and surfaces washed with tap water or with solutions containing calcium ions.

Alginate solution – alginate powder is put into sterile tubes of plastic with screw cap. Each tube contains 50 mg of alginate and can store 4 ml solution, resulting a solution with 1.25% (w/v) concentration. This volume is sufficient to dissolve two venom doses, so a tube with alginate solution is enough for 2 weeks (one dose of venom is injected per week), than other tube with alginate solution is prepared.

Add 2 ml of sodium chloride 0.9% into the tube containing alginate powder and shake slowly until dissolution (approximately 10 minutes). Add other 2 ml of sodium chloride 0.9% and shake for further 2 minutes. Leave the solution at room temperature for 20 minutes, then use the alginate solution to prepare the venom solution according to the corresponding instructions.

If alginate is not dissolved or a gel is obtained, the procedure is repeated.

2nd phase

Principle

Increased doses of venom dissolved in 0.9% sodium chloride solution were injected subcutaneously.

Reagents

Doses of 18 mg, respectively 36 mg crystallized venom each into 1.8 ml tubes.

0.9% sodium chloride solution.

Method

Doses of venom are subcutaneously injected twice a week.

1.8 ml solution of 0.9% sodium chloride is added with a single use syringe into the tube corresponding to the working week.

The volume of venom specified into the table below is injected subcutaneously.

Table 2

Timetable of the second phase of primary immunization

Week number	9		10		11	
Tube number	9	10	11	12	13	14
Volume of venom solution injected (mL)	0.1	0.3	1	1.8	1	1.8
Quantity of venom used (mg)	1	3	10	18	20	36

For the solutions of venom remained after inoculation, it is proceeded in the same way as in case of previous phase.

3rd phase

Two weeks pause is taken in order to give to the animal body a sufficient time interval to produce antivenomous antibodies.

Maintaining immunization – for the producers animals

The maintaining immunization is performed regularly during the period when the animal is used as source of antivenom serum (several years).

4th phase

6 doses, 2 doses /week, increasing, pure.

This phase is performed similarly with 2nd phase.

5th phase

Two weeks pause is taken based on the same reasons as in case of 3rd phase.

6th phase

Serum harwesting, 1–2 harwesting/week.

The vessel and instruments used should be sterile.

A volume of 0.1–0.5 L blood from the producer animal can be taken and it is left to coagulate for serum separation. The serum is separated (centrifugated) into a sterile flask and this can be stored in the refrigerator for maximum one week for further processing.

7th phase

One week pause is taken in order to give the animal some rest after 6th phase.

2. Preparation of the immunoglobuline concentrate by precipitation with caprylic acid

Principle

Caprylic acid 5% (w/v) concentration can action as precipitant for serum proteins, except for immunoglobulins [8]. This operation is performed in order to increase the antivenomous potential of the preparate.

Reagents and materials

Magnetic agitator with thermostate

Centrifuge

Berzelius, Erlenmeyer flasks, automatic pipette, glass vessel.

Serum provided by animals hyperimmunized with venom from *Vipera ammodytes*

Caprylic acid (analytical grade)

0.9% sodium chloride solution

4N sodium hydroxide solution.

Method

150 mL crude ovine serum (at 4°C–6°C) is put into an Erlenmeyer flask (with 200–250 mL capacity) and 7.9 mL of caprylic acid are added by dropping under continuous stirring (50 rotations/minute). The pH value should be maintained at 5.2 using 4N sodium hydroxide solution. The stirring is continued for other 60 minutes, at 4°C–6°C. The mixture is centrifuged at 3000 rotations/minute for 10 minutes and the supernatant is separated. Then, the supernatant is dialyzed against 2L of 0.9% sodium chloride for 24 hours (the sodium chloride solution is replaced 2 times in 24 hours).

The solution obtained by dialysis is concentrated up to 15–30 mL by placing the dialysis bag into a Berzelius flask that contains 300 g polyethylene glycol 4000. The dialysate that contains the concentrated antibodies is to be analyzed for its antivenomous capacity.

Based on the results of the determinations, the above-obtained solution is diluted with 0.9% sodium chloride solution until an antivenom with a certain efficacy is obtained.

3. Analytical characterization of the antivenom by determination of anti-L-aminoacidoxydase activity

Principle

It is determined the anti-L-amminoacidoxydase activity of well-defined mixture of venom and antivenom (immunoglobuline concentrate or crude serum) and it is observed the capacity of antivenom to inhybite the enzymatic activity. The L-amminoacid-oxydase activity is determined by spectrophotometric assay of H_2O_2 produced into the system[10–12] using peroxydase, phenol and 4-amminoantipyrine in certain reaction conditions.

Reagents and apparatus

Spectrophotometer, cells of L = 1 cm, micro-pipettes;

1 mg/ mL venom solution in 0.9% NaCl

Antivenom (crude serum or immunoglobuline concentrate)

1 mg/mL (cca. 100 I.U./mL) peroxydase solution in 0.2M TrisHCl buffer solution; pH=7.5

Chromogen solution (10 mM 4-amminoantipyrine, 30 mM phenol)

0.2 M TrisHCl buffer solution; pH=7.5

Standard solution of amminoacids, L-tyrosine of 0.1–2.5 mM, 30 mM concentration.

Method

The reagents were tempered at 25 °C for 45 minutes.

0.5 mL solution of 1 mg/mL crude dried venom in 0.9% NaCl was mixed with a volume of 0.0; 0.05; 0.1; 0.2; 0.3 mL crude serum or with a volume of immunoglobuline concentrate with a value of 0.0; 0.01; 0.02; 0.03; 0.04 mL. The

mixture was brought to the volume of 1 mL using Tris buffer solution, 0.2 M, pH 7.5 and incubated at 37 °C for 30 minutes.

0.1mL of the previous incubation mixture was mixed with 0.1 mL peroxidase solution (aprox. 100 I.U./mL) and 1.8 mL Tris buffer solution, 0.2 M, pH 7.5, containing 10 mM 4-aminoantipyrine, 30 mM phenol and 3 mM L-tyrosine. The absorbance of sample versus blank at λ = 507 nm was monitored for about 40 minutes in 1 cm width cell and the result was interpolated from the calibration curve previously obtained, using standard solutions of L- tyrosine and long reaction time (approx. 1–3 hours) or standard solutions of H_2O_2.

The (apparent) enzymatic activity of each incubation mixture sample was calculated as 0.4Absorbance (units) and expressed in micromoles aminoacid/min/mg venom.

All reagent solutions used for this determination were prepared with Tris buffer solution, 0.2 M, pH 7.5.

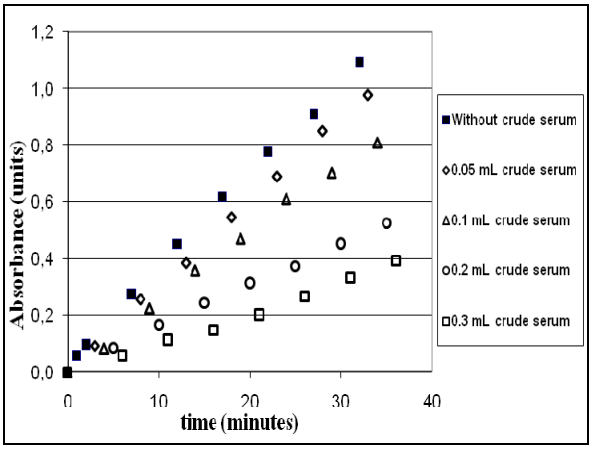

Fig. 1. Absorbance variation at the wavelenght λ = 507 nm during reaction catalysed by L-aminoacid oxydase from venom with addition of different volumes of crude serum.

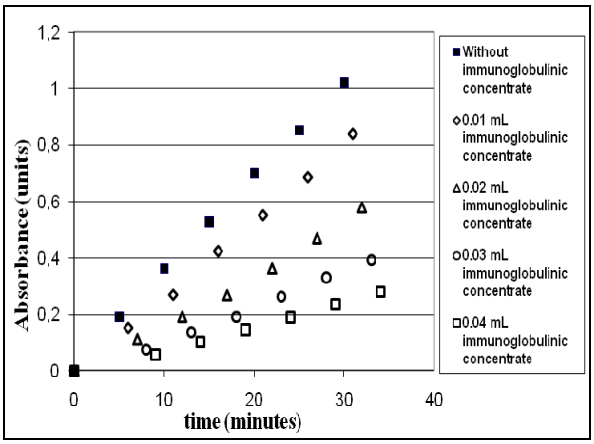

Fig. 2. Absorbance variation at the wavelenght λ = 507 nm during reaction catalyzed by L-aminoacid oxydase from venom with addition of different volumes of immunoglobuline concentrate.

The sequence of antibodies purification and concentration with caprylic acid is presented in Table 3. It is observed that immunoglobuline concentration can increase 5–7 times during this process.

Table 3

Purification of antibodies in serum from hyperimmunized animals

Phase	**Raw material**	**Resulted product**	**Protein concentration by Biuret method**
Serum harwesting	Male sheep blood	Crude serum	7.2–7.5 g /100 mL
Precipitation with 5% caprylic acid	Crude serum	Supernatant containing high antibodies level	1.5–2 g /100 mL
Concentration with PEG 4000	Dialyzed supernatant	Antivenom – Ig concentrate, experimental and functional model	8–10 g /100 mL
Dilution with 0.9% sodium chloride solution	Antivenom – Ig concentrate	Antivenom – Pharmaceutical model	7.2–7.9 g /100 mL

As can be observed in Figures 3 and 4 the apparent enzymatic activity of L-aminoacid oxydase from venom decreases with the increase of antivenom volume in incubation mixture (crude serum or immunoglobuline concentrate).

The variation of the apparent enzymatic activity with the incubation ratio between crude serum, respectively immunoglobuline concentrate and venom were plotted. The best fitting curve was found by nonlinear regression algorithms.

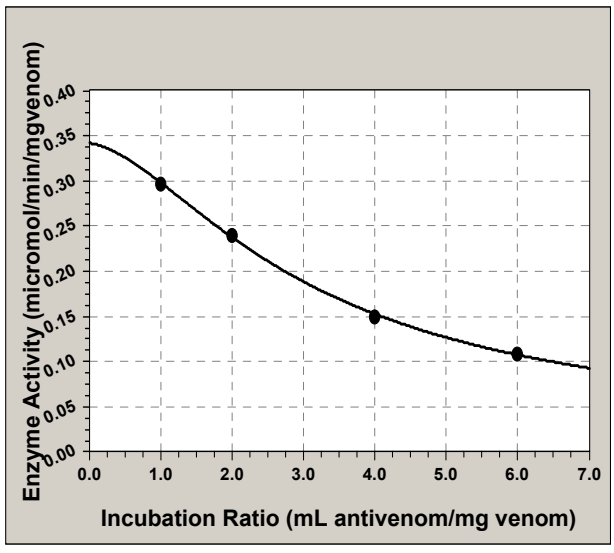

Fig. 3. Variation of L-aminoacidoxidase activity with the incubation ratio crude serum/venom.

The equation of the above graphical representation has the following form:

$$y = (a+bx)/(1+cx+dx^2)$$

where y = enzymatic activity, x = incubation ratio

Coefficients values are: a = 0.341; b = 0.088; c = 0.295; d = 0.148.

By data interpolation, it is observed that inhibition with 50% of the L-aminoacidoxidase activity in pure venom is obtained at an incubation ratio of 3.443 mL crude serum/mg venom, so the value of ED_{50} = 0.29 mg venom/ mL crude serum.

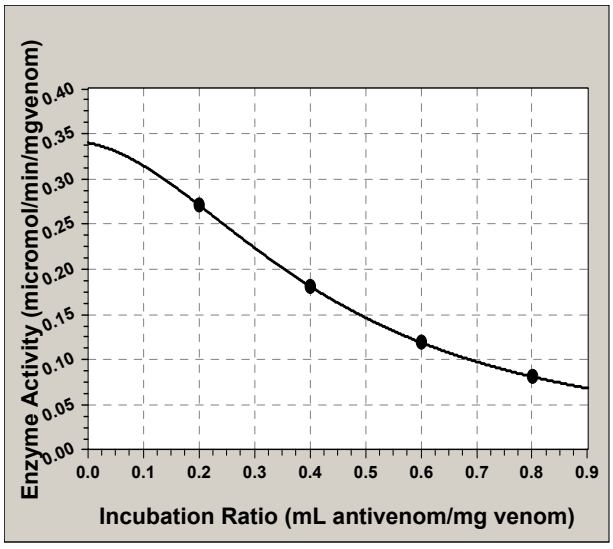

Fig. 4. Variation of L-aminoacidoxidase activity with the incubation ratio immunoglobuline concentrate/venom.

The equation of the above graphical representation has the following form:

$$y = (a+bx)/(1+cx+dx^2)$$

where y = enzymatic activity, x = incubation ratio.

Coefficients values are: a = 0.340; b = 0.013; c = 0.378; d = 4.725.

By data interpolation, it is observed that inhibition with 50% of the L-aminoacidoxidase activity in pure venom is obtained at an incubation ratio of 0.429 mL antivenom/mg venom, so the value of ED_{50} = 2.33 mg venom/ mL immunoglobuline concentrate.

CONCLUSIONS

It was issued and practically tested a procedure for hyperimmunization of the indigene sheeps (*Ovis aries*) with venom from *Vipera ammodytes*.

It was practically checked a method for purification and concentration of antivenomous antibodies from serum of producers animals.

It was determined, for an experimental antivenom batch, that immunoglobuline concentration, expressed by the protein concentration, increased for about 7 times in the immunoglobuline concentrated as reported to crude serum, while the anti-L-aminoacidoxidase activity increased for about 8 times reported to crude serum.

It was issued and practically tested a spectrophotometric method to calculate precisely the antivenom potency.

Based on the performance of the method, it can be concluded that determination of antivenom activity by determination of anti-L-amminoacid oxydase activity is suitable especially during phases of antibodies purification/concentration.

ACKNOWLEDGMENTS

This research was funded within of national program of the Ministry of Education and Research.

REFERENCES

1. Theakston, R.D., Warrell, D.A., Griffiths, E., Report of a WHO workshop on the standardization and control of antivenoms. *Toxicon* 41(5), **2003**, 541–557.
2. Fry, B.G., Winkel, K.D., Wickramaratna, J.C., Hodgson, W.C., Wuster, W., Effectiveness of Snake Antivenom: Species and Regional Venom Variation and Its Clinical Impact. *Journal of Toxicology,* **2003**, 22(1), 23–34.
3. Laing, G.D., Yarleque, A., Marcelo, A., Rodriguez, E., Warrell, D.A., Theakston, R.D.G.; Preclinical testing of three south American antivenoms against the venoms of five medically-important Peruvian snake venoms. *Toxicon* **2004**, 44 (1), 103–106.
4. Jones, R.G., Landon, J., A protocol for 'enhanced pepsin digestion': a step by step method for obtaining pure antibody fragments in high yield from serum. *J. Immunol. Methods,* **2003**, 275 (1–2), 239–250.
5. Jones, R.G., Landon, J.; Enhanced pepsin digestion: a novel process for purifying antibody F(ab')(2) fragments in high yield from serum. *J. Immunol. Methods,* **2002**, 263 (1-2), 57–74.
6. Chotwiwatthanakun, C., Pratanaphon, R., Akesowan, S., Sriprapat, S., Ratanabanangkoon, K.; Production of potent polyvalent antivenom against three elapid venoms using a low dose, low volume, multi-site immunization protocol. *Toxicon,* **2001**, 39(10), 1487–1494.
7. Ariaratnam, C.A., Meyer, W.P., Perera, G., Eddleston, M., Kuleratne, S.A., Attapattu, W., Sheriff, R., Richards, A.M., Theakston, R.D., Warrell, D.A.; A new monospecific ovine Fab fragment antivenom for

treatment of envenoming by the Sri Lankan Russell's viper (Daboia Russelii Russelii): a preliminary dose-finding and pharmacokinetic study. *Am. J. Trop. Med. Hyg.*, **1999**, 61(2), 259–265.
8. Leon, G., Stiles, B., Alape, A., Rojas, G., Gutierrez, J.M., Comparative study on the ability of IgG and F(ab')2 antivenoms to neutralize lethal and myotoxic effects induced by Micrurus Nigrocinctus (coral snake) venom. *Am. J. Trop. Med. Hyg.,* **1999**, 61(2), 266–271.
9. Theakston, R.D.G., Reid, H.A.; Development of simple standard assay procedures for the characterization of snake venoms. *Bull. W.H.O.*, **1983**, 61, pg. 949–956.
10. Donlon, J., Fottrell, P.F.; Quantitative determination of intestinal peptide hydrolase activity using L-Amino acid oxidase. *Clinica Chimica Acta,* **1971**, 33(2), 345–350.
11. Capitanescu, A., Capitanescu, C., Bisceanu, C.; Kit of analysis of amino acids using L-amino acid oxidase and peroxidase. *Roumanian Biotechnological Letters,* **2005**, 10(2), 2179–2184.
12. Capitanescu, A., Tanase, I. Gh., Capitanescu, C., Draghici, M.; Analytical characterization of antivenom pharmaceutical potential by assay of L-amino acid oxidase activity *Analele Universitatii din Bucuresti, Seria Chimie,* **2006**; XV; I, 11–66.