APPLICATION OF A NEURAL NETWORK FOR THE SIMULTANEOUS IDENTIFICATION OF SEVERAL ANALYTES

M. Yu. Rubtsova*, J. V. Samsonova*, A. A. Ezhov, and A. M. Egorov***

Competition chemiluminescent immunoassay was used in a combination with a neural network (NN) to identify and estimate amounts of three cross-reacting s-triazines (atrazine, terbythylazine and ametryn). Antibodies with different cross-reactivity towards s-triazines were immobilized in separate wells of 8-well microtitre strip or in separate spots of a single membrane strip. The data obtained with chemiluminescent ELISA and membrane immunoassay were processed by NN. The main objective for NN application was to find the best topology, learning method and its parameters for the correct estimation of the amount, as well as, the correct identification of an individual compound in a mixture. The necessity of additional normalization for native experimental data was examined. The correct s-triazine classification of environmental samples containing various analyte mixtures was possible in 74–92% of all cases depending on the type of analyte. The test developed can be proposed as an alternative field test for multianalyte environmental monitoring.

Introduction

Immunoassays relying on enzyme label for antigens or antibodies have played a major role in biomedical science for more than three decades. Such assays have been developed in different formats involving traditional ELISA tests which are performed in microtitre plates as well as dot-blot or dipstick tests on porous membranes which are suitable for home or on-site testing or as field tests for environmental monitoring. The main limitation for such tests is that as a rule immunoassays developed are mostly single analyte methods, while in most cases it would be desirable to measure more than one analyte in a sample or discriminate the analytes within a group of structurally closed compounds. So it is obvious that for practice multianalyte assays would be most attractive, and for this purpose the immunoassays can be evaluated as a sum of several single tests. Setting up the multiassay as a sum of separate tests with traditional data evaluation means the necessity to test a panel of standards for each of analyte together with samples, that in turn can be a laborous and expensive task. Another problem deals with the cross-reactivity of antibodies (even monoclonal antibodies) for the primary target analyte and its analogs and metabolites that limits the discrimination between small structural differences of molecular analogs. Careful choice of antibodies combining with appropriate statistical analysis or principle for data evaluation has the potential not only to overcome the cross-reactivity problem but to turn it into an advantage for multianalyte testing. Artificial neural networks represent a new radically different approach to the interpretation of multivariate data and for the pattern recognition [1–3]. NN do not require the data to be conformed to any particular mathematical model and do not depend on linear superposition and orthogonal functions, which are the base classical regression methods are based.

The aim of present study was in application of NN for the discrimination of several analytes in mixtures by chemiluminescent immunoassay developed in different formats. The main objective for NN application was to find the best topology, learning method and its parameters for the correct estimation of the amount, as well as, the correct identification of an individual compound in a mixture of three s-triazine pesticides (atrazine, terbuthylazine and ametryn). Our approach for the multianalyte assay consisted in the simultaneous immobilization of antibodies with different specificity towards pesticides in separate wells of 8-well microtitre strip or in separate spots of a membrane strip. The immunoassay was based on a competition scheme with different antibodies immobilized and one type of tracer for all antibodies. Horseradish peroxidase (HRP) was employed as a label for antigen, and it was detected with enhanced chemiluminescence (ECL) reaction. The ECL as a detection system was shown to be one of the most sensitive and expressive methods for medicine and analytical biochemistry [4–5]. Recently we showed its advantages as an effective detecting method for the purposes of ecological monitoring [6].

Materials and Methods

Horseradish peroxidase (HRP) (RZ 3.3) was obtained from Biozyme (Blaenavon, UK). Chemicals and substrates for HRP were supplied by Sigma (St. Louis MO, USA). S-triazine derivatives were kindly provided by Riedel-de-Haën AG (Seelze, Germany). Stock solutions of pesticides were prepared at a concentration of 1 mg/ml in methanol. The water used for dilution and preparations of buffers was obtained by distillation. Pyrogen-free purified water (Milli-Q

* Dept. of Chem. Enzymology, Faculty of Chemistry, MV Lomonosov Moscow State University, Vorobievy Gory, 119899 Moscow, Russia.
** Dept. of Mathematics, Troitsk Institute of Innovation and Fusion Research, 142092 Troitsk, Moscow Region, Russia.

System, Millipore, Watford, UK) was used to prepare the substrate mixture for chemiluminescence detection.

Conjugates of different pesticide derivatives with HRP were prepared following a modified carbodiimide/N-hydroxysuccinimide method accordingly [7].

Mouse monoclonal antibodies K4G2, S3 and P6A7 were kindly provided by Prof. B. Hock and Dr. Th. Giersch (Technical University of Munich, Freising, Germany). Polyclonal antibodies PS2 and PA3952 and PA3422 against s-triazines were produced in rabbits as described in [8]. Polyclonal antibodies P2060 against horseradish peroxidase were produced in Immunotek (Moscow, Russia).

Microtiter plate ELISA for a single pesticide. The competitive ELISA test with chemiluminescent detection was performed in breakable 8-well polystyrene strips (Dynatech, Andover MA, USA). The wells of microtitre plates and strips were coated with 0.2 ml of specific antibodies diluted in 0.05 M carbonate buffer pH 9.5 overnight at 4°C. All further steps were performed at 37°C. The wells were washed 3 times with PBST (phosphate buffered saline pH 7.4 supplied with 0.05% Tween 20). 100 μl of standard solution together with 50 μl of diluted tracer in PBST were added to the wells and incubated for 1 h. After a final washing step, the enzyme activity was determined by chemiluminescence method. 150 μl of the chemiluminescence substrate mixture was added to each well. The formula of the substrate mixture was as follows: 1.0 mM sodium luminol, 0.5 mM *p*-iodophenol, 1.0 mM hydrogen peroxide in 100 mM borate–NaOH buffer (pH 8.5). Each strip was inserted into the holder of the portable scanning luminometer (Immunotek, Moscow, Russia) and the light intensity was detected in all wells for 5 min. The value of maximum light intensity in each well was used for the calculations.

ELISA for the simultaneous determination of atrazine, terbuthylazine, and ametryn with 8-well strip. The test was performed in breakable 8-well polystyrene strips (Dynatech, Andover MA, USA). 0.2 ml of different specific antibodies diluted in 0.05 M carbonate buffer pH 9.5 were added to the wells of the strip: antibodies PS2—to the 1st well, antibodies PA3952—to the 2nd well, antibodies PA3422—to the 3rd well, antibodies S3—to the 4th well, antibodies K4G2—to the 5th well. The immobilization procedure continued overnight at 4°C. The wells were then washed 3 times with PBST. 0.2 ml of a solution of sacharose (1%) and casein (0.5%) in 0.05 M carbonate buffer pH 9.5 was added to the wells and incubated for 1 h at room temperature, then the strips were dried at room temperature and then stored at 4°C. 100 μl of standard or sample solution together with 50 μl of diluted tracer in PBST were added to all wells of the strip and incubated for 1 h at 37°C. All strips were washed 3 times with PBST, and the enzyme activity was determined by chemiluminescence as described above. The light emission was recorded for each strip continuously for 3 min, and the value of maximum light emission was determined for all wells.

Membrane immunoassay with a multispot membrane strip. Positively charged nylon (Hybond N+ with a pore size of 0.45 μm, Amersham Int. plc, UK) was modified with para-azidobenzoic acid as described in [9] and used as a support for covalent photoimmobilization of antibodies. Aliquotes of antibodies (5 μl) diluted with 0.1 M Tris-HCl buffer, pH 8.0 supplied with 0.15 M NaCl (TBS) were simultaneously spotted by a micropipette on the surface of a membrane. Antibodies PS2 were spotted in the 1st spot, antibodies PA3952—in the 2nd spotl, antibodies PA3422 - in the 3rd spot, antibodies S3—in the 4th spot, antibodies K4G2—in the 5th spot. All incubations and washing steps were performed in polystyrene strips at RT. The strips were dipped in a solution of 1.5 ml standard or sample mixed with 1.5 ml of triazine tracer diluted in TBS with 0.1% Tween 20 (TBST). After the immunoreaction, lasting 30 min, the strips were washed (3×10 min) with TBST and then were dipped into a TBS buffer. The activity of peroxidase was then measured with ECL detection. For this, each strip was dipped into a substrate solution containing 1.0 mM sodium luminol, 0.4 mM *p*-hydroxycinnamic acid, 2 mM hydrogen peroxide in 100 mM borate–NaOH buffer pH 9.5. The light emission was recorded for all spots on each strip continiously for 3–5 min, and the value of maximum light emission was determined for each of spots.

Neural networks. Training was performed using code CRES written in Microsoft Visual C++ 1.0 and operated under WINDOWS on Pentium-100 platform. Four different learning algorithms were examined for feedforward neural network training: standard backpropagation, batch mode of backprop, Silva and Almeida modification of backprop, Schmidhuber method). Neurons of hidden and outputs layers had sigmoidal characteristics with effective temperatures 0.5, 2.0, and 1.0 for the first and second (if exists) hidden layer neurons and output neurons correspondingly. Training have been done using stochastic presentations of examples.

Generalization of NN. Different mixtures of s-triazines, each in one of 6 concentration (0, 0.05, 0.1, 0.5, 1, and 10 μg/l) were tested with chemiluminescent ELISA and membrane assay. The result file prepared for NN contained the data for 322 patterns determined with ELISA test and the data for 184 patterns determined with membrane immunoassay. Each pattern consisted of 5 input (maximal light intensities) and 3 output (the concentrations of the analytes) values. For the validation initial data sets were divided into two parts: approximately 150 samples were taken as the training set and the others were used for the internal validation (testing set). Training have been done using stochastic presentations of examples and took as a rule up to 5000 learning cycles to achieve error value about 0.03–0.04. The result file contained the data for the determination of amounts of each of three analytes in all samples included into training or testing set and also the parameters of sensitivity and selectivity for each of analyte determined for the fixed threshold value.

Results and Discussion

In our work we aimed to develop multiassay with chemiluminescence detection for the for pattern recognition of three different triazine pesticides in mixtures. s-Triazine

pesticides atrazine, terbuthylazine and ametryn were chosen as target analytes. This choice has an importance form view point of practical application. Atrazine and terbuthylazine are chloro-containing triazines and ametryn is a thio-containing triazine. In order to develop a multiassay we examined different polyclonal and monoclonal antibodies in a search for a combination which could provide the differentiation between a group of selected s-triazines when detected with the same enzyme tracer. Atrazine derivative coupled to HRP at modified N-alkyl position was found as suitable tracer as it showed the better differentiation between atrazine, terbuthylazine and ametryn with the selected panel of antibodies and also provided the lower detection limit for analytes. Two polyclonal antibodies PS2 and PA3952 and monoclonal antibodies S3, K4G2 and P6A7 recognized 2-chloro-s-triazines much better then thio-s-triazines. Polyclonal antibodies PS2 and PA3952 were more specific to atrazine with some cross-reactivity to terbuthylazine. Monoclonal antibodies S3, K4G2, and P6A7 were more specific to terbuthylazine and cross-reacted in lower extent with other 2-chloro-s-triazines. The third polyclonal antibody (PA3422) had distinctively different pattern recognition and was highly specific to ametryn with negligible cross-reactivity to 2-chloro-s-triazines.

To develop chemiluminescent multi-ELISA we immobilized five different antibodies (PS2, PA3952, PA3422, S3, and K4G2) in separate wells of 8-well polystyrene strip. To develop chemiluminescent membrane assay we immobilized five antibodies (antibodies (PS2, PA3952, PA3422, S3, and P6A7) in separate spots of a single membrane strip. One another well on strip or spot on the membrane strip were used as a control. Polyclonal antibodies P2060 against horseradish peroxidase were immobilized in these wells and spots. The antibodies P2060 can bind tracers via molecule of the label. Changing the concentration of these antibodies, it was possible to select a concentration that provides the binding of tracer resulting in the light intensity value equal to the 50–70% of maximal light intensity detected for zero standard in first five wells.

Mixtures with different composition of analytes, each in one of 6 concentration (0, 0.05, 0.1, 0.5, 1, and 10 μg/l) were prepared and tested with chemiluminescent immunoassays in two formats. For this, each strip was incubated in a solution of one of samples mixed with fixed dilution of the tracer, and after the washing the light intensities were detected in all wells on the portable luminometer. The value of intensity obtained for sixth well (control) was used for the normalization of the light intensity values obtained on the 1st–5th wells or spots of each strip. We used two-step normalization which was performed as follows: relative intensities were obtained by normalization of light intensities to the value of positive control and then the relative intensity for each spot was expressed in percent of relative intensity on this spot obtained when "0" standard (standard which does not contain any analyte) was tested.

In order to find a suitable network topology different learning methods were studied, and for each of them different parameters (the learning rate, the number of neurons, the momentum) were varied. Before the training the program set the connection weights in the network randomly. The input values were then run through the network and an output was predicted for each of the training set vectors. Initially, the outputs were mostly incorrect because the connection weights were randomly assigned. During each pass of the training set through the network, the corrections to the connection weights were made to improve network performance. The network was considered trained when all training-set elements were correctly predicted within the preset tolerance. Result file contained the data for the determination of the concentrations of each of analytes for all samples in the set (training or testing). Also the NN estimated the parameters of sensitivity and specificity for each analyte in the set. We considered sensitivity as amount of correctly determined positive samples and specificity as amount of correctly determined negative samples. To discriminate samples between positive and negative we used a threshold value for analyte concentration of 0.1 μg/l which is of interest because it is a maximum permissible concentration for a single pesticide according to the European Drinking Water Regulation. The samples contained any of analytes in the concentration higher than the threshold value were considered as positive, and those that contained any of analytes in the concentration lower than the threshold value as negative towards the analyte.

Table 1 presents the data for sensitivity and specificity of each of three analytes determined with different immunoassay formats. The best topology for the data measured with ELISA was a net with two hidden layers with 20 neurons in the first and 20 neurons in the second layer. We did not reveal any markable effect of data normalization on the parameters of discrimination for ELISA test. In opposite, the discrimination between analytes with membrane test was improved when normalized data were used as input parameters. It mostly concerns the values of sensitivity and selectivity estimated for the testing set. The best topology for the data obtained with membrane assay was a net also with two hidden layers with 20 neurons

Table 1

The effect of data normalization on the sensitivity (s) and specificity (r) for s-triazines estimated with NN for the threshold value of 0.1 μg/l

Immunoassay format	NN topology*	Analyte	Training set		Testing set	
			s, %	r, %	s, %	r, %
ELISA, native data	6-20-20-3, 322, 153 2968, 0.038	Atr	84.2	79.2	82.8	73.1
		Tba	84.8	70.4	74.3	72.0
		Am	78.8	83.8	76.5	70.4
ELISA, normalized data	5-20-20-3, 322, 153, 2409, 0.035	Atr	85.1	79.2	73.4	70.5
		Tba	87.2	72.4	70.4	67.3
		Am	78.3	73.2	66.4	70.2
Membrane test, native data	6-20-25-3, 184, 154 7713, 0.035	Atr	81.4	93.4	67.2	78.6
		Tba	81.4	84.2	64.1	62.2
		Am	81.2	81.8	52.9	53.5
Membrane test, normalized data	5-20-25-3, 184, 154, 5118, 0.042	Atr	84.2	96.6	83.3	91.7
		Tba	85.0	90.7	89.5	81.8
		Am	86.5	86.0	73.9	85.7

* Number of neurons in each layer, total number of samples in the set, number of samples in training set, number of learning cycles and final squared error, correspondingly.

Table 2

Effect of the antibody combination on the specificity and selectivity of s-triazines discrimination with different formats of immunoassay (1—atrazine, 2—terbuthylazine, 3—ametryn)

	Training set					Testing set				
	$\delta(-A_1)$	$\delta(-A_2)$	$\delta(-A_3)$	$\delta(-A_4)$	$\delta(-A_5)$	$\delta(-A_1)$	$\delta(-A_2)$	$\delta(-A_3)$	$\delta(-A_4)$	$\delta(-A_5)$
ELISA										
s_1	−4.8	0	−9	4.8	0	8.2	8.2	−4.3	10.3	10
r_1	3.6	−14.6	−7.1	−16.4	−16.4	4.4	−11.1	−5.8	−17.8	−17.8
s_2	−3.8	1.3	−4.5	−2.5	−1.3	7.5	25.4	−2.9	−3	1.5
r_2	−31.4	−60	−1.2	−17.1	−34.3	−17.8	−50	3.4	−10	−6.8
s_3	8.7	−4.4	−23.7	11.6	5.8	10.9	1.6	−34.7	10.6	3.4
r_3	−10.8	−7.7	−34.8	−15.4	−2.4	−8.6	−5.7	−38.9	−8.3	−14.3
Membrane immunoassay										
s_1	−2.1	−1.1	0	−3.2	4.2	0	−5.5	−5.5	0	−11
r_1	−6.8	−3.4	−3.6	−3.4	−6.9	0	0	−0.1	0	−8.4
s_2	−2	−2	3	7	−5	−15.8	−15.8	−10.6	−5.3	−36.9
r_2	−11.4	−9.2	−5.6	−27.8	−33.3	−9.2	−18.2	0	0	−45.2
s_3	−2.9	−7.7	−16.5	−3.8	−4.8	−4.4	−3	−18.7	−4.3	−8.7
r_3	−6	−2	−26	−8	−4	−5.3	−8.6	−48.6	0	−10

on the first and 25 neurons on the second layer. We observed enough high sensitivity and specificity (of 74–92%) for the determination of all analytes. The selectivity for all analytes was higher for the data determined with membrane immunoassay. The main difference between two formats of immunoassay involved one change in antibodies set: monoclonal antibody K4G2 used in ELISA test was changed for monoclonal antibody P6A7 which was more specific to terbuthylazine. To elucidate the effect of this change and effect of each of antibodies on the parameters of discrimination we used the ability of NN to analyse the relative contributions of different input elements. Each of input parameters was consequently excluded from the training set and NN was trained again. Table 2 presents the parameters of selected s-triazines discrimination when one of antibodies was excluded from the training set. Antibodies PS2 and PA3953 were valuable for the determination of terbuthylazine, their combination—for the determination of atrazine, antibody PA 3422 was essential for the determination of ametryn. It was found that antibody S3 was valuable for the determination of terbuthylazine in ELISA test and it might be excluded from the set of antibodies for membrane test as the parameters were changed insignificantly especially for the testing set.

To conclude, we established the potential of the neural network application for the discrimination between these three analytes in mixtures. The certain incorrectness of the discrimination can be explained by matrix effects but also it is necessary to consider the effect of antibodies cross-reactivity. Nevertheless we can propose our approach for multianalyte assay for identification and estimation of concentration of structurally similar pesticides at low levels in water samples. Such test might be considered as a preliminary test for the screening of large amount of samples with the following detailed investigation of selected samples with the test based on another principle or with another mathematical approach for the quantitative data estimation.

Acknowledgement

This work was supported by Russian State Scientific Programs "Vaccines of new generation and medical diagnostic systems for future" (grant 02.04/25) and "Novel methods of bioengineering. Biocatalytic technologies" (2-26), which are gratefully acknowledged. We wish to thank Prof. B. Hock and Dr. Th. Giersch for kindly providing the monoclonal antibodies K4G2, S3, and P6A7 used in this study and also Prof. R. Schmid for scientific assistance.

References

1. Jansson, P.A. (1991) *Anal. Chem.*, **63**, 357A–361A.
2. Vertosik, F.T. and Rehr, T. (1993) *Clin. Chem.*, **39**, 2478–2482.
3. Wittmann, C., Schmid, R.D., Loffer, S., and Zell, A. (1997) in: *Immunochemical Technology for Environmental Applications ACS Symposium series* (Aga, D.S. and Thurman, E.M., eds.) ACS, Washington, pp. 343–360.
4. Thorpe, G.H.G. and Kricka, L.J. (1987) in: *Bioluminescence and Chemiluminescence: New Perspectives* (Scholmerich, J., Andreesen, R., Kapp, A., Ernst, M., and Woods, W.G., eds.). J. Wiley, Chichester, pp. 199–208.
5. Kricka, L.G. and Thorpe, H.G. (1983) *Analyst*, **108**, 1274–1296.
6. Rubtsova, M.Yu., Wittmann, C., Egorov, A.M., and Schmid, R.D. (1997) *Food Agric. Immunol.*, **9**, 235–247.
7. Wittmann, C. and Hock, B. (1989) *Food Agric. Immunol.*, **1**, 211–224.
8. Eremin, S.A. and Samsonova, J.V. (1994) *Anal. Letters*, **27**, 3013–3025.
9. Dobricov, M.I., Tenetova, E.D., and Schishkin, G.V. (1990) *Biotechnologia*, **6**, 80–83 (Russ.).