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ORIGINAL ARTICLE

USING THE PIG'S BRAIN ACETYLCHOLINESTERASE IMMOBILIZED ON A TEXTILE FIBER TO TEST THE ANTIDOTES OF NERVE AGENT POISONINGS – PART I

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Summary

An integral part of the research and development of new antidotes against nerve agents (herein referred to as NAs) is the verification of their efficacy by means of in vitro tests. The purpose of these tests is to verify the ability of an antidote to protect cholinesterases from inhibition or to reactivate inhibited cholinesterases. For this purpose cholinesterases of different species and with a different degree of purification are traditionally used. Using immobilized cholinesterases proves to be new and advantageous for a number of reasons. The authors of the presented work verified the usability of Detehit detector (Oritest Praha Ltd) containing the pig's brain acetylcholinesterase for this purpose. In this article we see the results of tests focused on verifying the effect of different reaction conditions on the activity of acetylcholinesterase, especially with regard to reproducibility of these measurements.

Key words: Detehit detector; cholinesterase; nerve agents; reactivator

INTRODUCTION

The antidote personnel protection from NAs effects is almost as old as the nerve agents themselves. In the past sixty years, various principles and instrumentations, in their essence mostly corresponding to the current level of technology, have been used for testing these means. From the point of view of current development, preparation and utilization of immobilized enzymes is not a new but certainly still a useful technology. In the existing sphere of enzymology there are numbers of different applications (even in relation to cholinesterases) differing in used carriers, enzyme sources and ways of their carrier anchorage. Some of these applications have reached such perfection that it is possible to utilize them in series production of, for example, detectors for detection of NAs and pesticides in air, water and material samples. A typical example is the Detehit detector as part of the equipment of the Armed Forces of the Czech Republic. This means belongs to a group of so called simple NAs detectors. For detection of these agents we use the pig's brain acetylcholinesterase immobilized on cotton cloth (1). That reacts with an artificial acetylthiocholine substrate whose degradation products react with a chromogenous DTNB reagent under the development of yellow coloring on the detection cloth. The Detehit detector acts fully autono-

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mously, independently of any other instrumentation. However, the use of this detector is possible also in connection with a more sophisticated technology such as photometers designed for the analysis of light reflected from tested surfaces, which enhances accuracy and reliability of this means because the influence of a subjective assessment of detector coloring by human eyes is excluded (2 - 4). In the presented work, however, a different variant of instrumentation is applied, i.e. common photometry in solutions where the Detehit strip is only an enzyme activity carrier. The advantage of this configuration is the possibility of using quite common laboratory equipment such as photometers operating in a visible area of electromagnetic radiation. Various devices can be used, ranging from wholly primitive ones up to highly sophisticated ones with a high degree of measurement and result processing automation. The authors verified a number of parameters which they considered as being able to influence enzyme activity and thus also reproducibility and accuracy of the measurement.

Experimental part

Instruments

Spectrophotometer HP8453, Hewlett-Packard, USA
Thermostat Julabo F25HE, JULABO Labortechnik GmbH, Germany
Peristaltic pump IPN8, Ismatec/ IDEX Health & Science SA, Switzerland
Electromagnetic stirrer Metrohm 728 Stirrer, Metrohm, Switzerland
Flow cell Hellma 178.010-QS, Hellma GmbH & Co. KG, Germany
Thermostated Franz cell for permeation measurement 4G-01-00-09-05, PermeGear, Inc., USA
Automatic titrator 751 GPD Titrino, Metrohm, Switzerland
Slide gauge 200 mm, Somet Inox, Bílina

Chemicals

Acetylthiocholine iodide, (ATCh) Sigma – Aldrich, Prague
5,5'-dithiobis-(2-nitrobenzoic acid), (DTNB) Sigma – Aldrich, Prague
Disodium hydrogen phosphate, dodecahydrate, Lachema Brno
Sodium dihydrogen phosphate, dihydrate, Lachema Brno
Detehit, Oritest, Prague, production batch 102002, the supplier's package contained serial numbers 010451 up to 010500 (being preserved for a period of two years in a refrigerator at the temperature of 5 – 6 °C).
Citric acid, Lachema Brno
Methanol, Lach – Ner, Neratovice
Sodium hydroxide, Penta Chrudim

Basic solutions

DTNB solution in phosphate buffer

The phosphate buffer with pH of 7.5 and phosphate ion concentration of 0.1M was prepared by dissolving the calculated weights of Na_2HPO_4 and NaH_2PO_4. The calculated DTNB weight was dissolved in 0.5 ml of methanol and this solution was mixed into the buffer solution so as to generate the 0.1M DTNB solution. The resulting pH of the solution was checked by the automatic titrator.

Acetylthiocholine iodide solution

The 0.01M citric acid solution was prepared by dissolving the substance in water; the solution pH was set by adding 0.1M NaOH to the value of 3. In this solution the ATCh weight was dissolved so as to generate the 0.01M solution. The resulting pH of the solution was checked by the automatic titrator.

Detehit modification for measurement in a flow apparatus

A part carrying the cloth with the immobilized enzyme including about a half of the length of the reference cloth was cut off from the detection strip. The rest of the reference cloth was ripped off from the cutting. A magnetic stirrer (diameter of 4.5 mm, length of 12 mm) in Teflon covering was attached with an adhesive tape to the cutting edge where the cut-off was made so that the cutting axis was at the level of the stirrer diameter.

Assembly and function of an apparatus for measurement of the Detehit acetylcholinesterase activity

The Franz cell was put on the plate of the electromagnetic stirrer and linked to an external water circuit of the thermostat. The cell was filled with 10 ml of buffer with DTNB. Through a cannula placed in the filler neck of the cell the solution was sucked in a peristaltic pump and transported to a flow cell of the photometer, also placed in the

thermostated holder. From the cuvette (cell) the solution was transported through another cannula back to the Franz cell. The solution flow through the system was kept by the pump at the value of 1.08 ml/min. After irrigating the apparatus a modified Detehit strip was inserted into the Franz cell and stirring was launched for a period of 0.5 minute. Subsequently a monitoring program of the photometer, designed for kinetic measurements, was launched. The absorbance recording was made at 415 nm. The reaction of the enzyme with the substrate was started by adding 1 ml of ATCh solution to the circulating solution in the permeation cell. The reaction was observed for a period of 10 minutes. The activity of the enzyme was expressed as absorbance of the sample after 10 minutes of reaction.

Mathematical processing of gross experimental data

The recording of absorbance dependence on time made by the photometer was transferred to MS Excel, to a point chart. A straight line was led through the displayed dependence and its slope, indicating the change of absorbance in unit time, was deducted. The enzyme activities defined in this way were further processed.

RESULTS

Checking the cholinesterase activity in one complete user package (tube)

10 Detehit strips were modified as it is described above and their activity was measured.

Table 1. Cholinesterase activity on strips of one Detehit package.

order number	enzyme activity
1	0.3758
2	0.4658
3	0.4641
4	0.3944
5	0.354
6	0.5073
7	0.377
8	0.4216
9	0.4925
10	0.4476

Average value of the enzyme activity 0.43
Standard deviation 0.05
Standard deviation as a percentage of the average value 11.7%
A check for outlying results was made by using the Dean – Dixon test, without excluding any value.

Deviations in geometry of cloth carrying the immobilized enzyme

It was the authors' effort to clarify which factors participated in dispersion of activities values. The inequality of the geometry of cloths with the enzyme proved obvious. That is why its dispersion was checked.

Table 2. Geometry of cloth with enzyme.

order number	lenght mm	width mm	area mm
1	14.85	10	148.5
2	15.55	11.2	174.16
3	14.75	11.2	165.2
4	14.3	11.5	164.45
5	14.4	10.6	152.64
6	16.2	10	162
7	14.1	10.7	150.87
8	14.6	10.1	147.46
9	16.45	10.75	176.84
10	15.2	9.75	148.2

Strip length

Average length 15.04 mm
Standard deviation 0.76 mm
Standard deviation as a percentage of the average value 5.05 %

Strip width

Average width 10.58 mm
Standard deviation 0.57
Standard deviation as a percentage of the average value 5.4%

Strip area

Average area 159 mm
Standard deviation 10 mm
Standard deviation as a percentage of the average value 6.6%

Effect of repeated application of the same Detehit strip on enzyme activity

With regard to the observed dispersion of values for a set of strips from one package there was an effort to eliminate that by a repeated application of one strip in more experiments if supported by the experiment methodology.

Table 3. Effect of repeated application of the same strip on enzyme activity in reaction with substrate.

order of use	enzyme activity
1	0.4512
2	0.4583
3	0.456
4	0.4508
5	0.4508

Average activity 0.453
Standard deviation 0.003
Standard deviation as a percentage of the average value 0.6 %

Effect of the strip rotation speed in developing solution on enzyme activity

Considering the fact that in the presented experimental arrangement it is a heterogeneous catalysis from the point of view of both the enzyme and the immobilization cloth, it is apparent that the dynamics of the liquid flow around the strip will be an influential parameter. It was the authors' effort to quantify this effect.

Table 4. Effect of the strip rotation speed on enzyme activity.

stirrer scale	strip revolution round per sec	enzyme activity
1	2.8	0.466
2	5.3	0.424
3	7.5	0.3845
4	10	0.4845

Average activity 0.44
Standard deviation 0.04
Standard deviation as a percentage of the average value 9.1%
A check for outlying results was made by using the Dean – Dixon test, without excluding any value.

Effect of the ionic strength of developig solution on enzyme activity

The ionic composition and concentration of salts in the solution influence the activity of a number of enzymes. In this experiment it was verified how the concentration of the used phosphate buffer would affect the enzyme activity.

Table 5. Effect of the ionic strength of phosphate buffer with pH of 7.5 on enzyme activity.

molar buffer conc. M	enzyme activity
0.01	0.3613
0.05	0.3711
0.1	0.3901
0.5	0.4631
1	0.3924

Average activity 0.39
Standard deviation 0.04
Standard deviation as a percentage of the average value 9.0%

Effect of Detehit cloth hydration on enzyme activity

Even though it is not explicitly specified in the detector operating manual, the effect of the cloth hydration time before launching the reaction with substrate was tested.

Table 6. Effect of detector cloth prehydration time on enzyme activity.

Hydration time minute	enzyme activity
0	0.3871
4	0.4259
6	0.3241
10	0.4019

Average activity 0.38
Standard deviation 0.04
Standard deviation as a percentage of the average value 9.8%
A check for outlying results was made by using the Dean – Dixon test, without excluding any value.

Effect of temperature on enzyme activity

Like in all reactions, the reaction kinetics of enzymes is influenced by temperature. The purpose of this test was to find a temperature at which the common enzyme kinetics passed to the protein degradation kinetics and to find out how steep the gradient of the reaction temperature dependence was at common temperatures.

Table 7. Effect of temperature on enzyme activity.

temperature °C	enzyme activity
10	0.3186
15	0.3905
20	0.4425
25	0.5357
30	0.573
35	0.6233
40	0.5568
45	0.4254
50	0.3024

Enzyme temperature limit: 35°C

Activation energy of enzyme reaction with substrate: 19.5 kJ/mol

Effect of pH on enzyme activity

Enzymes as protein character substances almost always contain a number of functional groups whose ionization degree is dependent on a concentration of hydrogen ions. If these groups are a part of binding or catalytic centre of an enzyme, the pH becomes a parameter significantly influencing the enzyme activity.

Table 8. Effect of hydrogen ions concentration on enzyme activity.

pH	enzyme activity	% of activity
6.0	0.1213	45
6.5	0.1787	66
7.0	0.2392	88
7.5	0.2673	99
8.0	0.2709	100
8.5	0.2273	84

Effect of DTNB concentration on the course of reaction

DTNB is added to the reaction of the enzyme with an artificial substrate as a chromogenous reagent. That is why a concentration, which would ensure a reliable transfer of the entire split substrate to a color product at the considered degree for mixture reaction, was searched for.

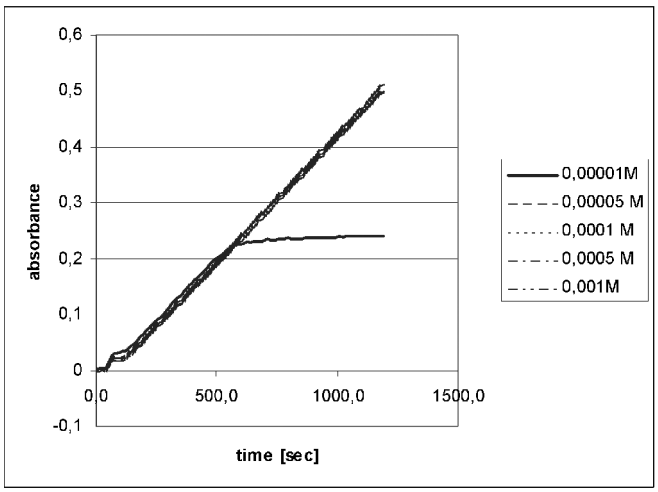

Figure 1. Dependence of absorbance of the reaction mixture in dependence on time and DTNB concentration.

Effect of pH on coloring of DTNB degradation products

Besides the effect of pH on the enzyme – substrate reaction itself, the effect of pH on coloring of a DTNB degradation product was also verified because this product contained ionizable functional groups being able to influence this quality.

pH value of 4.31 in curve inflexion point

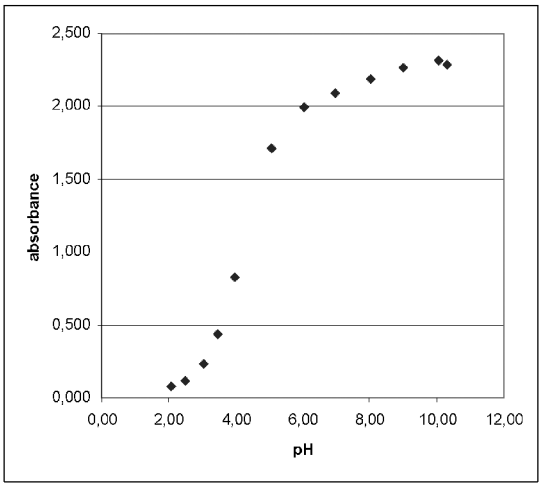

Figure 2. Dependence of absorbance of DTNB degradation products on pH at a wavelength of 415 nm.

DISCUSSION

Checking the cholinesterase activity in one complete user package (tube) (Table 1)

Considering the fact that a number of experiments were adjusted so that not more than ten Detehit strips, i.e. one user package, were used during their conduct, the effort was to verify how homogeneous values of the enzyme activity would be given by a set generated like that. The observed value of the standard deviation of an average activity expressed as a percentage of average makes 11.7% whereas the dispersion was not burdened with outlying values which would have to be excluded.

It can be thought that both deviations of an area enzyme activity on the strip and deviations in size of the strip area itself may participate in this dispersion.

Deviations in geometry of cloth carrying the immobilized enzyme (Table 2)

These deviations were quantified by measuring geometry of the cloth carrying the enzyme. Deviations in length and width of the cloth participate in resulting deviations of the cloth area in the same way. The value of the standard area deviation as a percentage of average makes 6.6%, which is ca. a half of the value reched in the experiment with ten strips. So it is obvious that the remaining value of deviation needs to be put into the account of other effects.

Effect of repeated application of the same Detehit strip on enzyme activity (Table 3)

In other experiments it was found out that it would be advantageous to use repeatedly the same Detehit strip in some of the experimental arrangements for the reasons of both savings and reaching a better accuracy of measurement. That is why it was verified whether there was any decline in enzyme activity on the strip due to a repeated application. The achieved results do not show, taking into the account the number of measurements made, that the enzyme activity would have a permanently downward tendency. The achieved standard deviation 0.6% of average can be considered as very good in the sphere of enzyme activities measurements. Since this set of measurements was not burdened with the permanently downward tendency in activity values, the achieved value of deviation can be considered as an inherent

measurement error given by the experimental arrangement and instruments used.

Effect of the strip rotation speed in developing solution on enzyme activity (Table 4)

The experimental arrangement used, when the immobilized enzyme on the stiff plate is sunk into liquid, is an analogy of arrangements which are used in heterogeneous catalysis. An influential parameter in these reactions is the intensity of liquid flow around a solid catalyst where it is the main factor which affects the disturbance of a surface layer of products of a reaction which prevents another substrate from approaching the catalyst (5). The influence of this parameter was verified also in the experimental arrangement used. Changes in the activity along with changes in revolutions showed a tendency neither towards a permanent growth nor decline whereas the standard deviation did not exceed the value reached in the experiment with ten strips. Besides that there was an orientation experiment which was to simulate a totally immovable catalyst. The strip was put into solution and kept inactive for 30 seconds; then it was stirred for 2 seconds. This was repeated for a period of 10 minutes. The step of stirring was included for the reasons of a regular homogenization of the cell content because this could not be reached by means of a simple diffusion. The achieved activity was ca. quarter compared to the result achieved by stirring. Thus a conclusion can be drawn that the liquid flow is an influential parameter also in this experimental arrangement but under the given experimental conditions its influence does not change any more.

Effect of the ionic strength of developing solution on enzyme activity (Table 5)

Ions present in enzyme solutions affect their activity by both quality and quantity. Some of them act as inhibitors; the others accelerate reactions with substrates. The cases when a metal ion becomes the cofactor, i.e. a substance absolutely necessary for the enzyme function, are not exceptional either (6). The phosphate ions are a majority component of the applied buffer and so the effect of their concentration on the enzyme activity was examined. The achieved results show the maximum at a concentration of 0.5 M. If, however, all the values have been averaged, this maximum does not prove to be an outlying value.

Effect of Detehit cloth hydration on enzyme activity (Table 6)

Even though it is not explicitly specified in the detector operating manual that conditioning in the solution should be done before measurement, the effect of the cloth hydration time before launching the reaction with substrate was tested. This experiment was conducted because it was almost regularly observed in the authors' workplace that enzymes, which were preserved in a frozen or lyophilized condition, often stabilized their activity after two hours of hydration. However, in the case of Detehit this did not prove because the strip hydration time did not have the tendency to increase the enzyme activity.

Effect of temperature on enzyme activity (Table7)

The number of works dealing with thermodynamics of cholinesterases reactions is not too high and the conditions, under which the reactions are conducted, are often hard to compare and therefore also the published values (for example of activation energies) are comparable only conditionally (7 - 9). Nevertheless, the authors verified the effect of temperature on enzyme activity. The reason was firstly the assessment of the effect of temperature stabilization in the experiment and secondly it was the interest to find out to what extent it was possible to raise the temperature of reaction without the risk of its inhibition. The temperature used as standard in the experiment, i.e. 25°C, is quite remote from the break at which the enzyme activity is inhibited. If the empirical experience in the area of thermodynamics of chemical reactions is taken into account, i.e. that the temperature rise by ten degrees mostly means the rise in reaction velocity twice up to four times (10), it can be said that the Detehit cholinesterase rather goes beyond that. The rise in reaction velocity at temperatures from 20 to 30°C is only 1.3-fold. Another quite a remarkable value is the temperature at which a break in the enzyme activity occurs, i.e. 35°C. It can be assumed that the enzyme reacting like that could cause certain problems in a living organism by its overheating extrinsically or intrinsically as a result of illness.

Effect of pH on enzyme activity (Table 8)

The effect of pH on Detehit cholinesterases does not go beyond the findings for cholinesterases of other species (11) and the process of

immobilization does not markedly affect this quality either.

The functional maximum is given by pH values of 7.5 – 8.

Effect of DTNB concentration on the course of reaction (Figure1)

For the reasons of economy of conducting the experiments and the effort not to complicate the applied reaction mixture, the authors' aim was to find out what DTNB concentration would be sufficient for visualization of all the dissolved substrate. For this experiment the reaction time was doubled from ten to twenty minutes. The only DTNB concentration, which was not satisfactory in the test, was 1.10^{-5} M.

Effect of pH on coloring of DTNB degradation products (Figure 2)

The pH of the reaction mixture does not affect only the enzyme activity but it can also affect the coloring of a DTNB degradation product. The resulting graft carries nitro, carboxyl and sulphhydryl groups where the last two have replaceable hydrogen. The presence of the nitro group on the aromatic skeleton will act as a factor increasing the dissociation, especially with the sulphhydryl group. In the conducted experiment it was found out that the pH of the half neutralization of a functional group causing the coloring of this compound was equal to 4.31, which was at the same time also the value of logarithm of the acid-base dissociation constant. If the pH is increased by two units, there are no noticeable changes in the coloring of this substance any more. The pH value of 6.31 is sufficiently remote from the pH value of 7.5 used in the experiment and so from this point of view the pH can be considered a non-influential parameter.

CONCLUSION

In this work the authors dealt with the effects of a number of factors which might affect the functionality of cholinesterases within the instrumentation used. Based on the achieved results we can say that the applied instrumentation is robust enough and while following at least the fundamental principles for working in the analytical laboratory this method can be used to obtain results not burdened with gross errors.

The subject of other experimental works will be identifying the functionality of enzymes used in Detehit construction because these will decide on the scope of usability of this means.

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