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Colorimetric dipstick for assay of organophosphate pesticides and nerve agents represented by paraoxon, sarin and VX

Miroslav Pohanka[a,b,*], Jana Zdarova Karasova[b], Kamil Kuca[a,b], Jiri Pikula[c], Ondrej Holas[b,d], Jan Korabecny[b,d], Jiri Cabal[b]

[a] Centre of Advanced Studies, Faculty of Military Health Sciences, University of Defense, Hradec Kralove, Czech Republic
[b] Department of Toxicology, Faculty of Military Health Sciences, University of Defense, Hradec Kralove, Czech Republic
[c] Department of Veterinary Ecology and Environmental Protection, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic
[d] Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Hradec Kralove, Czech Republic

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ABSTRACT

A dipstick for fast assay of nerve agents and organophosphate pesticides was developed. Indicator pH papers were used as detectors. The principle of the assay is based on enzymatic hydrolysis of acetylcholine into acetic acid and choline by acetylcholinesterase. Acidification of the reaction medium due to accumulation of acetic acid was visible. The colour changed from dark red to yellow as the pH indicator recognized pH shift. Presence of an organophosphate pesticide or a nerve agent results in irreversible inhibition of acetylcholinesterase intercepted on the dipstick. The inhibition stops the enzymatic reaction. The inhibition appears as no change of the medium pH. Three compounds were assayed: paraoxon-ethyl as representative organophosphate pesticides and nerve agents sarin and VX. The achieved limit of detection was 5×10^{-8} M for paraoxon-ethyl and 5×10^{-9} M for sarin and VX. Dipsticks were found stable for at least one month. Suitability of these dipsticks for routine assay is discussed.

1. Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7.) is an enzyme participating in the cholinergic neurotransmission through the neurosynaptic cleft. Its primary goal is hydrolysis of the neurotransmitter acetylcholine and termination of the transmission in this way [1]. The second important physiological role of AChE is the cholinergic antiinflammatory pathway in blood cells, especially macrophages [2,3]. AChE is a serine hydrolase. It is sensitive to inhibition by many compounds commonly known as organophosphorus inhibitors (OPIs). OPIs are represented by organophosphate pesticides and nerve agents [4]. OPIs bind to serine hydroxyl and prevent hydrolysis of acetylcholine in this way [5].

Typical OPIs are nerve agents historically used in chemical warfare. Well known agents such as sarin, soman, tabun and VX could be mentioned as typical examples [6]. The other group includes pesticides widely used in agriculture and epidemiologic events [7]. In comparison to nerve agents, only the so-called oxoforms of OPI pesticides are inhibitors in vitro. The most of OPI pesticides are thioforms. They are metabolically activated in order to be inhibitors and in vitro provide only minimal inhibitory potency [8].

The OPI potency to inhibit AChE is considered as a suitable way to construct applicable analytical device for OPI assay. A photometric assay based on the Ellman's reaction that is splitting 5,5′-dithiobis-(2-nitrobenzoic acid) into yellow coloured 5-thio-2-nitrobenzoic acid in the presence of hydrolyzed acetylthiocholine is of choice [9]. Colorimetric dipsticks such as DETEHIT (Oritest, Prague, Czech Republic) are based on the Ellman's reaction. A promising way to detect organophosphates and/or estimate the AChE activity in blood is based on the voltammetric principle [10,11]. Devices with intercepted AChE, biosensors, are extensively researched as a suitable tool for fast and low cost assay of pesticides as well as nerve agents [12–15].

Construction of simple detectors for OPI assay is a promising way for military as well as environmental and agricultural purposes. The current effort is focused on construction of simple dipsticks applicable for semi-quantitative assay of OPIs. Two major goals were followed throughout experiments. First, the dipstick should operate without any instrumental device. Second, colorimetric assay should provide a clear colour change. For the second reason, the Ellman's method is not considered as convenient due to

* Corresponding author at: Centre of Advanced Studies, Faculty of Military Health Sciences, University of Defense, Trebesska 1575, Hradec Kralove, Czech Republic.
E-mail address: rau@atlas.cz (M. Pohanka).

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the poorly visible change from white to yellow colour. Moreover, analytical parameters such as time needed per one assay and limits of detection are considered as final outputs.

2. Materials and methods

2.1. Chemicals

O-Isopropyl methylphosphonofluoridate (sarin; in NATO countries encoded also as GB) and S-[2-(diisopropylamino)ethyl] O-ethyl methylphosphonothioate (VX) were provided by the Military Technical Institute of the former Czechoslovak army in 1990s. Storage and manipulation with sarin was carried out under special technical conditions. Tubes with stock sarin and VX were kept in boxes filled with active carbon. Manipulation and experiments were carried out in fume chambers. Storage and manipulation was permitted by the governmental institution SUJB of the Czech Republic in accordance with the organisation for the prohibition of chemical weapons (OPCW) and the convention on the prohibition of the development, production, stockpiling and use of chemical weapons and on their destruction.

Acetylcholine chloride (AChCl) lyophilized powder, diethyl (4-nitrophenyl) phosphate (in following text paraoxon-ethyl) and AChE from human erythrocytes (20 mM HEPES pH 8.0 buffered, activity 500 U/protein mg) were purchased from Sigma–Aldrich (Prague, Czech Republic). All other chemicals were of standard analytical purity. Deionized water was prepared using Millipore system.

2.2. Estimation of AChE activity

Due to instability of AChE during storage, the activity of AChE was measured before AChE solution processing. A modified Ellman's method was chosen for these purposes [16]. A polystyrene cuvette was filled with 0.4 mg/ml of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB hereinafter the text)—0.4 ml, 25 μl of tested AChE solution and 500 μl of phosphate buffered saline (PBS). The mixture was gently shaken prior to the biochemical reaction. The reaction was started by immediate addition of 100 mM acetylthiocholine chloride (ATChCl)—100 μl in PBS. The yellow colour of 5-thio-2-nitrobenzoic acid was measured at 412 nm against blank (mixture of DTNB and ATChCl in the concentration equivalent to the sample) after 2 min of incubation and AChE activity in the original solution was calculated.

2.3. Dipstick preparation

A disposable pH indicator strip with pH range 6.0–7.7 (Macherey-Nagel, Duren, Germany) was covered with 5 μl of AChE with total activity of 0.25–1 U suspended in 20 mM HEPES, pH 8.0. The strip with AChE soaked pH indicative tissue was kept in 50 ml dry chamber with air saturated glutaraldehyde overnight. After that, dipsticks were ready for measurements and kept in a fridge or used immediately.

2.4. Assay procedure

Prior to assay, fresh solution of paraoxon-ethyl, VX and sarin in 5% isopropanol was prepared. The level of isopropanol was chosen according to the previous study; a higher level of isopropanol would inhibit AChE [17]. The content of paraoxon-ethyl and sarin was adjusted in a range from 10^{-10} to 10^{-3} M and in this way prepared sample was used during one day in order to avoid variability in the assay caused by spontaneous hydrolysis. The prepared dipstick was covered with 10 μl of 5 mM Tris–HCL and 5 μl of the analyzed sample. After 10 min of incubation, the dipstick was covered with 5 μl of 50 mM AChCl solution. The reaction was left to run over. The time was object of optimization.

2.5. Specific manipulation with highly toxic substances

Manipulation with sarin, VX and with the stock solution of paraoxon-ethyl was carried out in a fume chamber with exhaustion through filters with active carbon. Sarin and VX were stored in glass tubes placed into a teflon box filled with active carbon, too. Latex gloves were worn throughout samples manipulation. All contaminated tools and used disposable laboratory equipment as well as dipsticks were decontaminated in 1 M sodium hydroxide with 10% ethanol overnight (sarin) or in at least 30% calcium hypochlorite (VX).

3. Results and discussion

Disposable pH indicators with range from 6.0 to 7.7 were chosen as a convenient tool for the evaluation of AChE activity. The pH indicators were chosen for a sharp colour change between dark red (pH 7.7) and bright yellow (pH 6.0). This colour change is favourable when compared with the typical one provided by the Ellman's reaction. Typically, the positive Ellman's reaction (no OPIs present) is accompanied with no-colour-change (solution) or a change from white (textile matrix) into yellow. The colour change white–yellow is poorly visible on dipsticks in artificial light.

The dipsticks were performed for fresh solutions of OPIs represented by VX, sarin and paraoxon-ethyl. Visualization of the assay is depicted in Fig. 1. The colour changes were considered as sufficient. The original pH of the medium was slightly alkaline represented by red colour. It became bright red and yellow as the AChE-catalyzed reaction acidified the medium. In the first round, the activity needed per one strip and time of incubation was optimized.

Three levels of activities were chosen for tests: 0.25, 0.50 and 1.0 U per one dipstick. The tested activities are quite high in comparison with the one used for construction of devices such as biosensors [17]. The main advantage of this concept is that the overall assay time is shortened. The overall pH change needed for reaction visualization for human eye is quite high in comparison with devices providing electrical signal. The detected pH was considered as an output value. Etalon provided by the pH indicator producers was used throughout to estimate pH. The achieved pH value was measured at regular intervals of 2.5, 5, 10, 20, 40, 50, and 60 min after the AChCl addition on the sensitive surface. Paraoxon-ethyl rather than sarin or VX was used for optimization purposes due to its lower toxicity and thus higher safety during manipulation. Experimental data are depicted in Table 1.

The measured pH was not measured as a continuous distribution; on the contrary, it was measured as a discrete distribution when the pH value was detected as one of the following values:

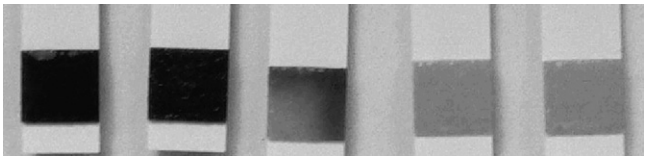

Fig. 1. Photograph of disposable dipsticks for assay of OPIs. The left dipstick was used for assay of only blank (deionized water). The second dipstick from left was performed for assay of paraoxon-ethyl in a lower concentration than the limit of detection (5×10^{-9} M). The third dipstick was performed for assay of paraoxon-ethyl solution in the amount equal to the limit of detection (5×10^{-8} M). The last two dipsticks were used for assay of paraoxon-ethyl in the concentration high above the limit of detection (5×10^{-7} and 5×10^{-6} M). The light grey colour corresponds to yellow, while the black corresponds to bright red.

Table 1
Optimization of the assay based on a dipstick with intercepted AChE. The concentration given in the table indicates the level of paraoxon-ethyl in the sample. The outputting value is pH recognized by colour indication on the dipstick tissue.

Time (min)	5×10^{-4} M	5×10^{-5} M	5×10^{-6} M	5×10^{-7} M	5×10^{-8} M	5×10^{-9} M	Control
0.25 U of AChE per one dipstick							
2.5	7.7	7.7	7.7	7.7	7.3	7.3	7.3
5	7.7	7.7	7.7	7.7	7.0	7.0	7.0
10	7.7	7.7	7.7	7.7	7.0	6.7	6.7
20	7.7	7.7	7.7	7.7	6.7	6.0	6.0
30	7.7	7.7	7.7	7.7	6.4	6.0	6.0
40	7.7	7.7	7.7	7.3	6.4	6.0	6.0
50	7.7	7.7	7.7	7.3	6.4	6.0	6.0
60	7.7	7.7	7.7	7.0	6.4	6.0	6.0
0.50 U of AChE per one dipstick							
2.5	7.7	7.7	7.7	7.7	7.3	6.7	6.7
5	7.7	7.7	7.7	7.3	7.0	6.0	6.0
10	7.7	7.7	7.7	7.0	7.0	6.0	6.0
20	7.7	7.7	7.7	6.4	6.4	6.0	6.0
30	7.7	7.7	7.7	6.4	6.4	6.0	6.0
40	7.7	7.7	7.7	6.0	6.0	6.0	6.0
50	7.7	7.7	7.7	6.0	6.0	6.0	6.0
60	7.7	7.7	7.7	6.0	6.0	6.0	6.0
1.0 U of AChE per one dipstick							
2.5	7.7	7.7	7.3	6.4	6.0	6.0	6.0
5	7.7	7.7	7.3	6.0	6.0	6.0	6.0
10	7.7	7.7	7.0	6.0	6.0	6.0	6.0
20	7.7	7.7	6.7	6.0	6.0	6.0	6.0
30	7.7	7.7	6.7	6.0	6.0	6.0	6.0
40	7.7	7.7	6.7	6.0	6.0	6.0	6.0
50	7.7	7.7	6.4	6.0	6.0	6.0	6.0
60	7.7	7.7	6.0	6.0	6.0	6.0	6.0

6.0, 6.4, 6.7, 7.0, 7.3, and 7.7. Due to this fact, the standard calculation that limit of detection is triplicate of signal to noise ratio was useless. On the other hand, the pH degrees were large enough to cover pertinent deviations. When experimental measurements were repeated, there was indicated the same pH value though slight colour discrepancies would occur. Here, limit of detection was considered as the lowest concentration of the substrate able to provide one step of pH change compared to the control (no inhibition). Paraoxon-ethyl was regularly detected when its concentration in the sample was at least 5×10^{-5} M. Limit of detection varies between 5×10^{-8} and 5×10^{-5} M according to the time of incubation and the applied activity of AChE. The best limit of detection was achieved for only intercepted activity of 0.25 and 0.50 U per one dipstick. The activity of AChE 1.0 U was not found as suitable for the construction of the dipstick. Activity 0.25 U per one dipstick was able to provide the best limit of detection 5×10^{-8} M. However, the change of pH was only one degree of pH: 6.0–6.4 when compared with the limit of detection of 5×10^{-8} M and the last not detected concentration (5×10^{-9} M). Unfortunately, the limit of detection was achieved after 10 and more minutes of incubation. Activity 0.5 U per one dipstick seems to be the best for practical application. The best limit of detection of 5×10^{-8} M was achieved in a short time interval from 2.5 up to 10 min. In this period, two respective three degrees of pH −6.7 to 7.3 respective 6.0–7.0 were observed. Above 20 min, the limit of detection of 5×10^{-8} M was achieved when the pH changed from 6.4 to 6.0. In a short note, we should emphasize that the assay is semi-quantitative so the exact value of pH is unknown. For this reason, time found to reach the lowest pH can be slightly different to the expected one.

From a practical point of view, activity 0.5 U per one dipstick is the best due to the very short time of assay needed for the best limit of detection. Time of 2.5 min for the reaction processing is quite short. The overall time needed per one measurement is less than 15 min (including incubation with an unknown sample). The incubation period was chosen as long enough to enable binding of OPIs to AChE. On the other hand, time of the incubation with the sample can be shortened up to 1 min so that the overall time of assay is less than 4 min. Binding kinetics is quite fast as presented in the previous study and a half-time inhibition would be around 1 min with OPIs present in sufficient amounts in the sample [18]. Here, incubation of OPIs with the dipstick was optimized for the intercepted overall AChE activity 0.5 U and incubation with AChCl for 2.5 min. No changes in the limit of detection were found for incubation of 5–10 min. The incubation shorter than 5 min leads to milder changes in pH. The pH change from 7.0 to 7.3 was found in the incubation for less than 5 min instead of the previously found range of 6.7–7.3. According to the achieved data, the shortening of incubation of OPIs with AChE is a possible way to improve the assay under some specific conditions where the overall time needed per one assay is an important factor.

The second of experiments was aimed at assay of highly toxic nerve agents VX and sarin. VX and sarin represent “V” and “G” type nerve agents, respectively [19]. The outcome from the optimization of paraoxon-ethyl assay was used also for assay of sarin and VX. A dipstick with 0.5 U of AChE per one strip was used for calibration of sarin and VX. The calibration is shown in Table 2.

Sarin as well as VX were assayed with the limit of detection ten times lower than the one found for paraoxon-ethyl, i.e. 5×10^{-9} M. Though the limit of detection was the same for sarin and VX, some differences were found in the pH change velocity. The difference of pH change between sarin levels 5×10^{-8} M and the control was one grade: 6.4–6.7 within the time interval 2.5–10 min. On the other hand, VX has the very same limit of detection but in the time interval of incubation of 2.5, the pH change was 6.4–7.0. In the time interval of 5–30 min, the grade of pH was 6.0–6.7 respective 6.0–6.4 when compared to the control.

It should be emphasized that the achieved limit of detection was nearly ten times higher when compared with the previously constructed biosensor [17], data presented by Schulze et al. for paraoxon extracted from spiked food [20] or paraoxon assay by a butyrylcholinesterase-based dipstick [21]. The AChE-based dipstick assay would be further improved by colour measurement in reflectometer [22]. The dipstick assay seems to be very sensitive. Unfortunately, the pre-concentration step for the sample is needed

Table 2
Assay of sarin and VX by a disposable colorimetric dipstick. Activity 0.5 U of AChE per one dipstick was used throughout the assay. Achieved values of pH are presented in the table, these values represent outputting signals.

Time (min)	5×10^{-5} M	5×10^{-6} M	5×10^{-7} M	5×10^{-8} M	5×10^{-9} M	5×10^{-10} M	Control
Sarin							
2.5	7.7	7.7	7.3	7.0	6.7	6.4	6.4
5	7.7	7.7	7.3	7.0	6.7	6.4	6.4
10	7.7	7.7	7.0	6.7	6.7	6.4	6.0
20	7.7	7.7	7.0	6.7	6.4	6.0	6.0
30	7.7	7.7	6.7	6.7	6.4	6.0	6.0
40	7.7	7.3	6.7	6.4	6.4	6.0	6.0
50	7.7	7.3	6.7	6.4	6.0	6.0	6.0
60	7.7	7.3	6.4	6.0	6.0	6.0	6.0
VX							
2.5	7.7	7.7	7.7	7.0	7.0	6.4	6.4
5	7.7	7.7	7.3	7.0	6.7	6.0	6.0
10	7.7	7.7	7.3	7.0	6.7	6.0	6.0
20	7.7	7.7	7.0	6.7	6.4	6.0	6.0
30	7.7	7.7	7.0	6.7	6.4	6.0	6.0
40	7.7	7.7	7.0	6.7	6.0	6.0	6.0
50	7.7	7.7	6.7	6.4	6.0	6.0	6.0
60	7.7	7.3	6.7	6.4	6.0	6.0	6.0

when e.g. the maximum limit of individual pesticides in drinking water of 0.1 μg/l demanded by the European Union (Drinking Water Directive 98/83/EC) is assayed.

The selectivity of the dipstick was also investigated. According to the previous study [17,23], organic solvents were recognized as inhibitors causing denaturation of captured AChE. Ethanol, methanol, acetone, dimethylsulfoxide, tetrahydrofuran, acetonitrile and polyethyleneglycol were significantly inhibiting AChE when exceeding 5% (v/v) concentrations. Dipsticks were fully suitable for assay of OPIs solved in the above-mentioned solvents not exceeding 5% in water. The sample with OPIs and higher than 5% concentration of any organic solvent should be assayed after water dilution.

The long term stability of AChE captured in the dipstick was investigated under room temperatures. Freshly prepared dipsticks were closed in a plastic box and remained in the laboratory (SATP conditions). The dipsticks were not exposed to OPIs. AChE activity represented by the pH shift due to the conversion of AChCl was measured daily. No decrease of the AChE activity was found during the first two weeks of storage. After that, the decrease was diminished to only 6.4 instead of 6.0. It appears that the dipstick was fully applicable for one month of storage.

Here should be also substantiated why the pH used measurement range was from 6.0 to 7.7 with the original pH value of reaction medium 8.0. There are two different factors influencing the assay. Firstly, the assay can be influenced by spontaneous hydrolysis of OPIs since OPIs are unstable in the higher pH [24]. Secondly, the assay should be carried out near pH optimum of AChE. The optimal pH quoted for human AChE are 9.0 [25], 8.1 [26], 8.0 [27], 7.5 [28] and 7.4 [29]. In this study, the initial pH 8.0 was used in experiment as the suitable one for OPI persistence in sample. Moreover, upper half part of used pH range can be marked as nearly optimal for AChE activity.

4. Conclusions

A colorimetric dipstick for assay of highly toxic organophosphates pesticides and nerve agents was developed. Dipsticks were successfully performed for assay of paraoxon-ethyl as a representative of toxic organophosphate pesticides and two military important nerve agents such as sarin and VX. The limit of detection was quite good: 5×10^{-9} M for nerve agents and 5×10^{-8} M for paraoxon-ethyl. The assay is quite fast when compared with similar devices based on the Ellman's method. The colorimetric dipsticks are promising detectors for low cost field assay with analytical parameters comparable to instrumental techniques. The assay could be also simply improved by distinguishing of inhibitor classes by oximes [9,30].

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