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Stability of acetylcholinesterase (AChE) activity in blood: the effect of the preservative agent

Zmiany aktywności acetylocholinoesterazy (AChE) we krwi: wpływ dodatku środka stabilizującego

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Abstract

Fluoride anions are believed to inhibit acetylcholinesterase activity, thus there is a possibility that the activity may be affected by blood sampling into vials containing sodium fluoride as an anticoagulant. The paper aims to draw attention to the effect of the anticoagulant used in blood collection tubes on acetylcholinesterase activity.

A stability study was conducted for 176 days on blood samples collected into tubes containing sodium fluoride or sodium EDTA. An improved Ellman procedure, utilizing spectrophotometric determination at 436 nm, was employed in order to determine acetylcholinesterase activity.

An immediate decrease in acetylcholinesterase activity was observed in blood samples containing sodium fluoride. A significant decrease was evident immediately after blood collection and on the first day, after which the activity stabilized at 84% of the initial value. Subsequently, the activity increased to a level comparable to the initial value. In samples collected with EDTA, acetylcholinesterase activity was comparable to that in the sample without anticoagulant, and the activity remained constant for about 2 months. In conclusion, it is recommended that acetylcholinesterase activity be tested immediately after blood collection to reduce enzyme inhibition by fluoride anions, or that tubes with another anticoagulant, such as EDTA, be used.

Keywords

acetylcholinesterase activity; UV-vis spectrometry; acetylcholinesterase inhibition; acetylcholinesterase stability; biological material preservation

Streszczenie

Uważa się, że aniony fluorkowe hamują aktywność acetylocholinoesterazy, dlatego istnieje możliwość, że na aktywność tę może wpływać pobieranie krwi do fiolek zawierających fluorek sodu jako środek stabilizujący. Artykuł ma na celu zwrócenie uwagi na wpływ substancji stabilizujących i przeciwkrzepliwych stosowanych w probówkach do pobierania krwi na aktywność acetylocholinoesterazy.

Przeprowadzono badania stabilności krwi pobranej do probówek z fluorkiem sodu lub EDTA przez 176 dni. W celu określenia aktywności acetylocholinoesterazy zastosowano ulepszoną procedurę Ellmana z oznaczeniem spektrofotometrycznym przy 436 nm.

Zaobserwowano natychmiastowy spadek aktywności acetylocholinoesterazy w próbkach krwi z fluorkiem sodu. Znaczący spadek był widoczny zarówno bezpośrednio po pobraniu krwi, jak i pierwszego dnia, po czym aktywność utrzymywała się na poziomie 84% wartości początkowej, a następnie wzrosła do poziomu porównywalnego z wartością początkową. W próbkach pobranych z EDTA aktywność acetylocholinoesterazy była porównywalna do tej w próbce bez antykoagulantu, a sama aktywność utrzymywała się na stałym poziomie przez około 2 miesiące. Podsumowując, zaleca się badanie aktywności acetylocholinoesterazy natychmiast po pobraniu krwi w celu zmniejszenia hamowania enzymu przez aniony fluorkowe lub stosowanie probówek z innym antykoagulantem, takim jak EDTA.

Słowa kluczowe

aktywność acetylocholinoesterazy; spektrometria UV-vis; inhibicja acetylocholinoesterazy; stabilność acetylocholinoesterazy; zabezpieczanie materiału biologicznego

Introduction

According to a 2017 United Nations report, acute poisonings with pesticides are responsible for approximately 200,000 deaths each year [1]. One way to confirm poisoning with an organophosphorus compound is to analyze acetylcholinesterase (AChE) activity in the blood. AChE is an enzyme belonging to the hydrolase group that catalyzes the hydrolysis of acetylcholine. Inhibition of AChE by organophosphorus compounds, among others, leads to the accumulation of acetylcholine in the synaptic gap and cholinergic hyperstimulation manifested by cholinergic crisis [2]. AChE analysis is crucial due to the growing threats of terrorist attacks, the use of war gases in armed conflicts, and the use of highly poisonous compounds from the "Novichok" group. Several authors have attempted to estimate the ranges within which erythrocyte acetylcholinesterase activity falls. Due to differences in units resulting from various measurement methods, it is difficult to determine a specific range. Arrieta et al. [3] reported a mean value of 0.74 delta pH units/hour. Other authors reported the values in relation to blood volume or hemoglobin count. Zlatković et al. [2] reported a range of 4037.7-11733.8 IU/L, Rathish et al. [4] a range of 290.4-669.1 mU/uM Hb, while Sanz et al. [5] reported values according to gender: 39.30 U/g Hb for men and 6.85 U/g Hb for women. Other studies, however, indicate that AChE activity does not significantly depend on gender. Instead, it is influenced by genetic factors [6], hematocrit and various red blood cell (RBC) count indices [7], and age [8], with a decrease in AChE activity observed as individuals age.

Some authors suggest that fluoride anions affect AChE activity, indicating a reversible inhibition mechanism of this enzyme by fluoride [9]. The proposed mechanism behind the inhibition of AChE by fluoride involves the formation of bonds between these anions and the amine residues of lysine, arginine and histidine, as well as the hydroxyl groups of serine, threonine and tyrosine within the protein structure [10]. The effect of fluoride ion, as well as hydrogen fluoride and hydrogen difluoride ions on AChE results in blocking acetylation and deacetylation of the enzyme [9]. Accordingly, it seems reasonable to consider the possibility that AChE activity may be affected by blood sampling into vials containing sodium fluoride as an anticoagulant. This one, on the other hand, is the most commonly used anticoagulant in Poland because in the vast majority of cases, blood analysis for ethanol is performed. Sodium fluoride is chosen because it prevents fermentation of glucose to ethanol [11]. However, it should be noted that routinely preserved material (for toxicological examinations) may not be suitable for accurately determining AChE activity and confirming or excluding possible exposure to organophosphorus compounds.

Aim of the study

The aim of the study was to highlight the effect of anticoagulants used in blood collection tubes on acetylcholinesterase

activity over time. Particular emphasis was placed on the impact of fluoride ions on the inhibition of acetylcholinesterase activity. A stability study for blood collected into tubes containing sodium fluoride or sodium ethylenediaminetetraacetate (EDTA) was conducted over a period of 176 days.

Materials and Methods

Chemicals and reagents

Water (Chromasolv® LC–MS), Triton™ X-100, potassium cyanide KCN, ethopropazine hydrochloride, 5,5'-dithiobis(2-nitrobenzoic acid) DTNB, acetylthiocholine iodide ASCh were purchased from Sigma-Aldrich (Steinheim, Germany). Phosphate buffer pH 7.4, potassium ferricyanide $K_3Fe(CN)_6$, sodium bicarbonate $NaHCO_3$, hydrochloric acid HCl 30% were purchased from Chempur® (Piekary Śląskie, Poland).

Biological material

Blood samples used for determining acetylcholinesterase activity were freshly collected into two tubes, each containing a different anticoagulant. In the first tube, the anticoagulant used was sodium fluoride NaF+sodium heparin (BD Vacutainer test tubes, Biomedico, Gdynia, Poland). In the other tube, sodium EDTA was present (Sarstedt, Nümbrecht, Germany). Blood samples were stored at 4°C.

Sample preparation

All reagents were prepared according to Worek et al. [12]. Each time, a set of fresh reagents was prepared. Briefly, the diluting reagent was prepared by dissolving 30 µL of Triton™ X-100 in 10 mL of phosphate buffer (0.1 M, pH 7.4). The transformation solution, necessary for the analysis of hemoglobin, was prepared by dissolving 40 mg $K_3Fe(CN)_6$, 10 mg KCN and 200 mg $NaHCO_3$ in water using a 200 mL volumetric flask. Then, 100 µL of Triton™ X-100 was added. The color reagent was prepared by dissolving 40 mg DTBN in 10 mL of water. The substrate solution was prepared by dissolving 16 mg of acetylthiocholine iodide in 2 mL of water. The ethopropazine solution was prepared by dissolving 10 mg of the substance in 5 mL of 12 mM HCl. All reagents were stored at -20°C except for the phosphate buffer and transformation solution (stored at room temperature), and the diluting reagent (stored at 4°C).

Hemoglobin determination

30 µL of the biological sample was transferred into a 10-mL plastic tube, and then 3 mL of the diluting reagent was added. The prepared solution was then vortex mixed and 1.5 mL was transferred into a quartz cell containing 1.5 mL of the transformation solution. The solution was then incubated for 10 minutes at room temperature. Subsequently, the absorbance at 546 nm was measured spectrophotometrically against a blank sample (consisting of 1.5 mL of the transformation solution and 1.5 mL of water). Hemoglobin concentration was then calculated according to Worek et al. [12].

AChE activity determination

2 mL of phosphate buffer (pH 7.4) was transferred into a 10-mL plastic tube and then 100 µL of DTNB solution was added. Next, 10 µL of ethopropazine solution and 1 mL of previously diluted blood (30 µL of blood and 3 mL of the diluting reagent) were added. The prepared solution was vortex mixed and the entirety was transferred to a quartz cuvette. The measurement was performed against a blank sample (consisting of all the abovementioned reagents but 1 mL of phosphate buffer was used instead of diluted blood). The spectrophotometric measurement involved recording absorbance at 37°C at 436 nm for 3 minutes with a data interval 0.1 min. Acetylcholinesterase activity was then calculated according to Worek et al. [12].

Instrumentation

A Shimadzu UV-1900i UV/VIS (Canby, Oregon, USA) spectrophotometer equipped with a quartz cell (Starna Scientific) with a10 mm path length was used. Measurement of absorbance spectra was performed for the specific wavelength. The spectrometer was controlled using LabSolutions UV-Vis version 1.03 software (2018 Shimadzu Corporation, Canby, Oregon, USA), which allowed for the collection of necessary data.

Results

Acetylcholinesterase activity and hemoglobin concentration were determined each time, one after another. The entire stability study was performed for 176 days and included 12 measurements for blood with each anticoagulant. The specified days and the corresponding values of AChE activity are presented in the Table I. The first noteworthy point is the difference in initial AChE activity values measured in blood with EDTA and NaF. AChE activity in blood collected into a tube with NaF was lower compared to that in blood with EDTA. A more reliable value appears to be the AChE activity obtained for blood with the addition of EDTA, as an activity analysis performed immediately after blood collection on a sample without the anticoagulant exhibited an AChE activity value of 601 mU/µmol Hb (data not shown). Although this difference is present and noteworthy, it did not affect the further course of the study, as subsequent activity values were compared to the initial AChE activity for each sample separately.

When observing the trend of AChE activity values over time it can be noted that AChE activity in the blood sample with EDTA addition (Figure 1) remained relatively stable until the 79th day of the study. After that, its value decreased to about 60% of the initial value. On the other hand, the plot of changes in AChE activity over time for blood with sodium fluoride (Figure 1) showed significant variations in the values obtained. Within the first 24 hours after blood collection, AChE activity dropped to 84% of the initial value. Over the next 44 days, it remained relatively stable, showing activity in the range of 84-94% of the initial value measured immediately after blood collection.

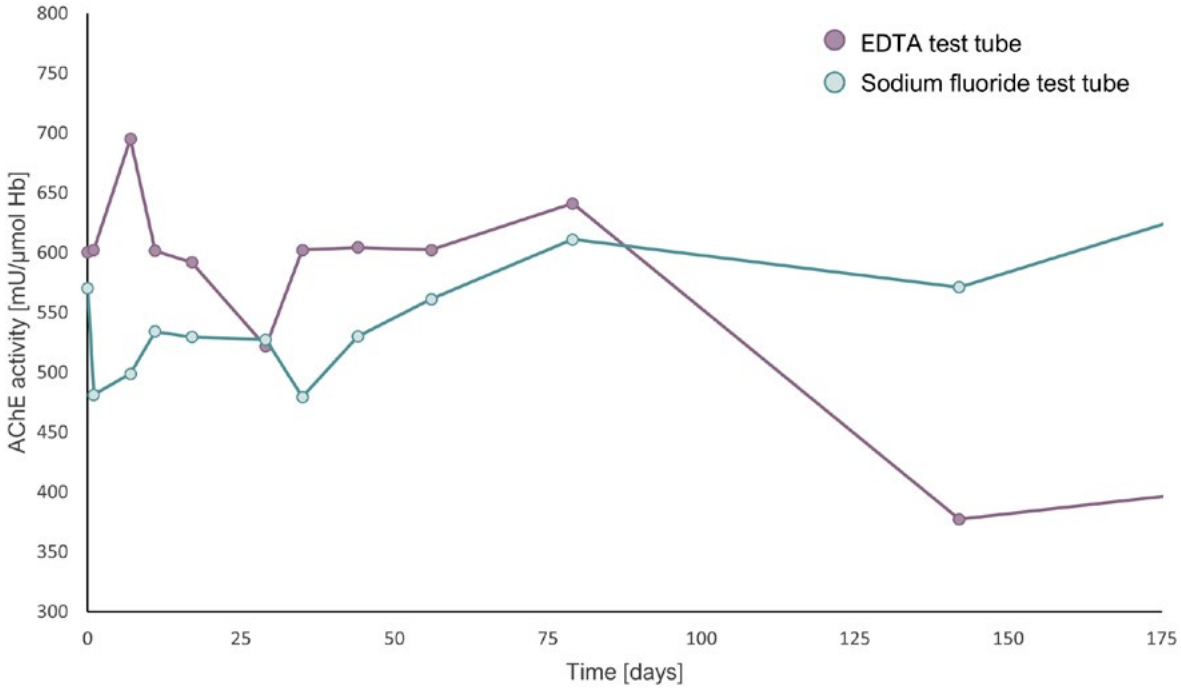

Figure 1. The relationship of acetylcholioesterase activity to the time that has passed since the blood sample was collected into the tube with EDTA and sodium fluoride
Rycina 1. Zależność aktywności acetylocholioesterazy od czasu jaki minął od pobrania próbki krwi do fiolki z EDTA oraz fluorkiem sodu

Table 1. Stability of acetylcholinesterase activity in blood collected to tubes with two preservative agents
Tabela 1. Aktywność acetylocholinoesterazy we krwi pobranej do fiolek z dwoma antykoagulantami

Day	Type of preservative agent used			
	EDTA		Sodium fluoride	
	AChE activity [mU/µmol Hb]	% of the initial activity	AChE activity [mU/µmol Hb]	% of the initial activity
0	600	100	570	100
1	602	100	481	84
7	695	116	499	87
11	602	100	534	94
17	592	99	529	93
29	522	87	527	92
35	602	100	479	84
44	604	101	530	93
56	602	100	561	98
79	641	107	611	107
142	377	63	571	100
176	397	66	625	110

EDTA – sodium ethylenediaminetetraacetate; AChE – acetylcholinesterase
EDTA – etylenodiaminotetraoctan sodu; AChE – acetylocholinoesteraza

Subsequently, a sudden increase in AChE activity to a level comparable to the initial value was observed.

Discussion

Inhibition of AChE activity by fluoride anions has been previously observed by other authors [13, 14]. Baselt et al. [14], who studied AChE activity in postmortem blood with and without sodium fluoride as a preservative agent, observed a 5-59% decrease in AChE activity in blood with NaF compared to the unfluoridated specimens. Kambam et al. [13] performed a study on heparinized blood to which a fluoride solution was added. The percentage of inhibition of AChE activity observed in this study ranged from 1.4 to 69.2%. The results obtained by the aforementioned authors seem to be in agreement with each other, while at the same time they depict a higher degree of AChE inhibition by fluoride anions than in the present study, where the highest percentage of observed inhibition was 16%. However, what is worth noting is the fact that the concentration of fluoride anions in each of the described cases differed significantly from each other. In the case presented in this paper, blood was collected into tubes with a NaF amount of 2-4 mg per 1 ml of blood. This results in a final fluoride ion concentration of 905-1810 µg/mL of blood. In the Baselt et al. study [14], the tubes contained between 0.7 and 31 mg/mL of NaF, which is equivalent to 317-14 024 µg/mL of fluoride anions. Finally, fluoride ion concentrations in blood analyzed by Kambam et al. [13] ranged from 0.19 to 95 µg/mL. As can be seen, although the results presented by Baselt et al. [14] and Kambam et al. [13] regarding the percentage of AChE inhibition were similar, the concentration of fluoride ions in the analyzed samples was remarkably different. As for the presented study, fluoride ion concentrations were in the range of those used by Baselt et al. [14] but the observed AChE inhibition was lower. Taking all of the above into account, it can be concluded that the described studies confirm the possibility of inhibition of AChE activity by the addition of fluoride ions to the sample. However, further studies are required to establish the effect of fluoride ion concentration on the potency of the enzyme inhibition.

For forensic toxicology analysis, especially in cases of organophosphorus poisonings, it is necessary to determine AChE activity. The presented study appears to shed light on the limitations of examining *post-mortem* biological material routinely collected in Poland into tubes containing sodium fluoride. First of all, an important aspect is the observed result of the first determination of AChE activity, immediately after the blood was collected for testing. In blood with sodium fluoride, AChE activity was lower compared to blood collected with EDTA and without anticoagulant, where the latter two values overlapped. This indicates a possible underestimation of even the initial activity value by fluoride ions. Additionally, measuring AChE activity 24 hours after sample collection is fraught with errors, as there is a decrease in enzyme activity that occurs almost immediately. Comparing AChE activity in blood containing NaF measured 24 hours after blood collection to the original value in blood not containing anticoagulant as a reference, it can be seen that the value is as much as 120 mU/µmol Hb lower, which may affect the final interpretation of the possibility of poisoning. So, as can be observed, even a sample examined just after collection or on the next day may already exhibit reduced AChE activity. This is especially important in forensic toxicology, where toxicological analysis is performed within first days of securing biological material at autopsy. There are, of course, other factors that confirm or exclude the possibility of poisoning, such as the presence of organophosphorus compounds or drugs in the biological material. However, considering only the activity of the enzyme AChE, one should be cautious in interpreting the obtained results. This is especially true for samples analyzed after a longer period of time, as the decrease in activity persists in subsequent days.

As studies show [9, 15, 16], fluoride anion is believed to be a reversible inhibitor of AChE, which has been employed in studies on the reactivation of this enzyme after poisoning with organophosphorus compounds. Perhaps this fact could explain the observed increase in AChE activity over time. However, much more research is needed in this area in order to fully understand the changes that AChE activity undergoes during storage of biological material collected using various anticoagulants.

In comparison to blood samples with sodium fluoride, blood with EDTA shows significantly better results for AChE activity. The first point to note is that AChE activity at the first measurement was consistent with the activity of this enzyme in blood collected without any anticoagulant. In addition, this activity remained practically unchanged over time, remaining relatively stable for more than 2 months. This may indicate that a potentially more appropriate anticoagulant for preserving blood for further testing for AChE activity when poisoning with, for example, organophosphorus compounds is suspected, would be EDTA. Although it is a much less commonly chosen method of preserving postmortem material for toxicological testing, the research presented in this paper aims to shed light on the potential for improving existing procedures. It should be noted that this type of preservation of the material for testing should be an additional procedure in cases where poisoning with compounds that inhibit AChE activity is suspected. In addition to such a sample, blood should be drawn according to routine procedures that allow for any other needed analysis to be performed, eliminating the possible effect of EDTA as an anticoagulant on other analyses in forensic or clinical toxicology. A significant limitation of the presented study is the fact that it was performed with the use of only one biological sample. The authors strongly believe that the topic addressed should be further investigated, as the literature data on this topic is very scarce. Forensic toxicology research would greatly benefit from further investigation in this area, using more samples of biological material and extending the study to other anticoagulants.

Conclusions

The described study has shown that acetylcholinesterase activity depends on the anticoagulant used. Tubes with sodium fluoride, which are routinely used, may be an improper way of securing biological material, since inhibition of the enzyme is observed immediately after preserving the material. It is therefore recommended that AChE activity should be tested immediately to reduce inhibition of the enzyme by fluoride anions, or that tubes with another anticoagulant, such as EDTA, should be used. However, the presented study is intended to outline the problem of securing biological material for the purpose of examining AChE activity, while much more research is needed to better understand the described issue.

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Ethical approval: All procedures performed in this study were in accordance with the ethical standards of the national committee and with the 1964 Declaration of Helsinki. Ethical approval was waived as it is not necessary for an anonymized case of forensic analysis requested by the judicial authorities. All examinations were conducted as part of analyses on behalf of the judicial authorities' request.

Conflicts of Interest: The authors declare no conflicts of interest (including financial and personal) that might appear to influence the work reported in this paper.

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