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Determination of reference values of acetyl and butyryl cholinesterase activities in Serbian healthy population

Određivanje referentnih vrednosti aktivnosti acetil i butiril holinesteraze kod zdrave populacije u Srbiji

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Abstract

Background/Aim. Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are important biomarkers of exposure to organophosphorus and carbamate insecticides. Since the estimation of the level of cholinesterase inhibition depends on the normal values which may vary in different populations, it is important to determine them in our population, which so far has not been done. Therefore, the aim of this study was to determine the reference values for AChE and BuChE in a healthy population of adults in the Republic of Serbia. **Methods**. The AChE activity was measured by spectrophotometry (λ = 412 nm), using a modified Ellman's method. BuChE activity was determined by the integrated chemical system (Dimension RxLMax) with ready-made reagent cartridge for analysis. The examinees were healthy voluntary blood donors from the Institute of Transfusiology and Hemobiology, Military Medical Academy in Belgrade, Serbia. Statistical Package for Social Sciences (SPSS) software program was used for data processing. **Results**. In the group of 851 persons, there were 728 males and 123 females. The mean age was 39.1 ± 11.6 years. For all of them, erythrocyte AChE activity was done while BuChE was determined in 205 persons (169 males and 36 females). Their mean value of acetylcholinesterase activity was 8,090.6 ± 1,976.7 IU/L, and of butyrylcholinesterase activity was 14,556.6 ± 4,078.1 U/L. Due to lack of normal data distribution in male group (both enzymes), reference ranges were estimated as 2.5 and 97.5 percentiles. **Conclusion**. The results of this pilot study on cholinesterase in healthy population in the Republic of Serbia which has now been done for the first time, indicate the need for considering their wider ranges of when estimating the severity of poisoning. However, further study for BuChE with the inclusion of a larger number of females and data for body weight of the examinees, in order to get more precise reference limits, is suggested.

Key words:
cholinesterases; butyrylcholinesterases; cholinesterase inhibitors; insecticides; poisoning; reference values; serbia.

Apstrakt

Uvod/Cilj. Acetilholinesteraza (AChE) i butirilholinesteraza (BuChe) su važni biomarkeri ekspozicije organofosfornim i karbamatnim insekticidima. S obzirom na to da procena nivoa inhibicije holinesteraze, koja odgovara težini trovanja ovim agensima, zavisi od normalnih vrednosti, koje mogu varirati u različitim populacijama, bilo je važno odrediti ih u našoj populaciji, što do sada nije urađeno. Stoga je cilj ovog rada bio da se odrede referentne vrednosti za aktivnost AChE i BuChE u zdravoj populaciji odraslih osoba na području Republike Srbije. **Metode**. Aktivnost AChE u eritrocitima merena je spekrofotometrijski (λ = 412 nm). Korišćena je modifikovana Ellman-ova metoda. Aktivnost BuChE u plazmi određivana je na integrisanom hemijskom sistemu (Dimenzion RxLMax) sa gotovim reagens uloškom za analizu. Ispitanici su bili dobrovoljni davaoci krvi iz Instituta za transfuziologiju i hemobiologiju Vojnomedicinske akademije. Za obradu podataka korišćen je SPSS (*Statistical Package for Social Sciences*) softverski program. **Rezultati**. U grupi od 851 osobe bilo je 728 muškaraca i 123 žene. Srednje životno doba ispitanika bilo je 39,1 ± 11,6 godina. Za sve ispitanike određena je aktivnost AChE, dok je aktivnost butirilholinesteraze određena kod 205 ispitanika (169 muškaraca i 36 žena). Srednja vrednost aktivnosti acetilholinesteraze iznosila je 8 090,6 ± 1 976,7 IJ/L, a za butirilholinesterazu 14 556.6 ± 4 078,1 U/L. Odsustvo normalne distribucije podataka kod muškaraca uslovilo je da referentne vrednosti enzima odredimo kao 2,5-ti i 97,5-ti percentil. **Zaključak**. Rezultati ove pilot studije o aktivnosti holinesteraza u zdravoj populaciji u Republici Srbiji, koja je sada urađena prvi put, ukazuju na potrebu da se pri proceni težine trovanja uzmu u obzir širi rasponi aktivnosti holinesteraza. Preporučuje se proširivanje studije za BuChE, sa uključenjem većeg broja ispitanika ženskog pola i podataka o telesnoj masi, u cilju dobijanja preciznijih referentnih vrednosti.

Ključne reči:
holinesteraze; butirilholinesteraze; holinesteraza, inhibitori; insekticidi; trovanje; referentne vrednosti; srbija.

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Introduction

Acetylcholinesterase (E.C. 3.1.1.7) (AChE) and butyrylcholinesterase (E.C. 3.1.1.8) (BChE or BuChE) are enzymes from the group of hydrolases, which catalyze the hydrolytic reaction of choline esters in the presence of water molecules [1–3]. The difference between the two enzymes is based on their different affinity for certain choline esters. AChE hydrolyzes acetylcholine (ACh) and acetyl-β-methyl choline, but unlike BuChE it does not hydrolyze benzoylcholine. Also AChE rate of hydrolysis decreases with the longer length of carbohydrate chain from ACh, than propionyl chloride to butyrylcholine. One molecule of AChE can hydrolyse 25,000 molecules of ACh *per* second, terminating its effect on muscarinic and nicotinic receptors and impulse transmission. This enzyme is found in erythrocytes, neuromuscular junctions, lungs, spleen and in all compartments of the brain. Inhibition of enzyme leads to accumulation of ACh in central and peripheral nervous system and cholinergic hyperstimulation, manifested as cholinergic crisis. Determination of the activity of AChE (true cholinesterase), along with the clinical picture, is therefore used to confirm cholinesterase inhibitors [(organophosphates (OP) and carbamates, nerve agents)] poisoning [3–6].

BuChE was known as plasmatic cholinesterase or pseudocholinesterase, and it is named according to its preference for the artificial substrate butyrylcholine. BuChE is also able to hydrolyze succinylcholine, adipoylcholine, benzoylcholine and propionylcholine [5]. The physiological role of this enzyme is still not known, but the clinical practice has shown that the use of suxamethonium in cases of genetic or acquired BuChE deficit may lead to neuromuscular block [7]. Although still under research, BuChE becomes increasingly promising therapeutic agent for detoxification of organophosphorus nerve agents and in cocaine abuse [6, 7]. BuChE is produced by the liver and is secreted into the circulation. BuChE is present in almost all tissues and in blood [6–12]. Reduced activity of this enzyme follows certain pathological conditions of the body (liver disease, malnutrition, acute infection, cancer, chronic anemia) [8]. Poisoning by OP pesticides can also cause a reduction of enzyme activity, as well as certain drugs (cimetidine, procainamide, androgens, estrogens, oral contraceptives, contrast agents, etc.) [9]. There are 2 types of pseudocholinesterases, normal (typical) and atypical. BuChE activity changes with age, with the lowest levels found in the newborn (about 60% of levels in healthy adults). According to some authors, a significant reduction of up to 30% BuChE activity occurs during pregnancy [11]. However, other authors have found that besides the genetic polymorphism, the difference in BuChE levels was not influenced by age, but only by body weight and height [12].

According to the World Health Organization, more than 3 million OPs poisonings, of which over 300,000 ends fatally, are registered annually. OPs irreversibly inhibit the enzyme AChE. The accumulation of the neurotransmitter ACh in the cholinergic synapses leads to the onset of symptoms and signs of acute poisoning. Besides reaction with AChE, OPs inhibit BuChE and other esterases [11].

Determination of cholinesterase activity is of great importance to confirm or rule out poisoning by OPs and as an indicator of the condition of patients and the success of the applied therapy. The degree of the enzyme inhibition, among other criteria (clinical picture, the level of OPs in blood, dose of atropine applied), determines the severity of poisoning [8, 11].

Methods for determination of cholinesterases activity in preventive and clinical diagnostics should be simple, rapid, reliable, sensitive and specific. There are different methods for their determination but most often spectrometric method and enzyme immunoassay tests are used [13–19].

The estimation of the level of cholinesterase inhibition, corresponding to the severity of OP poisoning, depends on the normal values which show biological variations among different populations, mainly due to genetic polymorphism [20–23]. Thus, it is important to determine their values in Serbian population, which has not been done so far. Therefore, the aim of this study was to determine the reference values for AChE and BuChE activities in a healthy population of adults in the Republic of Serbia.

Additionally, it was also intriguing to compare our results with the findings of the authors from other countries, and also to check the reliability of the previous estimation of ChE activity in our patients poisoned by OPs performed according to their findings.

Methods

The study was conducted on 851 volunteers, in order to determine the reference values of cholinesterases (AChE and BuChE) activity in the Republic of Serbia. The study was conducted from 31 March 2014 to 10 February 2015 under the project of the National Poison Control Centre, Military Medical Academy ("Assessment of the Efficacy of Standard Antidotes and Adjuvant Therapy in Acute Organophosphorus Insecticide Poisoning, MF/MMA 20/12-15).

Chemicals and Reagents for determination of the enzymes activity were Disodium phosphate, p.a. (Merck, Darmstadt, Germany); monopotassium phosphate p.a. [(Merck, Darmstadt, Germany); 5,5′-Dithiobis (2-nitrobenzoic acid, (Sigma-Aldrich, St. Louis Missouri, U.S.)]; acetylthiocholine iodide (Sigma-Aldrich, St. Louis Missouri, U.S); ethopropazine (10- [2-diethylaminopropyl] phenothiazine) hydrochloride (Sigma-Aldrich, St. Louis Missouri, U.S.); analytical standard human acetylcholinesterase (Sigma-Aldrich, St. Louis Missouri, U.S.) enzyme activity – 2,624 units / mg protein; Sigma, Flex® reagent cartridge, (Siemens, Munich, Germany).

Determination of AChE

As Reagents we used: Indicator erythrocyte cholinesterase (DTNB, 5 mM); the substrate for erythrocyte cholinesterase (Acetylthiocholine iodide, 0.34 M) Phosphate buffer (Na_2HPO_4/KH_2PO_4, 0.1 M – pH 7.4); Ethopropazine (6 mM).

Working conditions in the spectrophotometer GBC Cintra 10e: λ= 412 nm; recording speed range: 60 nm/min; slot width: 1.5 nm; total recording time: 180 s; the sample is taken via flow cuvette (cuvette length 10 mm).

The method of sample preparation

Not coagulated blood sample (anticoagulant K_2EDTA) was centrifuged for 10 min at 3,000 rev/min. After centrifugation, the upper layer was rejected (plasma). The test tube was charged with 1 mL of hemolysate (6 mL distilled water and 10 ml of washed erythrocytes in phosphate buffer), 0.8 mL of phosphate buffer, 0.1 mL of indicators, 10 uL ethopropazine and finally 0.1 mL of substrate. Measurement of erythrocyte cholinesterase activity was carried out at 412 nm.

Analytical measurement range of this method was 700–12000 IU/L, and reference values were 4 000–8 000 IU/L.

The method for determining the activity of AChE [18] was validated in the Department for Toxicological Chemistry, Military Medical Academy. The method was accredited (validated documented method number 43).

Determination of BuChE

BChE activity in plasma was determined by the integrated chemical system (Dimension RxLMax) with ready-made reagent cartridge for analysis [19].

Range of measurement was from 0 to 14 U/mL (0–14000 U/L); reference values: 7,000 to 19,000 U/ L [24].

SPSS, U.18 (USA), software programme was used for statistical analysis. Normality of data distribution was evaluated by using Kolmogorov-Smirnov test. Mann-Whitney test and Kruskal-Wallis test were used for comparison between groups.

Results

In the group of 851 examinees, there were 728 males and 123 females of different ages. For all of them, the erythrocyte cholinesterase activity was determined. Due to technical reasons, BuChE activity was determined in 205 subjects (169 males and 36 females).

Table 1 shows basic demographic characteristics of the examinees (gender, age) regarding AChE and BuChE determination. Although the examinees were predominantly males, there were no differences in relation to age structure and mean age for both sexes.

The basic parameters of descriptive statistics (mean, median, standard deviation, the minimum and maximum value of the selected variables with their difference - the scope of distribution) for AChE in both sexes are presented in Table 2. Kolmogorov-Smirnov test revealed that there was no normal distribution for data in the group of male examinees. Therefore, application of nonparametric statistics was necessary in further statistical analysis. Mann-Whitney test showed that there was no significant difference in average values of AChE activity between sexes, thus both gender groups were conjoined for further evaluation.

In accordance with the found data for AChE activity levels, the percentile distribution of AChE activity was calculated, and activity levels of 4,037.7 and 11,733.8 IU/L, corresponding to the position of 2.5 and 97.5 percentiles, respectively may be used for determination of lower and upper reference limits in a healthy population (Table 3).

Descriptive statistics for BuChE in both sexes was similar as the one for AChE, as Kolmogorov-Smirnov test revealed that there was no normal distribution for data in males, and nonparametric statistics showed that there was no significant difference in average activity levels of BuChE between genders, so the groups were further evaluated as one (Table 4).

The percentile distribution of BuChE activity levels of examinees was calculated. The activity of 9,053.7 U/L corresponding to the position of 2.5 percentiles, and 23,671.8 U/L corresponding to the position of 97.5 percentiles may be used for determination of lower and upper reference limits of plasma BuChE activity in a healthy population (Table 5).

The activity levels of AChE and BuChE of examinees of different age categories were also analyzed.

Kruskal-Wallis test did not reveal significant changes among age category regarding AChE ($\chi^2 = 1.13$; $p = 0.76$) as well as BuChE activity ($\chi^2 = 3.62$; $p = 0.30$) (Figure 1).

Table 1

Basic demographic characteristics of the examinees regarding with AChE and BuChE determination

Patients' characteristics	AChE	BuChE	Total
Gender, n (%)			
male	728 (85.5)	169 (82.4)	851 (100.0)
female	123 (14.5)	36 (17.6)	205 (100.0)
Age (years), $\bar{x} \pm$ SD (range)			
male	39.07 ± 11.28 (17–65)	39.10 ± 10.64 (17–65)	
female	39.63 ± 13.43 (16–63)	38.75 ± 14.92 (21–65)	
Total	39.15 ± 11.61 (16–65)	39.04 ± 11.46 (17–65)	

AChE – acetylcholinesterase; BuChE – butyrylcholinesterase; $\bar{x}$ – arithmetic mean; SD – standard deviation.

Table 2

Descriptive data of AChE (IU/L) samples (n = 851)

AChE activity (IU/L)	Gender		Total	Statistics (Mann-Whitney)
	male	female		
Mean	8,075.95	81,77.29[ns]	8,090.60	$z = 0.75$
Standar deviation	1,966.93	2,040.03	1,976.76	$p = 0.44$
Median	8,164.50	8,324.00	8,188.00	
Minimum	942.0	3,922.0	942.0	
Maximum	13,890.0	12,202.0	13,890.0	

Table 3

Percentiles distribution of acetylcholinesterase (AChE) activity levels of examinees

Activity of AChE (IU/L)	Percentiles
4,037.7	2.5
4,663.6	5
5,412.8	10
6,758.0	25
8,188.0	50
9,501.0	75
10,580.2	90
11,029.8	95
11,733.8	97.5

Table 4

Descriptive data of butyrylcholinesterase (BuChE) samples (n = 205)

BuChE activity (U/L)	Gender		Total	Statistics (Mann-Whitney)
	male	female		
Mean	14,938.15	12,765.56^{ns}	14,556.62	$z = 1.62$
Standard deviation	4,259.06	2,421.12	4,078.10	$p = 0.10$
Median	14,622.00	12,363.00	14,229.00	
Minimum	8,788.0	7,866.0	7,866.0	
Maximum	46,228.0	18,220.0	46,228.0	

Table 5

Percentiles distribution of butyrylcholinesterase (BuChE) values

Activity of BuChE (U/L)	Percentiles
9,053.7	2.5
9,605.8	5
10,374.6	10
12,201.0	25
14,229.0	50
16,045.0	75
17,816.6	90
20,308.4	95
23,671.8	97.5

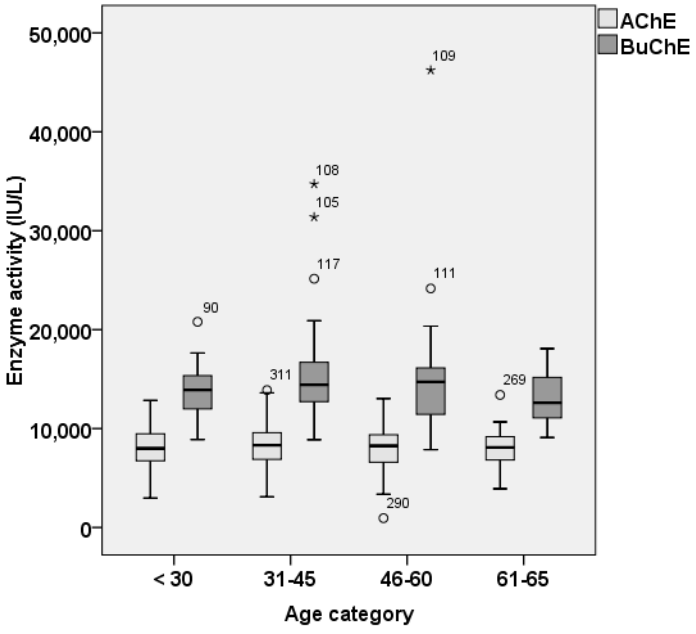

Fig. 1 – Mean values of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity in relation to age categories.

Based on this results, it seems reasonable to assume that unique reference intervals of AChE and BuChE activities are applicable for healthy subjects, regardless of their age.

Compared to reference values for AChE and BuChE activities used so far in our everyday practice, this pilot study has shown wider ranges for AChE (4,037.7–11,733.8 IU/L) and BuChE (9,053.7–23,671.8 U/L) activity.

Disscusion

Inhibition of AChE and BuChE is one of the pertinent diagnostic criteria in acute anticholinesterases poisoning. Whilst the role of AChE is thoroughly investigated, the precise role of BuChE still remains unknown despite extensive scientific research. Besides being a sensitive indicator for confirmation of exposure to anticholinesterases and hepatic biosynthetic capacity, there are new trends of BuChE use as a biomarker and detoxifying agent in OP nerve agents poisoning. Except in occupationally exposed workers who should have predetermined baseline activity levels of cholinesterases, in acute poisonings whether accidental or suicidal, they are not readily available. Thus, the degree of poisoning has to be estimated according to the reference values, that may differ among laboratories due to different methods and techniques, and different populations. There are also inter-individual and intra-individual variations caused by genetic polymorphism, age, body weight, sex, height [12]. Considering the reported biological varitions of cholinesterase activity levels in different populations, it was important to determine their values in our population, which so far have not been done.

Determining the reference values is extensive work that involves selection of suitable reference persons, preparing them for standardized sampling, analyzing of samples, statistical data analysis and presentation of the results [25–27]. The Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommends that the establishment of reference intervals requires a minimum of 120 individuals in each subgroup [28]. The IFCC recommends estimating a confidence range of 95% for each limit of the reference interval in both Gaussian and non-Gaussian distribution [26, 29]. Manufacturers of biochemical reagents recommend that each laboratory determines its own reference ranges, because of characteristics of the population covered by certain laboratories [24].

According to the number of subjects (851 and 205) our study meets the criteria with relevant standards, as compared to other studies [20, 24, 28]. Examinees were voluntary blood donors who have denied that they have any disease, and who had not taken any medicine that affects cholinesterase activity. The mean value of acetylcholinesterase activity was 8,090.6 ± 1,976.7 IU/L, and of butyrylcholinesterase activity was 14,556.6 ± 4,078.1 U/L. In this presented small pilot study of examinees in whom AChE and BuChE activity levels were determined, there were no differences according to age structure and ranges for both sexes. This is similar to results of other studies which failed to identify age as a significant factor for biological variations of cholinesterases [20, 30]. Although most of our examinees were males, nonparametric statistics showed no significant difference of average AChE and BuChE activity levels between sexes, so both gender groups were analyzed as one group. By using Kolmogorov-Smirnov test, the lack of normal data distribution was revealed in male (AChE as well as BuChE subgroups). After the percentile distribution of cholinesterases activity levels of our examinees was calculated, activity levels of 4,037.7 and 11,733.8 IU/L for AChE, and 9,053.7 and 23,671.8 U/L for BuChE, corresponding to the positions of 2.5 and 97.5 percentiles, respectively, were proposed to be used for determination of lower and upper reference limits in a healthy population.

Various laboratories in U.S. have different reference ranges for the values of AChE and BuChE activity in a healthy population. Mayo Medical Laboratory has reference range for BuChE activity for males > 18 years from 3,100 to 6,500 U/L and females at the age 18–49 years from 1,800 to 6,600 U/L and for those over 50 years from 2,550 to 6,800 U/L [31]. In Quest Diagnostics Laboratory this range for BuChE for males is 3,342–7,586 U/L, and for females 2,637–6,592 U/L. AChE activity levels for both sexes are in the range of 9,572–15,031 IU/L [32].

Reference values for BuChE activity in a Colombian population for people with different genotypes ranged between 4,796.3 – 10,321.1 U/L and 5,768.2 – 11,180.4 U/L [20]. The biological variations in cholinesterase activities in plasma were determined for a population of 3,372 subjects attending the Center of Preventive Medicine in Vandoeuvre-les-Nancy, France, for health examination in 1982. There were approximately equal numbers of examinees of both genders (1,732 males, 1,640 females), and 29% of the population were children 4–14 years old. The range of enzyme activity was 2,000–12,000 U/L [21].

The recommended normal values for cholinesterase activity vary according to values reported by other researchers, because tests were carried out on different populations (from various geographical areas, with racial, ethnic, nutritional status differences) [7, 10, 26, 29].

According to literature data, the reference values for AchE activity are in the range of 6,000 to 13,000 IU/L, and for BuChE for males 5,400–13,200 U/L and for female 3,700–9,300 U/L [33].

In our study, the activities of AChE and BuChE of examinees of different age categories were also analyzed. Kruskal-Wallis test did not reveal significant changes among age category regarding AChE as well as BuChE activity.

Based on this results, it seems reasonable to assume that unique reference intervals of AChE and BuChE are applicable for healthy subjects, regardless of their age.

With regard to the reference values for both cholinesterases, results of this pilot study are highly suggestive for the need to further evaluate the reference values for AChE and BuChE activity, expanding the study with other relevant parameters (including body weight) and a larger number of female patients for BuChE.

Conclusion

AChE and BuChE are important biomarkers of exposure to organophosphorus and carbamate insecticides. Inhibition of cholinesterase activity can also indicate the severity or course of poisoning and represents the useful gui-

de for the therapy. Due to this, it is very important to provide the correct reference values. The results of this small pilot study of these enzymes activities in healthy population in the Republic of Serbia which has now been done for the first time indicate the need for considering their higher ranges when estimating the severity of poisoning. However, further studies for BuChE with the inclusion of a larger number of females and data for the weight of the examinees, in order to get more precise reference limits, are necessary.

The limitation of this pilot study is a small number of female examinees and the fact that other parameters, such as body weight, have not been taken into account, so further study with larger number of female examinees and body weight, is recommended.

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