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COMPARISON OF PROTOCOLS FOR MEASURING ACTIVITIES OF HUMAN BLOOD CHOLINESTERASES BY THE ELLMAN METHOD

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This paper presents a protocol for routine assays of human blood cholinesterase activities which separates erythrocytes from plasma by centrifugation and measures acetylcholinesterase activity in unwashed erythrocytes and butyrylcholinesterase activity in the plasma. The recommended substrate for both enzymes is 1.0 mM acetylthiocholine. The protocol is compared with other two recommended protocols for the activity measurements of the two enzymes using the Ellman method. The paper discusses the advantages and disadvantages of each and concludes with a proposal for an international agreement between laboratories for the evaluation of a standardized protocol.

Key words:
acetylcholinesterase, butyrylcholinesterase, human erythrocytes, human plasma

Absorption of organophosphorus compounds or carbamates is commonly assessed by measuring the decrease in acetylcholinesterase (AChE; EC 3.1.1.7) or butyrylcholinesterase (BChE; EC 3.1.1.8) activities in human blood. The primary toxic effect of organophosphates and carbamates is inhibition of AChE. However, many compounds better inhibit BChE than AChE , in which case BChE is a better indicator of absorption. As there is no general rule to predict which enzyme will be more inhibited by a given compound, one should measure the activity of both cholinesterases in human blood in order to detect the absorption of inhibitors more reliably.

Erythrocyte AChE activities are usually measured in whole blood, and the most commonly used substrate is acetylthiocholine (ATCh). As whole blood also contains BChE, the rate of substrate hydrolysis in whole blood depends on the activity of both enzymes. The procedure for determination of BChE activity in human plasma or serum is well established, while this is not the case with the erythrocyte AChE.

In this paper we propose a procedure based upon separation of plasma from erythrocytes, followed by separate measurements of BChE in plasma and AChE in unwashed erythrocytes. The procedure has been prepared for presentation at the Third Chemical and Biological Medical Treatment Symposium (1, 2).

EXPERIMENTAL PROCEDURE AND CONDITIONS

The activities of AChE and BChE were measured by the spectrophotometric method of *Ellman and co-workers* (3).

Venous blood was collected into dried heparinised tubes. After blood centrifugation, plasma was separated from the erythrocytes. In that step it was essential to withdraw all plasma without withdrawing any erythrocytes. The erythrocytes were then suspended in deionised water in a volume corresponding to the initial volume of the whole blood, diluted 60-fold with a buffer (0.1 M phosphate buffer, pH=7.4), and then frozen for haemolysis. After thawing, the suspension was further diluted with the buffer (9-fold) and the thiol reagent 5,5'-dithio-bis(2-nitro benzoic acid) (DTNB) was added. The final DTNB concentration during the enzyme assay was 0.33 mM. Ten minutes after the addition of DTNB, the substrate ATCh was added (the final ATCh concentration during the enzyme assay was 1.0 mM) to the suspension and the increase in absorbance was read at 412 nm against a blank containing haemolysed erythrocytes suspended in buffer. The BChE activity in plasma was also measured with 1.0 mM ATCh using the same buffer and DTNB concentration. The final assay volume for both enzymes was 3.0 ml. Stock solutions of ATCh (100 mM) were prepared in deionised water whereas further dilutions were made in the buffer. Stock solutions of DTNB (10 mM) were prepared in the buffer. During the enzyme assay, the final dilution of erythrocytes was 600-fold and that of plasma 150-fold. The enzyme activities were measured at 25 °C or 37 °C.

The concentration of haemoglobin in the erythrocyte suspension was determined spectrophotometrically with a modified Zijlstra reagent containing potassium ferricyanide, potassium cyanide, sodium bicarbonate, and Triton X-100 (4). The final reaction volume was 1.5 ml and the final erythrocyte dilution was 120-fold. The absorbance was read at 546 nm against buffer at room temperature.

The activities of AChE were expressed as micromoles of hydrolysed ATCh per minute and per milliliter of whole blood, or per milligram haemoglobin or per micromole iron. The activities of BChE were expressed as micromoles of hydrolysed ATCh per minute and per milliliter of plasma. The molar coefficient of absorbance used in this study for the thiolate anion of the 5-thio-2-nitro benzoic acid was 13600 M^{-1} cm^{-1} (3) and for haemoglobin 10800 M^{-1} cm^{-1} (4).

RESULTS AND DISCUSSION

Evaluation of the suggested protocol

The described experimental conditions concerning the choice of the buffer and substrate were evaluated in an earlier study concerning routine measurements of cholinesterase activities in the human whole blood and plasma (5). The phosphate buffer was chosen because the effect of temperature on the pH of that buffer is small and the buffer can therefore be prepared at the room temperature and used at a temperature ranging from 10 °C to 40 °C. ATCh is a suitable substrate for both AChE and

BChE. For routine purposes it is convenient to use the same substrate concentration (1.0 mM) for both enzymes. At 1.0 mM of ATCh, AChE has its optimal activity and BChE about half of its maximum activity. The rate of spontaneous hydrolysis of 1.0 mM ATCh at pH=7.4 is slow and corrections for the non-enzymic substrate hydrolysis are required only at 37 °C.

The optical density of the haemolysed unwashed erythrocytes measured at 412 nm is not stable. The absorbance measured against buffer decreases about 0.006 absorbance units over 10 min at 25 °C or 37 °C (mean value of 12 measurements on erythrocyte samples from 7 individuals). That decrease is very small and need not be taken into account, particularly if the activities are measured against suspended haemolysed unwashed erythrocytes as a blank.

DTNB reacts not only with thiocholine, but also with thiol groups in the haemolysed erythrocytes. Under the above experimental conditions, the reaction between DTNB and thiol groups in the haemolysed erythrocytes is completed within 10 min, and this is why it is suggested to add the substrate 10 min after adding DTNB. The reaction between DTNB and thiol groups in the erythrocytes reduces the DTNB concentration by only about 0.5% (mean value of 20 measurements on erythrocyte samples from 10 individuals).

The suggested procedure is well reproducible. The within-run and between-run imprecision for the activity measurements of AChE in unwashed haemolysed erythrocytes was 2% each, and for the haemoglobin determination 1% each. The corresponding imprecisions for plasma BChE activity measurements were 1–3 and 6–7% (6 and this paper).

Comparison with protocols suggested by other authors

Several procedures have been suggested so far to measure the erythrocyte AChE in the whole blood. *Worek and co-workers* (4) suggest that the AChE activity be measured in the presence of 20 μM ethopropazine in order to inhibit plasma BChE in the whole blood. *Wicki* (7) and *Portmann* (8, 9) suggest that both AChE and BChE be measured in the whole blood by successive addition of ATCh and butyrylthiocholine (BTCh). *Reiner and co-workers* (5, 10) assessed the erythrocyte AChE activity by measuring the rate of hydrolysis of 1.0 mM ATCh in the whole blood, as under these conditions the rate of hydrolysis primarily depends on the erythrocyte AChE. Finally, one can separate erythrocytes from plasma by centrifugation, wash the erythrocytes with saline or buffer, and measure AChE activities in washed erythrocytes as described by *Ellman and co-workers* (3). Our procedure outlined in this paper also separates erythrocytes from plasma, but AChE is measured in unwashed erythrocytes.

The following is an assessment of advantages and disadvantages of each of the above protocols.

Unwashed erythrocytes might contain some residual plasma. In order to test whether BChE in the residual plasma affects the AChE activity in unwashed erythrocytes, we applied ethopropazine as a potent BChE inhibitor. Ethopropazine (20 μM) inhibited the hydrolysis of 1.0 or 0.5 mM ATCh by 8.8% in unwashed erythrocytes and by 8.5% in washed erythrocytes (mean values of 16 measurements on samples from 6 individuals). The difference between inhibition of washed and unwashed erythrocytes is very small and indicates that BChE in the residual plasma did not show up

in the assay, because otherwise the inhibition in unwashed erythrocytes would be higher than in washed erythrocytes. We therefore think that it is not necessary to wash erythrocytes before the AChE assay, particularly because washing might cause haemolysis. The above result further indicates that the 8–9% inhibition measured in erythrocytes must be attributed to the inhibition of AChE, which is in agreement with the 5% inhibition of AChE reported by *Worek and co-workers* (4).

We further tested the proposed protocol by measuring the cholinesterase activity of the whole blood with 1.0 mM ATCh without separating the erythrocytes from plasma. Knowing the haematocrit, the cholinesterase activity of the whole blood sample was calculated as the sum of separate measurements of AChE in unwashed erythrocytes and BChE in plasma. The mean values of the measured and calculated activities in samples from 15 individuals were in very good agreement: 7.16 and 6.97 μmol min^{-1} ml^{-1}, respectively.

We think that our protocol is suitable for routine assays, particularly because we suggest to measure both enzymes with the same substrate (ATCh) and with the same substrate concentration (1.0 mM). The protocol, however, requires centrifugation of whole blood and careful separation of plasma from erythrocytes. This is a disadvantage for the separation of stored blood samples which are usually haemolysed.

Worek and co-workers (4) suggest an AChE assay in whole blood with ATCh as substrate, but in the presence of ethopropazine (20 μM) in order to inhibit BChE. When ethopropazine is applied as a selective inhibitor of plasma BChE one should bear in mind that the usual BChE phenotype is 98% inhibited with 20 μM ethopropazine, while the atypical BChE phenotype is inhibited only 74%, and the heterozygots of the atypical variant between 94 and 85% (11). The occurrence of BChE phenotypes other than the usual is rare in Caucasian populations and the activities of BChE phenotypes other than UU are much smaller than the UU activities (12–14). The errors in estimated AChE activities due to the presence of BChE phenotypes other than UU will therefore be small and will rarely occur. *Worek and co-workers* (4) further suggest that plasma BChE is measured with BTCh as a substrate. Consequently, determination of AChE and BChE suggested by *Worek and co-workers* (4) requires two different substrates, one selective inhibitor, and centrifugation of the whole blood to obtain the plasma.

In the procedure of *Wicki* (7) and *Portmann* (8, 9) AChE and BChE are both measured in the whole blood. The activity of both enzymes is first measured with ATCh (1.0 mM) after which BTCh (5.0 mM) is added to the same cuvette and the activity measurement continued. The calculation of AChE and BChE activities is based on the assumption that BTCh is hydrolysed only by BChE and that the addition of BTCh stops the hydrolysis of ATCh. The advantage of this protocol is that it does not require blood centrifugation. One should, however, bear in mind that BTCh is not fully specific for BChE, but is also hydrolysed by AChE, although at a considerably slower rate. We obtained the following mean activities of washed erythrocytes in samples from 5 individuals: 4.35 μmol min^{-1} ml^{-1} measured with 1.0 mM ATCh and 0.31 μmol min^{-1} ml^{-1} measured with 5.0 mM BTCh.

The AChE activities can be expressed either per volume of whole blood, per haemoglobin content, or per iron content. We measured the AChE activities with 1.0 mM ATCh in unwashed erythrocytes obtained from 15 individuals. The mean activities ($\pm$SD) were 5.44 ± 0.68 mmol min^{-1} ml^{-1}_{BLOOD} and 0.661 ± 0.080 μmol min^{-1} μmol^{-1}_{IRON}.

This means that the relative standard deviations of the mean were 12–13% irrespective of how the activities were expressed. However, the mean haemoglobin (iron) concentrations in these 15 samples were 8.82 μmol_{IRON} ml^{-1} in the whole blood and 8.28 μmol_{IRON} ml^{-1} in unwashed erythrocytes. The difference is small, but it might point to a slight loss of haemoglobin due to withdrawal of plasma following centrifugation of blood.

CONCLUSION

A standardised protocol for measuring AChE and BChE activities in human blood is needed. That protocol should be simple in technical terms and should give reliable and well reproducible results. Each of the above described protocols has its own advantages and disadvantages. The protocol of choice should be based on an international agreement between laboratories following an interlaboratory quality control study on samples over a wide range of AChE and BChE activities in the whole blood.

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Sažetak

USPOREDBA PROTOKOLA ZA MJERENJE AKTIVNOSTI KOLINESTERAZA U LJUDSKOJ KRVI ELLMANOVOM METODOM

Mjerenjem aktivnosti acetilkolinesteraze i butirilkolinesteraze u krvi može se u osoba izloženih organofosfornim spojevima i karbamatima odrediti apsorpcija tih spojeva. Za mjerenje aktivnosti butirilkolinesteraze u ljudskoj plazmi postoji standardizirani protokol usvojen u velikom broju kliničkih i toksikoloških laboratorija, dok za mjerenje aktivnosti eritrocitne acetilkolinesteraze ima nekoliko predloženih protokola, ali ni jedan još nije opće privaćen. Zajednički princip svih predloženih protokola je spektrofotometrijska Ellmanova metoda, koja za mjerenje aktivosti kolinesteraza rabi tiokolinske supstrate. Autori ovoga rada predlažu jednostavni protokol u kojem se zasebno mjere aktivnosti u plazmi i u eritrocitima, koji nisu prani, a koji se odvoje centrifugiranjem. Aktivnost u eritrocitima mjeri se nakon hemolize eritrocita smrzavanjem. Mjerl se samo s 1,0 mM acetiltiokolinom. Drugi autori mjere aktivnost u punoj krvi koristeći se dvama supstratima (acetiltiokolinom i butiriltiokolinom), koji se u hemoliziranu krv dodaju sukcesivno i nakon svakog dodavanja supstrata mjeri se aktivnost. Neki autori predlažu uporabu etopropazina, specifičnog inhibitora butirilkolinesteraze, pa se mjerenje vrši u prisutnosti i odsutnosti toga inhibitora. U ovome su radu opisane prednosti i nedostaci pojedinog protokola te izvori mogućih pogrešaka u predloženim postupcima. Za usvajanje standardiziranog protokola za mjerenje aktivnosti eritrocitne kolinesteraze potreban je dogovor autora predloženih protokola nakon čega bi trebala uslijediti međulaboratorijska provjera toga protokola.

Ključne riječi:
acetilkolinesteraza, butirilkolinesteraza, ljudska plazma, ljudski eritrociti, preciznost mjerenja, etopropazin

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