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ACTIVITY OF CHOLINESTERASES IN HUMAN WHOLE BLOOD MEASURED WITH ACETYLTHIOCHOLINE AS SUBSTRATE AND ETHOPROPAZINE AS SELECTIVE INHIBITOR OF PLASMA CHOLINESTERASE*

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A procedure is suggested for measuring acetylcholinesterase and butyrylcholinesterase activities in human whole blood using acetylthiocholine as a substrate and ethopropazine as a selective inhibitor of butyrylcholinesterase. The procedure is suitable for screening cholinesterase activities in routine and/or field tests.

KEY WORDS: *acetylcholinesterase, activity assays in whole blood, butyrylcholinesterase, protocol for measurement in whole blood*

The inhibition of acetylcholinesterase (EC 3.1.1.7; AChE) and butyrylcholinesterase (EC 3.1.1.8; BChE) in human whole blood is an indicator of the absorption of cholinesterase inhibitors such as the highly toxic organophosphorus compounds. Erythrocyte AChE activity in human blood reflects the status of synaptic AChE, which is the target enzyme of anticholinesterases. However, most organophosphorus compounds inhibit plasma BChE faster than erythrocyte AChE, and the inhibition of plasma BChE is therefore a more sensitive indicator of the absorption of these agents. Consequently, more information about the exposure of an individual to anticholinesterases can be obtained if both AChE and BChE activities are determined.

Human blood contains BChE in plasma and AChE which is bound to the erythrocyte membrane. In order to determine the activity of each enzyme, erythrocytes and plasma have either to be separated or one has to apply selective substrates and/or inhibitors to assay only one enzyme in the presence of the other. Each approach has its advantages and disadvantages, and any assay of cholinesterases in whole blood is subject to some intrinsic error(s).

A widely used method for determining cholinesterase activities is the spectrophotometric method of *Ellman et al.* (1) with thiocholine esters as substrates and DTNB as the thiol reagent. Over the past ten years this method has repeatedly been modified in order to meet requirements for different applications (cf 2). Several authors described procedures that might enable assessment of both AChE and BChE enzymes in whole blood. *Meuling et al.* (3) used propionylthiocholine as substrate, and measured the enzyme activity in the presence and absence of the selective BChE inhibitor ethopropazine [10-(2-diethylaminopropyl) phenothiazine hydrochloride] by recording the absorbances of the reaction product at 415 and 450 nm. The activities of AChE and BChE were calculated from the differences between activities measured in the presence and absence of ethopropazine. These measurements require an instrument that can simultaneously measure the absorbance at two wavelengths. A group of Swiss authors (cf. 2) suggested acetylthiocholine (ATCh) and butyrylthiocholine (BTCh) as substrates to determine both AChE and BChE activities in whole blood. ATCh is a substrate for both enzymes, but the authors

considered BTCh primarily as a BChE substrate. *Worek et al.* (4) suggested a method for determining AChE activities in whole blood by measuring the activity with ATCh in the presence of ethopropazine. These authors measured BChE activities separately in plasma.

In this paper, we suggest a procedure that does not require separation of plasma from erythrocytes. The procedure is based on the method of *Worek et al.* (4), but it requires only one substrate (ATCh) and ethopropazine as a selective BChE inhibitor in order to evaluate both AChE and BChE activities in human whole blood.

GENERAL OUTLINE OF THE PROCEDURE

The activities of AChE and BChE are measured in whole blood with ATCh as substrate in the absence (v_T) and in the presence (v_E) of ethopropazine. The v_T activity is the sum of both AChE and BChE activities. The v_E should be the AChE activity, if ethopropazine is a sufficiently selective BChE inhibitor. The difference between v_T and v_E should therefore be the BChE activity.

To verify the suggested procedure, cholinesterase activities were measured with ATCh and BTCh as substrates in whole blood, in plasma, and in erythrocytes in the absence and presence of ethopropazine. The inhibitor was racemic ethopropazine.

EXPERIMENTAL

The enzyme source was heparinised whole blood from 10 healthy adults (4 female, 6 male). An aliquot of each blood sample was centrifuged to separate plasma from erythrocytes. The erythrocytes were washed with buffer to remove residual plasma, and diluted with buffer to the volume of the whole blood. Blood samples were taken from individuals who had the usual (UI) plasma BChE phenotype. The BChE had previously been phenotyped by measuring its activity with benzoylcholine as substrate and dibucaine as a differentiating inhibitor (5, 6). The dibucaine numbers were 80-82.

Activities were measured at 436 nm in 0.1 mol L⁻¹ phosphate buffer, pH=7.4 at 37 °C with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) as the thiol reagent (cf. 7 and 8 for details). The final concentrations during

enzyme assay were: DTNB 0.30 mmol L⁻¹, ATCh or BTCh 1.0 mmol L⁻¹, and ethopropazine 20 μmol L⁻¹. Final dilutions of whole blood, washed erythrocytes and plasma ranged between 120-fold and 600-fold, depending on the enzyme activities. The activities were calculated using the molar absorption coefficient 11280 L mol⁻¹ cm⁻¹ for the dianion of 5-thio-2-nitrobenzoic acid (9). Activities were corrected for spontaneous substrate hydrolysis.

In the method described by *Ellman et al.* (1), activities are measured at 412 nm. At that wavelength haemoglobin has a high absorption which decreases the sensitivity of measurements, particularly for haemolysed blood samples and at low enzyme activities (cf. 4). For that reason we followed the suggestion of *Worek et al.* (4) and measured activities at 436 nm.

RESULTS AND DISCUSSION

Ethopropazine is a potent BChE inhibitor and a poor AChE inhibitor. Due to this property, ethopropazine is used to selectively inhibit the BChE activity in samples where both AChE and BChE are present (3, 4, 8, 10, 11). The recommended ethopropazine concentration for this differential inhibition is 20 μmol L⁻¹, which was also used in the present study.

Table 1 shows the inhibition of ATCh hydrolysis by ethopropazine in 10 whole blood samples and in washed erythrocytes. The inhibition of ATCh hydrolysis by ethopropazine in the usual (UI) plasma BChE is known from previous studies, and it amounts to 98 % (8).

Table 1 Inhibition of AChE and BChE by ethopropazine measured with acetylthiocholine as substrate. Mean ± SD obtained in 10 blood samples.

Enzyme	Enzyme source	Percent inhibition	Range
AChE + BChE	Whole blood	22 ± 3	18-27
AChE	Washed erythrocytes	4.8 ± 1.6	2.5-7.7

We suggest ATCh as the substrate for measuring both AChE and BChE activities, because ATCh is well hydrolysed by both enzymes which is not the case with BTCh, as shown in Table 2.

Table 2 Mean activities (v) of AChE and BChE measured in 10 blood samples with acetylthiocholine (ATCh) and butyrylthiocholine (BTCh) as substrates in the absence of ethopropazine. Activities in whole blood and washed erythrocytes are expressed per mL of whole blood, and activities in plasma per mL of plasma.

Enzyme	Enzyme source	Substrate	v	
			$\mu\text{mol min}^{-1} \text{mL}^{-1}$	
AChE + BChE	Whole blood	ATCh	8.6	7.6-9.9
		BTCh	3.4	2.0-4.1
AChE	Washed erythrocytes	ATCh	6.6	5.7-7.6
		BTCh	0.17	0.07-0.34
BChE	Plasma	ATCh	3.1	1.8-4.1
		BTCh	5.7	2.8-7.8

Table 3 shows the rates of ATCh hydrolysis in 10 whole blood samples in the absence (v_T) and presence (v_E) of ethopropazine. The measured v_E rates should correspond to the AChE activity. However, AChE is slightly inhibited by ethopropazine (cf. Table 1). The v_E were therefore corrected for the degree of inhibition by ethopropazine, $v_{E,corr}$. Corrections were done using the mean percent of inhibition (4.8 %) given in Table 1. The calculated difference between v_T and $v_{E,corr}$ should be the activity of plasma BChE in whole blood.

Table 3 Mean activities (v) of AChE and BChE measured in 10 whole blood samples with acetylthiocholine as substrate in the presence and absence of ethopropazine (Etho). Activities are expressed per mL of whole blood or mL of plasma (indicated with an asterisk*)

Enzyme	$v \pm \text{SD} / \mu\text{mol min}^{-1} \text{mL}^{-1}$
v_T = activity without Etho	8.60 ± 0.77
v_E = activity in the presence of Etho	6.71 ± 0.75
$v_{E,corr}$ = activity corrected for the inhibition of AChE by Etho	7.03 ± 0.79
* $v_{P,calc}$ = calculated BChE activity in plasma from v_T and $v_{E,corr}$	2.90 ± 0.61
* $v_{P,meas}$ = measured BChE activity in plasma (cf. Table 2)	3.09 ± 0.67

The calculated plasma BChE activities (Table 3) were compared with the BChE activities measured in plasma (Table 2). The volume of plasma in whole blood depends on the haematocrit. The haematocrit mean values were 0.49 and 0.41 for male and female subjects respectively (own data on 8 male and 8 female individuals). Applying these haematocrits, BChE activities were expressed as activities per millilitre of plasma in order to have the calculated

BChE activities in Table 3 ($v_{P,calc}$) expressed in the same unit as the measured activities in Table 2 ($v_{P,meas}$). It follows from Table 3 that the $v_{P,calc}$ is on average 6 % lower than $v_{P,meas}$. This difference is not significant, as the mean values of these two activities overlap within one standard error. We therefore consider that $v_{P,meas}$ and $v_{P,calc}$ are in agreement. This in turn shows that BChE activities in whole blood can be determined as suggested in this paper.

In the above protocol no correction is suggested for the 2 % activity of the usual (UU) BChE which is not inhibited by ethopropazine, because it makes no significant contribution to the AChE activity measured in presence of ethopropazine. In Caucasian populations, 95 % of individuals have the usual (UU) phenotype (5, 12, 13). However, individuals with the atypical (AA) phenotype and heterozygotes of the A variant are less inhibited (74-87 %) than the UU phenotype (8) in which case the error due to incomplete inhibition of BChE by ethopropazine will be greater. Individuals with the AA phenotype have in Caucasian populations a frequency of less than 1 % (12).

In conclusion, we consider the procedure described above as suitable for screening AChE and BChE activities in human whole blood in routine and/or field tests. The procedure is also suitable for measuring activities in haemolysed blood samples.

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Sažetak

AKTIVNOST KOLINESTERAZA U PUNOJ LJUDSKOJ KRVI MJERENA ACETILTIOKOLINOM KAO SUPSTRATOM I ETOPROPAZINOM KAO SELEKTIVNIM INHIBITOROM KOLINESTERAZE U PLAZMI

Predložen je postupak mjerenja aktivnosti acetilkolinesteraze i butirilkolinesteraze u punoj humanoј krvi. Aktivnost se mjeri acetiltiokolinom kao supstratom u prisutnosti i odsutnosti etopropazina. Etopropazin je selektivni inhibitor butirilkolinesteraze te aktivnost mjerena u prisutnosti etopropazina predstavlja aktivnost acetilkolinesteraze. Razlika aktivnosti mjerenih u odsutnosti i prisutnosti etopropazina predstavlja aktivnost butirilkolinesteraze. Predloženi je postupak prikladan za rutinska mjerenja i/ili mjerenja na terenu.

KLJUČNE RIJEČI: acetilkolinesteraza, butirilkolinesteraza, mjerenje aktivnosti u punoj krvi, postupak mjerenja u punoj krvi

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