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AN IMPROVEMENT IN SEGREGATION OF HUMAN BUTYRYLCHOLINESTERASE PHENOTYPES HAVING THE FLUORIDE-RESISTANT VARIANTS

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Correct recognition of butyrylcholinesterase (BChE; EC 3.1.1.8) variants in human serum is essential if patients susceptible to a prolonged reaction following treatment with short acting muscle relaxants, like suxamethonium, are to be reliably identified. The dimethylcarbamate Ro 02-0683 is used in standard procedures for identification of BChE variant by measuring residual activity after two hours of inhibition. Such a long inhibition time distinguishes well between the usual (U) and atypical (A), but less successfully the fluoride-resistant (F) variant. In this paper, inhibition rate constants were determined from the initial time course of inhibition of homozygous (FF) and heterozygous (UF and AF) BChE phenotypes by Ro 02-0683; $1.6x10^6$, $2.7x10^6$ and $6.2x10^6$ $dm^3\ mol^{-1}\ min^{-1}$ for AF, FF and UF, respectively. After only 30 min of inhibition the resolution between the phenotypes was even better than after two hours. Hence, determination of the residual activity after 30 min inhibition is recommended for the segregation of the suxamethonium sensitive fluoride-resistant variants.

KEY WORDS: *fluoride-resistant variants, human butyrylcholinesterase, inhibition, Ro 02-0683, succinyldicholine*

The serum butyrylcholinesterase (BChE; EC 3.1.1.8) activity in humans is of major clinical importance in several therapeutic fields as well as in the toxicology of natural and synthetic poisons (1, 2). The importance of BChE in the hydrolysis of several drugs, especially the muscle relaxant suxamethonium (succinyldicholine) is well established (3-5). At least 20 different, naturally occurring BChE variants have been characterized by nucleotide substitution, deletion or insertion which result in alterations to the BChE protein giving different catalytic properties, and partial or complete absence of protein (6). The approximate frequency of homozygous BChE genotypes in Caucasian populations is 95 % for the usual (UU), 1 in 160000 for fluoride-resistant (FF) and 1 in 2500 for atypical (AA) enzyme. That for heterozygous BChE is 1 in 30 for usual-fluoride-resistant (UF) and 1 in 19000 for the atypical-fluoride-resistant (AF) (3). After receiving suxamethonium individuals with the fluoride-resistant (F) and atypical (A) BChE alleles may experience an apnea, in excess of the 3-5 min expected in individuals with usual BChE (4). Therefore, individuals with AF and FF phenotypes are at moderate risk (4).

Two fluoride-resistant variants have been recognised and characterized by nucleotide substitution changing Thr 243 to Met (Fluoride-1 variant) or Gly 390 to Val (Fluoride-2 variant) (7). Fluoride-2 occurs more frequently than the fluoride-1 variant (7).

The fluoride-resistant variant of human BChE owes its name to the observation that it is resistant to inhibition by 50 μM sodium fluoride in *in vitro* assays (8). Standard phenotyping methods require measurement of BChE activity and the degree of inhibition by sodium fluoride, dibucaine and the dimethylcarbamate, Ro 02-0683 (3). Due to the lack

of sharp boundaries between phenotyping data (the range of activities and inhibitor numbers overlaps) it is sometimes difficult to assign a definite phenotype, especially for heterozygous alleles. Dibucaine and sodium fluoride are reversible inhibitors of BChE, and the degree of inhibition depends on the type and concentration of substrate used in the assay. Moreover, sodium fluoride, although efficient for the detection of fluoride-resistant variants, is a difficult inhibitor to handle specially because its inhibition is strongly temperature dependent.

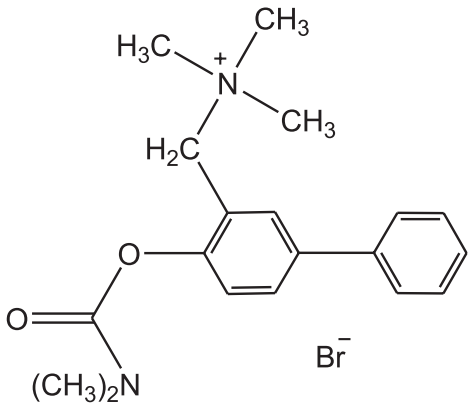

Figure 1 *Structural formulae of Ro 02-0683*

Dimethylcarbamate Ro 02-0683 (Figure 1) irreversibly inhibits BChE carbamoylating the enzyme catalytic centre, serine 198. Inhibition by Ro 02-0683 provides good supportive information on BChE phenotypes, but some workers find it inconvenient because of the required 2 h preincubation of enzyme with inhibitor (3). After 2 hours the reaction between BChE and Ro 02-0683 reaches a quasi steady-state phase. The initial phase of the inhibition follows the first order kinetics, wherefrom the rate constants of inhibition can be determined. Inhibition by Ro 02-0683 has been described in several previous papers (9-11), but without reference to the rate constants for fluoride-resistant variants. Therefore, in the present work we studied the time course of inhibition of human BChE by Ro 02-0683, and determined inhibition rate constants which enabled differentiation between homozygote and heterozygote fluoride-resistant BChE. Moreover, in order to make the phenotyping procedure shorter we suggest measurement of BChE activity after only 30 min of preincubation with 10 nM Ro 02-0683.

MATERIALS AND METHODS

Materials

Propionylthiocholine iodide (PTCh), benzoylcholine chloride (BzCh), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and dibucaine hydrochloride were purchased from Sigma Chemical Co., USA. Ro 02-0683 [dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)trimethylammonium bromide] was supplied by Hoffman La Roche, USA. Sodium fluoride was purchased from Kemika, Croatia.

The serum specimens were collected at the Cholinesterase Investigation Unit at St. James's University Hospital, Leeds, UK, during the Cholinesterase Proficiency Programme organized by Dr R. T. Evans between 1994 and 1998 (12).

All spectrophotometric measurements were performed on a Cary 300 spectrophotometer (Varian, Inc., Australia).

Determination of BChE phenotypes

Serum BChE specimens were phenotyped at 25 °C in 66.7 mM phosphate buffer (pH=7.4) by determining the activity and percentage inhibition (*i.e.* inhibitor numbers) of BzCh hydrolysis (50 μM) in the presence of dibucaine (50 μM), sodium fluoride (50 μM) or Ro 02-0683 (10 nM) (3). The inhibition by dibucaine and sodium fluoride was measured immediately after the addition of inhibitor and substrate to the reaction mixture, whereas the procedure for Ro 02-0683 inhibitor number determination required a 2 h preincubation of enzyme and inhibitor prior to the addition of substrate. The hydrolysis of benzoylcholine was determined from the decrease of absorbance measured at 240 nm. Phenotyping was complemented by measurement of activity with PTCh as described below.

BChE activity measurements

Enzyme activities in sera with PTCh (4.0 mM final concentration) were determined at 25 °C in 0.1 M phosphate buffer (pH=7.4) and 0.3 mM DTNB using the Ellman spectrophotometric method (13). The final serum dilution was 150- or 300-times. The increase in absorbance was read at 412 nm (ε_M=14220 M^{-1} cm^{-1}) (14).

Determination of inhibition rate constants

Sera were incubated with 2.5, 10 and 30 nM Ro 02-0683 in the absence of substrate. Inhibition

was stopped by the addition of PTCh (4.0 mM final concentration), and the extent of inhibition was determined as percentage of the control activity. The first-order rate constants (k_{obs}) were calculated by linear regression analysis at any given inhibitor concentration (i):

$$\ln \frac{\upsilon_0}{\upsilon_i} = k_{obs} \cdot t \qquad \text{(Eqn. 1)}$$

where v_0 and v_i stand for the enzyme activity in the absence and in the presence of inhibitor at time *t*. The second-order inhibition rate constant (k_i) was calculated from:

$$k_i = k_{obs} / i \qquad \text{(Eqn. 2)}$$

RESULTS AND DISCUSSION

Enzyme activities and dibucaine, sodium fluoride and Ro 02-0683 inhibitor numbers were determined in order to identify enzyme phenotype (Table 1). Phenotypes were classified according to published activities and inhibitor numbers for a great number of specimens and family studies coupled with DNA base sequence analysis (3, 12). The fluoride-resistant variant is less inhibited by sodium fluoride and dibucaine than usual enzyme. Consequently, fluoride and dibucaine numbers segregated well between homozygotes or heterozygotes for the fluoride-resistant and atypical variants and the usual variant. With the Ro 02-0682 inhibitor number, defined as the percentage inhibition following two hours incubation, the fluoride-resistant variant can only be identified when present as the atypical-fluoride-resistant heterozygote, AF (Table 1, Figure 2).

The initial time course of inhibition by Ro 02-0683 followed first-order kinetics for all inhibitor concentrations (Figure 3A). The first-order rate constant (k_{obs}) was a linear function of Ro 02-0683 concentration (Figure 3B), and the slope of the line is the second-order rate constant (Table 2). Ro 02-0683 strongly inhibited all BChE phenotypes studied except the atypical enzyme. The rate constants for usual and atypical homozygotes were in agreement with published data (9, 10). Neutralization of the charge obtained by the point mutation of aspartate 70 to glycine found in the atypical variant (15) results in a decreased reactivity of about 150-times towards the

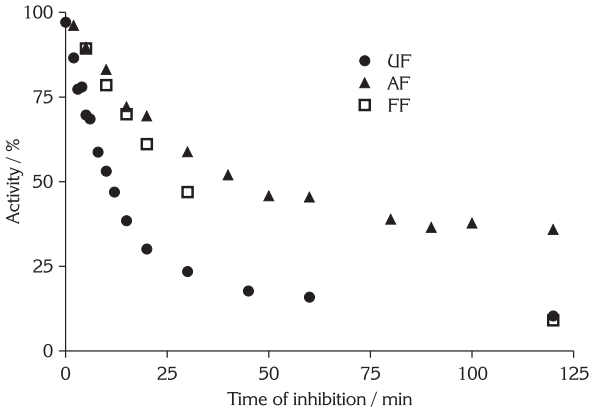

Figure 2 *Time course of inhibition of human BChE (UF, AF and FF phenotypes) by 10 nM Ro 02-0683. The points denote the mean of activities measured with PTCh in at least 3 experiments with at least 3 specimens*

Table 2 *Second-order rate constants, k_i, for the inhibition of human BChE phenotypes by Ro 02-0683, and the residual % activity after 30 min of inhibition by 10 nM Ro 02-683. Eqn. 2 was applied to calculate k_i (±SD) from k_{obs} (8-13 values) obtained in at least 3 experiments. The number of BChE specimens is given in brackets*

BChE		k_i / 10^6 dm^3 mol^{-1} min^{-1}	% Activity
UU	(6)	9.6 ± 1.5	11.5 ± 0.5
UF	(5)	6.2 ± 0.6	23.5 ± 1.1
FF	(3)	2.7 ± 0.6	47.0 ± 1.2
AF	(4)	1.6 ± 0.4	58.8 ± 1.1
AA	(3)	0.063 ± 0.004	~ 100

Table 1 *Activities (±SD) of human BChE phenotypes with benzoylcholine (BzCh) and propionylthiocholine (PTCh), and dibucaine (DN), fluoride (FN) and Ro 02-0683 (RoN) inhibitor numbers (±SD). The number of BChE specimens is given in brackets; SD denotes standard deviation*

BChE		Activity / μmol^{-1} min^{-1} ml^{-1}		Inhibitor numbers		
		BzCh	PTCh	DN	FN	RoN
UU	(6)	1.1 ± 0.3	5.3 ± 1.3	81.2 ± 1.4	60.5 ± 1.4	96.4 ± 1.1
UF	(5)	0.60 ± 0.08	2.6 ± 0.5	70.3 ± 4.0	46.3 ± 1.5	96.0 ± 1.0
FF	(3)	0.47 ± 0.09	2.0 ± 0.3	69.2 ± 6.0	33.7 ± 4.6	92.7 ± 3.1
AF	(4)	0.59 ± 0.11	2.8 ± 1.2	46.5 ± 1.2	31.1 ± 1.3	55.6 ± 2.5
AA	(3)	0.58 ± 0.09	1.4 ± 0.3	24.3 ± 0.6	26.0 ± 4.6	10.0 ± 3.5

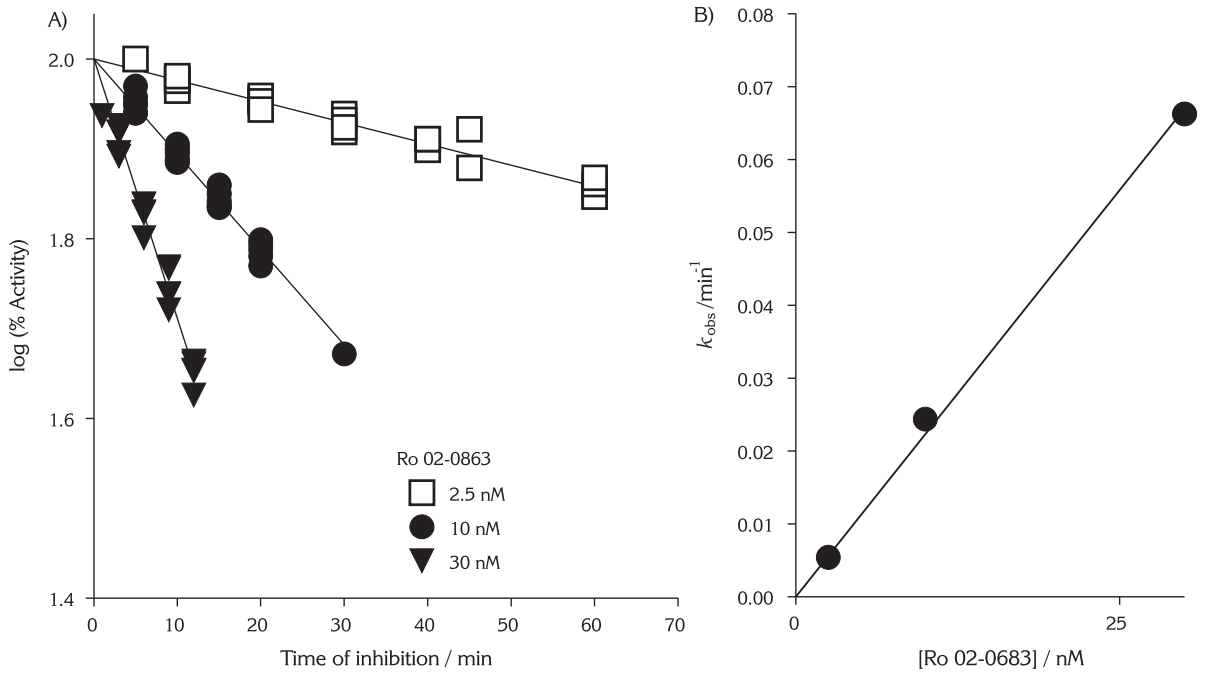

Figure 3 *Progressive inhibition of human fluoride-resistant BChE phenotype (FF) by Ro 02-0683. Points denote experimental values from experiments with 3 specimens. The slopes of the lines on plot (A), k_{obs}, were plotted as a function of Ro 02-0683 concentrations on plot (B). The overall inhibition rate constant, k_i, is the slope of the line on plot (B) (cf. Eqns. 1 and 2)*

positively charged Ro 02-0683. The inhibition potency of Ro 02-0683 towards fluoride-resistant homozygous BChE was about 3-times lower than for the usual enzyme. This relatively small effect compared to the atypical variant is due to the mutation found in the fluoride-resistant variant, threonine 243 to methionine or glycine 390 to valine at sites distant from the active serine of the enzyme (7, 16, 17) and away from the BChE active site gorge (17). However, both mutations induce conformational changes either close to the catalytic triad and the choline binding site (mutation glycine 390 to valine) or close to the entrance of the active site gorge by a conformational change in the backbone (mutation threonine 243 to methionine). These conformational changes may weaken the binding of charged ligands and reduce signal transduction capability along the active site (18).

Rate constants for the heterozygotes UF and AF fell between the corresponding constants for the homozygotes as one could expect. From the time course of inhibition, it is possible to differentiate well all three fluoride-resistant phenotypes after only 30 min of inhibition by 10 nM Ro 02-0683. Moreover, when inhibition of AF enzyme proceeded for a longer time with lower concentrations of Ro 02-0683, deviation from the kinetics described by Eqn. 1 was observed (Figure 2). This is primarily due to the mixture of two BChE variants, which have different inhibition rates, thus making different contributions to total activity (10). It is possible to assume that this deviation is the result of decarbamoylation of the catalytic serine, but that is probably not so, because it is known that both usual and atypical BChE phenotypes decarbamoylate spontaneously at a similar and very slow rate (the half-life for decarbamoylation was about 3.5 hours; cf. ref. 9). As seen from Figure 2, after 120 min of inhibition the good resolution between different fluoride-resistant phenotypes disappears, and it is possible to differentiate only AF from other fluoride-resistant phenotypes.

CONCLUSION

Ro 02-0683 is used in standard laboratory procedures for BChE phenotyping by measuring residual enzyme activity following two-hour inhibition. However, the time-dependent inhibition by Ro 02-0683 is a valuable aid for distinguishing between these phenotypes, especially UF and FF, and more reliable than reversible inhibition by dibucaine and sodium fluoride. Therefore, we suggest reducing the inhibition time by Ro 02-0683 from two hours to 30 min, when the time course of inhibition better reveals the difference between fluoride-resistant phenotypes.

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Sažetak

POBOLJŠANJE RAZLUČIVANJA FENOTIPOVA BUTIRILKOLINESTERAZE S FLUORID-REZISTENTNOM INAČICOM

Ispravno prepoznavanje inačica (varijanti) butirilkolinesteraze (BChE; EC 3.1.1.8) u ljudskom serumu nužno je kako bi se identificirali pacijenti osjetljivi na produljeno djelovanje kratkodjelujućih mišićnih relaksansa poput suksametonija (sukcinildikolina). Atipična (A) i fluorid-rezistentna (F) inačica normalne, obične (U) BChE veoma sporo razgrađuju suksametonij, pa njegova primjena kod pacijenta može izazvati produženu paralizu mišića. Da bi se odredile inačice BChE, odnosno fenotip, u serumu se određuje inhibicija aktivnosti BChE sa specifičnim inhibitorima. U standardnom postupku fenotipiziranja rabi se dvosatna inhibicija BChE dimetilnim karbamatom Ro 02-0683. Tako duga inhibicija omogućuje dobro razlikovanje normalne i atipične inačice BChE, ali ne i fluorid-rezistentne inačice. U ovom radu određene su konstante brzine inhibicije iz početnog dijela vremenskog toka inhibicije homozigota (FF) i heterozigota (UF i AF) BChE s Ro 02-0683: $1.6x10^6$, $2.7x10^6$ i $6.2x10^6$ $dm^3\,mol^{-1}min^{-1}$ za AF, FF i UF. Prema vrijednosti ovih konstanti fenotipovi se mogu dobro razlikovati. Nakon samo 30 minuta inhibicije razlučivanje između ovih fenotipova bolje je nego nakon dva sata. Stoga je preporuka da se u rutinskom postupku određivanja fenotipova BChE vrijeme inhibicije skrati s dva sata na 30 minuta.

KLJUČNE RIJEČI: *fenotipiziranje, fluorid-rezistentne varijante, konstante brzine inhibicije, ljudska butirilkolinesteraza, Ro 02-0683, suksametonij*

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