

## METHODOLOGY

### A METHOD FOR SERUM CHOLINESTERASE PHENOTYPING

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#### ABSTRACT

A method for the identification of serum cholinesterase phenotypes is proposed. It is based on methods already reported (Morrow and Motulsky, *J. Lab. Clin. Med.* 71: 350-356, 1968; Whittaker *et al.*, *Br. J. Anaesth.* 53: 511-516, 1981) and uses alpha-naphthyl acetate as substrate and DL-propranolol and RO2-0683 as inhibitors. Its application provides discrimination of the examined phenotypes (CHE1 U, CHE1 UF, CHE1 UA, CHE1 AK, CHE1 AF and CHE1 A). The advantages over other current methods are the following: clear identification of the CHE1 UF phenotype, no need of stringent control of temperature and reaction time, measurements at visible wavelength and adequacy for population studies.

#### INTRODUCTION

The genetic variability of serum cholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) at the *CHE1* locus is due to several alleles (*CHE1\*U* - usual, *CHE1\*A* - atypical, *CHE1\*F* - fluoride resistant, *CHE1\*S* - silent, *CHE1\*K*, *CHE1\*J*, *CHE1\*H*). It has been well documented that patients presenting non-usual phenotypes are more susceptible to develop prolonged apnea after the administration of the muscle relaxant suxamethonium (review in Whittaker, 1980). So, the identification of these

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phenotypes, based on the use of differential inhibitors of serum cholinesterase, is of both genetic and clinical interest.

A simple method which identifies the usual, atypical and CHE1 UA phenotypes is based on the use of alpha naphthyl acetate as substrate and RO2-0683 (dimethyl carbamate of (2-hydroxy-5-phenylbenzyl)-trimethyl-ammonium bromide) as inhibitor (Morrow and Motulsky, 1968). The addition of sodium fluoride as inhibitor in a previously described method (Kalow and Genest, 1957) which uses benzoylcholine as substrate allowed the discovery of the CHE1 UF and CHE1 AF phenotypes (Harris and Whittaker, 1961). The discrimination between the usual and CHE1 UF phenotypes proved to be difficult in view of the temperature influence on sodium fluoride inhibition (King and Dixon, 1970; King and Morgan, 1970). For this reason, the inhibitor DL-propranolol was included in this method and the consideration of propranolol, dibucaine and sodium fluoride inhibitions gave better differentiation of these two phenotypes (Whittaker *et al.*, 1981).

We decided to include DL-propranolol as inhibitor in the method of Morrow and Motulsky (1968) with the main intention of discriminating between the usual and CHE1 UF phenotypes. The procedure may be advantageous for some laboratories as the absorbances are read in 555 nm with no need of more sophisticated spectrophotometers that are required in the case of methods that use benzoylcholine as substrate (readings at 240 nm and stringent temperature control). Moreover, its use is very practical for population studies.

## MATERIALS AND METHODS

The non-hemolysed heparinized plasmas or sera belonged to 37 individuals, previously classified by other methods (Kalow and Genest, 1957; Harris and Whittaker, 1961) in the following phenotypes of serum cholinesterase: 10 CHE1 U, 9 CHE1 UF, 10 CHE1 UA, 5 CHE1 AK, 1 CHE1 AF and 2 CHE1 A. DL-propranolol concentration, reaction temperature and buffer pH were tested with 10 samples (3 CHE1 U, 3 CHE1 UA, 3 CHE1 UF and 1 CHE1 A).

The method is based on the principle that serum cholinesterase hydrolyses the substrate alpha naphthyl acetate, producing alpha naphthol that binds to the diazonium salt (5-chloro-o-toluidine) leading to a purple color (Bamford and Harris, 1964). The percentages of inhibition given by RO2-0683 and DL-propranolol identified the serum cholinesterase phenotypes at the *CHE1* locus.

All reagents were analytical grade. Alpha naphthyl acetate, fast red TR salt (F 1500) and sodium lauril sulphate were obtained from Sigma Chemical Co., St. Louis, MO 63178. RO2-0683 (dimethyl carbamate of (2-hydroxi-5-phenylbenzyl)-trimethyl-ammonium bromide) and DL-propranolol chloride were kindly provided

by Hoffmann-La Roche & Co. (Switzerland) and Laboratories Ayerst Ltd (Brazil), respectively. The reagents were prepared as follows:

*Phosphate buffer*, 0.2 mol/L, pH 7.1: Mix 670 ml of  $\text{Na}_2\text{HPO}_4$  (0.2 mol/L) to 330 ml of  $\text{NaH}_2\text{PO}_4$  (0.2 mol/L).

*Propranolol*,  $8.45 \times 10^{-4}$  mol/L: 25 mg of DL-propranolol chloride in 100 ml of phosphate buffer and stored at  $4^\circ\text{C}$ . For use it was diluted 1/10 with phosphate buffer.

*RO2-0683*,  $10^{-3}$  mol/L: 39.3 mg of RO2-0683 in 100 ml of phosphate buffer and stored at  $4^\circ\text{C}$ . It was diluted 1/1000 in phosphate buffer.

*Alpha naphthyl acetate stock solution*, 0.03 mol/L: 56 mg of alpha naphthyl acetate in 10 ml of 50% aqueous acetone (stable for one month at  $4^\circ\text{C}$ ). One ml of alpha naphthyl acetate stock solution in 20 ml of phosphate buffer and the volume completed to 100 ml with distilled water, maintained at  $37^\circ\text{C}$  for half an hour just before use.

*Color reagent*: 50 mg of fast red TR salt in 15 ml of distilled water and with 10 ml of 0.1 mol/L (30 g/L) sodium lauryl sulfate added just before use.

The procedure is summarized in Table I. The spectrophotometer was set to zero on water and the absorbance of the blank tube used as a control of spontaneous hydrolysis of substrate. Only one blank tube was sufficient because the inhibitors did not absorb at 555 nm. When the color reagent was added, some control tubes showed an orange color, which indicates high enzyme activity. In these cases, 0.2 ml of color reagent and 1.8 ml of distilled water were added to all three tubes of these samples and to the blank tube.

The percentages of inhibition of enzyme activity were calculated as:

$$\text{RON (RO2-0683 number)} = 100 (A_C - A_R)/A_C,$$

$$\text{PN (propranolol number)} = 100 (A_C - A_P)/A_C,$$

$A_C$ ,  $A_R$  and  $A_P$  being obtained by subtracting the blank absorbance from the absorbances of control, R and P tubes, respectively.

## RESULTS AND DISCUSSION

The discrimination between the CHE1 U and CHE1 UF phenotypes has been reported as difficult (King and Dixon, 1970). It was shown in the present work that these two phenotypes are better discriminated with final concentrations of DL-propranolol from approximately  $6.3 \times 10^{-6}$  mol/L to  $1.6 \times 10^{-5}$  mol/L. The chosen final concentration of DL-propranolol was  $9.39 \times 10^{-6}$  mol/L and the RO2-0683

concentration was used according to the previously described method (Morrow and Motulsky, 1968) on which the present procedure is based.

Table I - Protocol for the phenotyping method.

	Tubes (Vol. ml)			
	Control	R <sup>a</sup>	p <sup>b</sup>	Blank
Buffer	0.2	-	-	0.4
RO2-0683	-	0.2	-	-
DL-propranolol	-	-	0.2	-
Serum 1/100 (in buffer)	0.2	0.2	0.2	-
	Incubate for 5 min at 37°C			
Substrate	1.4	1.4	1.4	1.4
	Incubate for 60 min at 37°C			
	Remove from the water bath			
Color reagent	0.2	0.2	0.2	0.2
	Mix, avoiding foam			
	After 15 min, read absorbance at 555 nm			

<sup>a, b</sup> tubes with inhibitor, RO2-0683 and DL-propranolol, respectively.

The inhibitor DL-propranolol showed efficient discrimination of these two phenotypes in the range of temperatures tested in the present work (25°C-37°C). This is an advantage over sodium fluoride whose inhibition is influenced by temperature variation (King and Dixon, 1970; King and Morgan, 1970).

In the present study the buffer pH tested was from 6.5 to 8.0. Values below 7.1 lead to less accuracy in the separation of the CHE1 UF and CHE1 UA phenotypes. Although the variation from 7.1 to 8.0 did not modify the phenotype discrimination, the buffer pH was chosen as 7.1 as described elsewhere (Morrow and Motulsky, 1968) as at higher pH values the spontaneous substrate hydrolysis increased.

Table II shows that the use of DL-propranolol alone is sufficient to discriminate all the serum cholinesterase phenotypes examined in the present study. The comparison of the PN results obtained in the present study with those reported

previously (Whittaker *et al.*, 1981) indicates that the present method may be more efficient. It has already been applied for the phenotyping of a large population sample (1035 CHE1 U, 22 CHE1 UA, 11 CHE1 UF and 1 CHE1 AK), with reliable results (Alcântara *et al.*, 1990; 1991).

Table II - Inhibition numbers (mean  $\pm$  S.D. and range of variation) obtained with the use of the inhibitors DL-propranolol chloride (PN) and RO2-0683 (RON), as classified according to the serum cholinesterase phenotypes examined in the present study.

Phenotypes	N	PN		RON	
		Mean $\pm$ S.D.	Range	Mean $\pm$ S.D.	Range
CHE1 U	10	74.0 $\pm$ 1.9	71.8 - 76.9	94.4 $\pm$ 1.6	91.4 - 96.7
CHE1 UF	9	62.1 $\pm$ 3.7	56.4 - 67.8	91.5 $\pm$ 1.1	89.4 - 93.2
CHE1 UA	10	48.3 $\pm$ 2.1	45.0 - 51.4	68.5 $\pm$ 1.8	64.3 - 71.1
CHE1 AK	5	39.5 $\pm$ 1.4	37.8 - 40.9	55.3 $\pm$ 2.3	52.3 - 58.8
CHE1 AF	1	27.5		55.4	
CHE1 A	2	8.9 $\pm$ 0.1	8.8 - 8.9	26.5 $\pm$ 0.1	26.4 - 26.5

As an economic procedure for population screening, the sole use of DL-propranolol may be adopted at an initial stage. The non-usual phenotypes detected at this stage should then be retyped with the two inhibitors to sharpen the discrimination.

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### RESUMO

Um método para identificar fenótipos da colinesterase do soro é proposto. Baseia-se em métodos já relatados (Morrow e Motulsky, 1968; Whittaker *et al.*, 1981) e utiliza acetato de alfa naftila como substrato e os inibidores DL-propranolol e RO2-0683. Sua aplicação possibilita a discriminação dos fenótipos examinados (CHE1 U, CHE1 UF, CHE1 UA, CHE1 AK, CHE1 AF e CHE1 A). As vantagens

sobre outros métodos atualmente usados são as seguintes: clara identificação do fenótipo CHE1 UF, dispensa de rígido controle de temperatura e de tempo de reação, medidas em comprimento de onda visível e adequação para estudos populacionais.

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