THE INHIBITION OF CHOLINESTERASES BY PANCURONIUM

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SUMMARY

Pancuronium causes a powerful and highly selective inhibition of human serum cholinesterase in vitro. The inhibition was studied in serum from 14 individuals of both sexes (5-60 years of age) with normal reactions to suxamethonium. Pancuronium, in a concentration of $2.3 \times 10^{-7} \text{M}$, caused a 50% inhibition of the enzymatic hydrolysis of acetylcholine, when this substrate was present in a concentration of $10 \times 10^{-3} \text{M}$. The same $I_{50}$ value was also found for a commercial preparation of human serum cholinesterase. The inhibition was reversible and competitive in type. Pancuronium inhibition of the acetylcholinesterase in human red blood cells and from the electric eel was more than one thousand times weaker. Thus pancuronium is one of the most selective inhibitors of serum cholinesterase described so far. The in vivo activity of the serum cholinesterase in four patients receiving pancuronium 0.1 mg/kg decreased, during the first 3 min, by 60-80%, from the pre-induction value. After this a slow recovery occurred with 40% depression remaining at 45 min after the injection. The tachycardia produced by pancuronium may be related to this selective inhibition of serum cholinesterase. It is suggested that relaxants which selectively inhibit serum cholinesterase also selectively block the cardiac muscarinic receptors.

Values for in vitro inhibition of serum cholinesterase by pancuronium have been quoted by Katz (1971), but no reports on the constancy and types of inhibition of the various cholinesterases can be found in the literature. The in vivo activity of serum cholinesterase after the administration of pancuronium to a single patient with dystrophia myotonica has been reported by Dalai and colleagues (1972). No investigation of the activity of this enzyme in normal patients has been reported.

The present work reports the type and degree of inhibition produced by pancuronium in vitro, on human serum cholinesterase, and on human red blood cell acetylcholinesterase and a commercial preparation of electric eel acetylcholinesterase. The activity in vivo of serum cholinesterase was determined in patients after the administration of pancuronium 0.1 mg/kg and 0.02 mg/kg. The possible clinical significance of the results is discussed.

MATERIALS AND METHODS

In vitro inhibition

Principle of the method. The electrometric method of Michel (1949) has been used with the modifications suggested by Aldridge and Davies (1952). The method is based on the decrease of pH in a barbiturate buffer as a result of the production of acetic acid from acetylcholine after its hydrolysis by cholinesterase.

Human serum cholinesterase. This was obtained by sampling blood from 14 normal individuals whose ages ranged from 4 to 60 years. When exposed to suxamethonium on previous occasions none of them had shown a prolonged action. The freshly sampled human blood was allowed to clot and the serum was separated after centrifugation. A commercial preparation of human serum cholinesterase (Serumcholinesterase Behring) was also used. This consisted of ampoules containing 45 mg of desiccated protein. After dilution of one ampoule to 500 ml, the enzyme activity in the solution was similar to that of human blood serum.

Acetylcholinesterase. This was obtained from human red blood cells and from the electric eel, the two most commonly used sources of this enzyme. Blood samples were obtained from four healthy individuals. After centrifugation and washing three times with normal saline, the cells were haemolysed by shaking with saponin solution (0.01% w/w) added to a 4-times dilution. The enzyme activity of 0.1 ml of this haemolysate added to the reaction vessel
caused a decrease of pH of approximately one unit over 60 min. The acetylcholinesterase from the electric eel was a commercial preparation (Sigma No. 2754) and this was prepared by dissolving a batch of 200 micromolar units in 10 ml of distilled water. 0.1 ml of this solution, freshly prepared, gave an activity similar to the red cell haemolysate.

**Technical procedure.** To each reaction vessel 5 ml of distilled water with or without pancuronium as inhibitor was added. In the vessels with inhibitor an amount of pancuronium was dissolved which would give a final concentration of 1, 2, and $3 \times 10^{-7} \text{M}$ with serum cholinesterase as the enzyme. When acetylcholinesterase was the enzyme, an amount of pancuronium was used which would give a final concentration of 1, 2, 3, 4, 5, 10, 15, and $30 \times 10^{-4} \text{M}$ (the molecular weight of pancuronium was assumed to be 750). After this, barbiturate buffer appropriate to the enzyme to be used was added to the reaction vessel. 0.1 ml of the enzyme preparation was then added. Subsequently, the reaction vessels both with and without inhibitor were left for 30 min at room temperature to allow the inhibitor to react with the enzyme. Then 1 ml of the substrate, $10^{-2} \text{M}$ acetylcholine hydrochloride, was added. The pH was recorded with a centiscaled pH meter (Radiometer Copenhagen) at room temperature during the next 60 min. To test if the inhibition was progressive, a similar experiment was performed where the incubation time of the enzyme with the inhibitor was only 1 min.

The effect of the substrate concentration on the degree of inhibition was investigated in a series of experiments performed with a constant pancuronium concentration of $2.3 \times 10^{-7} \text{M}$, but with final concentrations of acetylcholine of 5, 10 and $20 \times 10^{-3} \text{M}$.

**Presentation of data.** In figures 1 and 2 the degree of inhibition (vertical axis) is plotted against the corresponding concentration of pancuronium (horizontal axis). The degree of inhibition was expressed as the ratio of the reaction rates of uninhibited and inhibited enzyme. The graph (Augustinsson, 1948) gives directly the $I_50$ value as the molar concentration of pancuronium when $V_0/V_{pnc}=2$ ($V_0$=uninhibited reaction rate; $V_{pnc}$=pancuronium inhibited reaction rate.) In figure 3, the degree of inhibition is plotted against different concentrations of the substrate with a constant amount of inhibitor present ($2.3 \times 10^{-7} \text{M}$).

**The reversibility of pancuronium inhibition.** The dilution technique described by Nachmansohn, Rothenberg and Feld (1947) for proving the reversibility of an inhibitor, was used. Normal serum was prepared in samples of 1.8 ml each. By adding acetylcholine (4 mg in 0.2 ml) directly to a serum sample the reaction rate could be recorded by observing the
INHIBITION OF CHOLINESTERASES BY PANCURONIUM

951
decrease in pH. Adding pancuronium to a concentration of 1.3 \times 10^{-4} \text{M} (2 \mu \text{g in 0.1 ml}) to a similar sample beforehand, inhibited the rate of hydrolysis of acetylcholine by at least 75\%. On dilution of such pancuronium-inhibited serum, the inhibition disappeared almost completely. Determination of the cholinesterase activity using the \textit{in vitro} method described above requires a hundredfold dilution of the serum. Consequently, an inhibition will be found which is much lower than the true \textit{in vivo} value if blood is sampled from a patient who has been given pancuronium for muscle relaxation and is subjected to a conventional \textit{in vitro} method which dilutes the serum. Therefore we had to record the hydrolysis rate directly in undiluted serum as described below.

\textbf{In vivo inhibition}

\textit{The principle of the method.} After adding acetylcholine to undiluted serum the rate of decrease of pH was recorded over a range in which it was found to be nearly linear (pH 7.5–7.1).

\textit{The patients studied.} Four adult patients, two males and two females, who presented for lower abdominal surgery, were studied. Blood samples (10 ml) were withdrawn from a forearm vein. The control sample was taken before any drug was given. After induction of anaesthesia with thiopentone, pancuronium 0.1 mg/kg was given and subsequent blood samples were withdrawn at 3, 5, 10, 15, 30 and 45 min following the injection of pancuronium. The samples were centrifuged and the cholinesterase activity in the serum was determined. In two additional female patients undergoing pelvic examination with uterine curettage, pancuronium 0.02 mg/kg was given. Blood was sampled before, and at 3 and 15 min after, the injection. All the patients had blood standard bicarbonate concentrations within the normal limits.

\textit{The initial pH of serum.} The pH of separated serum increases during storage as a result of loss of carbon dioxide. Passing a mixture of 4\% carbon dioxide in oxygen through the serum for 10 min decreases the pH to 7.5.

\textit{Technical procedure.} A solution containing acetylcholine hydrochloride 100 mg in 5 ml of distilled water was freshly prepared as substrate. Following the addition of 0.2 ml of this solution to 1.8 ml undiluted serum the pH decreased by about 1 unit in the course of 10–12 min. The first part of this decrease (7.5–7.1) was almost linear, and the reaction rate was calculated as: (0.4/time in sec). The reaction rate before operation was taken as 100\%, and the percentage inhibition of the subsequent samples was calculated.

\textbf{Non-enzymatic hydrolysis of acetylcholine.} This was investigated by inhibiting serum cholinesterase with neostigmine (7.5 \times 10^{-5} \text{M}). After the addition of acetylcholine the rate of decrease of the pH was now less than 0.01 unit/10 min and this source of error was disregarded.

\textbf{RESULTS}

Table I shows the age, sex, serum cholinesterase activity and pancuronium \(I_{50}\) values for the 14 individuals. The mean and standard deviation for \(I_{50}\) were 2.3 \times 10^{-7} \text{M} \pm 0.14. The same \(I_{50}\) was found also for a commercial preparation of serum cholinesterase. Figure 1 shows a linear relationship between inhibition and concentration of inhibitor and figure 3 an inverse linear relationship between inhibition and substrate concentration. No difference in degree of inhibition could be noted in experiments where the time of incubation of inhibitor with serum was shortened from 30 min to 1 min. Therefore the inhibition was not progressive with time.

\begin{table}
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\begin{tabular}{ccc}
\hline
\textbf{No.} & \textbf{Age (yr)} & \textbf{Sex} & \textbf{\(V_0\)} & \textbf{\(I_{50}\) (M \times 10^{-7})} \\
\hline
1 & 57 & male & 51 & 2.44 \\
2 & 32 & male & 54 & 2.24 \\
3 & 60 & female & 50 & 2.28 \\
4 & 56 & female & 53 & 2.22 \\
5 & 30 & female & 39 & 2.21 \\
6 & 57 & male & 42 & 2.44 \\
7 & 32 & female & 62 & 2.32 \\
8 & 43 & male & 72 & 2.05 \\
9 & 40 & female & 41 & 2.44 \\
10 & 33 & male & 69 & 2.10 \\
11 & 38 & male & 57 & 2.43 \\
12 & 8 & female & 55 & 2.44 \\
13 & 6 & male & 52 & 2.30 \\
14 & 5 & female & 60 & 2.22 \\
\hline
\end{tabular}
\end{table}

\(I_{50} = X \pm s = 2.30 \times 10^{-7} \text{M} \pm 0.14\)
\(V_0 = \text{uninhibited change in pH after 60 min.}\)

Figure 2 shows Augustinsson's graph for pancuronium inhibition of acetylcholinesterase from human red cells and from the electric eel. The \(I_{50}\) is 1.06 \times 10^{-3} \text{M} for commercial electric eel enzyme and 3 \times 10^{-4} \text{M} for human red cell enzyme.

The graph in figure 4 shows the inhibition \textit{in vivo} plotted as a function of time after the injection of pancuronium 0.1 mg/kg. It is seen that this dose caused a decrease in activity from 60 to 80\% during the first 3 min. Over the next 10 min, an increase in activity occurred with 50\% inhibition present at 15 min after the injection. Thereafter a slower recovery occurred with 40\% decrease still present 45 min after the injection.
It may be seen from table I and figure 1 that pancuronium is a powerful and consistent inhibitor of human serum cholinesterase. The normal values for the cholinesterase activity in all the 14 sera and the constancy of the inhibition by pancuronium point to the presence of genetically normal types of enzyme in all the individuals. This is consistent with the fact that none had shown prolonged action after the administration of suxamethonium on previous occasions. According to Kalow and Davies (1959) the higher the affinity of an inhibitor the larger the difference between affinity for normal and unusual enzyme variants. It would be interesting to see how the diquaternary pancuronium inhibits unusual variants of the serum cholinesterase.

Linear relationships in opposing directions, between the degree of inhibition and the concentrations of inhibitor and substrate, shown in figures 1 and 3, demonstrate that the inhibitor and the substrate compete for the active sites on the enzyme. Figure 2 shows pancuronium inhibition of acetylcholinesterase from human erythrocytes and the electric eel. It is seen that the concentrations are more than one thousand times greater than the $I_{50}$ value for serum cholinesterase. In a comprehensive study of anticholinesterase agents (Karczmar, Usedin and Wills, 1970) it was found that there is only one other inhibitor, the Astra 1397, which is more selective in its inhibition of human serum cholinesterase than that reported here for pancuronium.

Pancuronium did not cause a progressive inhibition, but rapidly reached equilibrium with the enzyme. For such inhibitors the dilution test is a valid indication of reversibility (Aldridge, 1950). The rapid disappearance of inhibition on dilution is, therefore, proof that pancuronium is a reversible inhibitor.

Testing the enzyme activity in vivo after the injection of reversible inhibitors raises the problem of using undiluted serum. This source of error was pointed out previously by Strauss and Goldstein (1943) and was re-emphasized by Barrow and Johnson (1966). The test-paper method used by Dalal and colleagues (1972) makes use of undiluted serum, but gave very poor results in our hands. It is possible that the agar-diffusion test would have been better (Harris and Robson, 1963). The rapid micromethod described by Johnson and Whitehead (1965) is similar to our method in that the standard bicarbonate in the serum represents the only buffer in the system. Consequently, only sera with reasonably normal standard bicarbonate values can be used. The enzyme activity is inhibited by a decrease in pH and, therefore, only the first part of the decrease in pH is linear. This difficulty is solved in the in vitro method of Michel (1949) by the barbiturate buffer which loses its buffering capacity with decreasing pH proportional to the decrease in enzyme activity. However, adding this buffer would dilute the serum. Our method demonstrates a considerable depression of the enzyme activity after the injection of pancuronium. At present no method can be designed which reproduces exactly the conditions in vivo. The actual inhibition occurring in vivo depends on the concentrations of enzyme, substrate and unbound inhibitor, factors unknown in the various tissues where serum cholinesterase is located.

![Fig. 4. In vivo serum cholinesterase after pancuronium 0.1 mg/kg. Substrate: acetylcholine $10^{-3}$M.](image)

The graph in figure 4 shows the depression in the activity of the serum enzyme in vivo after the injection of pancuronium. The graph is an inverse representation of the concentration of this drug in serum after a single dose (Agoston et al., 1973). The fact that the serum cholinesterase is decreased by 40% even at 45 min after the injection could be a result of the hydroxy derivatives of pancuronium having inhibitory actions. The serum cholinesterase is probably responsible for the biotransformation of pancuronium into three different hydroxy derivatives (Agoston, Kersten and Meijer, 1973). The 17 OH-derivative has been tried clinically under the name of dacuronium (Norman and Katz, 1971). The compound is a weak neuromuscular blocking agent, but a strong cholinesterase inhibitor (Foldes, 1969, personal communication). It is possible that the duration of pancuronium action is dependent to some extent on the activity of the serum cholinesterase.

A 40% decrease in serum cholinesterase activity would probably imply a prolonged action of suxa-
methonium. Correspondence between duration of suxamethonium apnoea and the enzyme activity has been shown by Foldes and colleagues (1956).

The 10% and 15% decreases found after pancuronium 0.02 mg/kg would hardly produce a prolongation of apnoea that could be detected clinically. This agrees with the findings of Cullen (1971) and Katz (1971), who pretreated their patients with small doses of pancuronium to prevent muscle fasciculations from subsequent doses of suxamethonium. No prolongation of the suxamethonium apnoea was found.

It is tempting to relate the pharmacological actions of pancuronium and its 17-OH derivative to the selective inhibition of serum cholinesterase exhibited by these compounds. Gallamine is the only other relaxant in use showing selective inhibition of this enzyme. Tachycardia as a result of vagal blockade occurs after gallamine (Riker and Wescoe, 1951) and also after the injection of dacuronium (Norman and Katz, 1971). The selective affinity to serum cholinesterase by drugs with antimuscarinic actions, like atropine, were shown by Todrick (1954). He advanced the theory that the muscarinic receptors in general resembled structurally the active sites on the serum cholinesterase molecule. The fact that serum cholinesterase is present in excess in organs with muscarinic innervation like the heart, intestine and glands (Ord and Thompson, 1950) supports this hypothesis.

The heart muscle has structural features in common with both skeletal muscle and smooth muscle. Therefore the cardiac muscarinic receptors might have features in common with both the nicotinic receptors in skeletal muscle and the muscarinic receptors in smooth muscle. This was the explanation offered by Saxena and Bonta (1970) for the finding that gallamine, pancuronium and dacuronium, in animals, blocked the cardiac muscarinic receptors in preference to other muscarinic receptors. These relaxants, however, differ from other relaxants in causing inhibition of the serum cholinesterase. It is suggested that the muscarinic receptors in the heart can be selectively blocked in preference to other muscarinic receptors by compounds which inhibit both serum cholinesterase and neuromuscular transmission.

By its inhibition of serum cholinesterase, pancuronium might preserve acetylcholine locally or systemically, causing such autonomic phenomena as meiosis or increased secretions. Such side effects have occasionally been described after large doses (Prokić, Dinicic and Petrovic, 1972; Bennett et al., 1971). Acetylcholine is also a strong releaser of catecholamines (Nahas, 1968). Liberation of catecholamines after pancuronium has been reported in man (Nana, Cardan and Domokos, 1973) and may explain the tachycardia. In general, however, we agree with Foldes (1973) when he commented on pancuronium tachycardia: "When used judiciously, pancuronium is capable of producing excellent muscular relaxation with little or no side effects."

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REFERENCES


**INHIBITION DE LA CHOLINESTERASE PAR LE PANCURONIUM**

**RESUME**

Le pancuronium provoque une inhibition puissante et hautement sélective de la cholinesterase du sérum humain *in vitro*. Cette inhibition a été étudiée sur le sérum prélevé sur 14 individus des deux sexes (silant de 5 à 60 ans) ayant des réactions normales au suxaméthonium. Le pancuronium en concentration de 2,3 × 10⁻⁷ M, a provoqué une inhibition à 50% de l'hydrolyse enzymatique de l'acétylcholine, lorsque ce substratum se trouvait dans une concentration de 10⁻⁵ M. La même valeur de I₅₀ a aussi été trouvée pour une préparation commerciale de cholinesterase de sérum humain. L'inhibition était de type reversible et compétitif. L'inhibition au pancuronium de l'acétylcholinesterase des globules rouges du sang humain et de l'anglelle électrique a été plus de mille fois inférieur. Ainsi, le pancuronium est l'un des modérateurs les plus sélectifs de la cholinesterase du sérum que l'on ait décrit jusqu'à maintenant. L'activité *in vivo* de la cholinesterase du sérum a diminué de 60 à 80% pendant les trois premières minutes, sur quatre malades recevant 0,1 mg/kg de pancuronium, à partir de la valeur de préinduction. On a constaté après cela une lente récupération, avec une dépression de 40% durant 45 minutes après l'injection. La tachycardie produite par le pancuronium peut être reliée à cette inhibition sélective de la cholinesterase du sérum. On suggère que les décontractants qui modèrent sélectivement la cholinesterase du sérum bloquent aussi sélectivement les récepteurs muscariniques cardiaques.

**DIE INHIBITION VON CHOLINESTERASE DURCH PANCURONIUM**

**ZUSAMMENFASSUNG**


**LA INHIBICION DE COLINESTERASAS POR EL PANCURONIO**

**SUMARIO**

El Pancuronio produce una inhibición poderosa y altamente selectiva de la colinesterasa de suero humano *in vitro*. Se estudió la inhibición en suero de 14 personas de ambos sexos (de 5 a 60 años de edad) con reacciones normales al suxametionio. El Pancuronio, en una concentración de 2,3 × 10⁻⁷ M, produjo un 50% de inhibición de la hidrolisis enzimática de la acetalcolina, cuando se encuentra este substrato en una concentración de 10⁻⁵ M. También se obtuvo el mismo valor I₅₀ para una preparación comercial de la colinesterasa de suero humano. La inhibición fue reversible y de tipo competitivo. La inhibición del Pancuronio de la acetalcolinesterasa en glóbulos rojos de sangre humana y de la anguilla eléctrica fue más de mil veces más débil. Así, el Pancuronio es uno de los inhibidores más selectivos de la colinesterasa de suero descritos hasta ahora. La actividad *in vivo* de la colinesterasa de suero, en cuatro pacientes que recibieron Pancuronio 0,1 mg/kg disminuyó, durante los 3 primeros minutos, en un 60-80% del valor de pre-inucción. Después de esto, se produjo una lenta recuperación con un 40% de depresión, que se mantuvo 45 minutos después de la inyección. La taquicardia producida por el Pancuronio puede estar relacionada con esta inhibición selectiva de la colinesterasa de suero. Se supone que los relajantes que inhiben selectivamente la colinesterasa de suero bloquean también selectivamente los receptores muscarinicos cardíacos.