コリンエステラーゼ阻害薬の体内動態と薬理作用に関する速度論的研究

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(1)

Pharmacokinetics, Pharmacodynamics and Toxicodynamics of Cholinesterase Inhibitors.

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Contents

Introduction

Reversible Cholinesterase (ChE) inhibitors are used as the first choice of therapy for myasthenia gravis. ChE inhibitors intensify contractile muscle tension by elevating the acetylcholine (ACh) concentration at the synaptic cleft of the neuromuscular junction by acetylcholinesterease (AChE) inhibition. However, the relationship between the concentration of ChE inhibitors in plasma and the enhancement of contractile muscle tension have not been clearly explained.

Chan and Calvey (1978) reported a positive correlation between the concentration of pyridostigmine, the most frequently used ChE inhibitor, in plasma and neuromuscular transmission function in five myasthenic patients. In contrast, Davison et al. (1981) investigated the same concentration-effect relationship in nine myasthenic patients, and found only two patients who showed a significant positive correlation between the pyridostigmine concentration in plasma and the clinical evaluation of muscle tension; a negative correlation was found in one patient. Aquilonius et al. (1983) reported on the relationship between the concentration of ChE inhibitors and the muscle response after i.v. administration of neostigmine and pyridostigmine. In their study, positive correlations were found between the drug concentration in plasma and responses at lower concentrations, but the effect declined with increases in plasma concentration at higher concentrations, suggesting a bell-shaped concentration-effect relationship.

Until now, neither pharmacological characteristics of ChE inhibitors nor *in vivo-in vitro* relationship of ChE inhibitors have been investigated, since determination of quaternary ammonium ChE inhibitors in biological samples are very difficult because of their hydrophilic property. Pharmacokinetic and pharmacodynamic property of ambenonium, a potent ChE inhibitor, is completely unknown since its quantitative analysis is difficult (Aquilonius et al., 1986).

The concentration-effect relationship of a drug is the essential information for the establishment of effective usage. Unfortunately information for pharmacokinetics / pharmacodynamics of ChE inhibitors are quite poor and the dosing schedule of ChE

inhibitors have decided empirically. To clarify the basic pharmacokinetic / pharmacodynamic property of ChE inhibitors, following studies were carried out.

I. Quantitative determination method for ambenonium was developed for ambenonium in biological samples by HPLC method.

II. More sensitive quantitation method for ambenonium using inhibitory activity to AChE was developed.

III. Basic pharmacokinetic properties of a very short-acting, a short-acting and two long-acting ChE inhibitors, edrophonium, neostigmine, pyridostigmine and ambenonium (Figure 1), were investigated after i.v. administration to rats.

IV. Time dependency of concentration-effect relationship for increasing of contractile muscle tension induced by ChE inhibitors were represented.

V. The time courses of increase in contractile muscle tension after i.v. administration of four ChE inhibitors to rats were pharmacodynamically analyzed as a principal effect. Further, Ki values for AChE inhibition, dissociation constants for nicotinic receptors estimated by *in vivo* pharmacodynamic study were compared to the value estimated by *in vitro* enzyme kinetics and binding study.

VI. The time courses of heart rate change after i.v. administration of four ChE inhibitors to rats were toxicodynamically analyzed as a side effect. Further, Ki values for AChE inhibition, dissociation constants for muscarinic receptors estimated by *in vivo* pharmacodynamic study were compared to the values estimated by *in vitro* enzyme kinetics and binding study.

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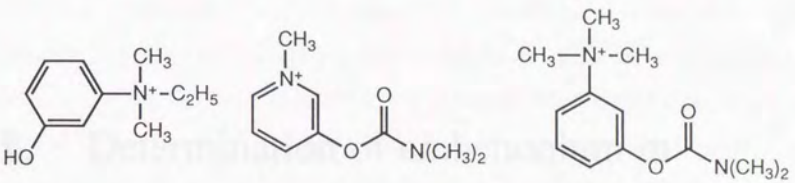
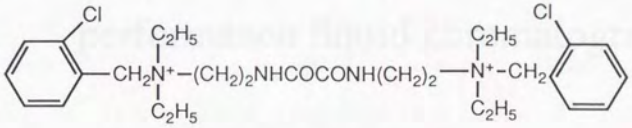


edrophonium pyridostigmine neostigmine



ambenonium

Figure 1 Chemical structure of ChE inhibitors used in this study.

I. Determination of ambenonium in biological samples by ion-pair high performance liquid chromatography

ABSTRACT

A sensitive and selective analytical method for the determination of ambenonium in biological samples is described. The procedure involved ion-pair extraction of the drug, followed by reversed-phase ion-pair chromatographic analysis with ultraviolet detection at 217 nm. The detection limits at a signal-to-noise-ratio of 5 were 100 nM using 0.2 ml of plasma and bile, 250 nM using 0.2 ml of urine and 200 pmol/g using 1 ml of tissue homogenates containing 0.1 g/ml of each tissue.

Introduction

Ambenonium chloride, a reversible cholinesterase inhibitor, is used for the treatment of myasthenia gravis, a neuromuscular disorder. There have been no reports on the pharmacokinetics or pharmacodynamics of ambenonium, since the measurement of ambenonium in biological samples is difficult.

For the measurement of other quaternary ammonium compounds with anti-cholinesterase activity, such as neostigmine, pyridostigmine and edrophonium in biological samples, gas chromatography (Cohan et al., 1976, Chan et al., 1976, Pohlmann and Cohan, 1977) and liquid chromatography (Yakatan and Tien, 1979, De Ruyter et al., 1980, Ellin et al., 1982, Shih et al., 1986, Matsunaga et al., 1987, Yturralde et al., 1987) are available. More sensitive and selective methods involving gas chromatography-mass spectrometry are also available for the determination of neostigmine (Aquilonius et al., 1979) and pyridostigmine (Aquilonius et al., 1980, Sørensen et al., 1984).

More recently, an ion-pair liquid chromatographic method for the determination of ambenonium in human plasma was reported (Tharasse-Bloch et al., 1987). It is the only method that can be used to measure ambenonium concentrations in biological samples. However, to study the pharmacokinetics and pharmacodynamics in detail, it is necessary to determine the drug concentration not only in plasma but also in other biological samples, such as bile, urine and tissues. Furthermore, only small amounts of plasma can be used for the measurement to avoid physical damage by frequent blood collection in a small animal such as a rat.

This article reports a high-performance liquid chromatographic (HPLC) method that involves ion-pair extraction of ambenonium from biological samples, followed by reversed phase separation and UV detection at 217 nm.

Methods

Chemicals and reagents

Ambenonium chloride was kindly supplied by Nippon Shoji Co. (Osaka, Japan) and benzilonium bromide, used as an internal standard, was from Sankyo (Tokyo, Japan). The chemical structures of these drugs are shown in Figure 1. Ambenonium chloride was used as a stock solution composed of 1 μmol/ml as ambenonium in physiological saline. Sodium alkylsulphonates were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and were of ion-pair chromatographic grade. Acetonitrile was purchased from Wako (Osaka, Japan) and was of liquid chromatographic grade. All other solvents and reagents used were purchased from commercial sources and were of reagent grade. All materials were used further purification.

Sample preparation

Blood samples were collected in heparinized containers through a polyethylene cannula inserted into a femoral artery of a rat, and centrifuged for 10 min at 2260 g to separate plasma. Bile and urine were collected through a polyethylene cannula inserted into the bladder of bile duct, respectively, and diluted as necessary with physiological saline.

To 0.2 ml of each samples, 1 nmol of benzilonium in 10 μl of water, 1 ml of 1 M hydrochloric acid and 4 ml of dichloromethane were added. The mixture was shaken with a mechanical shaker for 10 min and centrifuged at 1660 g for 5 min. A 1 ml volume of the upper aqueous phase was transferred to another tube, and 11 ml of dichloromethane and 0.2 ml of 1 M perchloric acid were added. The mixture was shaken for 10 min, then separated by centrifugation. After the upper aqueous phase had been removed with a Pasteur pipette, 10 ml of the organic phase was transferred to another tube and 0.2 ml of 1 M sodium perchlorate-1 M sodium hydroxide (4:3) solution was added. The mixture was shaken for 10 min, then separated by centrifugation. Then 9 ml of the lower organic phase was transferred to another tube and evaporated to dryness at room temperature. The dried residue was dissolved in 50-100 μl if mobile phase, and 20 μl was injected into the HPLC column.

Liver, kidney, spleen, lung, skeletal muscle and brain were immediately removed from a rat killed by decapitation, then gently rinsed with physiological saline solution and blotted with filter paper to remove excessive moisture. A 0.3-1.0 g aliquot was homogenized with saline in an ice-bath, and prepared as a tissue homogenate sample containing 0.1 g of tissue in 1 ml. To 1.0 ml of each sample, 1 nmol if benzilonium, 1.5 ml of 1 M hydrochloric acid and 4 ml of dichloromethane were added. The mixture was shaken for 10 min, then separated by centrifugation. A 2-ml volume of the upper aqueous phase was transferred to another tube and subjected to the procedure described above.

Chromatography

The HPLC apparatus used in this study was composed of Shimadzu LC-6A liquid chromatograph and a Shimadzu SPD-6AV spectrophotometer (Shimadzu, Kyoto, Japan). UV detection was at 217 nm. The column was a stainless-steel tube (250 mm x 4 m I.D.) packed with Senshu gel $7C_{18}H$ (Senshu Kagaku, Tokyo, Japan) by using a column-packing apparatus (Senshu Kagaku). The column temperature was maintained at 40 ℃ by a column jacket connected to a water-bath.

The mobile phase was 35% acetonitrile in water containing 20 mM sodium octanesulphonate, 2.5 mM tetramethylammonium chloride and 10 mM sodium dihydrogenphosphate, and the pH was adjusted to exactly 3.0 with concentrated sulfuric acid. The mobile phase was degassed before use and pumped at a flow-rate of 0.7 ml/min.

Results and Discussion

Chromatographic conditions and extraction

Under the conditions described, ambenonium has a relatively long retention time compared with some other quaternary ammonium compounds, such as pyridostigmine and neostigmine. The only method for the determination of ambenonium in plasma, recently reported by Tharasse-Bloch et al. (1987) was the absolute calibration method. To achieve further precision, the internal standard method was used. Of several compounds tested for use as an internal standard, benzilonium was found most suitable. The retention times of ambenonium and benzilonium are ca. 19 and 16 min, respectively.

Several kinds of mobile phases were investigated. Hexane-, heptane-, octane-, nonane- and decanesulphonate anions were tested for the ion-pairing reagent. Increasing the carbon chain-length of the alkyl group increased the retention times for ambenonium and benzilonium (Figure 1). Hexane- and heptanesulphonate did not retain ambenonium sufficiently to separate it from endogenous substances, but octane-, nonane- and decanesulphonate were well suited for ambenonium analysis. Octanesulphonate was superior to the others in purity, availability and price. Perchlorate was also tested, but was found inappropriate as the pairing ion in the mobile phase.

When the concentration of pairing ion, octanesulphonate is increased the capacity factors of ambenonium and benzilonium are decreased (Figure 2), because the retention of positively charged solute ion depends on the concentration of negatively charged pairing ion at the hydrophobic surface of the stationary phase. Thus, to achieve a good separation of ambenonium and benzilonium from endogenous substances, a relatively high concentration of octanesulphonate is desirable. However, little change in the retention was observed at concentrations above 20 mM (Figure 2) so this concentration was selected.

The pH of the mobile phase had affected on the retention of ambenonium and benzilonium in the range 2-4 (Figure 3). In a usual ion-pairing system, the pH value of mobile phase is very important because the changing pH can convert the solute into non-

ionized form. Quaternary ammonium compounds, however, cannot be in the non-ionized form, and thus the effect of the pH on their chromatographic behavior is usually limited. Therefore, the pH of the mobile phase was set at 3.0, as in the method of De Ruyter et al. (1980).

As shown in Figure 4, a small change in the proportion of acetonitrile yielded a relatively large change in the retention of ambenonium and benzilonium. A slight change in the acetonitrile concentration in the mobile phase would be fatal for the ambenonium analysis, because an increase results in incomplete separation between ambenonium and benzilonium, and decrease causes too long retention and too low sensitivity.

Perchlorate (Yakatan and Tien, 1979) and picrate (De Ruyter et al., 1980, Sørensen et al., 1984, Tharasse-Bloch et al., 1987) anions have frequently been used as ion-pair extraction reagents for the extraction of quaternary ammonium compounds. In this study, perchlorate anion was chosen, since it gave a higher extraction recovery than picrate anion in a preliminary study.

Prior to ion-pair extraction, biological samples were washed with dichloromethane to remove oil-soluble endogenous substances. Further clean-up under basic conditions decreased the amounts of endogenous substances.

Typical chromatograms obtained from each blank biological sample and the samples spiked with ambenonium chloride are shown in Figure 5. In the blank chromatogram, a slight interference with the ambenonium peak was found, but only in the urine sample. The detection limits for ambenonium were 100 nM in plasma and bile, 250 nM in urine and 200 pmol/g in each tissue, respectively, at a signal-to-noise ratio of 5.

A series of ambenonium standards containing 20, 50, 200, 500 and 2000 pmol in 0.2 ml of plasma, bile, urine, and 1 ml of each tissue homogenate was prepared, and these samples were analyzed by the described procedure. A calibration curve for each sample was obtained by plotting the peak-height ratio against the known amount of ambenonium in each sample. The calibration curve for each biological sample (except urine) was linear in the

range 20-2000 pmol (e.g., plasma: $y = 0.00235 \cdot x - 0.00116$, $r = 1.0000$; liver: $y = 0.00205 \cdot x - 0.00063$, $r = 0.9999$), and that for urine was linear in the range 50-2000 pmol ($y = 0.00238 \cdot x + 0.09661$, $r = 0.9998$). Furthermore, the calibration curves for bile and urine diluted with 9 volume of saline were also linear in the range 50-2000 pmol (e.g., diluted bile: $y = 0.00243 \cdot x + 0.01543$, $r = 1.0000$).

The reproducibility of the analysis was determined by repeating the procedure five times for each sample. The coefficients of variation (C.V.) of the peak-height ratio were sufficiently small, as shown in Table 1.

The extraction recovery of ambenonium from each sample was determined by comparing the peak height obtained from the extract of each sample with that resulting from direct injection of a known amount. Extraction recoveries from each sample at four different amounts (50, 200, 500 and 2000 pmol) were substantially constant, as shown in Table 2.

It is well known that some quaternary ammonium cholinesterase inhibitors are readily hydrolyzed in blood (Aquilonius et al., 1979, 1980), plasma (Shih et al., 1986, Yturralde et al., 1987, Aquilonius et al., 1979, 1980, Pfaff et al., 1985) and even buffer solutions (Pohlmann and Cohan, 1977, Ellin et al., 1982). This decomposition is probably related to the action of plasma cholinesterase, so, blood sampling and plasma separation should be performed under cool conditions. In a preliminary study, we examined the stability of ambenonium in stock solutions and in rat plasma. The concentration of ambenonium in stock solution remained constant for more than a year at room temperature. In plasma, a sample containing 2.5 nmol/ml ambenonium was incubated at 37 ℃, and analyzed. No decrease in the ambenonium concentration was found within 48 hr. Consequently, the decomposition of ambenonium in a biological sample at room temperature will be negligible.

In conclusion, a sensitive, specific and precise method for the determination of ambenonium in plasma, bile, urine and tissues by ion-pair HPLC was established. It would be useful in pharmacokinetic studies of ambenonium, since ambenonium concentration in these samples could be precisely determined in the range 20-50 pmol.

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Table 1 Reproducibility of ambenonium analysis in biological samples from rats.

Amount added (pmol)	Coefficient of variation (n = 5) (%)								
	Plasma	Bile	Urine	Liver	Kidney	Brain	Muscle	Lung	Spleen
20	3.4	3.1	-	5.5	4.2	4.0	7.0	5.0	4.7
50	6.0	2.5	7.8	5.5	5.0	4.5	4.7	4.7	5.5
200	2.5	3.1	6.2	6.0	4.2	4.3	4.7	5.0	6.2
500	5.4	5.4	5.6	4.6	3.4	7.8	5.8	3.1	4.3
2000	5.2	3.2	5.4	4.8	4.5	6.7	4.6	4.4	2.8

Table 2 Extraction recoveries of ambenonium from biological samples from rats.

Amount added (pmol)	Recovery (mean ± S.D., n = 5) (%)								
	Plasma	Bile	Urine	Liver	Kidney	Brain	Muscle	Lung	Spleen
50	80.2 ± 6.3	87.8 ± 6.3	93.0 ± 4.2	87.8 ± 3.3	103.4 ± 11.9	86.6 ± 2.3	95.7 ± 4.2	94.3 ± 9.3	96.9 ± 5.5
200	86.2 ± 1.3	90.2 ± 3.8	79.9 ± 4.6	81.1 ± 3.5	90.8 ± 9.1	82.2 ± 3.8	82.6 ± 3.8	97.9 ± 2.3	100.1 ± 4.1
500	77.9 ± 4.2	87.1 ± 6.6	86.5 ± 3.1	86.1 ± 8.2	99.7 ± 7.1	78.7 ± 4.7	85.4 ± 5.8	97.4 ± 3.8	97.6 ± 4.2
2000	80.8 ± 2.4	89.3 ± 2.3	82.2 ± 6.9	86.2 ± 7.4	95.0 ± 4.2	84.1 ± 6.7	92.5 ± 2.9	94.4 ± 6.6	101.2 ± 2.9

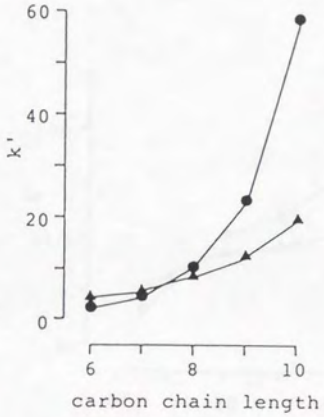

Figure 1 Influence of carbon chain-length of the paring alkylsulphonate anion on the retention of ambenonium (closed circle) and benzilonium (closed triangle). Other conditions are as described in the text.

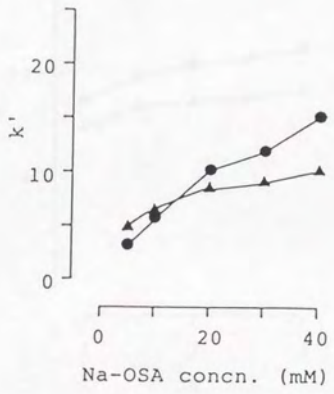

Figure 2 Influence of sodium octanesulphonate concentration on the retention of ambenonium (close circle) and benzilonium (closed triangle). Other conditions are as described in the text.

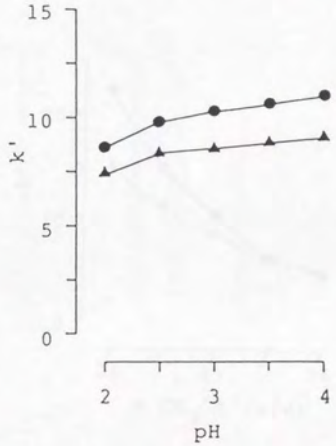

Figure 3 Influence of pH on the retention of ambenonium (closed circle) and benzilonium (closed triangle). Other conditions are described in the text.

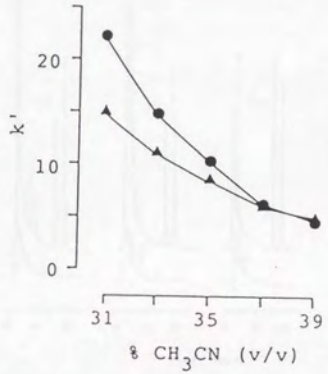

Figure 4 Influence of acetonitrile concentration on the retention of ambenonium (closed circle) and benzilonium (closed triangle). Other conditions are described in the text.

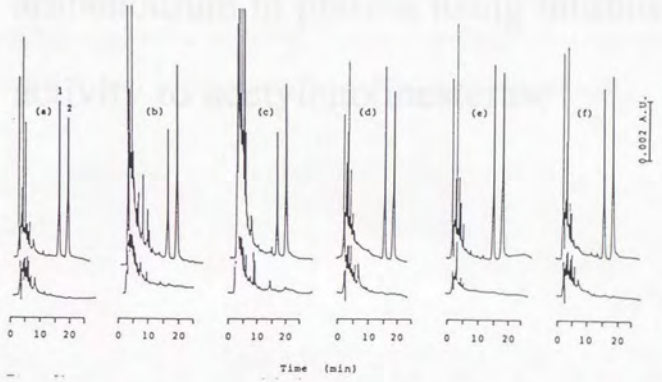

Figure 5 Chromatograms of plasma (a), bile (b), urine (c), liver (d), kidney (e) and spleen (f) from rats, spiked with 500 pmol of ambenonium chloride and 1 nmol of benzilonium bromide (upper chromatograms) and drug-free (lower ones). Peaks: 1 = benzilonium; 2 = ambenonium. Chromatographic conditions are as described in the text.

II. Sensitive determination of ambenonium in plasma using inhibitory activity to acetylcholinesterase

Abstract

A sensitive analytical method for the determination of ambenonium, a selective acetylcholinesterase inhibitor, in plasma was developed. The procedure involved ultrafiltration to remove endogenous plasma cholinesterase, followed by colorimetric measurement of the inhibitory activity to acetylcholinesterase by the thiocholine method. Coefficient of variation of within-day triplicate analysis is less than 20% at the concentration of 5 nM. Detection limit of this method is 1 nM, which is twice lower than the most sensitive HPLC method reported previously. This new method is rapid and simple and makes it possible to determine the ambenonium concentration in plasma with high sensitivity.

Introduction

Myasthenia gravis is a disease with abnormality in the neurotransmission by acetylcholine (ACh), caused by autoimmune response against acetylcholine receptor. Cholinesterase (ChE) inhibitors, which raise ACh concentration by the inhibition of acetylcholinesterase (AChE), are widely used for the treatment of patients with myasthenia gravis. However, there are wide interindividual variations of the therapeutic effect of ChE inhibitors, and the relationship between the dose or plasma concentration and the clinical response remains unclear because of the difficulty of quantitation of ChE in biological specimens. For the determination of edrophonium, neostigmine, pyridostigmine and ambenonium, gas-chromatography (Cohan et al., 1976, Chan et al., 1976, Pohlmann and Cohan, 1977), high-performance liquid chromatography (HPLC) (Yakatan and Tien, 1979, De Ruyter et al., 1980, Shih et al., 1986, Matsunaga et al., 1987, Tharasse-Bloch et al., 1987, Ohtsubo et al., 1989) and gas-chromatography-mass-spectrometric method (Aquilonius et al., 1979, Sørensen et al., 1984) have been reported. Previously, we reported the HPLC method for the determination of ambenonium (Yamamoto et al., 1988), a long-acting reversible ChE inhibitor. However, dose to obtain appropriate pharmacological response is quite low because ambenonium is very potent for AChE inhibition (Fujii and Namba, 1982), and the plasma concentration after administration of such dose cannot be determined. More sensitive determination method is required for pharmacokinetic / pharmacodynamic investigation of ambenonium. In this study, we developed a new method for the enzymatic determination of ambenonium using the AChE inhibition potency.

Methods

Chemicals and reagents

Ambenonium chloride was kindly supplied by Nippon Shoji Co. (Osaka, Japan). Neostigmine bromide and acetylthiocholine iodide were obtained from SIGMA (U. S. A.). Bovine erythrocyte AChE (EC 3.1.1.7) and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Other chemicals and reagents were purchased from the commercial source and of reagent grade, and used without further purification.

Inhibitory constants of ambenonium to AChE and plasma ChE

Esterase activity was measured by the thiocholine method reported by MacQueen et al. (1971) with slight modification. Bovine erythrocyte AChE (400 unit/L) in phosphate buffer (PB, 50 mM, pH 7.2) or rat plasma diluted five times by isotonic sodium chloride solution were used as the standard enzyme solution. To 3 ml of 0.1 mg/ml DTNB in PB, 0.1 ml of enzyme solution and 0.1 ml of ambenonium solution (0.15 - 62.5 nM and 0.15 - 62.5 μM as final concentration for AChE and plasma esterase, respectively) were added. After preincubation at 37 ℃ for 10 min, 0.1 ml of acetylthiocholine in water (2.7 mM or 5.4 mM as final concentration) was added, then incubated at 37 ℃. Exactly 10 min after the substrate solution was added, the enzyme reaction was stopped by addition of 3 ml of iced quinidine sulfate (1 mg/ml). Sample was stand on ice for 10 min, then absorbance at 412 nm was measured in a spectrophotometer (UV-3000, Shimadzu, Kyoto, Japan). Blank sample was prepared by substituting the PB instead of enzyme solution. Enzyme reaction velocity was calculated by subtracting the absorbance of blank from that of sample.

Provided the enzyme reaction velocity is expressed by the Michaelis-Menten equation with competitive inhibition (Eq. 1), rearrangement produces the Eq. 2, which shows the linear relationship between the inhibitor concentration [I] and the reciprocal of the reaction velocity 1/v (Dixon plot). Inhibitory constant Ki is calculated as the x-axis value of intersection point of the line obtained under the condition of different substrate

concentrations.

$$v = \frac{Vmax \cdot [S]}{Km \cdot (1 + \frac{[I]}{Ki}) + [S]} \quad (1)$$

$$1/v = (\frac{Km}{Vmax \cdot [S] \cdot Ki}) \cdot [I] + (\frac{1}{Vmax} + \frac{Km}{Vmax \cdot [S]}) \quad (2)$$

Assay procedure of ambenonium in plasma

Scheme of ambenonium assay procedure is shown in Figure 1. Endogenous plasma ChE was removed by filtration with MPS-3 centrifree micropartition system (Amicon, U. S. A.) at 4 ℃, 2000 g for 15 min. From 0.5 to 1 ml of plasma, 0.1 ml of plasma filtrate was obtained. Plasma filtrate was transferred to the plastic tube, then 25 μl of 1 mg/ml DTNB in PB, 0.1 ml of 200 unit/L AChE were added. After preincubation at 37 ℃ for 5 min, 25 μl of 25 mg/ml acetylthiocholine was added. Exactly 5 min after addition of substrate, enzyme reaction was stopped by addition of 0.6 ml of iced 0.1 μM neostigmine. Sample was stand for 5 - 10 min on ice, then absorbance at 412 nm was measured in a spectrophotometer. Blank sample was prepared by substituting the PB and drug free plasma instead of enzyme solution and sample plasma, respectively. Enzyme reaction velocity was calculated by subtracting the absorbance of blank from that of sample.

Plasma containing ambenonium at the concentration of 100, 50, 20, 10, 5, 2 and 1 nM was used for calibration curve preparation. Enzyme reaction velocity was measured as described above.

Effect of endogenous plasma ChE

Drug free plasma was drawn from 3 rats and plasma filtrate was obtained by filtration. A 0.1 ml of PB or 200 unit/L AChE was added to 0.1 ml of plasma filtrate, and enzyme reaction velocity was compared. Blank sample was prepared by substituting plasma filtrate by PB. Plasma from each rat was spiked with ambenonium, then the inhibitory activity to AChE in each rat was also compared.

Within-day precision

Within-day precision was determined by the triplicate analysis of the same plasma sample containing 1, 5, 20, 50 and 100 nM of ambenonium within a day using the same calibration curve.

Between-day precision

Between-day precision was determined by the triplicate analysis of the same plasma sample containing 1, 5, 20, 50 and 100 nM of ambenonium on the 3 sequential days. Calibration curves were prepared on each day, respectively.

Comparison of assayed value of enzymatic method with HPLC method

Concentration of plasma sample containing 100 - 5000 nM of ambenonium was determined by both enzymatic method and HPLC method (Yamamoto et al., 1988). For enzymatic determination, plasma sample was diluted with drug free plasma to avoid change of endogenous ChE activity and adsorption of ambenonium to ultrafiltration apparatus.

Recovery from aqueous solution and plasma

Ambenonium solution containing 5, 20 or 100 nM of ambenonium was ultrafiltrated at 2000 g for 3 min, then the concentration of ambenonium in the filtrate and the residue was measured. Calibration curve is prepared with ambenonium aqueous solution of the same concentration sequence as plasma calibration. For the measurement of recovery ratio from plasma, ambenonium concentration in plasma filtrate obtained from plasma containing 5, 20 or 100 nM of ambenonium was measured. Drug free plasma filtrate was spiked with ambenonium and was used for calibration curve preparation.

Results

Inhibitory effect of ambenonium on AChE and plasma ChE activity

Inhibitory activity of ambenonium to bovine erythrocyte AChE and rat plasma ChE was shown in Figures. 2 and 3, respectively. The values of inhibitory constants, Ki, obtained from Dixon plot were 2.99 nM for AChE and 18.2 μM for plasma ChE.

Effect of endogenous plasma ChE on the ChE activity in plasma filtrate

Figure 4 shows small interindividual variation of AChE activity in plasma filtrates obtained from 3 animals (C.V. = 5.2 %). Interindividual variation in the inhibitory activity of ambenonium to AChE added to plasma filtrates also rather small (C.V. = 1.8 - 6.9 %) (Figure 5).

Precision of determination method

Within-day precision and between-day precision were shown in Tables 1 and 2, respectively. Decrease of enzyme reaction rate could be detected at the concentration of 1 nM as a lower limit, but the variation of value was so large, especially in between-day precision study. The average value of the measurement at the concentration of 5 nM is substantially correct, and the coefficient of variation is less than 20%. Therefore, average of twice measurements should be used as the assayed value and the detection limit was set at 5 nM.

Comparison of assayed value of enzymatic method with HPLC method

Enzymatically assayed value was compared with the concentration determined by HPLC method (Figure 6). There is good correlation between these assayed values, suggesting the validity of the enzymatic method.

Recovery ratio from aqueous solution or plasma

Due to plasma protein binding or adsorption to the filter membrane, the concentration of ambenonium in plasma filtrate should be decreased. To confirm the validity of our assay method, the recovery ratio from the aqueous solution or plasma to filtrate was determined (Table 3). The concentration of ambenonium in filtrate and the residue of aqueous solution

is seriously decreased from the initial concentration, suggesting strong adsorption of ambenonium to the micropartition system. On the other hand, the ratio of concentration in plasma filtrate to that in plasma is 70% at the initial concentration of 5 nM and 110% at 20 nM and 100 nM, respectively. Since recovery ratio is close to unity at high concentrations (20 - 100 nM), the effect of plasma protein binding can be ignored.

Discussion

Though ChE inhibitors are widely used for the treatment of myasthenia gravis, neither pharmacokinetics nor pharmacodynamics are clearly explained because of the difficulty in monitoring of drug level in biological fluid. Edrophonium, pyridostigmine, neostigmine and ambenonium as ChE inhibitors are used in the clinical field for the treatment and/or diagnosis of myasthenia. Since inhibitory activity of ambenonium to AChE is stronger and more selective than the others, therapeutic dose of ambenonium is smaller than the others. Therefore, the therapeutic concentration of ambenonium to show appropriate pharmacological effect should be lower. In order to study pharmacokinetics and pharmacodynamics of ambenonium, it is necessary to develop the highly sensitive assay method. Moreover, simple and rapid quantitation is required for the dose adjustment to achieve the rational drug therapy in the clinical situation.

Until now, only three HPLC methods have been reported for the determination of ambenonium in biological samples. The first method reported by Tharasse-Bloch et al. (1987) has made possible to determine ambenonium concentration in plasma. The concentration of ambenonium in bile, urine and tissues became possible by more precise and selective method reported by the authors (Yamamoto et al., 1988). However, the detection limits of these HPLC methods are about 20 nM and seems insufficient to investigate pharmacodynamics. The more sensitive HPLC method, which requires the ion-exchange extraction of large volume of diluted plasma sample, were reported by Ohtsubo et al. (1989). The detection limit of this method is about 2 nM, but the reproducibility at this concentration is not shown. With the enzymatic method, 1 nM of ambenonium in plasma can be detected though the reproducibility is not enough to detailed pharmacokinetic study. Furthermore, this enzymatic method required simple procedure and short time, and considered useful for clinical application.

Enzymatic determination method for pyridostigmine in the presence of endogenous ChE has been reported previously (Shatkay, 1988). Since the inhibitory activity of

pyridostigmine to AChE and plasma ChE is less selective, the inhibition of plasma ChE activity can be used for the pyridostigmine assay at the therapeutic concentration (30 - 700 nM) (Cohan et al., 1976, Calvey and Chan, 1977, Chan and Calvey, 1978, Aquilonius et al., 1980, Aquilonius et al., 1983, Breyer-Pfaff et al., 1985, 1990). In order to determine the concentration of ambenonium enzymatically, endogenous ChE, which is less inhibited at the therapeutic concentration of ambenonium, should be removed to avoid interference by its esterase activity. After plasma ChE was removed by ultrafiltration using MPS-3 micropartition system (Figure 5), the effect of background activity of esterase based on endogenous plasma ChE can be neglected by addition of enough exogenous AChE. Variation of leakage of plasma ChE activity is so small that the inhibitory activity of ambenonium to AChE can be determined by subtracting the blank plasma ChE activity.

Since ambenonium is known to have adhesive characteristics (Ohtsubo et al., 1989), it is necessary to assay carefully. For of aqueous solution, the decrease of the concentration of ambenonium was observed, which means the adsorption of ambenonium to ultrafiltration apparatus. Considering the recovery ratio from plasma be close to unity, no serious adsorption may occur during plasma filtration. However, since recovery ratio is slightly decreased with the low concentration, calibration curves were prepared for both high and low concentrations to achieve higher accuracy. The reason of less adhesive behavior in the presence of plasma components remains still unknown.

In conclusion, our developed enzymatic determination method is rapid and simple, and makes it possible to assay the concentration of ambenonium in plasma with high sensitivity, and should be useful for pharmacokinetic and pharmacodynamic study.

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Table 1 Within-day precision of the enzymatic determination method

Ambenonium concentration	Assayed value	
(nM)	(nM, Mean ± S.D.)	(n = 3)
1	1.52 ± 1.26	
5	5.25 ± 0.91	
20	16.7 ± 1.9	
50	49.6 ± 3.9	
100	108.7 ± 7	

Table 2 Between-day precision of the enzymatic determination method

Ambenonium concentration	Assayed value
(nM)	(nM, Mean ± S.D.) (n = 3)
1	3.01 ± 2.02
5	5.14 ± 3.56
20	23.6 ± 4.4
50	55.5 ± 3.0
100	99.5 ± 22.1

Table 3 Recovery of ambenonium in filtrate

	Recovery (%: ratio to the initial concentration)	
	Mean	S.D. (n = 3)
Water (residue)		
5 nM	—	—
20 nM	34.0	7.2
100 nM	33.6	7.6
Water (filtrate)		
5 nM	—	—
20 nM	—	—
100 nM	6.1	0.57
Plasma (filtrate)		
5 nM	74.3[a)]	10.6[a)]
20 nM	113.7	0.9
100 nM	111.9	5.7

a) Assayed values were less than the detection limit.

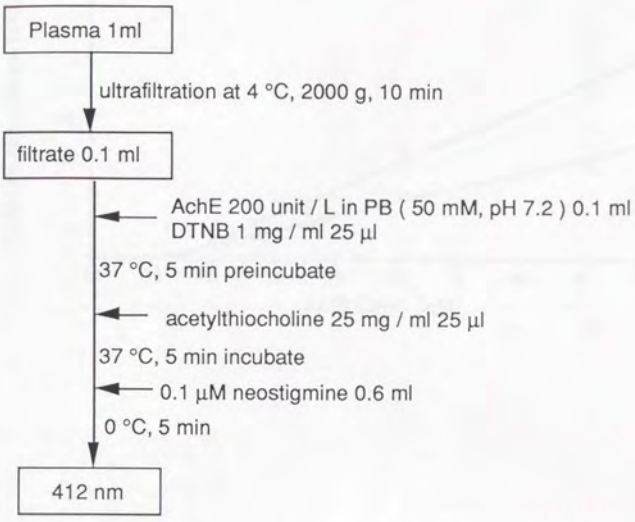

Figure 1 Scheme of the enzymatic determination procedure. Sample plasma was filtrated to remove endogenous plasma esterase. Acetylcholinesterase was added to the filtrate as standard enzyme, and the enzyme activity was measured by the thiocholine method. AChE: bovine erythrocyte acetylcholinesterase, PB: phosphate buffer, DTNB: 5, 5'-dithiobis-2-nitrobenzoic acid.

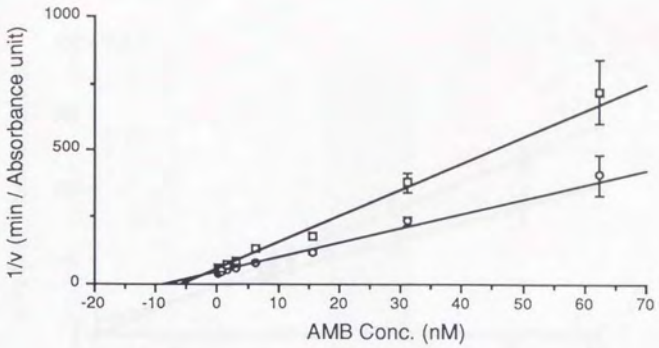

Figure 2 Dixon plot of AChE inhibition by ambenonium. Final acetylthiocholine concentrations are; ○: 5.4 mM, □: 2.7 mM. Each symbol and vertical bar represents mean and standard deviation of 3 experiments. Regression lines are; $y = 5.42 \cdot x + 46.8$ ($r^2 = 0.998$) for 5.4 mM, $y = 9.88 \cdot x + 60.1$ ($r^2 = 0.998$) for 2.7 mM. Ki is calculated as 2.99 nM.

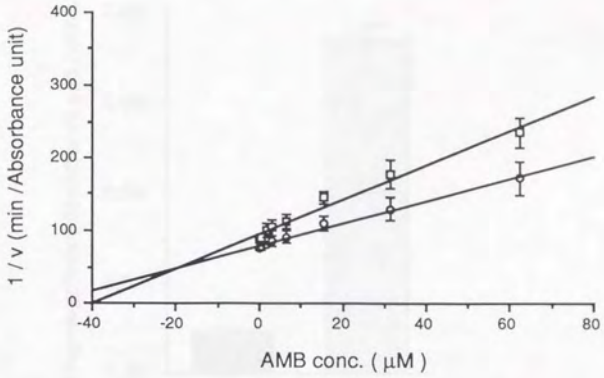

Figure 3 Dixon plot of plasma ChE inhibition by ambenonium. Final acetylthiocholine concentrations are; ○: 5.4 mM, □: 2.7 mM. Each symbol and vertical bar represents mean and standard deviation of 3 experiments. Regression lines are; $y = 2.39 \cdot x + 94.7$ ($r^2 = 0.978$) for 5.4 mM, $y = 1.54 \cdot x + 79.2$ ($r^2 = 0.990$) for 2.7 mM. Ki is calculated as 18.2 μM.

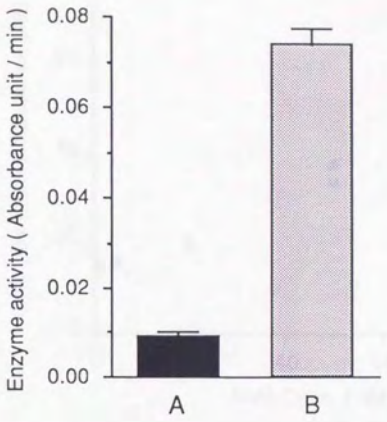

Figure 4 Interindividual variation of esterase activity in plasma filtrate with or without standard enzyme solution. A: Plasma filtrate without standard enzyme solution (200 unit/L AChE), B: Plasma filtrate with standard enzyme solution.

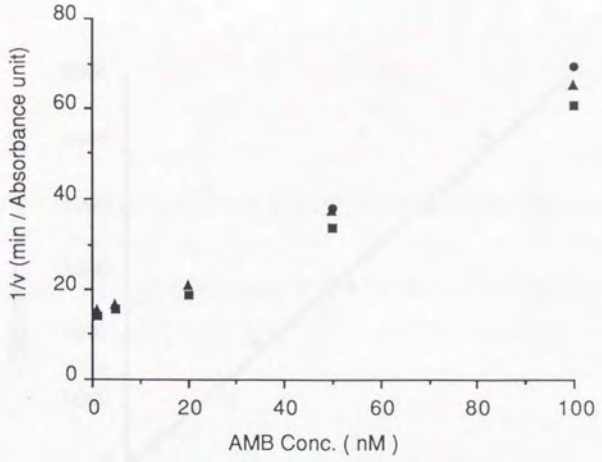

Figure 5 Interindividual variation of the effect of endogenous ChE activity in plasma filtrate on enzymatic determination of ambenonium. Drug free plasma obtained from rat A (●), rat B (■), rat C (▲): was spiked with ambenonium and the inhibitory activity to AChE was compared.

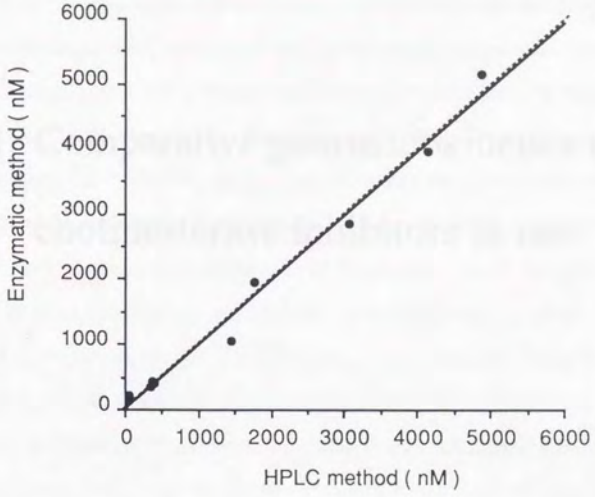

Figure 6 Comparison of HPLC and enzymatic method for the determination of ambenonium concentration in rat plasma. The dashed line represents the line of identity. Solid line represents the line fitted by least squares method ($y = 0.99 x + 15.6$, $r^2 = 0.987$).

III. Comparative pharmacokinetics of four cholinesterase inhibitors in rats

Abstract

A very short-acting, a short-acting and two long-acting cholinesterase (ChE) inhibitors, edrophonium, neostigmine, pyridostigmine and ambenonium, were administered to rats and pharmacokinetic characteristics of these ChE inhibitors were compared. No dose-dependency in pharmacokinetic behavior was observed within 2-10 μmole/kg for edrophonium, 0.5-2 μmole/kg for pyridostigmine, 0.1-0.5 μmole/kg for neostigmine and 0.3-3 μmole/kg for ambenonium, respectively. Neostigmine has the shortest elimination half life, and edrophonium, pyridostigmine and ambenonium are in the order. Four ChE inhibitors have similar Vdss values within the range of 0.3-0.7 L/kg, which is similar to muscle / plasma concentration ratio of these drugs. Liver or kidney to plasma concentration ratio of all ChE inhibitors at 20 min after i.v. administration ranged from 5 to 15. Based on the result of physiological pharmacokinetic analysis for ambenonium, it is suggested carrier-mediated, membrane-limited transport to kidney and liver is the determinant of the elimination rate of ChE inhibitors from plasma.

Introduction

Reversible ChE inhibitors are used as the first choice of therapy for myasthenia gravis. ChE inhibitors elevate the ACh concentration at the synaptic cleft of the neuromuscular junction by AChE inhibition, and intensify contractile muscle tension. However, the relationship between the concentration of ChE inhibitors in plasma and the enhancement of contractile muscle tension have not been clearly explained.

Several pharmacokinetic studies were reported for pyridostigmine, a frequently used ChE inhibitor. Pharmacokinetics after i.v., i.m. and p.o. administration of ^{14}C-pyridostigmine to rats and dogs were studied (Birtley et al.,1966, Husain et al., 1968, Taylor et al., 1991), and further, plasma concentration (Calvey and Chan, 1977, White et al., 1981, Sørensen et al., 1984, Cohan et al., 1976), bioavailability (Breyer-Pfaff et al, 1985, Aquilonius et al, 1980, Cohan et al., 1977), metabolism (Somani et al., 1972), excretion to breast milk (Hardess et al., 1982) in myasthenic patients were reported. Pharmacokinetic studies of ^{14}C-neostigmine were also carried out in rats (Roberts et al., 1963, Roberts et al., 1965a, Roberts et al., 1965b, Roberts et al., 1966). Plasma concentrations in myasthenic patients were determined in some cases (Nowell et al., 1962, Aquilonius et al., 1979). A few investigators reported the plasma concentration-time profiles of edrophonium in rats (Back and Calvey, 1972a, Back and Calvey, 1972b, Barber et al., 1976) and in human (Calvey et al., 1976). Pharmacokinetics of ambenonium is almost unknown (Aquilonius and Hartvig, 1986). Further, it remains unknown whether difference in pharmacokinetic behavior reflects on that in their pharmacological character.

In this study, a very short-acting, a short-acting and two long-acting ChE inhibitors, edrophonium, neostigmine, pyridostigmine and ambenonium, were administered to rats and pharmacokinetic behavior of ChE inhibitors were compared. Furthermore, physiological pharmacokinetic model was applied for ambenonium to investigate the determinant of pharmacokinetics of quaternary ammonium ChE inhibitors.

Methods

Chemicals and reagents

Ambenonium chloride was generously supplied by Nippon Shoji Co. (Osaka, Japan). Edrophonium chloride, neostigmine bromide and pyridostigmine bromide were purchased from SIGMA (U.S.A.). All other reagents were of analytical grade and used without further purification.

Animal experiments

Male Wistar rats weighing 250 - 330 g were used in all experiments. Under light ether anesthesia, polyethylene cannulas were inserted into left femoral artery and vein, bile duct and urinary bladder. The rats were left for 2 hr after the surgery to recover from anesthesia. At 1, 3, 5, 10, 15, 20 and 30 min after i.v. administration of edrophonium (2 - 10 μmole/kg) and neostigmine (0.1 - 0.5 μmole/kg), and at 2, 5, 15, 30, 45 and 60 min for pyridostigmine (0.5 - 2 μmole/kg) and ambenonium (0.3 - 3 μmole/kg), 0.5 ml of blood was taken and the plasma was obtained immediately by centrifugation at 1620 g for 5 min. Bile and urine were collected at appropriate intervals until 6 hr after administration.

To estimate the pharmacokinetic parameters, A, B, α and β, the concentration of ChE inhibitors in plasma obtained in all single bolus dose experiments were simultaneously fitted to Equation 1 by non-linear least squares method. The total body clearance (CL_{tot}), the steady state volume of distribution (Vdss) and the elimination half life ($t_{1/2}$) were calculated according to Equations 2, 3 and 4, respectively.

$$Cp = Dose \cdot (A \cdot \exp(- \alpha \cdot t) + B \cdot \exp(- \beta \cdot t)) \quad (1)$$

$$CL_{tot} = \frac{\alpha \cdot \beta}{A \cdot \beta + B \cdot \alpha} \quad (2)$$

$$Vd_{SS} = \frac{A \cdot \beta^2 + B \cdot \alpha^2}{(A \cdot \beta + B \cdot \alpha)^2} \quad (3)$$

$$t_{1/2} = \frac{\ln 2}{\beta} \qquad (4)$$

where Cp is the concentration of ChE inhibitors in plasma, Dose is the i.v. dose administered and t is the time after administration. Excretion ratios in urine and bile were estimated with extrapolation to the infinite time using the terminal slope of the semi-logarithmic plots of excretion rate versus time curve.

In tissue distribution experiments, rats were killed by exsanguination at 20 min after administration of drugs to collect tissues. Tissues were also collected at 40 min, 1, 3, 6, 12, 18 and 24 hr for ambenonium (1 μmole/kg). Sample was stored at -20 ℃ until analysis.

Blood cell to plasma concentration ratio

The drug free blood was withdrawn through the femoral arterial cannula. Hematocrit values were determined immediately after blood collection. To the fresh blood, ambenonium was spiked at 0.5, 1 and 2 μM as total blood concentration. After incubation at 37 ℃ for 30min, 0.2 ml of blood was taken for determination of the concentration of ambenonium in blood. Then plasma was separated from the rest by centrifugation at 1620 g for 5 min. The concentration of ambenonium in blood cell (C_{bc}) was estimated by the equation:

$$C_{bc} = \frac{C_b - (1 - H_t) \cdot C_p}{H_t} \qquad (5)$$

where H_t, C_p and C_b are the hematocrit value, the drug concentration in plasma and blood, respectively.

Plasma protein binding

Through the femoral arterial cannula, drug free blood was withdrawn and plasma was separated as described above. To the fresh plasma, ambenonium was spiked at 0.5, 1 and 2 μM as total plasma concentration. Spiked plasma was incubated at 37 ℃ for 30 min, then 1 ml aliquot was applied to a micropartition system MPS-3 (Amicon Corp., U.S.A.) for the ultrafiltration method at 2000 g for 10 min. The binding of ambenonium to plasma protein

was estimated by the measurement of the drug concentrations in the plasma and its filtrate.

Tissue binding

Rats are killed by exsanguination. The abdomens of the animals were opened immediately and liver was perfused with cold physiological saline via portal vein until the effusate became colorless. Tissue homogenate sample (10%) was prepared as described in animal experiments. To the liver homogenate, ambenonium was spiked at the concentration of 0.5, 1 and 2 μM. Spiked homogenate was incubated at 37 ℃ for 30 min, then 1 ml aliquot was used for the measurement of tissue binding of ambenonium by the same method as plasma protein binding.

Physiologically based pharmacokinetic analysis for ambenonium

Physiologically based pharmacokinetic model was developed to estimate the tissue concentration profiles of ambenonium as shown in Fig. 1. All tissues in which ambenonium concentration was determined are included and linked by the blood flow.

A blood flow-limited model was used for muscle compartment (Eq. 6),

$$V_{Muscle} \cdot \frac{d\,C_{Muscle}}{d\,t} = Q_{Muscle} \cdot (C_a - C_{Muscle} / Kp_{Muscle}) \quad (6)$$

where V_{Muscle}, C_{Muscle}, Q_{Muscle}, and Kp_{Muscle} are the volume, drug concentration, blood flow and tissue/blood partition coefficient for muscle compartment. A diffusion-limited model was used for other tissues since drug concentration-time profiles could not be described by blood flow-limited model. For tissue i, $V_{i,B}$ and $V_{i,T}$ are the volume of parenchymal tissue and the interstitial fluid, $C_{i,B}$ and $C_{i,T}$ are the drug concentration in parenchymal tissue and the interstitial fluid, and $CL_{inf,i}$ and $CL_{eff,i}$ are the influx and efflux clearance, respectively. The apparent tissue volume, Vi, and drug concentration, Ci are calculated as

$$V_i = V_{i,B} + V_{i,T} \quad (7)$$

$$C_i = (C_{i,B} \cdot V_{i,B} + C_{i,T} \cdot V_{i,T}) / V_i \quad (8)$$

The mass-balance equations for spleen and lung become Eq. 9-11 and Eq. 12-14, respectively,

$$V_{Spleen,B} \cdot \frac{d\,C_{Spleen,B}}{d\,t} = Q_{Spleen} \cdot (C_a - C_{Spleen,B}) - CL_{inf,Spleen} \cdot C_{spleen,e} + CL_{eff,Spleen} \cdot C_{Spleen,T} \quad (9)$$

$$V_{Spleen,T} \cdot \frac{d\,C_{Spleen,T}}{d\,t} = CL_{inf,Spleen} \cdot C_{Spleen,B} - CL_{eff,Spleen} \cdot C_{Spleen,T} \quad (10)$$

$$V_{Spleen} \cdot \frac{d\,C_{Spleen}}{d\,t} = Q_{Spleen} \cdot (C_a - C_{Spleen,B}) \quad (11)$$

$$V_{Lung,B} \cdot \frac{d\,C_{Lung,B}}{d\,t} = Q_{tot} \cdot (C_v - C_{Lung,B}) - CL_{inf,Lung} \cdot C_{lung,B} + CL_{eff,Lung} \cdot C_{Lung,B} \quad (12)$$

$$V_{Lung,T} \cdot \frac{d\,C_{Lung,T}}{d\,t} = CL_{inf,Lung} \cdot C_{lung,B} - CL_{eff,Lung} \cdot C_{Lung,T} \quad (13)$$

$$V_{Lung} \cdot \frac{d\,C_{Lung}}{d\,t} = Q_{tot} \cdot (C_v - C_{Lung,B}) \quad (14)$$

where Q_{tot} and C_v are the total blood flow and drug concentration in venous blood, respectively.

If renal excretion rate of ambenonium is governed by the sum of the rates of glomerular filtration (GFR), tubular secretion (CL_{SCR}) and reabsorption fraction (FR), the concentration in kidney and amount of urinary excretion (X_{Urine}) of ambenonium may be written as

$$V_{Kidney,B} \cdot \frac{d\,C_{Kidney,B}}{d\,t} = Q_{Kidney} \cdot (C_a - C_{Kidney,B}) - CL_{inf,Kidney} \cdot C_{Kidney,B} + CL_{eff,Kidney} \cdot C_{Kidney,T} - GFR \cdot C_a + FR \cdot (GFR \cdot C_a + CL_{SCR} \cdot C_{Kidney,T}) \quad (15)$$

$$V_{Kidney,T} \cdot \frac{d\,C_{Kidney,T}}{d\,t} = CL_{inf,Kidney} \cdot C_{Kidney,B} - (CL_{eff,Kidney} + CL_{SCR}) \cdot C_{Kidney,T} \quad (16)$$

$$V_{Kidney} \cdot \frac{d\,C_{Kidney}}{d\,t} = Q_{Kidney} \cdot (C_a - C_{Kidney,B}) - (1 - FR) \cdot (GFR \cdot C_a + CL_{SCR} \cdot C_{Kidney,T}) \quad (17)$$

$$\frac{d X_{Urine}}{d t} = (1 - FR) \cdot (GFR \cdot C_a + CL_{SCR} \cdot C_{Kidney,T}) \quad (18)$$

A compartment model for the hepatobiliary transport of ambenoium was considered. In this model, the intracellular space in the liver consists of two compartments: one is the precursor pool, the other is the storage compartment in the liver (Fig. 1).

$$V_{Liver,B} \cdot \frac{d C_{Liver,B}}{d t} = Q_{Liver} \cdot (C_a - C_{Liver,B}) - CL_{inf,Liver} \cdot C_{Liver,B} + k_2 \cdot X_{Liver,T} \quad (19)$$

$$\frac{d X_{Liver,T}}{d t} = CL_{inf,Liver} \cdot C_{Liver,B} + k_4 \cdot X_{Liver,Store} - (k_2 + k_3 + k_5) \cdot X_{Liver,T} \quad (20)$$

$$\frac{d X_{Liver,Store}}{d t} = k_3 \cdot X_{Liver,T} - k_4 \cdot X_{Liver,Store} \quad (21)$$

$$V_{Liver} \cdot \frac{d C_{Liver}}{d t} = Q_{Liver} \cdot (C_a - C_{Liver,B}) - k_5 \cdot X_{Liver,T} \quad (22)$$

$$\frac{d X_{Bile}}{d t} = k_5 \cdot X_{Liver,T} \quad (23)$$

where $X_{Liver,Store}$ is the drug amount in storage compartment, k_2, k_3, k_4 and k_5 are the inter-compartmental transport rate constants, respectively.

Physiological parameters such as the blood flow, GFR and the volume of tissues and interstitial fluid were obtained from the literature (Tsuji et al., 1983, Bernareggi and M. Rowland, 1991, Gallo et al., 1989) and listed in Table 1. In order to estimate the kinetic parameters, concentration-time profile for each tissue was fitted to the hybrid model using the arterial blood concentration as the input function. Then, all tissue compartment were linked by the arterial and venous blood compartment as shown in Fig.1.

$$V_{Artery} \cdot \frac{d C_a}{d t} = Q_{tot} \cdot (C_{lung,B} - C_a) \quad (24)$$

$$V_{Vein} \cdot \frac{d C_v}{d t} = \sum_{i=1}^{n} Q_i \cdot C_{i,B} - Q_{tot} \cdot C_v \quad (25)$$

where Cv and Ca are the drug concentration in venous and arterial blood, respectively. Profiles for concentration of ambenonium in blood and tissues, amount excreted in bile and urine were calculated according to the physiological model with the estimated parameters.

Analytical procedure

The concentration of ChE inhibitors in plasma were determined by the HPLC method (Ruyter and Cronnelly, 1980, Yamamoto et al., 1988).

Results and Discussion

Plasma concentration profiles after i.v. bolus administration of edrophonium (2 - 10 μmole/kg), pyridostigmine (0.5 - 2 μmole/kg), neostigmine (0.1 - 0.5 μmole/kg) and ambenonium (0.3 - 3 μmole/kg) to rats are shown in Figure 2. The disappearances of these drugs in plasma were described by biexponential curves in all cases. The pharmacokinetic parameters are listed in Table 2. No dose-dependency in pharmacokinetic parameters was observed within the dose range in this study. Four ChE inhibitors have similar Vdss values within the range of 0.3-0.7 L/kg. Neostigmine has the shortest $t_{1/2}$ and edrophonium, pyridostigmine and ambenonium were in the order. Plasma elimination half lives of ChE inhibitors in rats were reported as 6.3-13.5 min (Back and Calvey, 1972b) and 81-175 min for edrophonium (Calvey et al., 1976), 19 min for pyridostigmine (Birtley et al., 1966), 147 min for neostigmine (Roberts et al., 1965a). Elimination half life of ambenonium from plasma was determined only in dogs as 36 ± 2 min. However, they cannot be compared simply because experimental conditions such as dose and sampling schedule are different. Elimination half lives estimated in this study are rather short (Table 2), which may be due to the short experimental period of less than 90 min.

All ChE inhibitors are readily excreted in urine and 40-60% of dose were found as parent drug. About 25% of dose was excreted in bile for ambenonium, while biliary excretion of other drugs were negligible. This difference in hepatobiliary disposition may be due to the large molecular weight (608.5) and bisquaternary structure of ambenonium (Meyjer et al., 1990). Back and Calvey (1972a) reported that 5% of radioactivity was excreted in bile as glucronide after i.v. administration of ^{14}C-edrophonium to rats. Urinary excretion of edrophonium in rats has not been reported previously. The 90% of dose was excreted in urine after i.v. administration of ^{14}C-pyridostigmine, and about one third of radioactivity was related to a metabolite 3-hydroxy-N-methylpyridinium (Birtley et al., 1966). For ^{14}C-neostigmine, 50% of dose was excreted in urine within 2 hr after

intramuscular administration, and a half of radioactivity was related to hydrolyzed metabolite N-hydroxyphenyltrimethylammonium (Roberts et al., 1965b). It is well known that various quaternary ammonium compounds including ChE inhibitors are readily excreted in urine by renal tubular secretion (Roberts et al., 1963, Mintum et al, 1980, Neef and Meijer, 1984ab).

Contrary to rapid elimination of ChE inhibitors, they are highly accumulated in liver and kidney (Back and Calvey, 1972a, Roberts et al., 1965b, Birtley et al., 1966, Yamamoto et al., 1988). As shown in Figure 3, liver or kidney to plasma concentration ratio of all ChE inhibitors at 20 min ranged from 5 to 15, suggesting that ChE inhibitors may be transported into these tissues like other quaternary ammonium compounds (Mintum et al, 1980, Meyjer et al., 1990). Though approximately 50% of the dose was distributed into liver and kidney at 20 min after i.v. administration, the distribution volumes at steady state are relatively small value of 0.3-0.7 L/kg, which were somewhat larger than extracellular space and similar to muscle / plasma concentration ratio (Figure 4).

In order to estimate the tissue concentration profiles of ambenonium, a physiologically based pharmacokinetic model was developed (Figure 1). Blood to plasma partition coefficient and unbound fraction of ambenonium is substantially constant (Table 3). Transport clearance between blood and tissues/organs were estimated according to the hybrid models (Table 4) using blood concentration profile as the input function.

Since drug concentration-time profiles in organs/tissues except muscle could not be described by blood flow-limited model, a diffusion-limited model was used for other tissues. Simulation lines of all organs/tissues and biliary and urinary excretion showed good agreement with the observed value. Since binding of ambenonium to liver tissue was negligible, ambenonium is transported from blood to hepatocyte by carrier-mediated system. Theoretical tissue to blood partition coefficients (CL_{inf} / CL_{eff}) in tissues of which pharmacokinetics is described by diffusion-rate-limiting model are more than 100. Especially liver and kidney, efflux from tissue to blood was substantially negligible, therefore rapid disappearance of ambenonium from plasma may be due to the carrier-

mediated uptake to these tissues. On the other hand, distribution of ambenonium to muscle, a site of action, was blood-flow limited and readily equilibrated. Muscle to plasma partition coefficient was similar to the steady-state volume of distribution of ambenonium, suggesting that distribution to muscle, which contributes about 50% of the body weight, is the determinant of distribution volume. Since all ChE inhibitors showed similar distribution volume of 0.3-0.7 (Fig. 3), this characteristics is common in quaternary ammonium ChE inhibitors.

In conclusion, rapid disappearance of ChE inhibitors from plasma may reflect the uptake to the liver and kidney. Though ChE inhibitors are highly concentrated in liver and kidney, steady state volumes of distribution are relatively small value of 0.3-0.7, reflecting the distribution to muscle and extracellular space of other organs/tissues.

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Table 1. Physiological parameters for rats with the body weight of 250 g.

tissue	total volume (ml)	extracellular space[a] (ml)	blood flow rate (ml/min)
liver	9.59[b]	0.169[d]	11.8[c]
lung	1.05[b]	0.204[d]	43.0[c]
kidney	1.98[b]	0.196[d]	9.23[c]
GFR			1.06[d]
muscle	121.9[c]	0.119[d]	7.5[c]
spleen	0.43[b]	0.353[e]	0.63[c]
other tissues			14.47
arterial blood	5.6[c]		
venous blood	11.3[c]		

a) Plasma volume in capillary bed is assumed as inulin space x tissue volume.

b) Determined experimentally in this laboratory.

c) Based on the values in J. Pharm. Sci., 72: 1239-1252 (1983).

d) Based on the values in J. Pharmacokin. Biopharm., 19: 21-50 (1991).

e) Based on the values in J. Pharmacokin. Biopharm., 17: 305-326 (1989).

Table 2. Pharmacokinetic parameters of ChE inhibitors after i.v. administration to rats.

parameters	edrophonium	pyridostigmine	neostigmine	ambenonium
CL_{tot} (ml/min/kg)	47.8 ± 1.0	15.0 ± 0.2	44.4 ± 3.7	12.6 ± 0.1
Vd_{ss} (L/kg)	0.651 ± 0.290	0.353 ± 0.050	0.289 ± 0.067	0.381 ± 0.106
$T_{1/2}$ (min)	17.9 ± 8.1	24.2 ± 4.2	7.3 ± 1.3	35.9 ±13.7
Urinary excretion (%)*	38.0 ± 10.6	59.5 ± 11.2	38.0 ± 10.6	36.7 ± 4.7
Biliary excretion (%)*	0.93 ± 0.42	0.23 ± 0.22	0.56 ± 0.24	24.1 ± 7.0

Dose: edrophonium: 10, 5, 2 μmole/kg, pyridostigmine: 2, 1, 0.5 μmole/kg, neostigmine: 0.5, 0.2, 0.1 μmole/kg, ambenonium: 3, 1, 0.3 μmole/kg. (n=3-4, mean ± S.D.).

* Biliary and urinary excretion were determined after i.v. administration of: edrophonium: 5 μmole/kg, pyridostigmine: 1 μmole/kg, neostigmine: 0.5 μmole/kg, ambenonium: 1 μmole/kg.

Table 3. Blood cell (C_{bc}) to plasma (C_p) concentration ratio and the unbound fractions in plasma (f_p) and in liver homogenate (f_H)

ambenonium concentration (μM)	C_{bc} / C_p (n = 3)	f_p (n = 3)	f_H (n = 3)
0.5	0.11 ± 0.14	0.89 ± 0.04	1.00 ± 0.01
1.0	0.04 ± 0.03	0.91 ± 0.04	1.05 ± 0.05
2.0	0.15 ± 0.11	0.80 ± 0.03	1.00 ± 0.03

Table 4. Pharmacokinetic parameters of ambenonium by physiological model.

parameters	value	
$CL_{inf,liver}$ (ml/min)	1.24	Influx clearance from interstitial fluid to liver precursor compartment
k_2 (min^{-1})	5.48×10^{-6}	Efflux rate constant from liver precursor compartment to interstitial fluid
k_3 (min^{-1})	0.111	Transport rate constant from liver precursor compartment to storage compartment.
k_4 (min^{-1})	4.30×10^{-3}	Transport rate constant from storage compartment to liver precursor compartment.
k_5 (min^{-1})	2.82×10^{-2}	Biliary excretion rate constant
$CL_{inf,lung}$ (ml/min)	1.44×10^{-2}	Influx clearance from interstitial fluid to lung compartment
$CL_{eff,lung}$ (ml/min)	6.42×10^{-4}	Efflux clearance from lung compartment to interstitial fluid
$CL_{inf,kidney}$ (ml/min)	0.348	Influx clearance from interstitial fluid to kidney compartment
$CL_{eff,kidney}$ (ml/min)	3.12×10^{-7}	Efflux clearance from kidney compartment to interstitial fluid
CL_{SCR} (ml/min)	9.51×10^{-3}	Tubular excretion clearance
FR	0.380	Reabsorption ratio
$K_{p,muscle}$	0.436	Muscle/ blood partition coefficient
$CL_{inf,spleen}$ (ml/min)	6.52×10^{-3}	Influx clearance from interstitial fluid to spleen compartment
$CL_{eff,spleen}$ (ml/min)	2.41×10^{-5}	Efflux clearance from spleen compartment to interstitial fluid

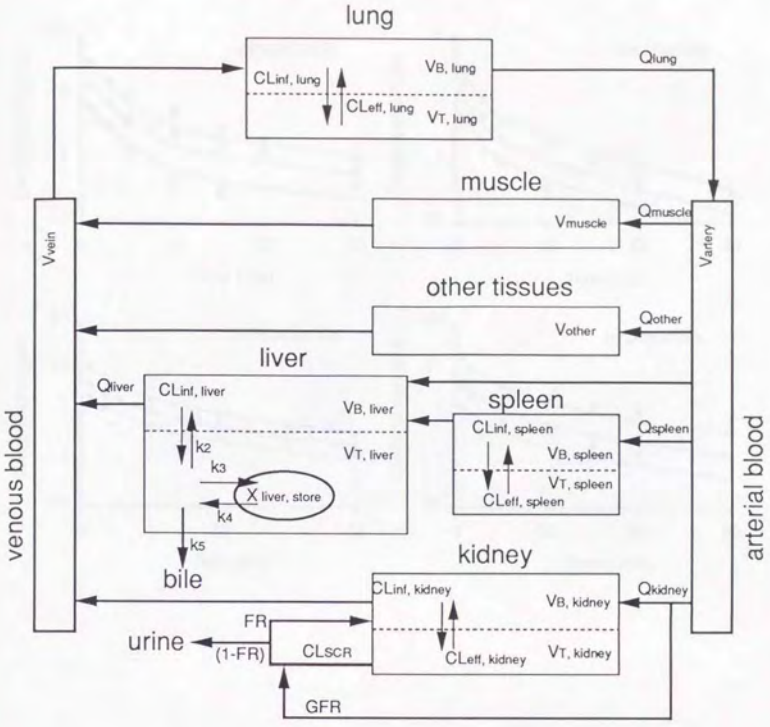

Figure 1 Physiologically based pharmacokinetic model for ambenonium.

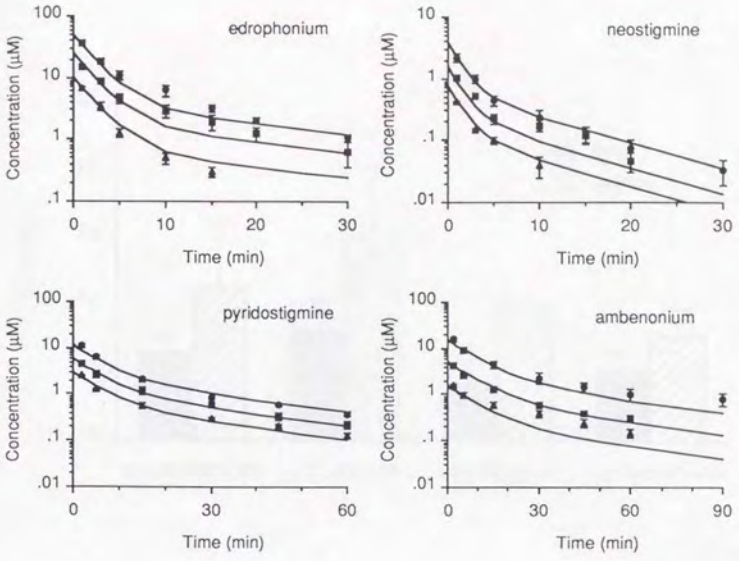

Figure 2 Plasma concentration-time profiles of ChE inhibitors after i.v. administration to rats. Edrophonium: 10 (●), 5 (■), 2 μmole/kg (▲), Pyridostigmine: 2 (●), 1 (■) , 0.5 μmole/kg (▲) , Neostigmine: 0.5 (●) , 0.2 (■) , 0.1 μmole/kg (▲) , Ambenonium: 3 (●) , 1 (■) , 0.3 μmole/kg (▲) . (n=3-4, mean ± S.E.)

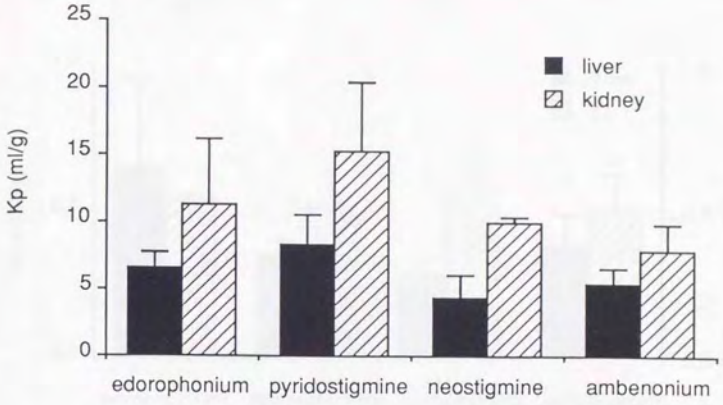

Figure 3 Apparent tissue / plasma partition coefficients (Kp) of ChE inhibitors. Drug concentration at 20 min after i.v. administration of edrophonium 10 μmole/kg, pyridostigmine 1 μmole/kg, neostigmine 0.2 μmole/kg or ambenonium 1 μmole/kg were determined (n = 3, mean ± S.D.).

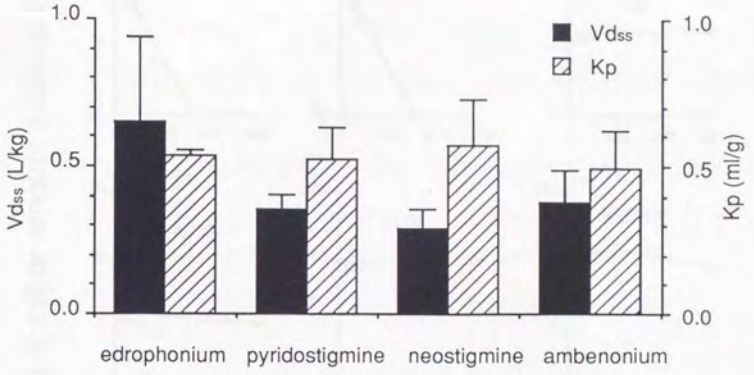

Figure 4 Steady-state distribution volume and apparent muscle / plasma partition coefficients of ChE inhibitors. Drug concentration at 20 min after i.v. administration of edrophonium 10 µmole/kg, pyridostigmine 1 µmole/kg, neostigmine 0.2 µmole/kg or ambenonium 1 µmole/kg were determined (n = 3, mean ± S.D.).

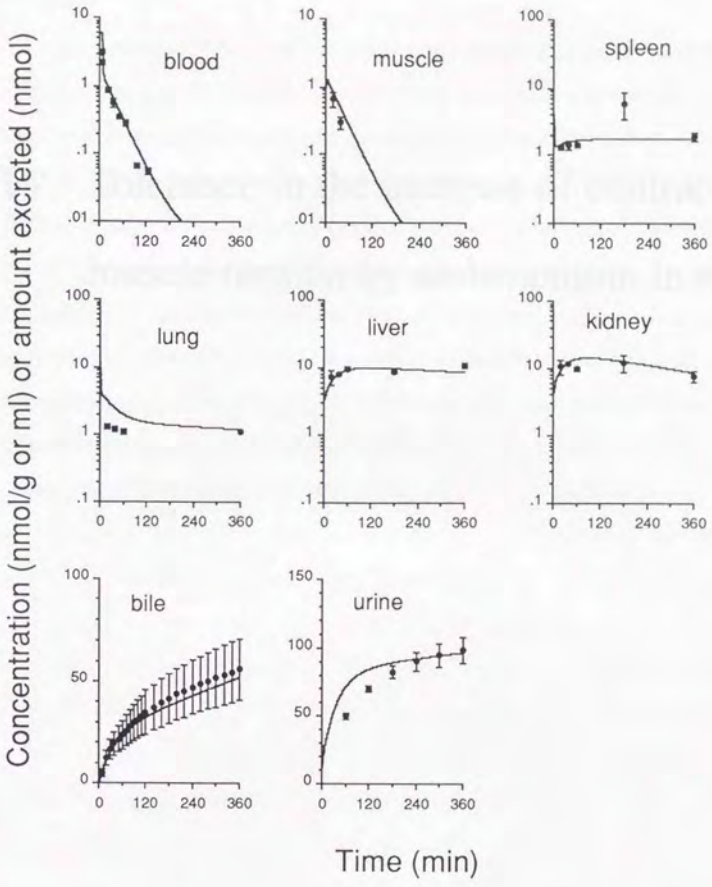

Figure 5. Tissue concentration, biliary and urinary excretion profiles after i.v. administration of ambenonium (1 μmole/kg, n = 3, mean ± S.E.). Solid lines are the simulation lines according to the physiologically based pharmacokinetic model.

IV. Tolerance in the increase of contractile muscle tension by ambenonium in rats

Abstract

The relationship between the concentration of ambenonium, a selective and reversible acetylcholinesterase (AChE) inhibitor, in plasma and the potentiation of contractile muscle tension was investigated using a sciatic nerve-muscle preparation of rat. The developed isometric contraction was enhanced dose-dependently after i.v. administration of low doses (5-20 nmol/kg) of ambenonium, but the contraction was weakened when ambenonium was administered at high doses (100-1000 nmol/kg), and the concentration-effect relationship was bell-shaped. The muscle contraction profile after 50 nmol/kg administration without previous administration, with 20 nmol/kg and with 50 nmol/kg administered previously were quite different from each other. These findings suggest that the potentiation of contractile muscle tension by ambenonium may be acutely tolerated and the concentration-effect relationship may change time-dependently.

Introduction

Reversible cholinesterase (ChE) inhibitors are used as the first choice of therapy to treat myasthenia gravis. ChE inhibitors elevate the acetylcholine (ACh) concentration at the synaptic cleft of the neuromuscular junction by AChE inhibition, and intensify contractile muscle tension. However, the relationship between the concentration of ChE inhibitors in plasma and the enhancement of contractile muscle tension have not been clearly explained. Chan and Calvey (1978) reported a positive correlation between the concentration of pyridostigmine in plasma and neuromuscular transmission function in five myasthenic patients. In contrast, Davison et al.(1981) investigated the same concentration-effect relationship in nine myasthenic patients, and found only two patients who showed a significant positive correlation between the pyridostigmine concentration in plasma and the clinical evaluation of muscle tension; a negative correlation was found in one patient. Aquilonius et al. (1983) reported on the relationship between the concentration of ChE inhibitors and the muscle response after i.v. administration of neostigmine and pyridostigmine. In their study, positive correlations were found between the drug concentration in plasma and responses to lower concentrations, but the effect declined with increases in plasma concentration at the higher concentration, again suggesting a bell-shaped concentration-effect relationship. However, the severity of disease states and dose of drugs administered were not controlled in these clinical studies with myasthenic patients. Therefore, basic animal studies under controlled conditions are required.

In this study, we investigated the time-dependent relationship between the concentration of ambenonium, a potent AChE inhibitor (Hodge et al., 1992), in plasma and the enhancement of contractile muscle tension after i.v. administration to rats.

Methods

Chemicals and reagents

Ambenonium chloride was generously supplied by Nippon Shoji Co. (Osaka, Japan). Benzilonium bromide was used as the internal standard for the HPLC method and was supplied by Sankyo Co. (Tokyo, Japan). Bovine erythrocyte AChE (EC 3.1.1.7) was used for the enzymatic determination of ambenonium and was purchased from Wako Pure Chemical Industries Co. (Osaka, Japan) for biochemical research use. All other reagents were of reagent grade and used without further purification.

Animal experiment

The general procedure for the sciatic nerve-muscle preparation was similar to that reported by Van Maanen (1950). Male Wistar rats purchased from Nippon Ikagaku Dobutsu Co. (Tokyo, Japan) weighing 300-330 g were used in all experiments. The animals had free access to a standard pellet diet (Oriental Yeast Co., Tokyo, Japan) and tap water before the experiments. Anesthesia was induced by the i.p. administration of 1000 mg/kg of urethane and 25 mg/kg of alpha-chloralose. The right femoral artery and vein were canulated for blood sampling and drug administration, respectively. The proximal end of the left sciatic nerve was ligated and Ag:AgCl electrodes were applied to its distal end. The interelectrodal distance was 3 mm. The distal end of the Achilles tendon was cut and tied to a string connected to the strain gauge (TB-611T, Nihon Kohden, Tokyo, Japan). The isometric tension was measured by a carrier amplifier (RP-5, Nihon Kohden, Tokyo, Japan) and the resting tension was adjusted to 20 g. The sciatic nerve was stimulated by an electronic stimulator (SEN-3201, Nihon Kohden, Tokyo, Japan) with square pulses at the rate of 0.067 Hz, with 0.1msec duration and 3-7 V.

The basal developed tension was monitored for 5 min before drug administration. Then 5, 10, 20, 50, 100, 300 or 1000 nmol/kg of ambenonium in saline was infused over 30 sec into the femoral vein. Contractile muscle tension was recorded continuously for 60 min and 0.5 ml of blood was taken at 2, 5, 15, 30, 45 and 60 min after i.v. administration.

In the additional dose experiments, the second dose of 50 nmol/kg was administered by 30 sec i.v. infusion following a preceding dose of 20 or 50 nmol/kg. The contractile muscle tension was recorded continuously for 30 min and the blood was taken at 7.5, 15, 22.5 and 30 min after the first administration. The plasma sample was obtained immediately by centrifugation and was stored at -20 ℃ until analysis.

Pharmacokinetic Analysis

To estimate the pharmacokinetic parameters, A, B, α and β, the concentration of ambenonium in plasma obtained in all single bolus dose experiments were simultaneously fitted to Equation 1 by the non-linear least squares method. The total body clearance (CL_{tot}), the steady state volume of distribution (Vd_{SS}) and the elimination half life ($t_{1/2}$) were calculated according to equations 2, 3 and 4, respectively.

$$Cp = Dose \cdot (A \cdot \exp(- \alpha \cdot t) + B \cdot \exp(- \beta \cdot t)) \quad (1)$$

$$CL_{tot} = \frac{\alpha \cdot \beta}{A \cdot \beta + B \cdot \alpha} \quad (2)$$

$$Vd_{SS} = \frac{A \cdot \beta^2 + B \cdot \alpha^2}{(A \cdot \beta + B \cdot \alpha)^2} \quad (3)$$

$$t_{1/2} = \frac{\ln 2}{\beta} \quad (4)$$

where C_p is the concentration of ambenonium in plasma, Dose is the i.v. dose administered and t is the time after administration.

Assay of ambenonium in Plasma

The concentration of ambenonium in plasma after administering 300-1000 nmol/kg was determined by the HPLC method (Yamamoto et al., 1988). Samples obtained from the lower dose studies (5-100 nmol/kg) were assayed by the enzymatic method (Yamamoto et al., 1993).

Results

The plasma concentration profiles of ambenonium and the increasing ratios of contractile muscle tension after i.v. bolus administration are shown in Figures 1 and 2, respectively. The concentration-time curves could be fitted to the two exponential equation (see Method), and no dose dependency was observed (Figure 1). The CL_{tot}, Vd_{SS} and $t_{1/2}$ were 6.01 ± 1.81 ml/min/kg, 0.494 ± 0.176 L/kg, and 63.5 ± 43.2 min, (optimal estimates ± S.E., n = 20) respectively.

The increase in tension in the contractile muscle following the administration of low doses was slow, and the increasing ratio was positive and dose dependent (Figure 2A). In contrast, a transient increase followed by a rapid decrease of the contractile muscle tension was observed after the administration of high doses, and the contractile tension at 60 min was decreased as the dose increased (Figure 2B). Figure 3 shows the relationship between the plasma concentration and the potentiation of muscle tension after the i.v. bolus administration of ambenonium. A bell-shaped relationship was observed, suggesting the depressive effect of ambenonium at high concentrations.

To confirm the decrease in contractile potency of ambenonium at high concentrations, additional doses of 50 nmol/kg of ambenonium were administered intravenously 15min after the first administration of ambenonium. The concentration time profiles of ambenonium in plasma after the additional administration were very close to the simulation lines of the pharmacokinetic parameters estimated by a single bolus administration study (Figure 4). On the other hand, the pattern of the contractile muscle tension was considerably different from the single dose study. Following a preceding administration of 20 nmol/kg, a slight increase in muscle tension was observed after the second administration, though the extent of tension increase was much smaller than that after single doses of 50 - 100 nmol/kg. Furthermore, a decrease in contractile muscle tension was observed after the second administration following the preceding administration of 50 nmol/kg of ambenonium (Figure 5).

Discussion

Reversible ChE inhibitors, such as ambenonium, are the first choice for the therapeutic treatment of myasthenia gravis. However, the relationship between the clinical effects of these drugs and their concentration in plasma has not been clearly explained. A positive correlation was found between the effect and the concentration in plasma in some patients, but not in other patients (Davison et al., 1981). To establish a rational drug therapy, it is necessary to understand the concentration-effect relationship based on the controlled study of the pharmacokinetics and pharmacodynamics of these inhibitors in experimental animals, without variations due to the difference in disease states, drug dosage, and/or other factors that complicate human studies.

As shown in Figure 3, a bell-shaped relationship between plasma concentration and the increasing ratio of contractile muscle tension after the i.v. bolus administration of ambenonium was observed. Aquilonius et al. (1983) also reported a similar relationship in myasthenic patients after the i.v. bolus administration of pyridostigmine or neostigmine. In their study, a positive relationship between plasma concentration and effect was seen when the plasma concentration was lower than 30-60 ng/ml for pyridostigmine and 5-15 ng/ml for neostigmine, but higher concentrations gave a negative correlation between plasma concentration and effect. Similarity of the bell-shaped concentration-effect relationship among these drugs suggested that it is a general characteristic of acetylcholinesterase inhibitors.

Thus, the plasma concentration after an additional dose of 50 nmol/kg ambenonium is substantially the same as the estimated value from the single dose study (Figure 3). Therefore, the pharmacokinetic behavior of ambenonium is not thought to be changed by any preceding administration of ambenonium. By contrast, the profiles of contractile muscle tension were considerably different between the first and the second dose. Especially, the increasing ratio of muscle tension after the additional administration of ambenonium following the preceding administration of 50 nmol/kg was slightly decreased (Figure 5).

One possibility for this difference may be a desensitization of the ACh receptor induced by the first administration of ambenonium. Acute desensitization of an ACh receptor at a high concentration of ambenonium may lead to the bell-shaped and time-dependent concentration-effect relationship. Another possibility could be due to between difference of the drug concentration in plasma and in the receptor site, as Aquilonius et al.(1983) discussed. If the transport rate of the drug to the effective site is slow, the concentration of the drug at the effective site would be high and show a direct antagonistic effect on the ACh receptor, similar in its bell-shape and time dependent relationship. A small volume of distribution (0.49 L/kg) was inconsistent with the accumulation of drug in the deep tissues, but the possibility of accumulation to very small effective site such as synaptic cleft was not excluded. Therefore, the mechanism of change in the concentration-effect relationship remains to be clarified.

The reduction of the ratio of potentiation of contractile muscle tension after the first administration of ambenonium in this study may play a role in the concentration independency of the clinical effect of ChE inhibitors. Pyridostigmine potentiated a muscle twitch caused by the electronic stimulation of a nerve at 0.2-0.4 mM and depressed it at more than 0.8 mM (Pascuzzo et al., 1984). In that study, pyridostigmine did not affect membrane potential or muscle action potential, but a change in the number and/or properties of conducting channels at the synaptic cleft were indicated. Pyridostigmine was also found to inhibit the binding of ACh; therefore, the mechanism of the twitch depression caused by pyridostigmine was thought to involve a weak agonist action or the formation of desensitized receptor -complex intermediates.

In this study, the plasma concentration of ambenonium at 15 min after 20 nmol/kg administration was similar to those in myasthenic patients reported previously (Tharasse-Bloch et al., 1987, 1991). However the plasma concentration of ambenonium is about 10 times higher than values reported by other investigators (Ohtsubo et al., 1989, 1992). For other AChE inhibitors, pyridostigmine and neostigmine, bell-shaped plasma concentration-

effect relationships were observed during the clinical treatment of myasthenic patients. The pyridostigmine concentration that induced the desensitization of ACh receptors (50-100 μM) (Bradley et al., 1986) was considerably higher than the plasma concentration in myasthenic patients (30-700 nM) (Aquilonius et al., 1980, Breyer-Pfaff et al., 1990, Calvey and Chan, 1977, Cohan et al., 1976). Furthermore, neuromuscular function did not change after repeated doses of pyridostigmine at a dose of 90 mg/day for 8 days (Glikson et al., 1991). It may be necessary to consider the possibility that adverse effects relate to the wide variations in the effective concentrations of ChE inhibitors.

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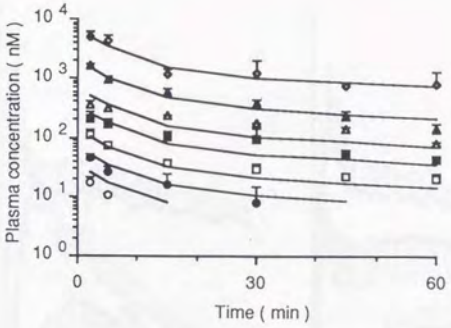

Figure 1 The plasma concentration profile of ambenonium after i.v. bolus administration. ○: 5 nmol/kg, ●: 10 nmol/kg, □: 20 nmol/kg, ■: 50 nmol/kg, △, 100 nmol/kg, ▲: 300 nmol/kg、◇: 1000 nmol/kg. Data represent the mean ± S.E., n=3-4. The solid lines are the regression lines obtained by simultaneous least squares fitting of all the data to the equation: $Cp = Dose \cdot (A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(- \beta \cdot t))$. Cp (nM), Dose (nmol/kg), t (min), A = 5.41 ± 0.544 (kg/L), $\alpha = 0.181 \pm 0.047$ (min^{-1}), B = 1.38 ± 0.416 (kg/L), $\beta = 0.0101 \pm 0.0064$ (min^{-1}). Parameter values are the optimal estimates and the S.E., n=20.

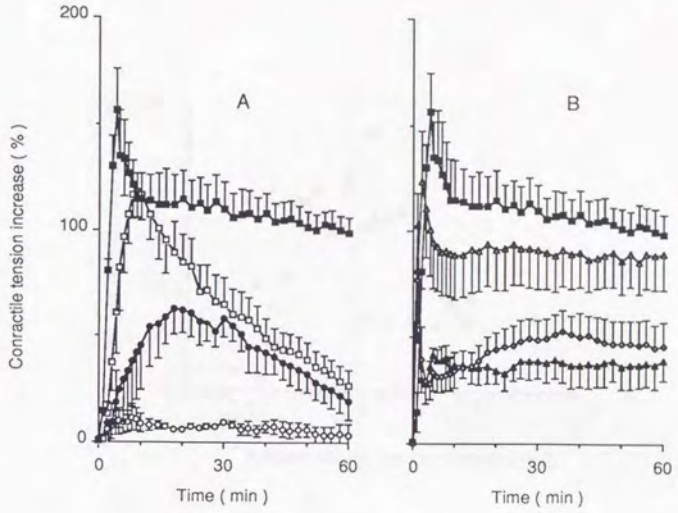

Figure 2 The increase of muscle tension after i.v. bolus administration of ambenonium. ○: 5 nmol/kg, ●: 10 nmol/kg, □: 20 nmol/kg, ■: 50 nmol/kg, △: 100 nmol/kg, ▲: 300 nmol/kg, ◇: 1000 nmol/kg. Data represent the mean ± S.E., n = 3-4.

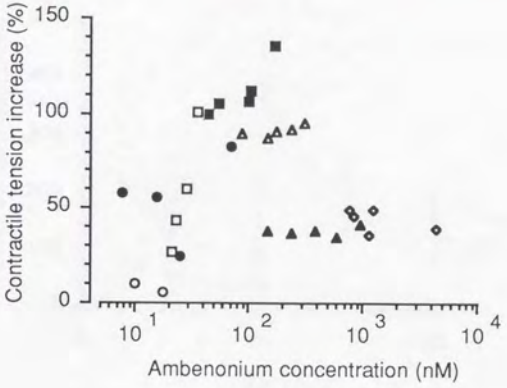

Figure 3 The relationship between the ambenonium concentration and the potentiation of muscle tension after i.v. bolus administration of ambenonium. An increasing ratio of muscle tension was plotted according to the estimated plasma concentration of ambenonium at 2, 5, 15, 30, 45 and 60 min after i.v. bolus administration of ambenonium. ○: 5 nmol/kg, ●: 10 nmol/kg, □: 20 nmol/kg, ■: 50 nmol/kg, △: 100 nmol/kg, ▲: 300 nmol/kg, ◇: 1000 nmol/kg. Data represent the mean value of 3-4 rats.

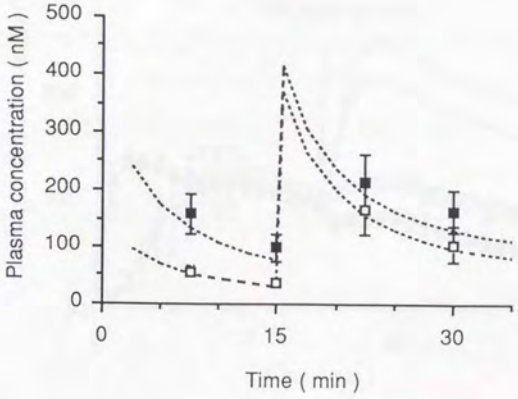

Figure 4 The plasma concentration of ambenonium after additional administration. □: An additional 50 nmol/kg was administered at 15 min after the preceding 20 nmol/kg administration ■: An additional 50 nmol/kg was administered at 15 min after the preceding 50 nmol/kg administration. Data represent the mean ± S.E., n=3. The solid lines are the simulation curves calculated using the pharmacokinetic parameters obtained from the single dose experiments shown in Figure 1.

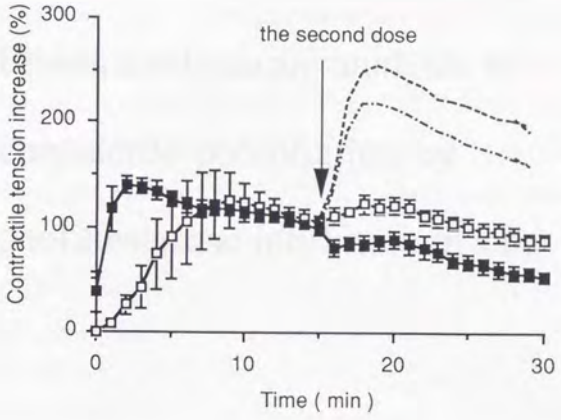

Figure 5 The increase of muscle tension after additional i.v. administration of ambenonium □: Additional 50 nmol/kg was administered at 15 min after the preceding 20 nmol/kg administration ■: Additional 50 nmol/kg was administered at 15 min after the preceding 50 nmol/kg administration. Data represent the mean ± S.E., n=3. The lines are the simulation curves calculated by the addition of effect profiles observed in single dose experiments. The dotted line: 50 nmol/kg administered at time 0 min and 20 nmol/kg administered at time 15 min. The broken line: 50 nmol/kg administered at time 0 min and 15 min.

V. Pharmacodynamic analysis of contractile potentiation by cholinesterase inhibitors in rats

ABSTRACT

Pharmacological profiles of four cholinesterase (ChE) inhibitors, edrophonium, pyridostigmine, neostigmine and ambenonium after i.v. administration to rats were analyzed by the pharmacodynamic model considering acetylcholinesterase (AChE) inhibition, direct antagonism to the nicotinic receptor and desensitization of nicotinic receptor. Pharmacokinetics of these drugs were dose-independent and had similar volume of distribution at steady state (Vdss) values of 0.4-0.6 L/kg among various doses. Neostigmine has the shortest elimination half life ($t_{1/2}$) and edrophonium, pyridostigmine and ambenonium were in the order. Inhibitory constants of ChE inhibitors to bovine erythrocyte AChE determined *in vitro* were 2010, 276, 26 and 3.7 nM for edrophonium, pyridostigmine, neostigmine and ambenonium, respectively. The maximum value of contractile tension after i.v. administration decreased at high dose of each drug and the dose-response curves were bell-shaped. Time courses of plasma concentration and contractile muscle tension were fitted to the pharmacodynamic model to estimate the association/dissociation rate constants to AChE and nicotinic receptor. Significant correlation between inhibitory constants of ChE inhibitors to AChE estimated by *in vivo* pharmacodynamic analysis and those determined by *in vitro* enzyme kinetic study was shown, while relationship between dissociation constants to nicotinic receptor estimated by *in vivo* pharmacodynamic analysis and those measured by *in vitro* binding study were unclear. It seems that the contribution of other process such as desensitization induced by endogenous acetylcholine (ACh), diffusion rate of drugs into synaptic cleft, action of presynaptic receptors, etc., cannot be neglected to explain the dose-effect relationship of ChE inhibitors. In conclusion, pharmacodynamics of ChE inhibitors may be described by the developed model considering the AChE inhibition and desensitization of nicotinic receptor, though antagonistic mechanisms for muscle contraction remain unclear.

Introduction

Reversible ChE inhibitors are used as the first choice of therapy for myasthenia gravis. ChE inhibitors elevate the ACh concentration at the synaptic cleft of the neuromuscular junction by AChE inhibition, and intensify contractile muscle tension. However, the relationship between the concentration of ChE inhibitors in plasma and the enhancement of contractile muscle tension have not been clearly explained.

Chan and Calvey (1978) reported a positive correlation between the concentration of pyridostigmine in plasma and neuromuscular transmission function in five myasthenic patients. In contrast, Davison et al. (1981) investigated the same concentration-effect relationship in nine myasthenic patients, and found only two patients who showed a significant positive correlation between the pyridostigmine concentration in plasma and the clinical evaluation of muscle tension; a negative correlation was found in one patient. Aquilonius et al. (1983) reported on the relationship between the concentration of ChE inhibitors and the muscle response after i.v. administration of neostigmine and pyridostigmine. In their study, positive correlations were found between the drug concentration in plasma and responses at lower concentrations, but the effect declined with increases in plasma concentration at the higher concentration, suggesting a bell-shaped concentration-effect relationship. In our previous study (Yamamoto et al., 1994), we investigated the relationship between the concentration of ambenonium, a potent selective AChE inhibitor, in plasma and the enhancement of contractile muscle tension after i.v. administration to rats. The developed isometric contraction was enhanced dose-dependently after i.v. administration of low doses (5-20 nmol/kg) of ambenonium, but the contraction was weakened when ambenonium was administered at high doses (100-1000 nmol/kg), and the concentration-effect relationship was bell-shaped. There was quite a difference in the muscle contraction profile after 50 nmol/kg administration with 20 nmol/kg, with 50 nmol/kg and without pretreatment of ambenonium. These findings suggested that the potentiation of contractile muscle tension by ambenonium may be acutely tolerated and the

concentration-effect relationship may change time-dependently.

Kordas et al. (1975) showed that low concentration of prostigmine, with which activity of AChE was not completely inhibited, increased the amplitude, rise time and the half time of the end-plate current, while higher concentration of prostigmine depressed the end-plate current. Slater et al. (1986) showed the potentiation of ACh-induced current at the low concentration of neostigmine and pyridostigmine and antagonized at higher concentration using voltage-clamped *Aplysia* neurons. Edrophonium, pyridostigmine and neostigmine interacted directly with ACh-activated ion-channels and altered their kinetics in cell-attached patches of BC3H1 mouse tumor cell (Wachtel, 1990), and similar results were obtained with frog sartorius muscle (Pascuzzo et al., 1984). These actions may be distinct from AChE inhibition caused by the drugs, and three mechanism of action, a weak agonist action, the formation of desensitized receptor-complex intermediates and the alteration of the conductance properties of active channels were suggested (Pascuzzo et al., 1984).

Many *in vivo* and *in vitro* studies described above suggested the bell-shaped pharmacological characteristics of ChE inhibitors, but comparative pharmacodynamics and *in vivo-in vitro* relationship of ChE inhibitors have never been investigated. In order to clarify the determinant of pharmacodynamics of ChE inhibitors, a very short-acting, a short-acting and two long-acting ChE inhibitors, edrophonium, neostigmine, pyridostigmine and ambenonium, were administered to rats and the time course of plasma concentration and contractile muscle tension were monitored. Then, pharmacological profiles of these drugs were analyzed by the pharmacodynamic model considering AChE inhibition, direct antagonism to the nicotinic receptor and desensitization of nicotinic receptor. Furthermore, comparison between Ki value estimated under *in vivo* condition and those under *in vitro* condition was carried out.

Methods

Chemicals and reagents

Ambenonium chloride was generously supplied by Nippon Shoji Co. (Osaka, Japan). Edrophonium chloride, neostigmine bromide and pyridostigmine bromide were purchased from SIGMA (U.S.A.). Bovine erythrocyte AChE (EC 3.1.1.7) was used for the enzymatic determination of Ambenonium and was purchased from Wako Pure Chemical Industries Co. (Osaka, Japan) for biochemical research use. All other reagents were of analytical grade and used without further purification.

Pharmacokinetic study

The plasma concentration-time profiles of edrophonium (2 - 20 μmole/kg), neostigmine (0.1 - 0.5 μmole/kg) and pyridostigmine (0.2 - 5 μmole/kg) after i.v. administration to rats were studied in this study. The pharmacokinetic parameters after i.v. administration of ambenonium (0.005 - 1 μmole/kg) in rats estimated in previous study was used for further analysis. Male Wistar rats purchased from Nippon Ikagaku Dobutsu Co. (Tokyo, Japan) weighing 300-330 g were used in all experiments. The animals had free access to a standard pellet diet (Oriental Yeast Co., Tokyo, Japan) and tap water before the experiments. Anesthesia was induced by the i.p. administration of 1000 mg/kg of urethane and 25 mg/kg of alpha-chloralose. The right femoral artery and vein were canulated for blood sampling and drug administration, respectively. At 1, 3, 5, 10, 15, 20 and 30 min after i.v. administration of edrophonium and neostigmine, and at 2, 5, 15, 30, 45 and 60 min for pyridostigmine, 0.5 ml of blood was taken and the plasma was obtained immediately by centrifugation. Sample was stored at -20 ℃ until analysis. The concentration of edrophonium, neostigmine and pyridostigmine in plasma was determined by the HPLC method (Ruyter and Cronnelly, 1980). Unbound fraction in plasma was determined by ultrafiltration method using MPS-3 centrifree system (Amicon, U.S.A.).

To estimate the pharmacokinetic parameters, A, B, α and β, the concentration of ChE

inhibitors in plasma obtained in all single bolus dose experiments were simultaneously fitted to Equation 1 by the non-linear least squares method. The total body clearance (CL_{tot}), the steady state volume of distribution (Vdss) and the elimination half life ($t_{1/2}$) were calculated according to Equations 2, 3 and 4, respectively.

$$Cp = Dose \cdot (A \cdot \exp(- \alpha \cdot t) + B \cdot \exp(- \beta \cdot t)) \quad (1)$$

$$CL_{tot} = \frac{\alpha \cdot \beta}{A \cdot \beta + B \cdot \alpha} \quad (2)$$

$$Vd_{SS} = \frac{A \cdot \beta^2 + B \cdot \alpha^2}{(A \cdot \beta + B \cdot \alpha)^2} \quad (3)$$

$$t_{1/2} = \frac{\ln 2}{\beta} \quad (4)$$

where Cp is the concentration of ChE inhibitors in plasma, Dose is the i.v. dose administered and t is the time after administration.

Pharmacodynamic study

The increase of muscle tension after i.v. bolus administration of edrophonium (0.5 - 10 μmole/kg), neostigmine (0.02 - 0.5 μmole/kg) and pyridostigmine (0.2 - 5 μmole/kg) after i.v. administration to rats were determined in this study. For ambenonium, data after i.v. 0.01 - 0.1 μmole/kg in rats in previous study (Yamamoto et al., 1994) was used for pharmacodynamic analysis. Animals for pharmacodynamic study were treated surgically under the same procedure for pharmacokinetic study. The general procedure for the sciatic nerve-muscle preparation was carried out as reported previously (Yamamoto et al., 1994). Briefly, the proximal end of the left sciatic nerve was ligated and Ag:AgCl electrodes were applied to its distal end. The interelectrodal distance was 3 mm. The distal end of the Achilles tendon was cut and tied to a string connected to the strain gauge (TB-611T, Nihon Kohden, Tokyo, Japan). The isometric tension was measured by a carrier amplifier (RP-5,

Nihon Kohden, Tokyo, Japan) and the resting tension was adjusted to 20 g. The sciatic nerve was stimulated by an electronic stimulator (SEN-3201, Nihon Kohden, Tokyo, Japan) with square pulses at the rate of 0.067 Hz, with 0.1msec duration and 3-7 V. The basal developed tension was monitored for 5 min before drug administration, drug was infused over 30 sec into the femoral vein, then contractile muscle tension was recorded continuously during experiments.

Inhibitory constants of ChE inhibitors to AChE

Esterase activity was measured by the thiocholine method as reported previously (Yamamoto et al., 1993). Briefly, bovine erythrocyte AChE (400 unit/L) in phosphate buffer (50 mM, pH 7.2) was used as the standard enzyme solution. To 3 ml of 0.1 mg/ml 5,5'-dithiobis-2-nitrobenzoic acid in phosphate buffer, 0.1 ml of enzyme solution and 0.1 ml of ChE inhibitor solution were added. After preincubation at 37 ℃ for 10 min, 0.1 ml of acetylthiocholine in water (2.7 mM or 5.4 mM as final concentration) was added, then incubated at 37 ℃. Exactly 10 min after the substrate solution was added, the enzyme reaction was stopped by addition of 3 ml of iced neostigmine bromide (0.1 mM). Sample was stand on ice for 10 min, then absorbance at 412 nm was measured in a spectrophotometer (UV-3000, Shimadzu, Kyoto, Japan). Blank sample was prepared by substituting the phosphate buffer instead of enzyme solution. Enzyme reaction velocity was calculated by subtracting the absorbance of blank from that of sample.

Provided the enzyme reaction velocity is expressed by the Michaelis-Menten equation with competitive inhibition (Equation 5), rearrangement produces the Equation 6, which shows the linear relationship between the inhibitor concentration [I] and the reciprocal of the reaction velocity 1/v (Dixon plot). Inhibitory constant Ki is calculated as the x-axis value of intersection point of the line obtained under the condition of different substrate concentrations.

$$v = \frac{Vmax \cdot [S]}{Km \cdot (1 + \frac{[I]}{Ki}) + [S]} \quad (5)$$

$$1/v = (\frac{Km}{Vmax \cdot [S] \cdot Ki}) \cdot [I] + (\frac{1}{Vmax} + \frac{Km}{Vmax \cdot [S]}) \quad (6)$$

Development of pharmacodynamic model

A pharmacodynamic model was developed considering inhibitory activity to AChE and competitive antagonism of ChE inhibitors to nicotinic receptor, and desensitization of nicotinic receptor (Figure 1). Further, following propositions were assumed.

1) Concentration of ChE inhibitors at effective site

Quaternary ammonium compounds, including ChE inhibitors, can pass membrane of cells by the carrier-mediated transport and highly accumulated in the liver, kidney and some other tissues (Meijer et al., 1990). On the other hand, quaternary ammonium compounds are highly hydrophilic and may poorly permeate through lipid membrane. Since molecular weight of ChE inhibitors are within the range of 200 to 700, these drugs may readily distribute from blood capillary to extracellular fluid but not into tissue cells in many tissues such as muscle and skin. Therefore, the drug concentration in synaptic cleft of neuromuscular junction, a site of action of ChE inhibitors, is assumed to be equal to the plasma unbound concentration (Cpf) in this model.

$$Cp \cdot fp = Cpf = Ce \quad (7)$$

where Cp, fp and Ce are the drug concentration in plasma, plasma unbound fraction and the drug concentration in site of action, respectively. Since amount of drugs bound to AChE and ACh receptor is very small, drug concentration in site of action does not change by association and dissociation to the enzyme and the receptor, therefore Cpf can be used as the input function.

2) Concentration of active ChE

Total concentration of ChE and active ChE, which is not bound to ChE inhibitors and

have ACh hydrolysis activity, are defined as E_0 and [E], respectively. The concentration of active ChE is expressed as Equation 8,

$$\frac{d[E]}{dt} = - k_{on1} \cdot Cpf \cdot [E] + k_{off1} \cdot (E_0 - [E]) \quad (8)$$

where k_{on1} and k_{off1} are association and dissociation rate constants of ChE inhibitors to ChE, respectively. ChE are also occupied by endogenous ACh, but it may be negligible since the basal concentration of ACh is much lower than the Michaelis constant of ACh hydrolysis, and the elevation of ACh concentration caused by the release of ACh from the synapse is a transient process.

3) Concentration of nicotinic receptor

Three species of nicotinic receptor are assumed in this model; [R], active receptor that can be bound to ACh and open the ion-channel, followed by the contraction of muscle fiber, [R-D], reversibly bound to ChE inhibitors, and [R*], desensitized receptor. Concentrations of [R] and [R-D] are presented as Equations 9 and 10, respectively,

$$\frac{d[R]}{dt} = - k_{on2} \cdot Cpf \cdot [R] + k_{off2} \cdot [R\text{-}D] \quad (9)$$

$$\frac{d[R\text{-}D]}{dt} = k_{on2} \cdot Cpf \cdot [R] - k_{off2} \cdot [R\text{-}D] - k_{des} \cdot [R\text{-}D] \quad (10)$$

where k_{on2}, k_{off2}, and k_{des} are the association and dissociation constants of [R] and ChE inhibitors, the desensitization rate constant from [R-D] to [R*], respectively. Occupation of receptor by endogenous ACh was neglected since the basal concentration of ACh is much lower than the dissociation constant of ACh to nicotinic receptor. Desensitization of nicotinic receptor induced by ACh was neglected, since the constant muscle contraction was produced by repetitive stimulation of sciatic nerve with a frequency of 0.067 Hz within 2 hr in preliminary study without drug administration. Further, the restoration of [R] from [R*] is assumed to be negligible in this model for the simplicity of the model.

4) ACh concentration at synaptic cleft and the increasing ratio in muscle tension

Actually, ACh is released by the deporalization of nerve to elevate the ACh

concentration in the synaptic cleft and associated with ACh receptor to open the ion-channel and depolarized the post synaptic membrane, before ACh is quickly hydrolyzed by AChE and ACh concentration returns to the basal level. ChE inhibitors elevate the open probability of ion-channel by prolongation of ACh turn-over. However, it is substantially impossible to simulate these mili-second order process for an hour order period, a usual time-span for pharmacokinetic/dynamic studies. Therefore, the changes of ACh concentration and contractile muscle tension are approximated by following pseudo-steady state model in this study.

The release of ACh from the presynaptic membrane is assumed to be the constant rate of k_0, and ACh is eliminated by ChE. If the elimination rate of ACh is proportional to the active ChE concentration [E] and ACh concentration, the ACh concentration in synaptic cleft is expressed as Equation 11,

$$\frac{d[ACh]}{dt} = k_0 - k_e \cdot [E] \cdot [ACh] \tag{11}$$

where k_e is the second order elimination rate constant of ACh. Before drug administration [E] equals to E_0, and the ACh concentration is retained at the constant level of ACh_0 as shown in Equation 12.

$$ACh_0 = K_0/ke/E_0 \tag{12}$$

Considering a short period of a few seconds, the ACh concentration may reach the pseudo-steady state condition depending on [E], since turn-over of ACh is a mili-second-order process and the change of drug concentration is a minute-order process. With this assumption, the pseudo-steady state ACh concentration is approximated in Equation 13.

$$[ACh] = \frac{k_0}{k_e \cdot [E]} = [ACh_0] \cdot \frac{[E_0]}{[E]} \tag{13}$$

Contractile muscle tension is proportional to the amount of nicotinic receptor-ACh complex or open ion-channel, therefore the increase ratio (Effect) of contractile tension is

$$\text{Effect} = \left(\frac{\frac{[ACh]}{Kd + [ACh]}}{\frac{[ACh_0]}{Kd + [ACh_0]}} \cdot \frac{[R]}{[R_0]} - 1\right) \cdot 100\ (\%) \qquad (14)$$

where Kd is the dissociation constant of ACh to the nicotinic receptor.

Data analysis

The mean drug concentration in plasma after each dose administration was fitted to the Equation 1 by simultaneous least squares method. The mean increasing ratio of contractile muscle tension after each dose administration of ChE inhibitors are fitted to Equations 7-14 by simultaneous least squares method to estimate the k_{on1}, k_{off1}, k_{on2}, k_{off2}, k_{des} and Kd/ACh_0. Plasma concentration profile calculated by the pharmacokinetic parameters estimated in pharmacokinetic study was used as the input functions. Further, the inhibition constant of AChE under *in vivo* condition Ki1 ($= k_{off1}/k_{on1}$) and the dissociation constant of ChE inhibitors to ACh receptor under *in vivo* condition Ki2 ($= k_{off2}/k_{on2}$) were calculated.

Results

Pharmacokinetics of ChE inhibitors

Plasma concentration profiles and pharmacokinetic parameters after intravenous administration of edrophonium (2 - 20 μmole/kg), pyridostigmine (0.2 - 5 μmole/kg), neostigmine (0.1 - 0.5 μmole/kg) and ambenonium (0.005 - 1 μmole/kg) to rats are shown in Figure 2 and Table 1, respectively. Plasma concentration profiles of these drugs are represented by sum of 2 exponential. No dose-dependency was observed within the dose range in pharmacokinetic experiments. Four ChE inhibitors have similar Vdss values within the range of of 0.4-0.6 L/kg. Neostigmine has the shortest $t_{1/2}$ and edrophonium, pyridostigmine and ambenonium are in the order.

Inhibitory effect of ChE inhibitors on AChE activity

Dixon plot concerning inhibitory activity of ChE inhibitors to bovine erythrocyte AChE determined *in vitro* were shown in Figure 3. Inhibitory constants are 2010, 276, 26 and 3.7 nM for edrophonium, pyridostigmine, neostigmine and ambenonium, respectively.

Pharmacodynamics of ChE inhibitors

Time courses of contractile muscle tension after intravenous administration of edrophonium (0.5 - 10 μmole/kg), pyridostigmine (0.2 - 5 μmole/kg), neostigmine (0.02 - 0.5 μmole/kg) and ambenonium (0.01-0.1 μmole/kg) to rats are shown in figure 4. The maximum value of contractile tension decreased at high dose of each drug and dose-response curves are bell-shaped (Figure 5). The time to reach maximum effect is shortened with increase of dose in all drugs (Figure 4). Fitting lines according to the developed model and the estimated parameters are shown in Figure 4 and Table 2.

Theoretically, Ki1 ($= k_{off1}/k_{on1}$) value of each drug represents the inhibitory constant to AChE under *in vivo* condition. Relationship between Ki1 value and the inhibitory constant of each ChE inhibitors to AChE determined by *in vitro* experiment was shown in figure 6. Significant correlation was observed between these parameters, but the slope is

smaller than 1. On the other hand, significant correlation was not found between Ki2 (= k_{off2}/k_{on2}) value and the dissociation constant of each ChE inhibitor to nicotinic receptor determined by previous *in vitro* binding study (Seifert and Eldefrawi, 1974) (Figure 7).

Discussion

Myasthenia gravis is a disease with abnormality in the neurotransmission by acetylcholine, caused by autoimmune response against the nicotinic receptor. ChE inhibitors, which raise the ACh concentration by inhibition of AChE, are widely used for the treatment of patients with myasthenia gravis. However, there is wide interindividual variation of the therapeutic effect of ChE inhibitors, and the relationship between the dose or plasma concentration and the clinical response remains unclear.

Most of ChE inhibitors are readily excreted in urine and their plasma elimination half lives in human are 24-45 min for edrophonium (Calvey et al., 1976), 1.52 hr for pyridostigmine (Aquilonius et al., 1980), 0.87 hr for neostigmine (Aquilonius et al., 1979). Elimination half life of ambenonium in human has not been reported. On the other hand, variation of their duration of the clinical effect (edrophonium: very short acting, neostigmine; short acting, pyridostigmine and ambenonium: long acting) is so large that we cannot explain the duration of action by disappearance of drugs from plasma. Therefore, we tried to clarify the relationship between the plasma concentration of ChE inhibitors, AChE inhibition activities and the potentiation of muscle contraction in this study.

Plasma elimination half lives of ChE inhibitors in rats are reported as 81-175 min for edrophonium (Calvey et al., 1976), 19 min for pyridostigmine (Birtley et al., 1966), 147 min for neostigmine (Roberts et al., 1965a) and 22-24 min for ambenonium (Yamamoto et al., 1991). However, they cannot be compared simply because experimental conditions such as dose and sampling schedule are different. Elimination half lives estimated in this study under anesthetizing condition are rather short (Table 1), which may be due to the short experimental period of less than 90 min.

Though several investigations on the relationship between the plasma concentration of ChE inhibitors and clinical responses were reported in myasthenic patients, it remains unclear because of the large interindividual difference. Breyer-Pfaff et al. (1990) reported that 100 ng/ml or higher plasma concentration of pyridostigmine was effective, while White

et al. (1981) showed that the plasma concentration in uncontrolled group (46-370 ng/ml) was higher than that of controlled group (21-155 ng/ml). Pyridostigmine concentration in plasma was well correlated to the clinical effect in the study of Chan and Calvey (1978), while only two patients showed a significant positive correlation between the pyridostigmine concentration in plasma and the clinical evaluation of muscle tension in other study (Davison et al., 1981).

Aquilonius et al. (1983) investigated the plasma concentration of neostigmine or pyridostigmine and the muscle contraction after i.v. administration of these drugs in myasthenic patients. The concentration-effect relationships for both drugs were bell-shaped and the maximum effect was obtained with 30-60 ng/ml and 5-15 ng/ml for pyridostigmine and neostigmine, respectively. They discussed the possibility that the drug concentration in plasma did not reflect the concentration in effective site. We previously investigated the relationship between the plasma concentration of ambenonium and the potentiation of muscle contraction in rats, and found the relationship between the plasma concentration and effect was bell-shaped (Yamamoto et al., 1994). Furthermore, pharmacokinetics of ambenonium did not change in repeated administration, while muscle potentiation after repeated dose of ambenonium was quite different from that after single dose, which suggested the development of acute tolerance time-dependently.

Kordas et al. (1975) showed that the reversible ChE inhibitors eserine and prostigmine increased the amplitude of the end-plate current at low concentrations that did not inhibit the ChE completely, while they depressed it at high concentrations that almost completely inhibited the ChE. They discussed that the change in the end-plate current observed at a relatively high concentration of reversible ChE might relate to a presynaptic or postsynaptic curare-like action. Pascuzzo et al., (1984) reported that pyridostigmine at concentration of 0.2-0.4 mM potentiated the indirect evoked muscle twitch and at more than 0.8 mM depressed the indirect twitch with an IC_{50} of about 2 mM in frog sartorius muscle. They suggested that pyridostigmine directly influenced on neuromuscular transmission by at least

three mechanisms distinct from AChE inhibition, that is, the weak agonist action, the formation of desensitized receptor-complex intermediates, and alteration of the conductance properties of active channels. Slater et al. (1986) investigated the mechanism of action of ChE inhibitors in *Aplysia* neurons, and concluded that both neostigmine and pyridostigmine showed the cholinergic action caused by AChE inhibition at low concentrations, while these drugs showed the antagonistic action, which may be associated with activation and desensitization of the ACh response. In their study, the relationships between the concentration of ChE inhibitors and ACh-induced currents were bell-shaped, and maximal potentiation was obtained at 100 nM and 10 μM for neostigmine and pyridostigmine, respectively.

In the present study, a pharmacodynamic model for potentiation of muscle contraction was developed considering elevation of ACh level by AChE inhibition, competitive inhibition to ACh binding to nicotinic receptor, and the formation of desensitized drug-receptor complex. Presynaptic action of ChE was neglected for simplicity. A good correlation was observed between the inhibitory constant determined by *in vitro* enzyme kinetic study and estimated from *in vivo* pharmacodynamic study (Figure 6). Bimolecular association (k_{on}) and dissociation (k_{off}) rate constant of ambenonium to AChE estimated by Hodge et al. (1992) were $5.2 \times 10^7 M^{-1}sec^{-1}$ and $0.013 sec^{-1}$. The dissociation constant estimated in the present study was similar to the reported value, while the association rate constant in the present study was 1000 times larger than that estimated by Hodge et al. (1992). The inhibitory constants estimated by pharmacodynamic analysis were different from those determined by *in vitro* study, which may be due to the difference of morphological condition of AChE. A good correlation between onset of action and affinity to ACh receptors of neuromuscular blocking agents (Kopman, 1989, Donati and Meistelman, 1991, Min et al., 1992), of which site of action is also on post synaptic membrane in synaptic cleft, and not association/dissociation rate but diffusion rate of drugs in synaptic cleft is considered as the rate limiting step of these drugs (Glavinovic et al.,

1992). A neuromuscular blocking agents with higher affinity interacts with ACh receptor more frequently, then the diffusion rate through synaptic cleft and onset time of action is slower. Assuming similar process for ChE inhibitors, the association rate of a drug with higher affinity to AChE may be underestimated. Since ambenonium is the most potent for AChE inhibition, the diffusion rate through synaptic cleft may be slow, and as a result, the inhibitory constant may be overestimated (Figure 6).

Affinity of ChE inhibitors to nicotinic receptor estimated by pharmacodynamic analysis were ambenonium < neostigmine < edrophonium < pyridostigmine in the ascending order, and they did not correlate to the *in vitro* value determined by Seifert and Eldefrawi (1974) (Figure 7). The inhibitory activity of edrophonium to AChE was weaker than that of pyridostigmine, while the estimated affinity to nicotinic receptor was stronger than that of pyridostigmine, which was consistent with the previous *in vitro* study (Seifert and Eldefrawi, 1974). The EC_{50} values for reduction of the time constant of open time distributions in cell-attached patches of BC3H1 mouse tumor cell were 3.8, 4.6, 97 μM for edrophonium, neostigmine and pyridostigmine, respectively (Wachtel, 1990). Dissociation constants of ambenonium to muscarinic receptors were reported as 1 μM (Kenakin and Beek, 1985), but the affinity to nicotinic receptor has not been reported.

To explain the mechanism of bell-shaped concentration-response curve, decrease of ACh release due to direct action of ChE inhibitors to presynaptic muscarinic receptor was proposed (Duncan and Publicover, 1979). Quantal content was decreased by neostigmine in frog neuromuscular junction (Duncan and Publicover, 1979) and ACh release was decreased by pyridostigmine in rat muscle (Anderson, 1987). Further, frequency of miniature-end-plate potentials was suppressed by tetraisopropylpyrophosphoramide, a non-specific ChE inhibitor, but not by ambenonium, a specific AChE inhibitor(Duncan and Publicover, 1979). They suggested the possibility that ACh release is controlled by presynaptic muscarinic receptor, and the ACh level around the presynaptic membrane is controlled by non-specific ChE. Assuming this mechanism, it is considered that antagonistic action

should be well correlated to the inhibitory activity to non-specific ChE. However, the inhibitory activity of edrophonium to non-specific ChE is 100 times weaker than that of pyridostigmine (Fujii and Namba, 1982), which is not consistent with the result in this study. Morphological change of neuromuscular junction developed by long-term administration of ChE inhibitors are well known, but it is considered that ChE inhibitors are not affecting on muscle in a short period (Anderson, 1987).

Several pharmacodynamic analyses of tolerance was found for cocaine (Chow et al., 1985), nicotine (Porchet et al., 1988) and frosemide (Hammarlund et al., 1985). In the model for pharmacodynamics of cocaine and nicotine, the effect compartment model involving hypothetical antagonistic metabolites were considered. In the pharmacodynamic model of frosemide, effect of the drug assumed to decrease time-dependently. For ChE inhibitors, pharmacodynamic analysis of reversal effect to neuromuscular blocking reagents was studied (Verotta et al., 1991, Morris et al., 1981), however, limited information of concentration-effect relationship of ChE inhibitors could be obtained since concentration and effect of neuromuscular reagent also changed time dependently.

Inhibitory constant to AChE estimated from *in vivo* effect well correlated to that measured in *in vitro* enzyme kinetic study, though absolute values were much different. Significant correlation could not be observed between dissociation constants of ChE inhibitors to nicotinic receptor determined by model analysis and by *in vitro* binding study reported previously. It may be necessary to consider the desensitization induced by ACh, the diffusion rate of drugs into synaptic cleft, action of presynaptic receptors, etc., to establish the dose-effect relationship of ChE inhibitors.

In conclusion, pharmacodynamics of ChE inhibitors may be described by the developed model considering the AChE inhibition and desensitization of nicotinic receptor, though the antagonistic mechanism for muscle contraction remain unclear.

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Table 1 Pharmacokinetic parameters of ChE inhibitors in rats.

Parameters	edrophonium	neostigmine	pyridostigmine	ambenonium*
A (μM/(μmol/kg))	5.51 ± 1.03	5.62 ± 0.61	6.42 ± 0.11	5.41 ± 0.54
$\alpha(min^{-1})$	0.64 ± 0.18	0.74 ± 0.07	0.25 ± 0.05	0.18 ± 0.05
B (μM/(μmol/kg))	0.59 ± 0.29	0.83 ± 0.07	0.97 ± 0.27	1.38 ± 0.42
β (min^{-1})	0.049 ± 0.028	0.076 ± 0.049	0.019 ± 0.007	0.010 ± 0.006
CL_{tot} (ml/min/kg)	48.4 ± 4.2	53.8 ± 1.1	12.9 ± 1.5	6.0 ± 1.8
Vdss (L/kg)	0.608 ± 0.283	0.446 ± 0.037	0.483 ± 0.099	0.494 ± 0.172
$t_{1/2}$ (min)	14.1 ± 8.1	9.1 ± 0.6	37.5 ± 13.6	68.6 ± 43.1

* Pharmacokinetic parameters of ambenonium are obtained from the previous report (Yamamoto et al., 1994)

Table 2 Pharmacodynamic parameters of ChE inhibitors in rats.

	edrophonium	pyridostigmine	neostigmine	ambenonium
A_0/Kd	0.0538 ± 0.0298	0.193 ± 0.036	0.155 ± 0.079	0.179 ± 0.070
k_{on1} ($min^{-1} \cdot \mu M^{-1}$)	0.517 ± 0.008	0.112 ± 0.006	1.42 ± 0.08	2.44 ± 1.04
k_{off1} (min^{-1})	0.468 ± 0.060	0.0631 ± 0.0323	0.0354 ± 0.0032	0.0397 ± 0.0215
Ki1 (=k_{off1}/k_{on1}, μM)	0.905	0.563	0.025	0.016
k_{on2} ($min^{-1} \cdot \mu M^{-1}$)	0.234 ± 0.046	0.0170 ± 0.0032	0.425 ± 0.137	0.639 ± 0.186
k_{off2} (min^{-1})	1.316 ± 0.511	0.199 ± 0.023	0.127 ± 0.033	0.106 ± 0.014
Ki2 (=k_{off2}/k_{on2}, μM)	5.62	11.7	0.299	0.166
k_{des} (min^{-1})	0.0773 ± 0.0140	0.0101 ± 0.0019	0.0466 ± 0.0132	0.00869 ± 0.00181

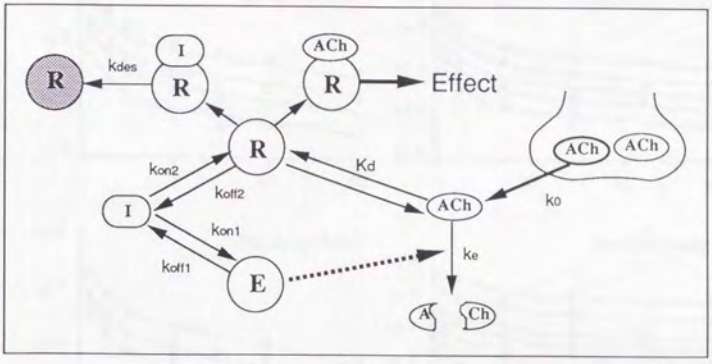

Figure 1 Schematic illustration of the proposed pharmacodynamic model for the change of contractile muscle tension after i.v. administration of ChE inhibitors to rats. A; acetic acid, ACh; acetylcholine, Ch; choline, E; acetylcholinesterase, I; cholinesterase inhibitor, R; nicotinic receptor.

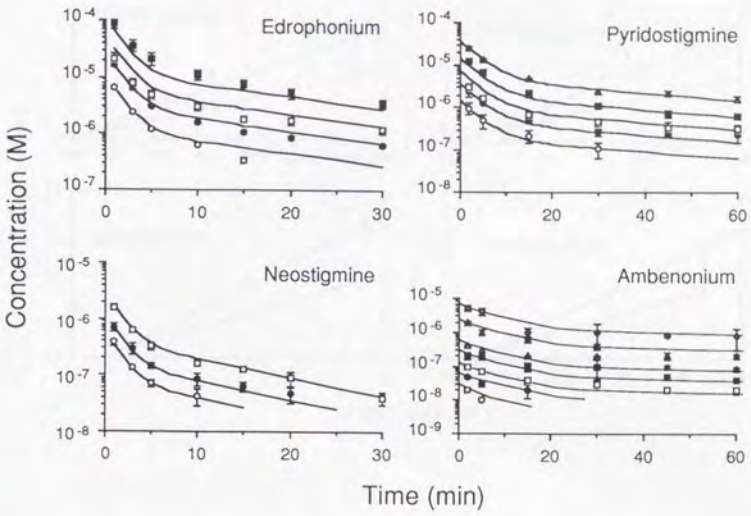

Figure 2 Time courses of concentration in plasma after i.v. administration of ChE inhibitors. Edrophonium (○: 2, ●: 5, □: 10 and ■: 20 μmole/kg), pyridostigmine (○: 0.2, ●: 0.5, □:1, ■: 2 and △: 5 μmole/kg) and neostigmine (○: 0.1, ●: 0.2 and □: 0.5 μmole/kg) were administered intravenously to rats. The data for ambenonium (○: 0.005, ●: 0.01, □: 0.02, ■: 0.05, △: 0.1, ▲: 0.3 and ◇: 1 μmole/kg) is obtained in previous study (Yamamoto et al., 1994). Each symbol and vertical bar represents mean and standard deviation of 3-6 experiments.

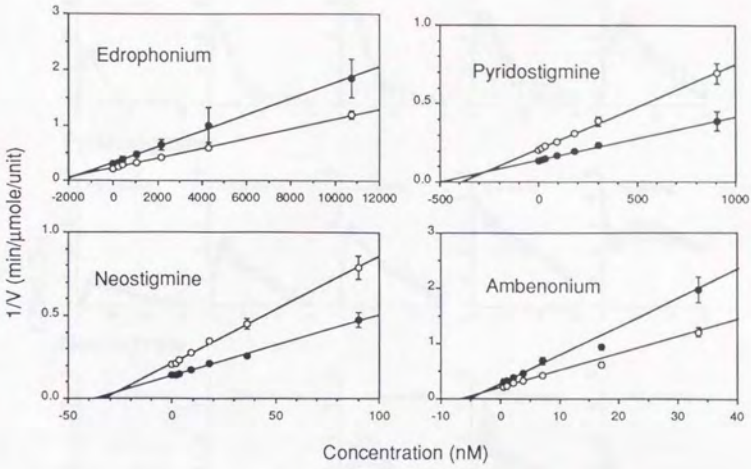

Figure 3 Dixon plot of AChE inhibition by ChE inhibitors. Final acetylthiocholine concentrations are ●: 30 mM and ○: 15 mM. Each symbol and vertical bar represents mean and standard deviation of 3 experiments.

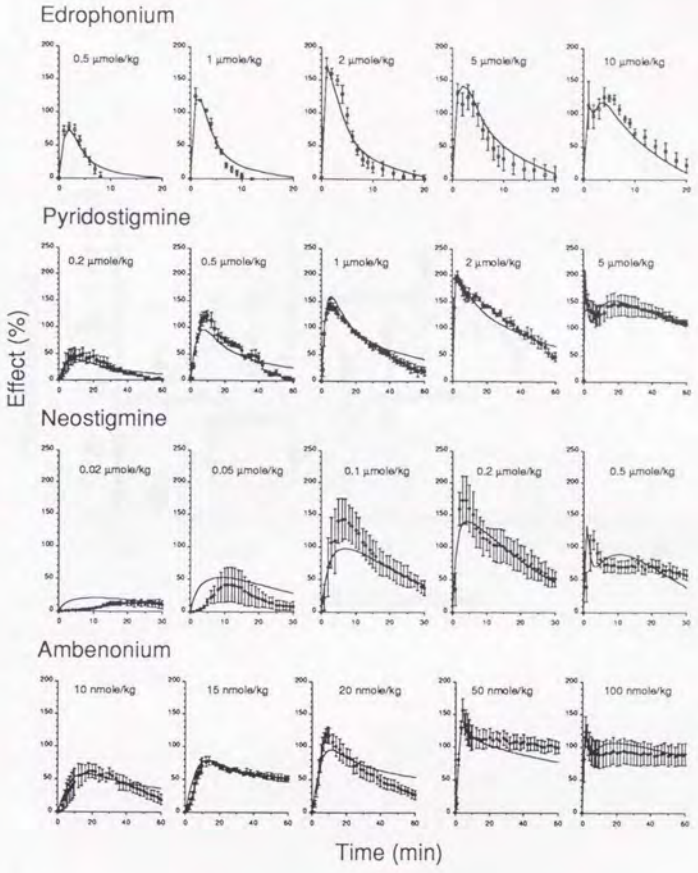

Figure 4 Time courses of increase in muscle tension after i.v. administration of ChE inhibitors. Edrophonium (2 - 20 μmole/kg), pyridostigmine (0.2 - 5 μmole/kg) and neostigmine (0.1 - 0.5 μmole/kg) were administered intravenously to rats. The data for ambenonium is obtained in previous study (Yamamoto et al., 1994). Each symbol and vertical bar represents mean and standard deviation of 3-6 experiments. □: Edrophonium, ■: Pyridostigmine, ○: Neostigmine, ●: Ambenonium.

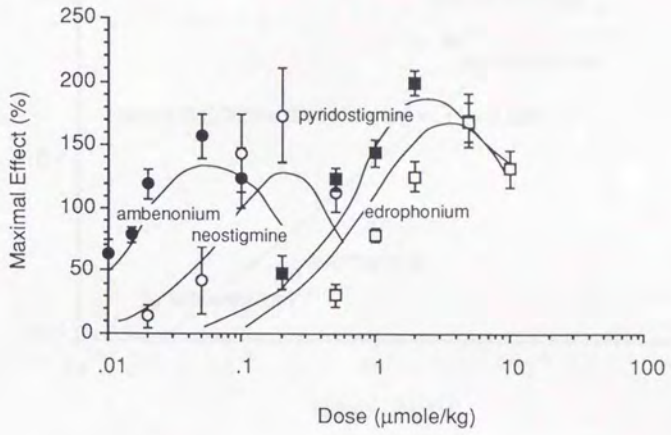

Figure 5 Dose-response relationship of ChE inhibitors. Maximal potentiation of muscle contraction versus i.v. dose of each ChE inhibitor was plotted. The data for ambenonium is obtained in previous study. Symbols are the mean of 3-6 experiments. Lines are the estimated line according to the pharmacodynamic model. Parameters are listed in Table 2. □: Edrophonium, ■: Pyridostigmine, ○: Neostigmine, ●: Ambenonium.

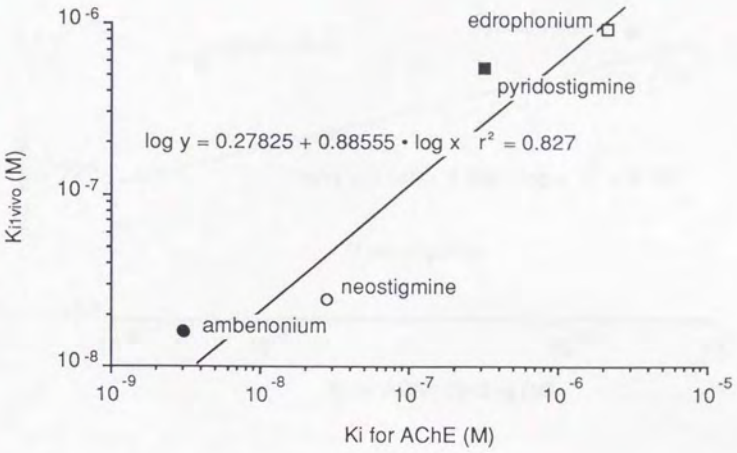

Figure 6 In vitro-in vivo relationship of AChE inhibition. Inhibitory constants of ChE inhibitors to AChE determined by in vitro enzyme inhibition study versus in vivo Ki value estimated from potentiation of contractile muscle tension is plotted. □: Edrophonium, ■: Pyridostigmine, ○: Neostigmine, ●: Ambenonium.

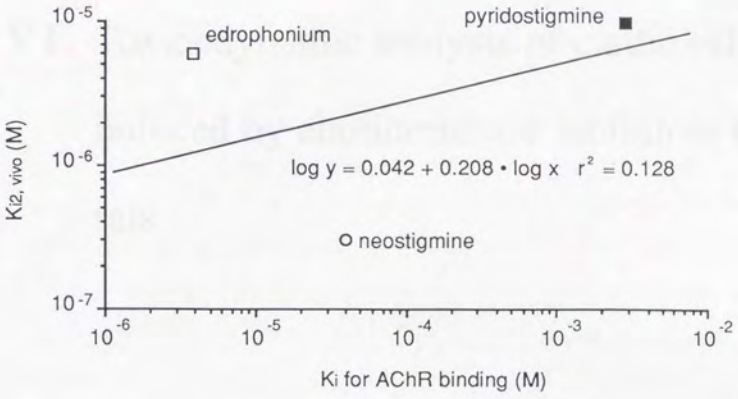

Figure 7 In vitro-in vivo relationship of ChE binding to nicotinic receptor. Binding affinity of ChE inhibitors to nicotinic receptor reported by Seifert and Eldefrawi in 1974 determined by in vitro binding study and in vivo Ki2 value estimated from pharmacodynamic study. □: Edrophonium, ■: Pyridostigmine, ○: Neostigmine, ●: Ambenonium.

VI. Toxicodynamic analysis of cardic effect induced by cholinesterase inhibitors in rats

Abstract

Cardiac effect of edrophonium (2 - 20 μmole/kg), pyridostigmine (0.5 - 5 μmole/kg), neostigmine (0.05 - 0.5 μmole/kg) and ambenonium (0.02-0.3 μmole/kg) was investigated after i.v. administration to rats. After administration of pyridostigmine and neostigmine, the heart rate decreased with dose dependent manner, and then gradually recovered to the basal level at about 10 min. Rapid decrease of heart rate was observed after edrophonium and ambenonium administration, and rapidly recovered to the basal level within a minute. As for ambenonium, dose-dependent tachycardiac response was observed thereafter. The time-course of heart rate change was analyzed by effect compartment model. Significant correlation was observed between bradycardiac EC_{50} value obtained by effect compartment model analysis and inhibitory constant (Ki) to acetylcholinesterase in vitro, suggesting that bradycardiac response was induced by inhibition of this enzyme and following elevation of acetylcholine concentration in synaptic cleft. On the other hand, tachycardiac EC_{50} of edrophonium and ambenonium based on effect compartment model analysis were similar to dissociation constants (Kd) of these drugs to muscarinic receptors in vitro, suggesting that the tachycardiac activity of these drugs may be associated with antagonistic activity to postsynaptic muscarinic receptors.

As clinical implication, we could discuss effectiveness and safety of four drugs from point of view of pharmacokinetics, pharmacodynamics and toxicodynamics.

Introduction

Cholinesterase (ChE) inhibitors are used for the reversal from the postoperative neuromuscular blockade or for the treatment of myasthenia gravis. ChE inhibitors elevate the acetylcholine (ACh) level in the synaptic cleft by inhibition of acetylcholinesterase (AChE), and potentiate the skeletal muscle contraction (Aquilonius and Hartvig, 1986, Fujii and Namba, 1982). One of common side effects of ChE inhibitors is bradycardia. It is suggested that ChE inhibitors produce bradycardia by preventing the hydrolysis of ACh released from the parasympathetic neurons and following stimulation of cardiac muscarinic M_2 receptor (Blumental et al., 1968, Roberts and Konjovic, 1969). Recently, Backman et al. (1993) reported that pirenzepine, a selective M_1 antagonist, could not block bradycardia produced by vagul nerve stimulation at the dose that blocked the neostigmine-induced bradycardia. They concluded that the neostigmine-induced bradycardia may result from the presynaptic muscarinic receptor stimulation, which leads the ACh release. Furthermore, ambenonium, a selective AChE inhibitor, has antagonistic effect to muscarinic receptor (Kanekin & Beek, 1985) and induces tachycardia (Brown et al., 1982). The mechanisms of chronotropic effect of ChE inhibitors are unclear and the intensity of cardiac action has not been evaluated among ChE inhibitors. It is necessary to know what is the determinant of chronotropic effect of ChE inhibitors for the appropriate drug selection. In this study, toxicodynamics concerning chronotropic effect of four ChE inhibitors (edrophonium, neostigmine, pyridostigmine and ambenonium) after i.v. administration to rats was investigated. The time-course of plasma concentration and heart rate change was analyzed by effect compartment model (Sheiner et al., 1979). Then, relationship between the toxicodynamic parameters and the in vitro biochemical parameters (i.e., AChE inhibition and affinity to muscarinic receptor) was discussed.

Methods

Chemicals and reagents

Ambenonium chloride was generously supplied by Nippon Shoji Co. (Osaka, Japan). Edrophonium chloride, neostigmine bromide and pyridostigmine bromide were purchased from SIGMA (U.S.A.). All other reagents were of analytical grade and used without further purification.

Animal experiments

Male Wistar rats purchased from Nippon Ikagaku Dobutsu Co. (Tokyo, Japan) weighing 300-330 g were used in all experiments. The animals had free access to a standard pellet diet (Oriental Yeast Co., Tokyo, Japan) and tap water before the experiments. Anesthesia was induced by the i.p. administration of 1000 mg/kg of urethane and 25 mg/kg of alpha-chloralose. After that, polyethylene cannula SP-31 (Natsume, Tokyo) was inserted into right carotid artery and connected onto pressure transducer (TNF-R, Viggo-Spectramed, Singapore).

The left femoral veins were cannulated with PE-50 (Becton Dickinson, U.S.A.) for drug administration. Heart rate was enumerated by electrically amplified arterial pressure signal with a heart rate counter (AT-621G, Nihon-Kohden, Tokyo). The body temperature was maintained at 37.5 ± 0.3°C by means of temperature controller CMA/150 (Stockholm, Sweden). Edrophonium (2 - 20 μmole/kg), pyridostigmine (0.5 - 5 μmole/kg), neostigmine (0.05 - 0.5 μmole/kg) or ambenonium (0.02-0.3 μmole/kg) was administered intravenously. Higher dose study could not be done, since decrease of heart rate to less than 50% caused severe toxicity to animals. Blood pressure and heart rate were monitored throughout the experiments.

Data analysis

The chronotropic effect was described as the percent change in heart rate change from the basal value. Toxicodynamic analysis was carried out by effect compartment model analysis (Sheiner et al., 1979). Pharmacokinetic parameters of edrophonium (0.1 - 0.5

μmole/kg), pyridostigmine (0.2 - 5 μmole/kg), neostigmine (0.1 - 0.5 μmole/kg) and ambenonium (0.005 - 1 μmole/kg) after i.v. administration were determined previously. The plasma concentration profiles of ChE inhibitors used as the input functions for effect compartment analysis were obtained from previous report.

For edrophonium and ambenonium, two effect compartments (Figure 1) were assumed, one for bradycardiac effect compartment and the other for tachycardiac effect compartment (Equations 1 and 2). Concentration-effect relationship for both effect was assumed to be represented by Emax model (Equations 3 and 4), and heart rate observed was assumed as the sum of both effect (Equation 5).

$$\frac{dC_{e,\,Brady}}{dt} = k_{1e,Brady} \cdot C_P - k_{e0,\,Brady} \cdot C_{e,\,Brady} \quad (1)$$

$$\frac{dC_{e,\,Tachy}}{dt} = k_{1e,Tachy} \cdot C_P - k_{e0,\,Tachy} \cdot C_{e,\,Tachy} \quad (2)$$

$$E_{Brady} = \frac{E_{max,\,Brady} \cdot C_{e,\,Brady}}{EC_{50,\,Brady} + C_{e,\,Brady}} \quad (3)$$

$$E_{Tachy} = \frac{E_{max,Tachy} \cdot C_{e,\,Tachy}}{EC_{50,\,Tachy} + C_{e,\,Tachy}} \quad (4)$$

$$E = E_{Brady} + E_{Tachy} \quad (5)$$

where $C_{e,Brady}$ and $C_{e,Tachy}$ are the drug concentration in bradycardiac and tachycardiac effect compartment, $E_{Brady}(\%)$ and $E_{Tachy}(\%)$ are the bradycardiac and tachycardiac effect, respectively.

For pyridostigmine and neostigmine, only bradycardiac effect compartment was considered for these drugs (Equation 6), since clear bradycardiac action was not observed.

$$E = E_{Brady} \quad (6)$$

Maximal bradycardiac effect was fixed to 100% in all cases. Heart rate change from basal level is fitted to Equations 5 or 6 by nonlinear least squares method (Yamaoka et al., 1981) to estimate the pharmacodynamic parameters.

Results

The mean values of heart rate, systolic and diastolic pressure of rats before drug administration under urethane-chloralose anesthesia were 400.5 ± 37.5 beat/min, 109.4 ± 12.9 mmHg and 49.0 ± 10.6 mmHg (n = 48, mean ± S.D.), respectively. The profiles of heart rate change after i.v. administration of ChE inhibitors are shown in Figure 2. Each ChE inhibitor showed quite different pattern from each other. Rapid and dose-dependent decrease of heart rate was observed after edrophonium administration, and rapidly recovered to the basal level within a minute. After administration of pyridostigmine and neostigmine, the heart rate decreased dose-dependently, followed gradual return to the basal level within 15 min. Sustained and dose-dependent tachycardia was observed after the transient bradycardia induced by ambenonium. From these findings, two effect compartments regarding bradycardia and tachycardia were considered for edrophonium and ambenonium, while only bradycardiac effect compartment was assumed for pyridostigmine and neostigmine.

The estimated toxicodynamic parameters were listed in Table 1, and the simulation lines calculated with these parameters were shown as solid lines in Figure2. The profiles of heart rate change might be expressed by these effect compartment models. As shown in Figure 3, the EC_{50} values of four drugs for bradycardiac effect determined by in vivo toxicodynamic study were significantly correlated with the slope of unity to their inhibitory constants to AChE determined by in vitro enzyme kinetic study determined previously. The EC_{50} values of edrophonium and ambenonium for tachycardiac effect determined by in vivo toxicodynamic study were similar to the dissociation constants of these drugs to muscarinic receptor (Brown et al., 1982) determined by in vitro binding study as shown in Figure 4.

Discussion

It has been generally considered that ChE inhibitors induce bradycardia by preventing the hydrolysis of ACh released from parasympathetic neurons and following stimulation of cardiac M_2 receptor (Blumental et al., 1968, Roberts and Konjovic, 1969). Gardner and Allen (1977) also reported that ChE inhibitors decreases the muscarinic receptor-mediated cAMP production to induce bradycardia. Recently, the role of ChE inhibitors as agonists for muscarinic receptor has been noticed. Backman et al. (1993) reported that bradycardia was attenuated after ACh depletion in the cardiac parasympathetic pathway, suggesting that the release of ACh from parasympathetic neuron is essential for bradycardia induced by ChE inhibitors. They discussed that neostigmine evokes bradycardia by activation of muscarinic receptors on cardiac ganglion cells producing ACh release. Since atropine and pirenzepine blocked neostigmine-induced bradycardia but McN-A-343 (muscarinic M_1 agonist) did not produce bradycardia, the receptor on cardiac ganglion cells should not be an M_1 type. Further, the dose of pirenzepine that blocked the neostigmine-induced bradycardia was lower than that for blocking bradycardia produced by vagul nerve stimulation, and the pharmacological identity of this receptor remains unclear. Ambenonium, a selective AChE inhibitor, acts as antagonist to muscarinic receptors on post synaptic membrane (Kenekin & Beek, 1985). In fact, Brown et al. (1982) showed the tachycardiac activity of ambenonium after i.p. administration to rats, which is consistent with the results of our study.

Bradycardiac response after administration of pyridostigmine or neostigmine lasted for about 15 min, while the bradycardia induced by edrophonium and ambenonium disappeared within a minute. In case of ambenonium, dose-dependent tachycardiac change was observed thereafter. In order to describe these chronotropic profiles, two effect compartments were assumed for edrophonium and ambenonium, while only bradycardiac effect compartment was considered for pyridostigmine and neostigmine.

Significant correlation with the slope of unity was observed between EC_{50} value

estimated in this study and inhibitory constants to AChE of these drugs determined by previous in vitro enzyme kinetic study as shown in Figure 3, suggesting that bradycardiac activity of these drugs may be resulting from AChE inhibition and following elevation of ACh concentration in synaptic cleft. The dose-dependency of bradycardiac response after i.v. administration of ambenonium was unclear, which may be due to substantial complete inhibition of AChE by ambenonium in the dose range studied.

Tachycardiac EC_{50} of edrophonium and ambenonium were close to dissociation constant of these drugs for muscarinic receptors (Figure4), suggesting that the tachycardiac activity of these drugs may be associated with antagonistic activity to postsynaptic muscarinic receptors. Dissociation constants of neostigmine and pyridostigmine (140 μM, Brown et al., 1982) were much higher than the plasma concentration in this study, therefore it is not surprising to evoke undetectable bradycardiac effect for these drugs.

The inhibitory potency to AChE was smaller, the $k_{e0,\ Brady}$, a parameter representing transfer rate of drug from central compartment to bradycardiac effect compartment was smaller. In case of edrophonium and ambenonium, $k_{e0,\ Brady}$ and $k_{e0,\ Tachy}$ were substantially the same, suggesting the site of action to be the same. For neuromuscular blocking agents, good correlation is reported between the onset of action and the affinity to ACh receptors (Kopman, 1989, Donati and Meistelman, 1991, Min et al., 1992), and their site of action is also on post synaptic membrane in synaptic cleft. It is not association / dissociation rate but diffusion rate of drugs in synaptic cleft is considered as the rate limiting step of these drugs (Glavinovic et al., 1992). A neuromuscular blocking agents with higher affinity interacts with ACh receptor more frequently, then diffusion rate through synaptic cleft and onset time of action is slower. Assuming similar process for ChE inhibitors, transfer rate of a drug with higher affinity to AChE from central compartment to effective site may be delayed.

Figure 5 shows the dose-response relationship of ChE inhibitors for bradycardiac and

tachycardiac effect as side effects, and muscle contraction as a pharmacological effect estimated in previous pharmacodynamic study. Maximum values of contractile tension increase are substantially the same among drugs, suggesting the same potency as the anti-myasthenic drugs. Increase of contractile tension is, as well as bradycardiac effect, associated with the AChE inhibition, therefore bradycardia is considered as an essential side effect for ChE inhibitors. Change of heart rate was, however, not induced by edrophonium and ambenonium at the dose which causes the maximum increase of contractile tension, since they have anti-muscarinic potency. From these findings, it can be thought that edrophonium and ambenonium are safer than pyridostigmine and neostigmine, at least concerning with muscarinic side effects including bradycardia.

In conclusion, pharmacological effects of ChE may appear by various mechanisms, including inhibition of AChE and non-specific ChE, direct agonistic or antagonistic action to nicotinic and/or muscarinic receptors. In this study, heart rate change after i.v. administration of four ChE inhibitors could be described by effect compartment model considering indirect agonistic action as the result of AChE inhibition and direct antagonistic action to muscarinic receptors. Contribution of presynaptic muscarinic receptors should be evaluated in further investigation. As clinical implication, we could discuss effectiveness and safety of four drugs from point of view of pharmacokinetics, pharmacodynamics and toxicodynamics.

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Table 1 Toxicodynamic parameters of ChE inhibitors in rats.

	Edrophonium	Pyridostigmine	Neostigmine	Ambenonium
Bradycardiac effect				
$k_{e0,Brady}$ (min^{-1})	11.6 ± 8.5	1.03 ± 0.28	0.761 ± 0.165	0.205 ± 0.043
$E_{max,Brady}$ (%)	-100	-100	-100	-100
$EC_{50,Brady}$ (μM)	314 ± 154	65.6 ± 5.6	3.11 ± 0.29	0.114 ± 0.019
Tachycardiac effect				
$k_{e0,Tachy}$ (min^{-1})	12.5 ± 8.5	—	—	0.217 ± 0.034
$E_{max,Tachy}$ (%)	14.3 ± 12.4	—	—	143.1 ± 3.3
$EC_{50,Tachy}$ (μM)	30.4 ± 154	—	—	0.217 ± 0.034

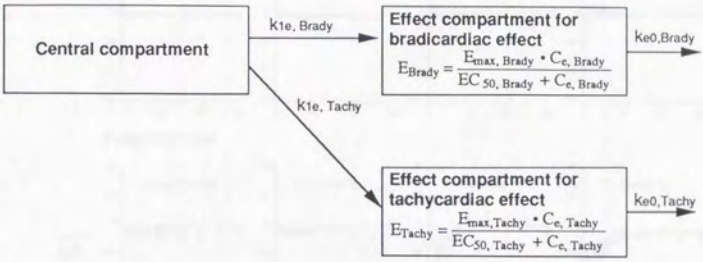


Figure 1 Effect compartment model for heart rate change induced by ChE inhibitors

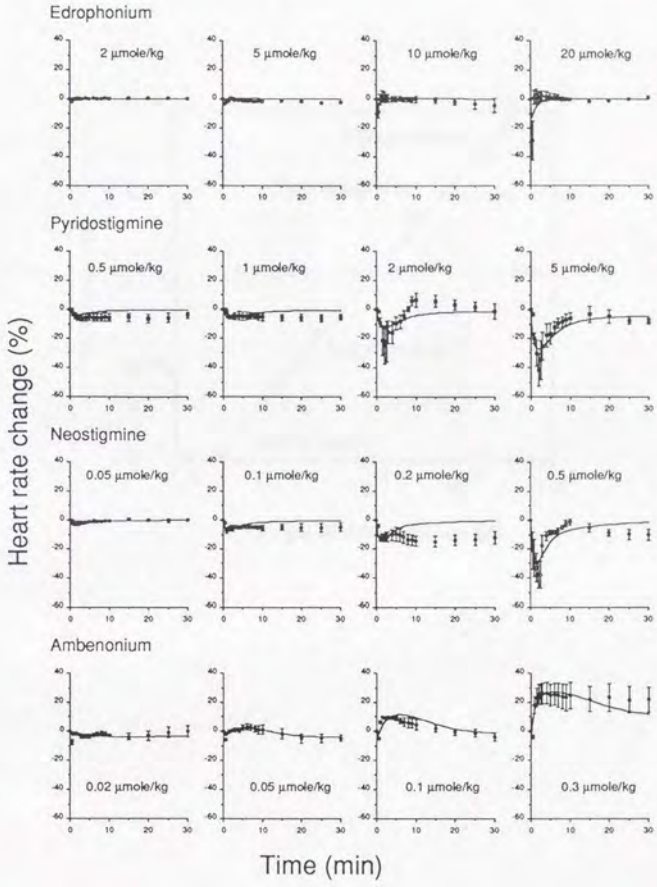


Figure 2 Time course of heart rate change after i.v. administration of ChE inhibitors. □: edrophonium, ■: pyridostigmine, ○: neostigmine and ●: ambenonium. Each symbol and vertical bar represents the mean ± S.E. of observed data (n = 3). The solid lines are the fitting line calculated according to the equation 1-6.

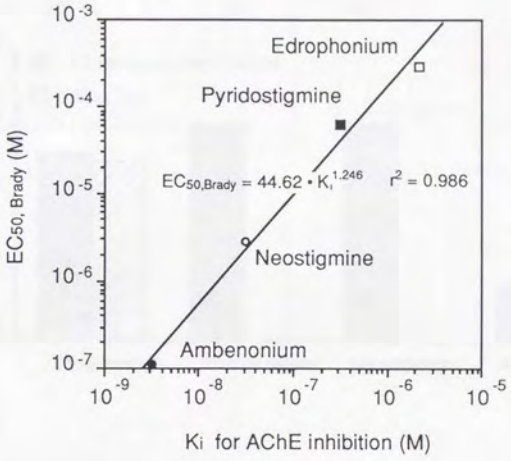


Figure 3 Relationship between the inhibitory constants of ChE inhibitors to AChE determined by in vitro enzyme kinetic study in previous pharmacodynamic study. □: edrophonium, ■: pyridostigmine, ○: neostigmine and ●: ambenonium.

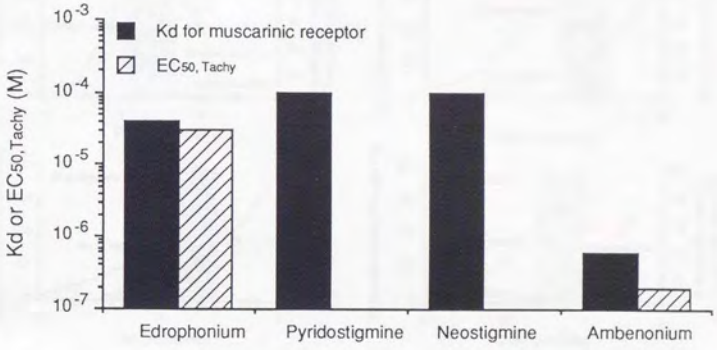


Figure 4 Comparison between the dissociation constant of ChE inhibitors to muscarinic receptor (closed column) determined by in vitro binding study (Brawn et al., 1982) to $EC_{50,\ Tachy}$ determined by in vivo pharmacodynamic study (hatched column).

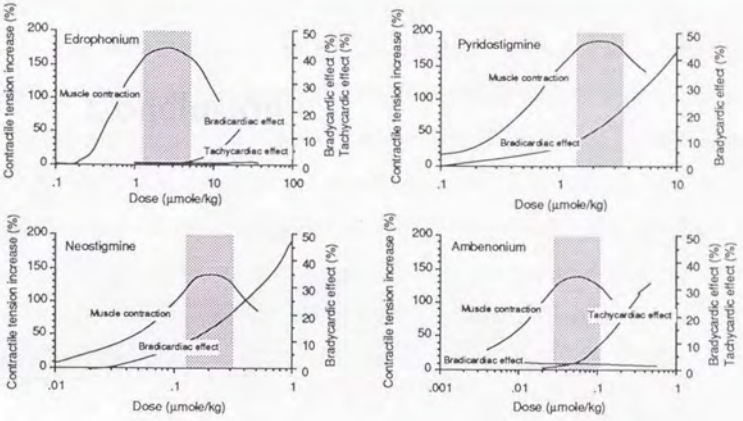


Figure 5 Dose-response relationship of ChE inhibitors for muscle contraction, bradycardiac and tachycardiac effect. Maximum value of each response was plotted. Dose-muscle contractile response is simulated according to the previous pharmacodynamic analysis.

Conclusion

Cholinesterase (ChE) inhibitors elevate the acetylcholine (ACh) concentration at the synaptic cleft of the neuromuscular junction by acetylcholinesterase (AChE) inhibition, and intensify contractile muscle tension. However, it remains unknown whether difference in pharmacokinetic behavior among ChE inhibitors reflects on that in their pharmacological characteritics because determination of ChE inhibitors in biological samples are very difficult. In this study, an HPLC and an enzymatic determination method for ambenonium, a potent ChE inhibitor, was newly developed. Furthermore, a very short-acting (edrophonium), a short-acting (neostigmine) and two long-acting ChE inhibitors (pyridostigmine and ambenonium) were administered to rats and pharmacokinetic, pharmacodynamic and toxicodynamic behavior of ChE inhibitors were compared. The results and discussion were as follows;

I. A sensitive, specific and precise method for the determination of ambenonium in plasma, bile, urine and tissues by ion-pair HPLC was newly established. It would be useful for basic pharmacokinetic study of ambenonium.

II. A rapid and simple enzymatic quantitation method for ambenonium in plasma with high sensitivity was newly developed. It should be useful for pharmacokinetic and pharmacodynamic study.

III. Rapid disappearance of ChE inhibitors from plasma may reflect the uptake to the liver and kidney. On the other hand, the main determinant of distribution volume at steady state of ChE inhibitors may be the distribution to muscle and extracellular space of other organs/tissues.

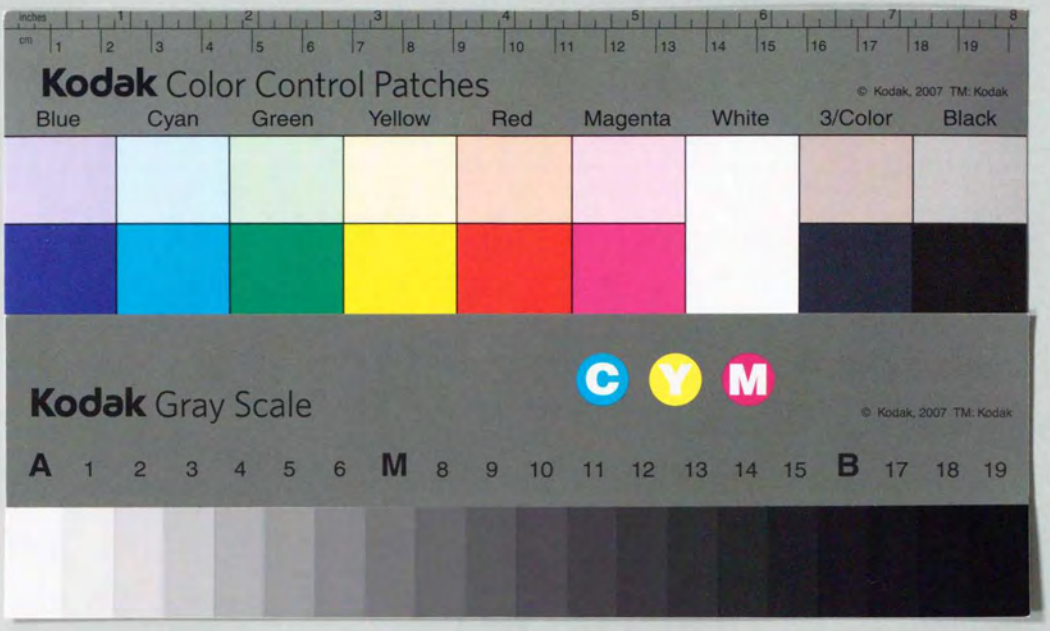
IV. A bell-shaped relationship between plasma concentration and the increasing ratio of contractile muscle tension after the i.v. bolus administration of ambenonium was demonstrated. This finding may not be due to change in pharmacokinetic behavior after preceding administration of ambenonium, while pharmacological profiles were considerably different between the first and the second dose, suggesting the time-

dependent change of concentration-effect relationship.

V. Pharmacodynamic analysis of ChE inhibitors was carried out, based on the newly developed model considering the AChE inhibition and desensitization of nicotinic receptor. Potentiation of contractile tension may be induced mainly by the inhibition of AChE at synaptic clefts, while the antagonistic mechanisms for muscle contraction remain unclear.

VI. Heart rate change after i.v. administration of four ChE inhibitors could be described by effect compartment model considering indirect agonistic action as the result of AChE inhibition and direct antagonistic action to muscarinic receptors. Change of heart rate was, however, not induced by edrophonium and ambenonium at the dose which causes the maximum increase of contractile tension, which may be due to their anti-muscarinic potency. Edrophonium and ambenonium may be safer than pyridostigmine and neostigmine, at least concerning with muscarinic side effects including bradycardia.

In this study, time courses of a primary effect and a side effect of ChE inhibitors in rats were quantitatively analyzed. However, information is quite poor about pharmacokinetics, pharmacodynamics and toxicodynamics of ChE inhibitors in human, and the dosing schedule of ChE inhibitors to myasthenic patients are designed in empirical way. To establish the effective usage of ChE inhibitors, it is essential to know pharmacokinetic, pharmacodynamic and toxicodynamic behavior of ChE inhibitors in human. Such information will be obtained by the similar way used in this animal study.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