Sensitive Determination of Ambenonium in Plasma Using Inhibitory Activity to Acetylcholinesterase

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A sensitive analytical method for the determination of ambenonium, a selective acetylcholinesterase inhibitor, in plasma was developed. The procedure involves ultrafiltration to remove endogenous plasma cholinesterase, followed by colorimetric measurement of the inhibitory activity to acetylcholinesterase by the thiocoline 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 assay procedure is applied to the pharmacokinetic study of ambenonium after intravenous administration of low dose (10—20 nmol/kg) to rat. This new method is rapid and simple and makes it possible to determine the ambenonium concentration in plasma with good accuracy.

Keywords ambenonium; acetylcholinesterase; determination; cholinesterase inhibitor; pharmacokinetics

Myasthenia gravis is a disease with abnormality in acetylcholine (ACh) neurotransmission, caused by autoimmune response against ACh receptors. Cholinesterase (ChE) inhibitors, which raise the ACh concentration by inhibiting acetylcholinesterase (AChE), are widely used for the treatment of patients with myasthenia gravis. However, there are broad 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 in quantitation of ChE inhibitors in biological specimens. Determination of edrophonium, neonigmine, pyridostigmine and ambenonium (AMB) has been reported by gas-chromatography, high-performance liquid chromatography (HPLC) and gas-chromatography—mass-spectrometric method. We used the HPLC method for the determination of AMB, a long-acting reversible ChE inhibitor, and studied its pharmacokinetic properties. The dose required to obtain the appropriate pharmacological response is quite low, however, because AMB is very potent for AChE inhibition, and the plasma concentration after administration of such a dose cannot be determined. A more sensitive means is required for pharmacokinetic/pharmacodynamic investigation of AMB. In this study, we developed a new method for the enzymatic determination of AMB using the AChE inhibition potency, and applied it to find the concentration of AMB in rat plasma after intravenous administration of a dose as low as 10—20 nmol/kg.

Experimental

Chemicals and Reagents AMB chloride was generously supplied by Nippon Shoji Kaisha (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 a commercial source, were of reagent grade and used without further purification.

Inhibitory Constants of AMB to AChE and Plasma ChE. Esterase activity was measured by the thiocholine method reported by MacQueen et al. 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 were added 0.1 ml of enzyme solution and 0.1 ml of AMB solution (0.15—62.5 nm and 0.15—62.5 μM as final concentration for AChE and plasma esterase, respectively). After preincubation at 37°C for 10 min, 0.1 ml of acetylthiocholine in water (2.7 or 5.4 mm as final concentration) was added, then the mixture was incubated at 37°C. 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 allowed to 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 for the 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), the rearrangement produces 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 the intersecting point of the line obtained under the condition of different substrate concentrations.

\[
v = \frac{V_{max} [S]}{K_m + \left(1 + \frac{[I]}{K_i}\right)} [S]
\]

\[
1/v = \frac{K_m}{V_{max} [S] \cdot K_i} \left(1 + \frac{1}{V_{max} + \frac{K_m}{V_{max} [S]}}\right)
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Assay Procedure of AMB in Plasma The scheme of AMB assay procedure is shown in Fig. 1. Endogenous plasma ChE was removed by ultrafiltration at 4°C, 2000 g, 10 min. After filtration and ultrafiltration, the concentration of AMB was determined, and the AMB concentration in plasma was calculated from the concentration of the filtrate.

![Fig. 1. Scheme of the Enzymatic Determination Procedure](image-url)
filtration with the MPS-3 centrifuge micropartition system (Amicon, U.S.A.) at 4°C, 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 a plastic tube, then 25 μl of 1 mg/ml DTNB in PB and 0.1 ml of 200 units/μl AChE were added. After preincubation at 37°C 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 left to 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 for the enzyme solution and sample plasma, respectively. Enzyme reaction velocity was calculated by subtracting the absorbance of blank from that of sample.

Plasma containing AMB at a concentration of 1, 2, 5, 10, 20, 50 and 100 mM 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/μl AChE was added to 0.1 ml of plasma filtrate, and enzyme reaction velocity was compared. Blank sample was prepared by substituting plasma filtrate with PB. Plasma from each rat was spiked with AMB, then the inhibitory activity of AChE in each rat was compared.

**Within-Day Precision** Within-day precision was determined by the triplicate analysis of the same plasma sample containing 1, 5, 20, 50 or 100 nm of AMB 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 or 100 nm of AMB on the 3 sequential days. Calibration curves were prepared each day.

**Comparison of Assayed Value of Enzymatic Method with HPLC Method** Concentration of plasma sample containing 100—5000 nm of AMB was determined by both enzymatic method and HPLC method.123 For enzymatic determination, plasma sample was diluted with drug free plasma to avoid a change in endogenous ChE activity and adsorption of AMB to the ultrafiltration apparatus.

**Recovery from Aqueous Solution and Plasma** AMB solution containing 5, 20 or 100 nm of AMB was ultrafiltered at 2000 × g for 3 min, then the concentration of AMB in the filtrate and the residue was measured. Calibration curve is prepared with AMB aqueous solution of the same concentration as plasma sample. To determine recovery ratio from plasma, AMB concentration in plasma filtrate obtained from plasma sam was compared. Drug free plasma filtrate was spiked with AMB and was used for calibration curve preparation.

**Intravenous Administration of AMB to Rats** Male Wistar rats weighing 250—330 g were anesthetized by intraperitoneal administration of 1000 mg/kg ethyl carbamate and 25 mg/kg α-chloralose. Left femoral vein and artery were catheterized for the drug administration and blood sampling, respectively. AMB was administered at the dose of 10 or 20 mmol/kg, and blood samples were taken at 2, 5, 15, 30, 45 and 60 min after administration. Blood was centrifuged and the concentration of AMB in plasma was measured by the enzymatic method. The concentration of AMB in plasma after 1000 mmol/kg administration was also measured by HPLC. Plasma concentration data were simultaneously fitted to Eq. 3 by nonlinear least squares method, where \( C_p \) (nm) is the plasma concentration and \( Dose \) (mmol/kg) in the AMB dose administered. \( A \) (kg/l), \( B \) (kg/l), \( \alpha \) (min⁻¹) and \( \beta \) (min⁻¹) are the pharmacokinetic parameters, respectively.

\[
C_p = Dose \cdot (A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t))
\]  

**Results**

**Inhibitory Effect of AMB on AChE and Plasma ChE Activity** Inhibitory activity of AMB to bovine erythrocyte AChE and rat plasma ChE is shown in Figs. 2 and 3, respectively. The values of inhibitory constants, \( K_i \), 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 minimal interindividual variation of AChE activity in plasma filtrates obtained from 3 animals (C.V. = 5.2%). Interindividual variation in the inhibitory activity of AMB to AChE added to plasma filtrates was also rather small (C.V. = 1.8—6.9%, Fig. 5).

**Precision of Determination Method** Within-day precision and between-day precision are shown in Tables I and II, respectively. Reduction in enzyme reaction rate could be detected at the concentration of 1 nm as lower limit, but the variation of value was large, especially in the 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, the average of two measurements was 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 (Fig. 6). There was 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 AMB in plasma filtrate should be lower. To confirm the validity of our assay method, the recovery ratio from the aqueous solution or plasma to filtrate was determined (Table III). The concentration of AMB in filtrate and the residue of aqueous solution is much lower than the initial concentration, suggesting strong adsorption of AMB to the microparticulate 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 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.
Pharmacokinetic Analysis after Intravenous Administration of AMB to Rat

Plasma concentration–time profiles of AMB after intravenous administration is shown in Fig. 7. These profiles showed a good fit to the two exponential equations (Eq. 3).

Discussion

Though ChE inhibitors are widely used in the treatment of myasthenia gravis, neither pharmacokinetics nor pharmacodynamics has yet been clearly elucidated because of the difficulty in monitoring drug level in biological fluid. Edrophonium, pyridostigmine, neostigmine and AMB as ChE inhibitors are used clinically for the treatment and/or diagnosis of myasthenia. Since the inhibitory activity of AMB to AChE is stronger and more selective than the others, a therapeutic dose of AMB is smaller than the others. Therefore, the therapeutic concentration of AMB to elicit an appropriate pharmacological effect should also be lower. To study the pharmacokinetics and pharmacodynamics of AMB requires a highly sensitive assay method. Moreover, simple and rapid quantitation for the dose adjustment must be used to achieve the rational drug therapy in the clinical situation.

To date, only three HPLC methods have been reported for the determination of AMB in biological samples. The first reported by Tharasse-Bloc et al. in 1987 has made it possible to determine AMB concentration in plasma. Knowledge of the concentration of AMB in bile, urine and tissues became possible by the more precise and selective method reported by the authors. The detection limit of these HPLC methods is only about 20 nm, however, which seems inadequate to investigate pharmacodynamics. A more sensitive HPLC method, which requires the ion-exchange extraction of a large volume of diluted plasma sample was reported by Ohtsubo et al. The detection limit of this method is about 2 nm, but reproducibility at this concentration has not been shown. With the enzymatic method, 1 nm of AMB in plasma can be detected though the reproducibility is not enough for detailed pharmacokinetic study. This enzymatic method employs a simple procedure and a short time, and is considered useful for clinical application.

An enzymatic determination method for pyridostigmine in the presence of endogenous ChE was reported previously. 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). For the enzymatic determination of AMB concentration, endogenous ChE, which is less inhibited at the therapeutic concentration of AMB, should be removed to avoid interference by its esterase activity. After plasma ChE was removed by ultrafiltration using the MPS-3 micropartition system (Fig. 5), the effect of background activity of esterase based on endogenous plasma ChE could be neglected by addition of enough exogenous AChE. Variation in leakage of plasma ChE activity is so small that the inhibitory activity of AMB to AChE can be determined by subtracting the blank plasma ChE activity.

Since AMB is known to have adhesive characteristics, it must be assayed carefully. In aqueous solution, a decrease in the concentration of AMB was observed, which implies the adsorption of AMB to the ultrafiltration apparatus. Considering the recovery ratio from plasma is close to unity, no serious adsorption may occur during plasma filtration. However, since the recovery ratio is slightly decreased at low concentration, calibration curves were prepared for both high and low concentrations to achieve higher accuracy. The reason for less adhesive behavior in the presence of plasma components is still unknown.

As one application of this assay method to pharmacokinetic study, the plasma concentration–time profile after intravenous administration of AMB (10 or 20 nmol/kg) to rat was measured. Plasma concentration–time profiles at these doses were decreased in parallel with that assayed by HPLC after administration of AMB (1000 nmol/kg, Fig. 7), suggesting that there is no dose dependency in pharmacokinetics of AMB. Our new enzymatic determination method is rapid and simple, and makes it possible to assay the concentration of AMB in plasma with high sensitivity, so that it should be useful in pharmacokinetic and pharmacodynamic study.

References