Role of DMSO and Tween 20 in Acetylcholinesterase Inhibitory Activity of Donepezil Hydrochloride

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Abstract
Improving the solubility of non-polar tested compounds is essential for evaluating *in vitro* acetylcholinesterase (AChE) activity using modified Ellman's method. Unfortunately, dimethyl sulfoxide (DMSO), a widely used solvent for dissolving hydrophobic compounds, displayed the inhibitory activity against AChE, resulting in its limited application. For this reason, Tween 20 was considered as a cosolvent. The aim of this study was to optimize the final concentration of DMSO in combination with Tween 20 in AChE activity determination. Donepezil HCl (0.01 mg/mL) was used as the reference for investigating AChE inhibitory activity in 11 different combined solvents. The results showed that 2%v/v Tween 20 had no significant change in AChE inhibition of donepezil HCl. Furthermore, the maximum concentration of DMSO which can be used in the reaction mixture without significant interference was 0.1%v/v. Accordingly, the solution of 0.1%v/v DMSO and 2%v/v Tween 20 is suitable for improving non-polar materials solubility in modified Ellman's test.

Keywords: *AChE; Donepezil; DMSO; Ellman; Tween 20*

1. Introduction

Alzheimer's disease (AD), a chronic neurodegenerative disease, is the most common cause of dementia with severe economic and social impact. The symptoms of AD are difficulties with memory, language, problem-solving and other cognitive skills that affect a person's ability to perform everyday activities (Alzheimer's Association, 2017: 325-373).

According to cholinergic hypothesis, the loss of cholinergic neurons leading to the reduction levels of acetylcholine (ACh) neurotransmitter causes impairment in cognitive function (Hoffman Snyder and Facchiano, 2011: 201-206). Acetylcholinesterase (AChE), the enzyme at cholinergic synapse, is involved in the metabolic hydrolysis of ACh. Therefore, using acetylcholinesterase inhibitors (AChEIs) could increase the duration of action and concentration of ACh that result in improving some symptoms and slowing down the progression of AD (Saify and Sultana, 2014: 387-425). Currently, AChEIs (donepezil, rivastigmine and galantamine) are indicated for AD treatment (Slattum et al., 2008: 1051-1065). Several methods for evaluation the activity of AChE have been described including the colorimetric methods. Among them, the rapid colorimetric modified Ellman's method is the most widely used for AChE activity determination (Silva et al., 2014: 116-145). This method is based on the reaction between thiols and chromogenic 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). As presented in Figure 1, AChE hydrolyzes acetylthiocholine (ATCh) into an intermediate thiocholine (TCh). Then TCh reacts with DTNB and provides a yellow product named 2-nitro-5-thiobenzoic acid (TNB) which can be quantified by its absorbance at the wavelength of 405 nm. The intensity of the absorption is proportional to the level of TCh. The presence of AChEI inhibits the hydrolysis of ATCh by AChE leading to the reduction of TCh as well as the intensity of the absorption (Ellman et al., 1961: 88-95; Mertens et al., 2016:161-166).

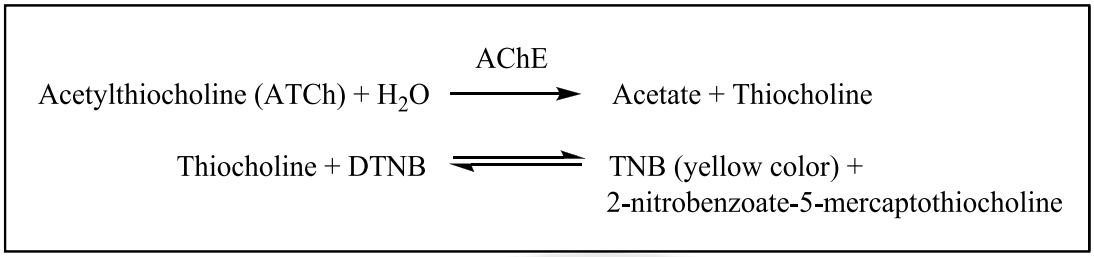

Figure 1 Principle of Ellman's method

Since this hydrolysis reaction is monitored by UV spectrophotometric readings, the test solution must be transparent. Accordingly, dimethyl sulfoxide (DMSO), one of the most commonly organic solvent has been used in biochemical and cellular assays for dissolving the tested compounds during drug discovery programs. Unfortunately, DMSO exhibits a highly AChE inhibitory effect at practical concentrations ranging from 0.6 to 10%. For this reason, DMSO is not the best choice of organic solvent for this enzyme assay (Obregon et al., 2005: 379-384).

However, some herbal materials are practically soluble in water, slightly soluble in other polar aprotic solvents and sparingly soluble in DMSO. In this case, use of DMSO is inevitable. Nevertheless, only DMSO utilization may not be sufficient to prevent the precipitation of solute from the test solution during this modified Ellman's assay. Thus, other cosolvents or surfactants may be considered as the solubilizers. One of commonly used solubilizer in laboratory assays is polysorbate 20 (Tween 20), a nonionic surfactant which is composed of 20 repeated units of polyethylene glycol via ethoxylation (Ayorinde et al., 2000: 2116-2124). However, the effect of Tween 20 on AChE activity still has not been reported. In this study, the impact of application DMSO with Tween 20 as cosolvent in the modified Ellman's method was investigated.

2. Method

2.1 Chemicals

AChE from *Electrophorus electricus* (*Ee*AChE) (E.C.3.1.1.7, Sigma C 2888), ATCh, DTNB, bovine serum albumin (BSA) and the reference compound, donepezil hydrochloride (donepezil HCl), were purchased from Sigma-Aldrich Chemical (St. Louis, MA, USA). DMSO was purchased from Carlo Erba (Milan, Italy). Tween 20 was obtained from Sino-Japan Chemical (Taipei, Taiwan). Tris(hydroxymethyl)aminomethane was acquired from Merck Millipore Corporation, Calbiochem (San Diego, CA, USA).

2.2 AChE assay

An assessment of *in vitro* AChE inhibition was assayed in conventional, flat-bottomed 96-well microplates (Thermo Fisher Scientific, Waltham, MA, USA) using the modified Ellman's method (Miao and Zhu, 2010: 5216-5234). The reaction mixture consisted of 25 µL of tested compound solution, 25 µL of 15 mM ATCh, 50 µL of 0.1%w/v bovine serum albumin in 50 mM Tris(hydroxymethyl)aminomethane pH 8, 125 µL of 3 mM DTNB and 25 µL of 0.22 units/mL *Ee*AChE. The final volume of this test was 250 µL. After 20 minutes, the reaction was incubated at 25°C and the absorbance was measured at 405 nm from microplate reader UV scan (EZ read 2000 microplate reader, Biochrom, UK). As a control, the tested compound solution was replaced with solvent that used for dissolving sample. The control and tested samples were assayed in triplicate. To monitor any nonenzymatic hydrolysis in the reaction mixture, two blanks for each run were prepared in triplicate. Percentage of AChE inhibition was calculated by the following equation;

$$\%\text{AChE inhibition} = \frac{(A-B) - (C-D)}{A-B} \times 100 \qquad (1)$$

where,
A is absorbance of control,
B is absorbance of blank,
C is absorbance of sample, and
D is absorbance of sample blank.

In this reaction, Tween 20 was used as cosolvent. According to preliminary test, using higher than 2%v/v of Tween 20 resulted in bubble formation that interfered the measurement. On the contrary, using less than 2%v/v of Tween 20 was not enough for enhancing the solubility of non-polar compounds. Therefore, the optimal concentration of Tween 20 for this reaction was 2%v/v. Donepezil HCl stock solution was prepared by dissolving in 11 different mixture of solvents as shown in Table 1. The final concentration of the tested compound solution, donepezil HCl, was 0.01 mg/mL.

Table 1 The final concentrations of DMSO, Tween 20, and water in 11 different combined solvents

Combined solvent No.	DMSO (%v/v)	Tween 20 (%v/v)	Water (%v/v)
1	0	0	10
2	10	0	0
3	0	2	8
4	0.1	2	7.9
5	0.2	2	7.8
6	0.5	2	7.5
7	0.8	2	7.2
8	1	2	7
9	2	2	6
10	5	2	3
11	8	2	0

2.3 Statistical analysis
Statistical significance was determined using ANOVA test with LDS post hoc analysis at 95% confidence interval using SPSS Statistics v17.0 program.

3. Results
Donepezil HCl, an approved AChE inhibitor for AD treatment was tested for AChE inhibitory activity using modified Ellman's colorimetric assay in 96-well microplate. The eleven different mixed solvents were used for preparing donepezil HCl stock solution. The final concentrations of combined solvents No.1 to 11 are demonstrated in Table 1. In this study, the absorbance of control, blank, sample and sample blank solutions as shown in Figure 2 were measured and the AChE inhibitory activities of donepezil HCl were calculated by using Equation 1.

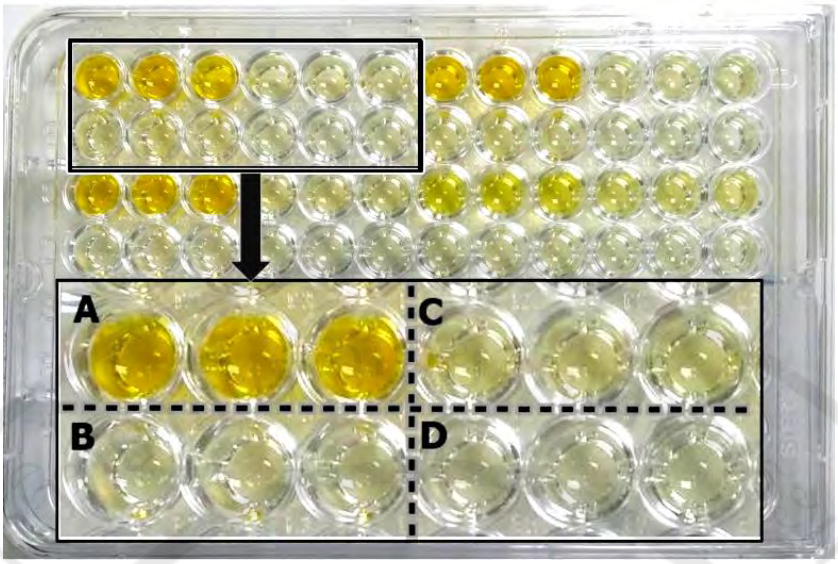

Figure 2 The reaction mixture of A = control (AChE with no sample), B = blank (no AChE with no sample), C = sample (AChE with donepezil HCl) and D = sample blank (no AChE with donepezil HCl)

The effect of DMSO and Tween 20 to the modified Ellman's colorimetric test was determined by comparing the AChE inhibitory activity of 0.01 mg/mL donepezil HCl prepared in the different solvents. The influences of these solvents on AChE inhibitory effect of donepezil HCl were indicated in Figure 3. It was found that donepezil HCl in combined solvent No.1 (10%v/v water) exhibited the highest AChE inhibition at 98.0±1.15% whereas this inhibitor in combined solvent No.2 (10%v/v DMSO) showed a significance reduction in AChE inhibitory activity. This result indicated that the utilization of 10%v/v DMSO in the reaction mixture should be restricted. In the presence of 2%v/v Tween 20 (combined solvent No.3), donepezil HCl exhibited lower activity than in combined solvent No.1. However, no significant differences in AChE activity were found with combined solvent No.1 or No.3. Therefore, using 2%v/v Tween 20 as solubilizer was suggested in this test while using 10%v/v DMSO should be limited.

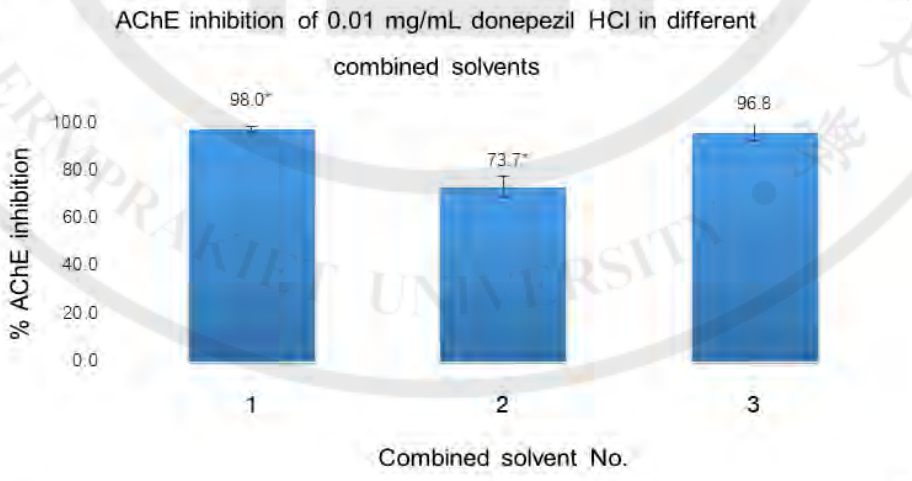

Figure 3 The AChE inhibitory activities of 0.01 mg/mL donepezil HCl in combined solvent No.1 (10%v/v water), No.2 (10%v/v DMSO) and No.3 (8%v/v water and 2%v/v Tween 20). Error bar represented standard deviation (SD). *$P < 0.05$ versus solvent No.1

To figure out the suitable final concentration of DMSO with cosolvent (Tween 20) in this assay, donepezil HCl was dissolved in DMSO ranging from 0.1 to 8%v/v in the presence of 2%v/v Tween 20. As presented in Figure 4, AChE inhibition of 0.01 mg/mL donepezil HCl in combined solvent No.5 to 11 which composed of DMSO 0.2, 0.5, 0.8, 1, 2, 5, and 8%v/v, respectively were significantly decreased from combined solvent No.3 (no DMSO). Only the inhibitory effect of tested compound in combined solvent No.4 which consisted of 0.1%v/v DMSO had no significant change in AChE inhibitory activity. This experiment indicated that the final concentration of DMSO in the reaction mixture of this modified Ellman's assay should be not more than 0.1%v/v when using 2%v/v Tween 20 as cosolvent.

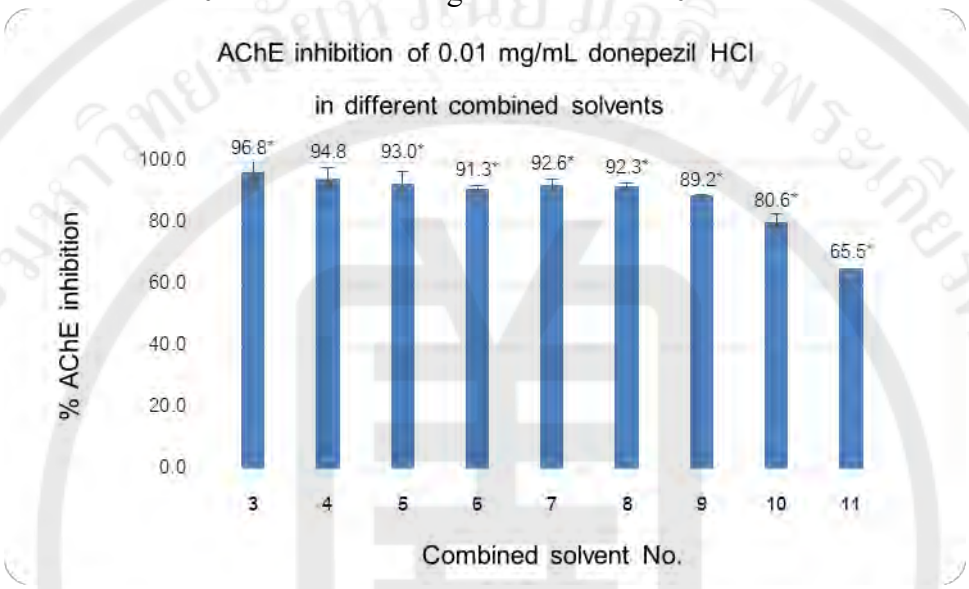

Figure 4 The AChE inhibitory activity of 0.01 mg/mL donepezil HCl in combined solvent No.3 to 11 (Data of combined solvent is shown in Table 1) *P value < 0.05 versus combined solvent No.3

4. Conclusion and Discussion

To carry out the AChE inhibitory activity of non-polar materials, concomitantly used of cosolvents or surfactants for the assay was practical. These solvents should have no interferences to the assay. In the current modified Ellman's method, DMSO and Tween 20 have been used as solvent of 0.01 mg/mL donepezil HCl, an AChE inhibitor. The appropriate final concentration of Tween 20 in the reaction mixture was 2%v/v because of no significant change in the activity of donepezil HCl. However, the reaction mixture should be gently mixed to avoid bubble formation.

DMSO is usually the solvent of choice for preparing stock solutions of the tested compounds for biological and pharmacological screening experiments. However, using of this solvent should be restricted because AChE inhibition of 0.01 mg/mL donepezil HCl significantly decreased with the increasing percentages of DMSO (0.2 to 10%v/v). As previously described, the influence of DMSO on the activity of the AChEIs was found to be significant. The tested compounds were less active in the presence of 0.6 and 1.6%v/v of DMSO (Di Giovanni et al., 2008: 109-119). Moreover, Kumar and Darreh-Shori (2017: 2618-2625) reported that DMSO is a considerably potent and highly selective irreversible mixed-competitive inhibitor of human AChE, as well as 1 to 4%v/v DMSO showed 37 to 80% inhibition of human AChE activity. Thus, developing stringent protocols in the presence of DMSO should be cautious. Comparison among different concentrations of DMSO (0 to 8%v/v) and 2%v/v Tween 20 solutions, only 0.1%v/v DMSO, 2%v/v Tween 20 and 7.9%v/v

water used in the reaction mixture had no statistically significant effect on AChE inhibition of the tested compound. This data suggested that the final concentration of DMSO in the presence of 2%v/v Tween 20 should not more than 0.1%v/v for using in this experiment. Therefore, 0.1%v/v DMSO in combination with 2%v/v Tween 20 and 7.9%v/v water could be the proper aids for improving solubility of hydrophobic tested compounds in modified Ellman's assay.

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