# Effect of combination of extracts of ginseng and ginkgo biloba on acetylcholine in amyloid beta-protein-treated rats determined by an improved HPLC[1]

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**KEY WORDS** ginseng; ginkgo biloba; acetylcholine; high pressure liquid chromatography; amyloid beta-protein

## ABSTRACT

**AIM:** To determine the concentration of acetylcholine (ACh) in amyloid beta-protein (Aβ) treated rats and offer a method determining ACh as well. **METHODS:** A 1-month combination of extrats of ginseng and ginkgo biloba (Naoweikang) ig administration to rats was performed daily after bilateral injection of $A\beta_{1\text{-}40}$ (4 g/L, 1 μL for each side) into hippocampus. After decollation, homogenizing, and centrifuging and extracting, a high pressure liquid chromatographic (HPLC) method using electrochemical detection (ECD) combined with two immobilized enzyme reactors was used to determine ACh in rat whole brain. **RESULTS:** With a mobile phase consisting of disodium hydrogen orthophosphate, tetramethylammonium chloride (TMACl), octanesulfonic acid sodium salt (OSA) and "Reagent MB" at a final pH of 8.0, ACh was determined while removing the interfering choline in less than 10 min at a flow rate of 0.35 mL/min on a platinum (Pt) working electrode at a potential of +300 mV *vs* a solid-state palladium (Pd) reference electrode. Linear regression analysis of peak area *vs* concentration demonstrated linearity in the 28.01 to 1400.06 μg/L injection range. The *r*-value was 0.9978. The limit of detection (LOD) is 0.28 ng on column. ACh in whole brain decreased by 20.34 % (from 162.1±32.7 to 134.7±14.0 μg/L, $P<0.05$) after bilateral injection of Aβ into rat hippocampus. After Naoweikang administration (31 and 15.5 mg/kg, respectively), ACh increased by 19.97 % (from 134.7±14.0 to 161.6±26.2 μg/L, $P<0.05$) and 18.56 % (from 134.7±14.0 to 159.7± 22.9 μg/L, $P<0.05$), respectively. **CONCLUSION:** Naoweikang significantly increased the level of ACh in whole brain of Aβ treated rats. And a sensitive, selective and reliable method for routinely determining ACh in rat whole brain was established in this study.

## INTRODUCTION

Much of the recent progress in elucidating the pathogenesis of Alzheimer's disease (AD) has centered on the apparent role of amyloid beta-protein (Aβ). It was reported that intracerebral injection of Aβ or core protein from senile plaques from AD brain caused many pathological changes including neurodegeneration and memory deficiency in rodents[1-3]. Meanwhile, the importance of ACh as a very important neurotransmitter in the nervous system was realized over 80 years ago. It is involved in sleep and behavior, learning and memory, and a number of neuronal diseases including

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AD, Huntington's disease and Parkinson's disease with prominent dementia. Furthermore, since acetylcholinesterase (AChE) inhibitors are the most important treatment options for AD at present, the quantification of ACh as a functional parameter of the cholinergic system is of special importance[4,5]. Historically, numerous approaches have been used to measure ACh such as bioassays, enzymatic assays, and techniques based on gas chromatography-mass spectrometry and HPLC[6-8]. However, despite modern analytical methods, ACh quantification remains difficult due to its very fast hydrolysis. Moreover, the use of AChE inhibitor, the need for specific and sensitive detective systems and the reproducibility of many approaches make ACh detection problematic.

Naoweikang is a combination of the extracts of Ginseng and Ginkgo biloba (ginsenoside $Rg_1$ and ginsenoside Re: 35 %; ginkgolides: 20 %; ginkgoflavones: 16 %). Four ingredients (components), including ginsenoside $Rg_1$, ginsenoside Re, ginkgolides and rutin hydrolyzed from ginkgoflavones on the acid condition, were determined and quantified by HPLC as markers. Our previous studies suggest that Naoweikang improves the behavioral impairment of Aβ treated rats (unpublished observations). However, the mechanism of this effect remains to be proved.

In this study, ACh in whole brain homogenates of Aβ treated rats was determined after Naoweikang administration using an improved HPLC-ECD combined with two immobilized enzyme reactors.

## MATERIALS AND METHODS

**Chemicals and reagents** All chemicals were of the analytical grade available. The AR grade TMACl was from Beijing Xingfu Refined Chemical Co (Beijing, China). And the HPLC grade ACh chloride, OSA, disodium hydrogen orthophosphate, and $A\beta_{1-40}$ were purchased from Sigma Chemical Co (St Louis, MO, USA). The microbicide "Regent MB" was from ESA (Bedford, MA, USA). The HPLC grade phosphoric acid (85 %) used to adjust the pH of mobile phase was purchased from Fisher Scientific (Fairlawn, NJ, USA). Polished water was obtained by first passing deionized water through an ultrapure water system (Purelab Plus, Pall, USA) and then filtered (0.2 μm, Millipore, Bedford, USA) to remove trace organics. The combination of extracts of ginseng and ginkgo biloba (Naoweikang) was provided by the pharmaceutical Lab of the research center (Xiyuan Hospital, Beijing, China).

**Animals** Male Sprague-Dawley rats (weighing 380±50 g, Grade II, Certificate No SCXK (Jing) 2002-0003, Center of Experimental Animals, Beijing, China) were caged individually with *ad libitum* access to water and food and maintained on a 12-h-light-dark cycle. The rats were divided into 4 groups of 12 each: control group, model group, and two Naoweikang-administrated groups (31 and 15.5 mg/kg, respectively).

**Surgery and drug administration** $A\beta_{1-40}$ was dissolved in saline at the concentration of 4 g/L and the solution was incubated at 37 ºC for 24 h before use. Rats were anesthetized with a 10-mL/kg ip dose of chloral hydrate (3.6 %); the depth of anesthesia was maintained by additional doses when necessary. Rats were placed on a stereotaxic apparatus (Stoelting Co, Illinois, USA). Bilateral injection was performed (coordinates: AP -3.0 mm, ML ±2.0 mm, DV 4.0 mm from bregma determined using the atlas of Paxinos and Watson as a guide). One microliter of Aβ was injected over 5 min through a 26-gauge Hamilton syringe into rat hippocampus (model group and Naoweikang-administrated groups). The needle was kept in place for 5 min before withdrawing slowly. A second injection was given at the same coordinate in the opposite hemisphere. Rats in control group were injected with saline of the same volume. After surgery, the rats were returned to the cages. When the rats had recovered from anesthesia, those in Naoweikang-administrated groups received Naoweikang ig administration (31 and 15.5 mg/kg, respectively; Research Center of Xiyuan Hospital, Beijing, China) per day for one month, whereas those in the other two groups (control group and model group) were administrated saline of the same volume. The doses used in this study were chosen on the basis of those used in experiments and clinical trials by other researchers.

**Sample preparation** All animals were sacrificed after 1 month by decollation. The whole brains were removed on an ice-plate and weighed, quickly frozen in liquid nitrogen and stored at -80 ºC until extraction. On the day of extraction, the whole brains were homogenized for 20 s in ice-cold perchloric acid (PCA) 0.1 mol/L (1 mL per 0.1 g brain tissue) containing 0.04 % (w/v) $Na_2S_2O_5$ and 0.04 % (w/v) edetic acid. The homogenates were centrifuged at 14 000×*g* at 4 ºC for 20 min. The supernatants were then filtered (0.2 μm, Millipore, Bedford, USA), aliquoted (1 mL into 1.5 mL vials), and then stored at -80 ºC for the detections of

ACh, monoamines, and amino acids, respectively. The sample aliquots for ACh detection were diluted 1:2 (v/v) in buffer (consisted of disodium hydrogen orthophosphate 100 mmol/L, TMACl 0.5 mmol/L, and OSA 2.0 mmol/L) before injected into the HPLC system.

**Chromatographic and detector conditions** The HPLC system was from ESA Inc (Bedford, MA, USA) that consisted of a pump (Model 582), an autosampler (Model 542), a column oven (CH 150) and a Coulochem II Model 5600A electrochemical detector equipped with a solid-state analytical cell (Model 5040), containing a PEEK/Pt target working electrode with a solid-state Pd reference electrode. Chromatograms were recorded and integrated on a data station (Coularray for Windows 3.2, Version 1.10) that also provided remote PS-232 control of the autosampler and detector. A 0.1-mm thick Teflon gasket was used providing an analytical cell volume less than 3 μL. The mobile phase consisting of disodium hydrogen orthophosphate 100 mmol/L, TMACl 0.5 mmol/L, and OSA 2.0 mmol/L and 0.005 % (v/v) "Reagent MB" at a final pH of 8.0. The mobile phase was filtered (0.2 μm, Millipore, Bedford, USA) before use and changed on a weekly basis. ACh was measured in a 10-μL injection volume at a flow rate of 0.35 mL/min when maintaining the polymeric reversed-phase column (ACH-3, 5 μm, 150 mm×3 mm ID) equipped with an ACH-3-G guard cartridge, a pre-column and a post-column immobilized enzyme reactors at 35 ºC. The potential was +300 mV *vs* Pd reference.

**External standard preparation** ACh chloride 140.06 mg was dissolved in polished water and then diluted 1×10 (v/v) in polished water to provide standard solution which was then diluted in mobile phase to provide the 280.12-μg/L working standard. All solutions should be prepared rapidly due to the hygroscopic nature of ACh chloride.

**Validation study** Five different levels of standard solutions (1400.60, 700.30, 280.12, 140.06, and 28.01 μg/L, respectively) were prepared to determine the linear range. ACh standard solution 280.12 μg/L 5 μL was added into the sample of the same volume whose concentration had been detected ($n$=3). The ratio between the increased concentration of the mixture *vs* the average concentration of the sample and the concentration of the working standard was determined to study the recovery of the assay ($n$=3). The 280.12 μg/L working standard was also used to detect the stability of the system within day and between days. Meanwhile, the limit of detection (LOD) was determined (S/N= 3:1). The 280.12 μg/L working standard should be analyzed on an hourly basis in order to monitor the system efficiency.

**Statistics** All results were expressed as mean±SD. Between-group differences were analyzed by Student's $t$-test. $P<0.05$ was considered significant.

## RESULTS

ACh was determined with a mobile phase consisting of disodium hydrogen orthophosphate, TMACl, and OSA and "Reagent MB" at a final pH of 8.0 in less than 10 min at a flow rate of 0.35 mL/min on the Pt working electrode at the potential of +300 mV *vs* a solid-state Pd reference electrode. All samples were detected once within 3 d. The chromatograms of mobile phase and 280.12 μg/L ACh working standard are presented in Fig 1. The chromatograms showed sharp ACh peak with no interfering choline peak normally seen when there was no pre-column enzyme reactor in the HPLC system.

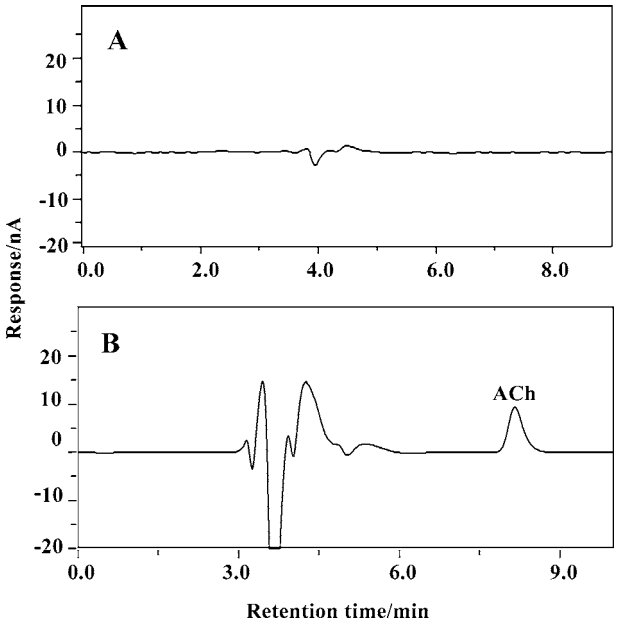


**Fig 1. With a mobile phase consisting of disodium hydrogen orthophosphate, TMACl, OSA and "Regent MB" at a final pH of around 8.0. ACh was determined within 10 min at a flow rate of 0.35 mL/min on a Pt working electrode at a potential of +300 mV *vs* a solid-state Pd reference electrode. A) the blank graph of mobile phase; B) the standard graph of 280.12 μg/L ACh standard.**

Linear regression analysis of concentration *vs* the peak area showed linearity for ACh in the range of 28.01 to 1400.60 μg/L (Fig 2). The $r$-value was 0.9978. The

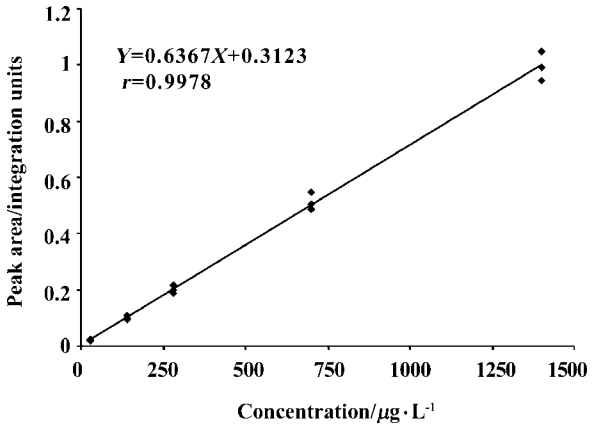


**Fig 2. The relationship between the concentration of ACh and the peak area in the range of 28.01 to 1400.60 μg/L.**

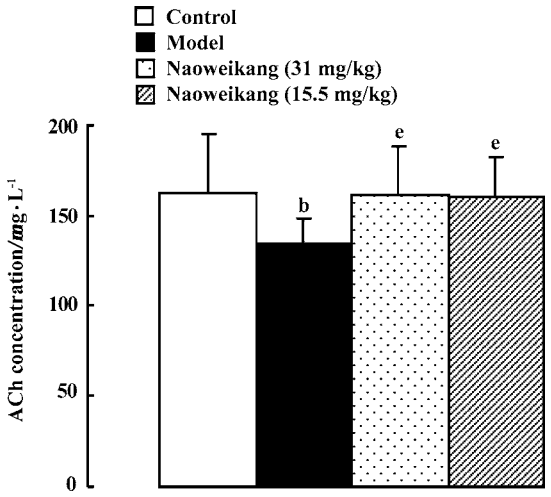


**Fig 3. Effect of Naoweikang on ACh in Aβ-treated rats. *n*=12. Mean±SD. $^{b}P<0.05$ *vs* control group; $^{e}P<0.05$ *vs* model group.**

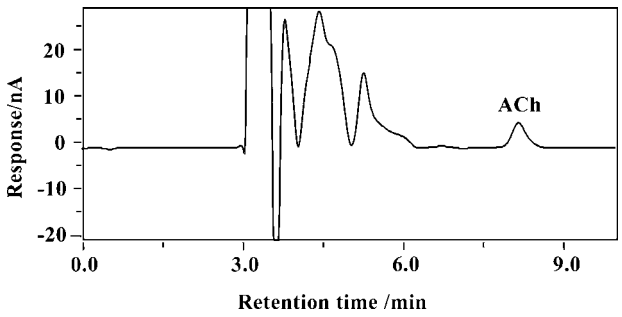


**Fig 4. Typical high performance liquid chromatogram of the samples.**

LOD was determined to be about 0.28 ng on column (S/N=3:1). The intra-day and inter-day variations in retention time and in concentration (analyzed over a 12-h period and a 3-d period, respectively) are shown in Tab 1. The average recovery was 91.37 %±2.60 %.

**Tab 1. Average recovery, intra-day and inter-day variations of ACh.**

| Concentration/μg·L$^{-1}$ | Recovery (*n*=3)/% | Variation in retention time (RSD)/% | | Variation in concentration (RSD)/% | |
|---|---|---|---|---|---|
| | | Intra-day (*n*=6) | Inter-day (*n*=12) | Intra-day (*n*=6) | Inter-day (*n*=12) |
| 280.12 | 91.37±2.60 | 0.16 | 0.57 | 3.70 | 4.46 |

ACh decreased by 20.34 % (from 162.1±32.7 to 134.7±14.0 μg/L, $P<0.05$) after bilateral injection of Aβ into rat hippocampus. After Naoweikang administration (31 and 15.5 mg/kg, respectively), ACh increased by 19.97 % (from 134.7±14.0 to 161.6±26.2 μg/L, $P<0.05$) and 18.56 % (from 134.7±14.0 to 159.7±22.9 μg/L, $P<0.05$), respectively (Fig 3). The typical chromatogram of the samples is presented in Fig 4.

## DISCUSSION

To determine the functional activity of cholinergic system which has already been proved to play a particularly important role in pathology of AD[9,10], the direct measurement of ACh is thought to be more sensitive than measuring the activity of the synthetic enzyme choline acetyltransferase and more specific and reliable than measuring the degrading enzyme AChE. However, of all available approaches at present, there are two common problems. One is that the ACh peak often overlaps with those of closely eluting peaks, including that of choline, which are commonly large enough to interfere with the ACh measurement. Another is the need for AChE inhibitors, which is now strongly discouraged. The use of AChE inhibitors may lead to an artificial elevation in ACh levels that can be relatively easily measured. However, many researchers have suggested that the use of them, especially at high levels, may also lead to an abnormal physiological state and altered pharmacological responses, which is problematic for the interpretation of drug effects and for the study on alterations in ACh release associated with certain pathological states[11,12].

Based on the approaches described previously[12,13], a second enzyme reactor, a pre-column immobilized enzyme reactor, was adopted for the selective assay of

ACh in this HPLC system. The assay of ACh followed the flaw sequence of pre-column enzyme reactor, polymeric reversed-phase column, and post-column enzyme reactor. The pre-column immobilized enzyme reactor (containing choline oxidase and catalase) was used to remove choline and the resulting hydrogen peroxide from injected samples before the post-column reactor in order to prevent the large choline peak from overlapping that of ACh. ACh was enzymatically converted to hydrogen peroxide by the post-column reactor (containing acetylcholinesterase and choline oxidase). And hydrogen peroxide was then detected electrochemically on the Pt target.

In the present study, ACh was detected while other closely eluting peaks, occurring especially when the performance of the analytical column deteriorates, were eliminated. Meanwhile, the determination was performed in the total absence of AChE inhibitors, which provided the opportunity to study ACh under conditions closer to the normal physiological state and the pharmacological alterations in ACh levels. Consistent enzymatic efficiency and minimum changes in retention time were achieved by maintaining the analytical column, the guard cartridge and two enzyme reactors at 35 ºC. In short, the system in this study represented an improved approach characterized by higher sensitivity, stability and selectivity compared with those of former reports.

The supernatant of the whole brain homogenate processed according to the above procedures can be used not only for ACh detection, but for the detections of monoamines and amino acids as well. It is of special practical meaning for the studies of neuroscience and pharmacotherapy on some neuronal disorders, such as AD. As the enzymes used in this study were very sensitive to the alterations of pH and represented a maximum efficiency in a pH range from 8.0 to 8.5, the samples were diluted 1:2 (v/v) in buffer to obtain a pH of around 8.0 before being injected into the HPLC system.

However, a successive decrease of ACh response was also observed every 8 to 12 injections in this study. It is also a serious limitation of HPLC-ECD methodology that might be caused by the decreasing sensitivity of the Pt electrode. To overcome this problem, frequent calibration and external standardization were adopted in order to improve the accuracy of the assay.

As reported, the impairments in animals after intracerebral injection of Aβ is likely to those in aging and AD patients[1-3]. Therefore, it is suggested that the injection of Aβ into rat hippocampus may offer a model for study on pathological mechanism of AD. In this study, bilateral intrahippocampal injection of Aβ significantly decreased the level of ACh. This result confirms the observations of previous studies on Aβ and lends further credence to the importance of cholinergic neurotransmitter system in AD development. The mechanism of ACh reduction induced by intracerebral injection of Aβ might be due to the increased AChE activities.

There is a trend in the interest and the use of natural medicinal plants and traditional Chinese medicines as remedies nowadays. Abundant evidence suggests that due to their complicated constituents, natural medicinal plants and traditional Chinese medicines might beneficially affect various systems with less side effects and more clinical results compared with synthetic drugs. It makes them the preferable choice in treating some chronic or complicated disorders, such as AD, which involves many unknown mechanisms and various systems in the pathogenesis. Meanwhile, certain active constituents or extracts have been proved to be essential to the efficacy and effectiveness of particular traditional Chinese medicine. Thus, it is of special academic and practical importance for the investigation and development of natural medicinal plants, traditional Chinese medicines and their extracts, which are realized as an interesting potential for the future medicinal world. Naoweikang is a combination of extracts of traditional Chinese medicines: ginseng and ginkgo biloba. In the present study, Naoweikang significantly increased the level of ACh in Aβ treated rats. The result lends further credence to the observations of our previous studies. The improvement of behavioral impairment in Aβ treated rats may be due to the increased ACh level. The candidate mechanism of Naoweikang in increasing ACh concentration in Aβ treated rats might be due to the decreased AChE activities.

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