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Research Article

Simultaneous determination of galantamine, rivastigmine and NAP 226-90 in plasma by MEKC and its application in Alzheimer's disease

A simple and sensitive MEKC with UV detection was developed and validated for the simultaneous determination of acetylcholinesterase inhibitors including galantamine, rivastigmine and major metabolite NAP 226-90 in plasma. A sample pretreatment by liquid–liquid extraction with diethylether and subsequent quantification by MEKC was used. The optimum separation for these analytes was achieved in <10 min at 25°C with a fused-silica capillary column of 30.2 cm $\times$ 50 μm id (effective length 20 cm) and a run buffer containing 25 mM Tris buffer (pH 5.0) with 160 mM sodium octanesulfonate, 20% ACN and 0.01% PVP as a dynamic coating to reduce analytes' interaction with the capillary wall. For sensitivity consideration regarding the determination of linearity, LOD, quantitation and monitoring drugs concentration in patients, the detection wavelengths for galantamine or rivastigmine and NAP 226-90 were set at 214 or 200 nm, respectively. One male volunteer (26-year-old) was orally administered a single dose of 4.5 mg rivastigmine (Exelon®, Novartis) in capsule, and blood samples were drawn over a 12 h period for concentration–time profile study. The method was also successfully applied for monitoring galantamine or rivastigmine and its metabolite NAP 226-90 in 11 Alzheimer's disease patients' plasma after oral administration of the commercial products Reminyl® (8 mg galantamine/capsule) or Exelon® (3 mg rivastigmine/capsule), respectively.

Keywords:
Alzheimer's disease / Liquid–liquid extraction / MEKC / Rivastigmine and NAP 226-90 in plasma / Simultaneous determination of galantamine
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1 Introduction

Alzheimer's disease (AD) with its associated loss of activity in the brain is the most common cause of dementia among people aged 65 years and older. It shows the typical characteristic of a progressive cognitive and functional decline in patients [1]. Degradation of cholinergic neurons in the cerebral cortex and other areas of the brain, with resulting deficits in cholinergic transmission and acetylcholine (ACh) levels, is considered to be a primary cause of cognitive decline [2]. It is well known that AD imposes a substantial burden on caregivers, family members and society and is associated with high economic costs. Strategies to reduce various burdens of AD by means of pharmacotherapy are therefore important. Although no pharmacologic treatments have been proved to alter the pathology of AD, acetylcholinesterase inhibitor (AChEI) therapy offers symptomatic improvements or delays in the progression of cognitive, behavioral and functional deficits. Recently, preclinical studies indicate that AChEIs could prevent damage and protect neurons from death [3].

AChEIs are known to inhibit AChE, which catalyzes the breakdown of ACh in the synaptic cleft, thus enhancing ACh. Donepezil, rivastigmine and galantamine are the best agents studied in this class. Two cholinesterases hydrolyze ACh in the human brain, AChE and butyrylcholinesterase (BuChE); current evidence indicates that AChE activity declines with the progressive loss of cortical neurons in AD, and at the same time BuChE levels increase and

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Abbreviations: ACh, acetylcholine; **AChEI,** acetylcholinesterase inhibitor; **AD,** Alzheimer's disease; **BuChE,** butyrylcholinesterase; **IS,** internal standard; **LLE,** liquid–liquid extraction; **PEO,** polyethylene oxide; **RE,** relative error; **SOS,** sodium octanesulfonate

may take over the function to metabolize ACh at the synapse [4]. Moreover, the rate of cognitive decline has been shown to correlate with BuChE levels in the temporal cortex.

Rivastigmine is a carbamate-type dual inhibitor of brain AChE and BuChE, which is widely used in the symptomatic treatment of AD with mild-to-moderate dementia associated with idiopathic Parkinson's disease. Therefore, rivastigmine has the theoretical advantage of being a dual inhibitor of AChE and BuChE over the selective AChEI, donepezil. It exhibits log linear pharmacokinetics at dosages up to 6 mg daily; however, the area under the concentration–time curve increases disproportionately to dose at higher dosages [5]. The drug rivastigmine is extensively metabolized to NAP 226-90 *via* the target enzymes, cholinesterases, and thus the concentration of NAP 226-90 more reliably reflects the extent of enzyme inhibition. It was shown that an increase in NAP 226-90 concentration correlated well with enzyme inhibition [6]. The mechanism of action of galantamine, different from that of the other AChEIs, has an allosteric modulating activity at nicotinic receptors in addition to its ability to inhibit AChE. Nicotinic receptor modulation may have beneficial effects in different symptomatic domains of AD. AChEI therapy may be switched for various reasons, including perceived inefficacy of current therapy or intolerable side effects. These drugs have varying pharmacodynamic and pharmacokinetic properties, safety, and tolerability profiles that can affect patient outcomes. Metabolic parameters can affect a drug's tolerability and become important when a switch from one agent to another is contemplated. For these reasons, a reliable determination of the plasma concentrations of galantamine, rivastigmine and the major metabolite of rivastigmine NAP 226-90 is very important in evaluating therapeutic efficacy, monitoring drug concentration for patient safety and studying the mechanism of drug metabolism.

Literature studies of the concentration of rivastigmine and/or its metabolite NAP 226-90 have been done using capillary gas chromatography with mass spectrometric detection [7], HPLC with fluorometric [8] or mass spectrometry [6, 9–11] in human plasma samples. Reports of HPLC with UV detection [12], fluorometric [13] and mass spectrometry [14–18] for the determination of galantamine in biological samples have been published. Powerful mass spectrometry detectors can improve detection sensitivity. However, they are not widely applicable and very expensive for the common laboratory.

CE is a powerful separation technique for the determination of ionic and neutral components. Compared with HPLC, only one CE method has been reported for the determination of galantamine [19] in biological fluids (LOD 35 ng/mL). We previously developed a sensitive CE method for the determination of donepezil and its application in clinic [20]. So far, no CE method has been developed for the simultaneous determination of galantamine, rivastigmine and its major metabolite NAP 226-90 in human plasma and in order to monitor drug concentrations in patients with AD under treatment with commercial preparations of AChEIs.

The aims of the study are not only to develop a sensitive CE method for the simultaneous determination of galantamine, rivastigmine and its major metabolite NAP 226-90 in plasma, and evaluate the time-concentration courses of rivastigmine and its metabolite for healthy volunteer plasma after oral administration of 4.5 mg rivastigmine (Exelon®) in a single dose, but also to evaluate the concentrations of galantamine, rivastigmine and the major metabolite NAP 226-90 in AD patients at steady state. In this study, we used liquid–liquid extraction (LLE) with diethylether for plasma pretreatment combined with MEKC, which was first described by Terabe *et al.* [21]. The concentrations of galantamine or rivastigmine and the major metabolite NAP 226-90 in 11 different AD patients who were administered 8 mg galantamine (Reminyl®) orally once daily or 3 mg rivastigmine (Exelon®) orally twice daily at steady state were evaluated.

2 Materials and methods

2.1 Chemicals

Rivastigmine, (*S*)-*N*-ethyl-3-[1-(dimethylamino)ethyl]-*N*-methyl-phenylcarbamate hydrogentartrate and NAP 226-90 (Fig. 1) were kindly supplied by Novartis pharmaceuticals (Spain), and atenolol, 4-(2′-hydroxy-3′-((1-methylethyl)amino)propoxy)-benzene-acetamide, used as an internal standard (IS), (Fig. 1) was purchased from MP Biomedicals (Germany). Galantamine (Fig. 1), PVA, polyethylene oxide (PEO) and PVP were from Sigma (St. Louis MO, USA). Sodium hydroxide, Tris, diethylether and phosphoric acid (H_3PO_4, 85%) were supplied by Merck (Darmstadt, Germany). Sodium octanesulfonate (SOS) was from Mallincrodt Baker. Isopropanol was purchased from Fluka (Germany). Other agents were analytical-reagent grade. Milli-Q-treated water (Millipore, Bedford, MA, USA) was used for the preparation of buffer and related aqueous solutions. Reminyl® (8 mg galantamine/capsule)

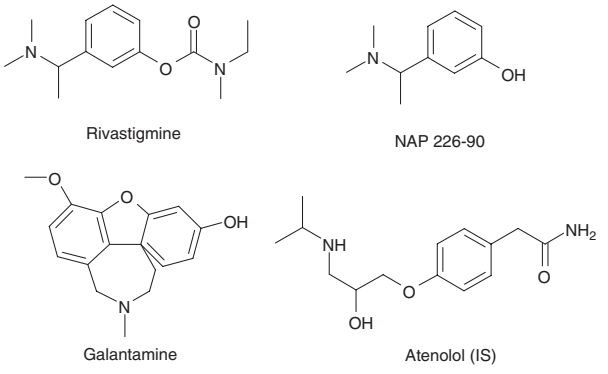

Figure 1. Chemical structures of galantamine, rivastigmine, NAP 226–90 and atenolol (IS).

(Janssen, Italy) and Exelon® (3 mg rivastigmine/capsule) (Novartis, Spain) are commercial preparations.

2.2 CE conditions

A Beckman P/ACE MDQ system (Fullerton, CA, USA) equipped with a UV detector and a liquid-cooling device were used. CE was performed in an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 30.2 cm (effective length 20 cm) with 50 μm id. The temperature of the separation was controlled at 25°C by immersion of the capillary in cooling liquid circulating in the cartridge, and the temperature of the sample tray was maintained at a room temperature of about 25°C. The detector was set at 200 or 214 nm (cathode at the detection side). The routine conditioning between runs each day was carried out using pressure with 1 N HCl (3 min), de-ionized water (2 min), 1 N NaOH (2 min), de-ionized water (2 min) and rinse buffer (5 min) under positive pressure applied at the injection end. After the capillary has been filled with BGE, 0.01% PVP in buffer electrolyte, Tris buffer (25 mM, pH 5.0) containing 160 mM SOS and 20% ACN, sample loading was achieved by electrokinetic injection at a positive voltage of 7 kV for 50 s. A constant voltage of +12 kV was applied throughout the run under BGE, and the average current was approximately 75 μA. The temperature of separation was maintained at 25°C. The Beckman 32 Karat 7.0 Software System was used for data processing.

2.3 Plasma extraction procedure

To prevent high protein concentrations in samples from affecting the CE separation, LLE was employed for clean-up and pre-concentration of the samples taken from plasma. Samples were prepared for CE analysis as follows: a 100 μL aliquot of patient's plasma or plasma spiked with three analytes was pipeted into a 2 mL Eppendorf vial, and extracted with 400 μL of diethylether, and then vortexed for 3 min. A 320 μL aliquot of supernatant was evaporated by centrifugal vaporizer (EYELA CVE-200 D, Japan) and reconstituted with 20 μL of ACN/0.25 mM H_3PO_4 (80:20 v/v; pH 6.6) that contained 100 ng/mL of atenolol (IS). Then, the sample was transferred to a 0.2 mL mini-vial that was placed into the sample tray of a Beckman P/ACE MDQ system for CE analyses.

2.4 Sample preparation and method validation

Stock solutions of galantamine and rivastigmine of reference standard at 10.0 and 4.0 μg/mL were prepared in de-ionized water and suitably diluted by de-ionized water; NAP 226-90 of reference standard at 4.0 μg/mL was prepared in 50% ACN and suitably diluted by de-ionized water. Plasma sample standards were prepared by spiking various levels of reference standard into drug-free plasma. The quantification ranges of plasma samples were the final galantamine, rivastigmine and NAP 226-90 concentrations over the range 1.0–120.0 ng/mL of galantamine and 0.5–50.0 ng/mL of rivastigmine and NAP 226-90 and a fixed concentration of 100 ng/mL of atenolol (IS). Calibration curves for galantamine, rivastigmine and NAP 226-90 in biological matrix were obtained with their corrected peak area ratios to atenolol (IS) as ordinate (y) *versus* their corresponding concentrations (ng/mL) as abscissa (x). The intra- and inter-day precision and accuracy examinations of galantamine, rivastigmine and NAP 226-90 assay in plasma were tested by analyzing three concentration levels of galantamine at 10.0, 30.0 and 120.0 ng/mL and rivastigmine and NAP 226-90 at 2.0, 20.0 and 50.0 ng/mL. The precision and accuracy of the method were estimated from the back-calculated standard concentration. The intra-day precision and accuracy was defined by RSD and relative error (RE) from analyses on the same days. The inter-day precision and accuracy were calculated from repeated analyses of identical samples on consecutive days for these concentrations of galantamine, rivastigmine and NAP 226-90 and expressed as RSD and RE. The LOQ is the minimum injected amount that gives precise measurements. The LOQ in plasma was defined as the sample concentration generating a peak height ten times higher than the level of the baseline noises (S/N of 10). The LOD was determined by spiking the reference standard with decreasing concentrations of galantamine, rivastigmine and NAP 226-90 until the S/N equaled 3 ($S/N = 3$, sampling 50 s at 7 kV). The absolute recoveries based on 5.0, 10.0 and 50.0 ng/mL of galantamine, rivastigmine and NAP 226-90 spiked in plasma were determined by comparing the calculated concentrations with the standard.

2.5 Application

The study protocol was approved by the Ethics Committee of the Kaohsiung Medical University Hospital. One healthy male volunteer (26-years-old) was administered a single dose of 4.5 mg rivastigmine capsule (Exelon®, Novartis) orally. Venous blood samples were collected in heparinized tubes before dosing and at 0, 0.5, 1.0, 2.0, 4.0, 8.0 and 12.0 h after dosing. The plasma collected before dosing was employed as a blank. All blood samples were centrifuged immediately at 500*g* and the plasma was separated and stored at −70°C until analysis. In addition, 11 different patients with AD had continuously received oral administration of AChEI to control their symptoms for at least 1 year: 7 AD patients took 8 mg galantamine capsule once daily (Reminyl®, Janssen) and the other 4 AD patients took 3.0 mg rivastigmine capsule (Exelon®, Novartis) two times daily. We measured the concentrations of galantamine or

rivastigmine and its major metabolite NAP 226-90 in the patients' plasma at steady state. Blood samples were collected in heparinized tubes and centrifuged immediately to monitor drug concentrations. Plasma fraction was separated immediately.

3 Results and discussion

Samples from biological origin frequently contain significant amounts of salts or buffer ions. These sample matrices can dramatically influence resolution and detectability when injected into the CE instrument. It is not unusual to find that separations performed on standards in relatively clean sample matrices are superior to the separations in more complex environments such as plasma. Thus, LLE with diethylether, dichloromethane and ethyl acetate was tested as sample pretreatment for detectability enhancement. Higher extraction recovery was obtained with diethylether and dichloromethane. However, a clean electropherogram was observed using diethylether as the extraction solvent. Diethylether was chosen as sample pretreatment for the extraction of the analytes from human plasma. After samples clean up, experiments were performed to examine several parameters that would affect the assay method, including the type and composition of the sample and BGE system (concentration and pH of Tris buffer, amount of PVP, SOS and organic modifier ACN).

3.1 Effect of sample matrix and injection

3.1.1 Influence of the nature of sample solvent

It appears that the composition of the sample is important with respect to the peak efficiency of the analyte. The phenomenon is related to the relative conductivities of the sample zone and the BGE: the greater the difference in conductivity between the sample zone and the run buffer, the greater the sample stacking. Therefore, after treating plasma samples with LLE (diethylether) and evaporation, we tried to enhance the detection sensitivity by changing the nature of reconstructed sample solvents. It has been reported that the addition of organic modifier to the sample solvent drastically influences stacking efficiency due to a modification of conductivity [22]. In this case, the electric field in the sample zone is relatively high, causing the analytes to migrate rapidly until they reach the interface between the sample buffer and run buffer and the sample to be stacked at that interface. Therefore, we studied the influence of three organic solvents (methanol, isopropanol and ACN) on the sample solvent in electrokinetic injection mode by injecting the analyte dissolved in three different hydro-organic mixtures: water–methanol (20:80 v/v), water–isopropanol (20:80 v/v) and water–ACN (20:80 v/v). The heights of the tested drugs give a better response in the presence of water–ACN than any other. The sensitivity of the three analytes in water–ACN mixture could be higher by a factor of about 1.6- and 6.7-fold in water–methanol and in water–isopropanol, respectively. Thus, the water–ACN mixture was selected as the sample solvent for further study.

3.1.2 Influence of the addition of phosphoric acid to the sample solvent

The stacking efficiency of on-column injection was affected by sample matrix. With electrokinetic sample introduction, the amount of solute injected is proportional to the effective electrophoretic mobility. Although pure solvent has the lowest conductivity, neither water nor ACN has the ability to act as a proton donator for galantamine (pK_a 8.2), rivastigmine (pK_a 8.9) or NAP 226-90. It has already been demonstrated that the addition of acid to the sample solvent may enhance the protonation of the cationic drugs and improve the sensitivity detection during electrokinetic injection because a larger amount of higher-mobility ions is introduced [23]. Thus, to charge the analytes, phosphoric acid and hydrochloric acid were added to the sample. The results showed that phosphoric acid can confer better sensitivity and higher theoretical plate number than hydrochloric acid. For the maximum stacking efficiency, the impacts of the phosphoric acid concentrations (0.01, 0.05 and 0.10 mM) on the water–ACN mixture (20/80 v/v) to dissolve the sample residue were studied. Figure 2 indicates the effect of the concentration of phosphoric acid added to the sample solvent upon the sensitivity of galantamine, rivastigmine and NAP 226-90. Peak height increased as the concentration of phosphoric acid increased due to an enhanced protonation of positively charged galantamine, rivastigmine and NAP 226-90. The peak heights of tested drugs were improved when the phosphoric acid content in water–ACN mixture increased from 0.01 to 0.05 mM and then decreased until 0.1 mM; the sensitivity ratios of three analytes were around 0.69, 1.0 and 0.89 for 0.01, 0.05 and 0.1 mM of phosphoric acid in the reconstructed solvent, respectively. The optimal H_3PO_4 concentration was found to be 0.05 mM. Thus, for robust operation with high sensitivity, the galantamine, rivastigmine and NAP 226-90 need to be extracted from plasma samples by LLE and reconstructed in ACN/0.25 mM H_3PO_4 aqueous solution (80:20 v/v). The reconstructed sample solution was electrokinetically injected at a positive voltage of 7 kV for 50 s.

3.2 Optimizing of separation buffer

Owing to the positive charge of galantamine, rivastigmine and NAP 226-90 under acidic BGE, it is important to consider the interaction of the analytes with the wall of fused-silica capillary. The migration time of analyte gradually became longer and less repeatable, when the BGE was Tris 25 mM (pH 5.0) and 160 mM SOS without

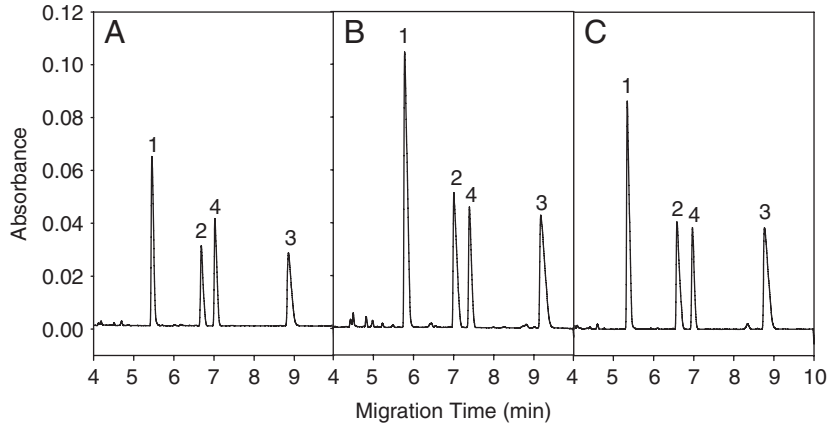

Figure 2. Effect of phosphoric acid concentration in hydro-ACN (20:80 v/v): (A) 0.01 mM H_3PO_4; (B) 0.05 mM H_3PO_4; (C) 0.1 mM H_3PO_4. CE conditions: 25 mM Tris (pH 5.0) with 160 mM SOS, 20% ACN and 0.01% PVP. Peaks: 1, rivastigmine; 2, galantamine; 3, NAP 226-90; 4, atenolol (IS), respectively. Applied voltage, 12 kV (detector at the cathode side); uncoated fused-silica capillary, effective length 20 cm × 50 μm id; sample injection, 7 kV, 50 s; wavelength, 200 nm.

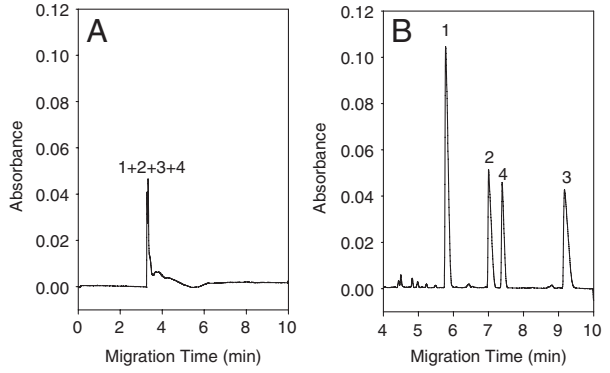

Figure 3. Electropherograms for the simultaneous analysis of galantamine, rivastigmine, NAP 226-90 and atenolol using different modes. (A) CZE, Tris 25 mM (pH 5.0) with 20% ACN and 0.01% PVP; (B) MEKC, Tris 25 mM (pH 5.0) with SOS 160 mM, 20% ACN and 0.01% PVP. Peaks: 1, rivastigmine; 2, galantamine; 3, NAP 226-90; 4, atenolol (IS), respectively. Other CE conditions are as in Fig. 2.

PVP. In order to minimize the adsorption of analytes, three kinds of 0.01% of polymers including PEO, PVP and PVA were added into BGE with the aim of dynamically modifying the capillary wall. A broader peak of rivastigmine metabolite, NAP 226-90, was observed when PEO was used as a capillary modifier, compared with the other polymers. An unstable baseline appeared when PVA was used as an additive in the rinse buffer. The results show that PVP can provide better reproducibility of migration. On the other hand, ;the results showed that PVP can also confer better sensitivity and higher theoretical plate number than PEO and PVA. The sensitivity ratios are around 0.75, 0.9 and 1.0 for PEO, PVA and PVP, respectively. The effect of PVP concentration (0.01–0.05%) on the repeatability of migration time and sensitivity was studied. With the concentration of PVP $\geq$ 0.01% in the BGE buffer, a reproducible migration of the drugs and similar resolution was observed and sensitivity ratios varied in the range from 1.0 to 0.82, the best result was found to be 0.01%. PVP was shown to rely on hydrophobic interaction or hydrogen-bonding to dynamically bind or adsorb to the capillary wall and the repeatability of migration time of the analytes was obtained. Finally, PVP was selected as the dynamic coating on the surface wall of capillary to reduce analyte adsorption in this study. Without PVP, lesser repeatability of migration time of rivastigmine metabolite NAP 226-90 was observed. Experiments were performed to determine the optimum conditions including Tris buffer system (concentration and pH), surfactant SOS and organic modifier ACN.

3.2.1 Effect of SOS

Galantamine (100 ng/mL), rivastigmine (40 ng/mL) and NAP 226-90 (40 ng/mL) extracted from human plasma by simple CZE with 25 mM Tris buffer, 0.01% PVP and 20% ACN under pH 5.0 were studied as rinse and separation buffers. Lower sensitivity with complete overlapping was observed (Fig. 3A). This was probably because the stacking effect for galantamine, rivastigmine and NAP 226-90 was insufficient for detection and pK_a values are very close to each other. Therefore, the surfactant, SOS or SDS, was added to Tris buffer (25 mM, pH 5.0) as rinse and separation buffers to modify the conductivity of BGE to enhance sample stacking and separation efficiency. In the preliminary test, we used surfactant SDS or SOS at 160 mM in run buffer; SOS can provide shorter migration of the tested drugs. Moreover, the peaks of galantamine and NAP 226-90 were not appeared in electropherogram even using a long period of 15 min when SDS was used as the surfactant. For reducing the migration times of the analytes, the lower concentration of SDS (40 mM) was used for separation and sample stacking; however, lower sensitivity of the tested drugs than SOS at 160 mM was observed. The results of separation using SOS are shown in Fig. 3B. Moreover, the donepezil peak did not appear in electropherogram even with a long period of 15 min. SOS has better separation efficiency, indicating that anionic surfactant SOS provided a suitable carbon chain length for separation. The negative charge of SOS may

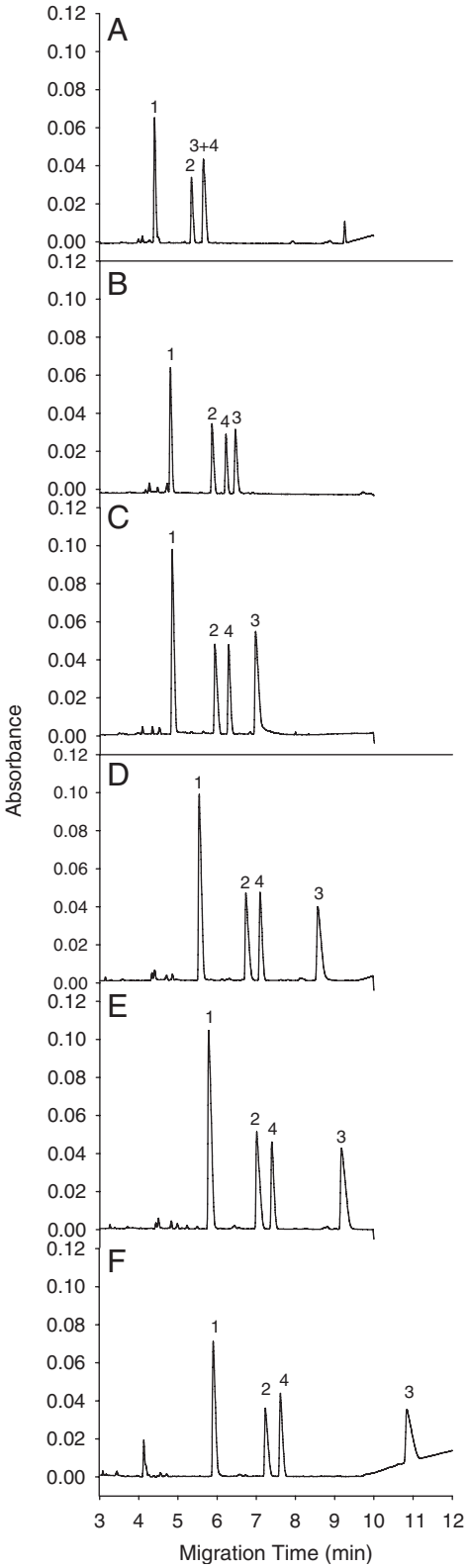

Figure 4. Effect of SOS concentrations (80–180 mM) in Tris buffer (25 mM, pH 5.0) with 20% ACN and 0.01% PVP on the sensitivity of galantamine, rivastigmine and NAP 226-90. (A) 80 mM; (B) 100 mM; (C) 120 mM; (D) 150 mM; (E) 160 mM; (F) 180 mM. Peaks: 1, rivastigmine; 2, galantamine; 3, NAP 226-90; 4, atenolol (IS), respectively. Other CE conditions are as in Fig. 2.

increase the sensitivity owing to the modified conductivity of BGE or to a strong interaction with a cationic analyte through electrostatic force. This allowed the increase in the sample introduced and stacked in interface under the lower pH value. As a consequence, simple parameters affecting the CE separation using SOS as the surfactant for simultaneous determination of galantamine, rivastigmine and NAP 226-90 were studied, including concentrations of the buffer, SOS and pH. The effects of SOS at a concentration range from 80 to 180 mM in Tris buffer (25 mM; pH 5) with 0.01% PVP and 20% ACN on the separation are shown in Fig. 4. The peak height of galantamine, rivastigmine and NAP 226-90 increased with the increase in SOS concentration and significant improvement in sensitivity was obtained under the 120–160 mM SOS in Tris buffer with 0.01% PVP. It has been shown that surfactant concentration does change conductivity, the EOF velocity, and consequently the sensitivity [24]. Therefore, 160 mM of SOS was used in this study, which is higher than its critical micellization concentration (CMC). The CMC values were determined by a procedure reported by Xu *et al.* [25], using pyrene as the fluorescent probe. The results showed the CMC values of SOS in water and in run buffer with 20% ACN were 80 and 140 mM, respectively. The micelles were formed under this tested condition. Micelles of ionic surfactant migrate electrophoretically due to their surface charge; therefore, the SOS micelles with negative charge go to the anode. If a solute is introduced into a micellar system, it will partition between the hydrophobic micellar phase and the aqueous phase with a particular partition coefficient P depending on the polarity of the analytes. Based on differential solubilization in micellar phase, the partition between the slow-moving micelles and the fast-moving aqueous phase causes differential retention and resolution of the solute. Owing to good solubility properties in organic modifier, the tested drugs in ACN can give better separation efficiency when performed in Tris buffer with SOS (160 mM) and ACN. Therefore, 20% ACN as an organic modifier was added in Tris buffer with 160 mM SOS on the simultaneous separation of galantamine, rivastigmine and NAP 226-90 for further studied.

3.2.2 Concentration and pH of Tris buffer

Because of limited sample loadability and a short detector path-length, CE is limited in trace analysis. Research has been carried out to improve the detection limits in CE. To achieve this goal, one strategy makes use of sample pre-concentration techniques through suitable BGE modification including increasing the difference in conductivity between the sample zone and run buffer. The effects of the concentration of Tris buffer 5–45 mM (pH 5.0) containing 160 mM SOS, 20% ACN and 0.01% PVP were investigated in plasma spiked with galantamine, rivastigmine and NAP 226-90 standards. Comparing the Tris concentrations (from 5 to 45 mM) for the effect of the sensitivity of galantamine, rivastigmine and NAP 226-90, the produced sensitivity ratios varied in the range from 0.8 to 1.0 and the best result was found to be 25 mM. On

the other hand, varied concentrations of Tris buffer 5–45 mM (pH 5.0) on the separation can give similar resolutions. Therefore, 25 mM of Tris buffer was chosen for the separation.

The degree of protonation of species present in the BGE system depends on the pH of the solution. Differences in the degree of ionization give rise to differences in electrophoretic mobilities. The analytes are weak basic compounds (pK_a 8.2–8.9) due to having the tertiary amine group. The effect of pH (4.0, 4.5, 5.0, 5.5 and 6.0) of 25 mM Tris buffer with 160 mM SOS, 20% ACN and 0.01% PVP on the separation and sensitivity of analytes were studied. The pK_a of atenolol (IS) was around 9.6, which was close to the pK_a value of the tested drugs. The analytes and IS dominate as the cationic species in electrolyte solution at the tested pH. Their cationic properties mean that they can be introduced simultaneously into capillary under positive voltage. Good resolution of the tested drugs at various pH values was obtained. Migration time decreased with increasing pH and a shorter migration time of the drugs was obtained at high pH. However, the pH values of buffer slightly influenced the peak height as shown in Fig. 5. Comparing the pH values of Tris buffer from 4.0 to 6.0 for the effect of the peak height sensitivity of rivastigmine, the pH 5.0 of Tris buffer gave a better response than any other. The sensitivity ratios of rivastigmine according to the peak height were 0.9, 0.9, 1.0, 0.9 and 0.87 at pH 4.0, 4.5, 5.0, 5.5 and 6.0, respectively. The best result was found to be pH 5.0. Therefore, pH 5.0 of Tris buffer was chosen for the simultaneous determination of galantamine, rivastigmine and NAP 226-90 in plasma. The 25 mM Tris buffer (pH 5.0) with 160 mM SOS, 20% ACN and 0.01% PVP was chosen as the optimal separation buffer. The typical electropherogram of the simultaneous separation of galantamine, rivastigmine, NAP 226-90 and atenolol (IS) in plasma is shown in Fig. 6B. Repeatability of migration velocity of galantamine, rivastigmine and NAP 226-90, and atenolol in plasma were investigated ($n = 20$), and the observed migration times were 7.03 ± 0.05, 5.70 ± 0.05, 9.17 ± 0.09 and 7.25 ± 0.08 min for galantamine, rivastigmine, NAP 226-90 and atenolol, respectively.

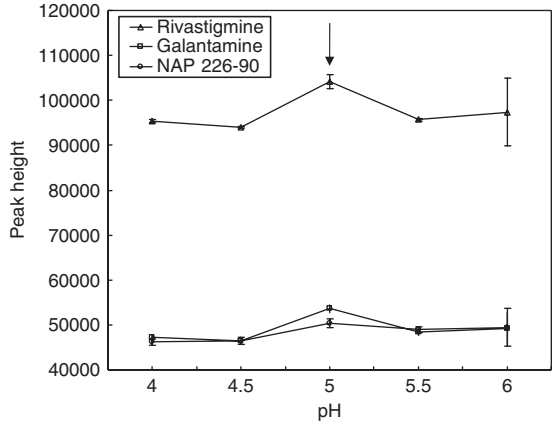

Figure 5. Effect of pH values (4.0–6.0) in Tris buffer (25 mM) with SOS 160 mM, 20% ACN and 0.01% PVP on the sensitivity.

Methanol was used for EOF determination. The observed migration time of EOF was 15.49 min. The apparent mobility (μA) was calculated according to the equation $\mu A = \mu E + \mu \mathrm{EOF} = (lL/tV)$, where l is the length along the capillary (cm) to the detector, V is the voltage, t is the migration time (s) and L is the total length (cm) of the capillary [26]. Under optimized CE conditions, the electrophoretic mobility values (μE) of galantamine, rivastigmine, NAP 226-90 and atenolol were 1.19×10^{-4}, 1.47×10^{-4}, 9.15×10^{-5} and $1.16 \times 10^{-4}\,\mathrm{cm^2\,V^{-1}\,s^{-1}}$, respectively.

3.3 Method validation in human plasma

To evaluate the quantitative application of the method, five different concentrations over the range 1.0–120.0 ng/mL of galantamine, 0.5–50.0 ng/mL of rivastigmine and NAP 226-90 and atenolol (100 ng/mL) (IS) were analyzed. The linear regression equations in plasma samples are listed in Table 1. The correlation coefficients of intra- and inter-day regression equations were all above 0.998. These data demonstrate the high linearity of this method for intra- and inter-day assays. The precision of the proposed method was evaluated on spiked plasma sample and estimated by the RSD value. The results in Table 2 show that the intra- and inter-day RSD of different concentrations from high, medium to low were all below 8.8%. The accuracy of galantamine, rivastigmine and

Table 1. Regression analyses for determination of spiked galantamine, rivastigmine and NAP 226-90 in human plasma

	Regression equations	Correlation coefficient (r)
Galantamine		
Intra-day[a)]		
1.0-120.0 ng/mL	$Y = (0.0492 \pm 0.0005)X + (-0.0212 \pm 0.0147)$	0.999
Inter-day[b)]		
1.0–120.0 ng/mL	$Y = (0.0488 \pm 0.0004)X + (-0.0168 \pm 0.0127)$	0.999
Rivastigmine		
Intra-day		
0.5–50.0 ng/mL	$Y = (0.0535 \pm 0.0003)X + (0.0247 \pm 0.0117)$	0.999
Inter-day		
0.5–50.0 ng/mL	$Y = (0.0539 \pm 0.0017)X + (0.0226 \pm 0.0151)$	0.998
NAP 226-90		
Intra-day		
0.5–50.0 ng/mL	$Y = (0.0407 \pm 0.0006)X + (0.0093 \pm 0.0113)$	0.999
Inter-day		
0.5–50.0 ng/mL	$Y = (0.0411 \pm 0.0011)X + (-0.0027 \pm 0.0137)$	0.998

a) Intra-day data were based on three replicate analyses.
b) Inter-day data were from five consecutive days.

NAP 226-90 was obtained from the RE values at three concentrations, which were all below 9.4% for intra- and inter-day assays. Compared with peak area ratio of the standard galantamine, rivastigmine and NAP 226-90, the absolute recoveries at three level concentrations are above 85%. The LOQ of galantamine was 1.0 ng/mL; and for rivastigmine and NAP 226-90 it was 0.5 ng/mL. The LODs were about 0.25 ng/mL for galantamine and 0.125 ng/mL for rivastigmine and NAP 226-90. Under the present MEKC conditions, a complete separation of commonly used AChEI such as galantamine, rivastigmine and NAP 226-90 was obtained as shown in Fig. 6. Peaks 1, 2, 3 and 4 represent rivastigmine, galantamine, NAP 226-90 and atenolol (IS), respectively. Many older adults have chronic cardiovascular and cerebral vascular disorders and are commonly treated with vasodilators. Atenolol, a beta-adrenergic blocker, is used for hypertension treatment. Therefore, galantamine or rivastigmine can be used, instead of atenolol, as an IS for quantification of the rivastigmine or galantamine when atenolol is co-administrated with galantamine or with rivastigmine. In our study, the 11 AD patients were not found to have used the atenolol for vasodilation.

Table 2. Precision and accuracy for the recovery of spiked galantamine, rivastigmine and NAP 226-90 in human plasma

Concentration known (ng/mL)	Concentration found (ng/mL)	RSD (%)	RE (%)
Galantamine			
Intra-day[a)]			
10.0	9.5±0.4	4.6	−4.6
30.0	30.4±1.0	3.3	1.4
120.0	120.2±1.5	1.2	0.2
Inter-day[b)]			
10.0	9.4±0.3	2.6	−5.8
30.0	30.8±1.1	3.4	2.7
120.0	120.3±1.9	1.6	0.3
Rivastigmine			
Intra-day			
2.0	1.8±0.0	2.4	−9.4
20.0	20.7±0.7	3.3	3.6
50.0	50.1±1.1	2.3	0.3
Inter-day			
2.0	1.9±0.0	2.4	−7.4
20.0	20.9±0.6	3.0	4.3
50.0	50.4±1.3	2.5	0.9
NAP 226-90			
Intra-day			
2.0	1.8±0.2	8.1	−8.6
20.0	10.4±0.7	3.3	1.8
50.0	50.4±1.0	2.0	0.7
Inter-day			
2.0	1.9±0.2	8.8	−5.9
20.0	21.2±0.5	2.5	5.8
50.0	50.2±1.2	2.4	0.4

a) Intra-day data were based on three replicate analyses.
b) Inter-day data were from five consecutive days.

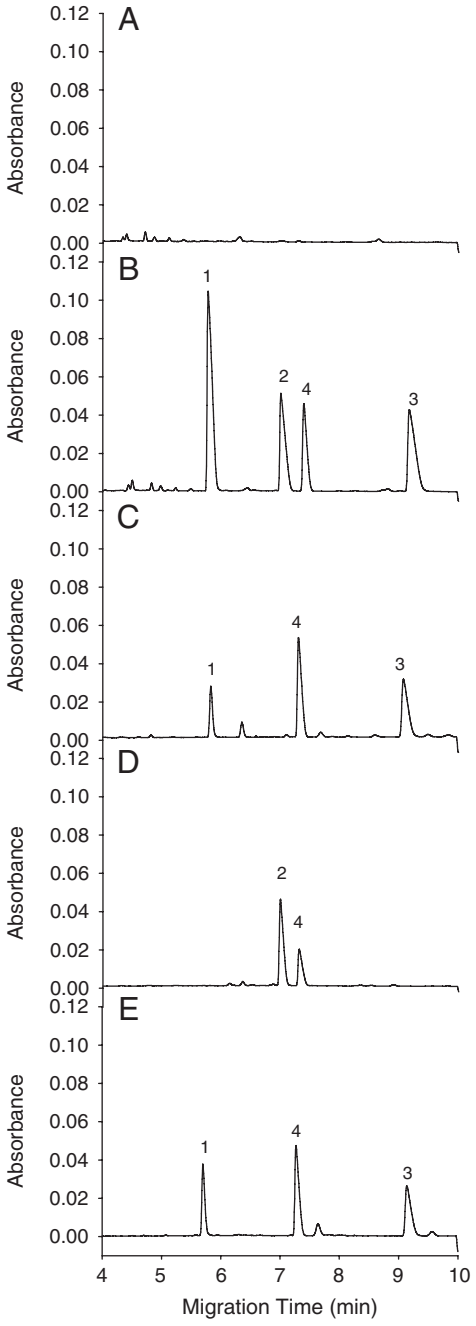

Figure 6. Electropherograms of galantamine, rivastigmine and NAP 226-90 in plasma determinations. (A) Plasma blank; (B) plasma spiked galantamine (100 ng/mL), rivastigmine (40 ng/mL), NAP 226-90 (40 ng/mL) and atenolol (100 ng/mL); (C) oral administration of 4.5 mg rivastigmine (Exelon®) single dosing after 2 h in healthy volunteer plasma; (D) plasma sample from AD patient who was orally administered 8.0 mg galantamine (Reminyl®) at steady state; (E) plasma sample from AD patient who was orally administered 3.0 mg rivastigmine (Exelon®) at steady state. Peaks: 1, rivastigmine; 2, galantamine; 3, NAP 226-90; 4, atenolol (IS), respectively. Other CE conditions are as in Fig. 2.

3.4 Application to human plasma and clinical study

One male volunteer (26-years-old) was administered one 4.5 mg rivastigmine (Exelon®) capsule orally. The electropherogram resulting from the analysis of plasma samples at 2 h after dosing is shown in Fig. 6C. To study the concentration–time profiles of rivastigmine, the plasma samples from the volunteer were measured at different time intervals. The concentration–time curve of rivastigmine in plasma after single dose oral administration is shown in Fig. 7. On the other hand, seven plasma samples from different AD patients who received oral administration of 8.0 mg galantamine (Reminyl®) and four plasma samples from different AD patients who received oral administration of 3.0 mg rivastigmine (Exelon®) at steady state were studied and the observed concentrations are summarized in Table 3. The electropherograms of extracted plasma samples obtained from one AD patient (46.4 ng/mL, patient number 1 in Table 3) receiving 8.0 mg/daily of galantamine (Reminyl®) and another AD patient (10.3 ng/mL, patient number 8 in Table 3) receiving 3.0 mg two times daily of rivastigmine (Exelon®) are shown in Fig. 6D and E, respectively, and these did not show interference peaks.

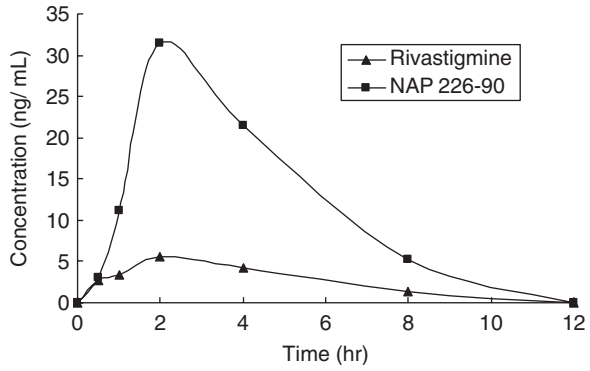

Figure 7. Concentration–time curve of rivastigmine in plasma after oral administration of single dose 4.5 mg of rivastigmine capsule (Exelon®). Data are expressed as mean ± SD; $n = 3$.

Table 3. The concentrations of galantamine, rivastigmine and NAP 226-90 in AD patients' plasma

Patient number	Drug concentration (ng/mL)		
	Galantamine	Rivastigmine	NAP 226-90
1	46.4		
2	0.9		
3	103.4		
4	2.4		
5	4.2		
6	92.5		
7	1.0		
8		10.3	18.9
9		12.0	23.0
10		1.4	5.1
11		17.2	52.1

Galantamine can be metabolized by hepatic cytochrome p450 isozymes CYP2D6. From the results (Table 3), the plasma concentration of galantamine was observed to be quite different in AD patients at steady state. The concentrations of rivastigmine in patients' plasma higher than the therapeutic range (rivastigmine: 1–10 ng/mL) were observed in patients 9 and 11. This may in part reflect the variability associated with pharmacogenetic factors, metabolite enzyme induce or patient compliance. Some researchers have hypothesized that the cognitive improvement was in proportion to the concentration of AChEI in plasma [5]. In an individualized efficacy therapy, which minimizes toxicity for patient safety, dose-adjustment of ACEIs in AD patients can obtain better therapeutic efficacy, based on individual pharmacokinetic parameters. Therefore, physicians should take the concentration of medicine into consideration when making a decision regarding treatment responses or switch to another AChEI medicine used in patients.

4 Concluding remarks

The LLE coupling MEKC method described above allowed the simultaneous determination of galantamine, rivastigmine and NAP 226-90 in plasma in a sensitive and efficient manner. Validation of the method for quantification of galantamine, rivastigmine and NAP 226-90 in plasma showed that the method had high sensitivity and accuracy. The method was sensitive to ng/mL level measurement. Moreover, the method was suitable for the analysis of rivastigmine and its metabolite, NAP 226-90, in plasma collected during pharmacokinetic investigations in humans and successfully applied to plasma samples from AD patients after orally administered commercial preparations, Reminyl® or Exelon®. This analytical method might be applicable to therapeutic drug monitoring of galantamine, rivastigmine and NAP 226-90 and it might be used to investigate the relationship of concentration of AChEIs in plasma and therapeutic effectiveness in AD patients.

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The authors have declared no conflict of interest.

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