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Research Article

Simultaneous determination of memantine and amantadine in human plasma as fluorescein derivatives by micellar electrokinetic chromatography with laser-induced fluorescence detection and its clinical application

A nonionic surfactant MEKC method with LIF detection was developed for the simultaneous determination of memantine, an anti-Alzheimer's disease agent, and amantadine, an anti-Parkinson's disease drug, in human plasma. Before analysis, the plasma samples were pretreated by liquid–liquid extraction with ethyl acetate, and derivatized with 6-carboxyfluorescein *N*-hydroxysuccinimide ester. The chemical derivatization is performed with 6-carboxyfluorescein *N*-hydroxysuccinimide ester in ACN – 5 mM pH 9.0 borate buffer (40:60, v/v) at 35°C for 3 h. After the derivatization reaction, hydrodynamic injection (0.5 psi, 8 s) was used to introduce the derivatized solution, and the separation was performed in borate buffer (30 mM, pH 9.5) with the nonionic surfactant Brij-35® (0.07%, w/v); the separation voltage was 6 kV. The linear ranges of the method for the determination of memantine and amantadine in human plasma were over a range of 2.0–60.0 ng/mL. The detection limit was 0.5 ng/mL ($S/N = 3$). This method was applied successfully to monitor the concentration of memantine or amantadine in patients with Alzheimer's disease or Parkinson's disease.

Keywords:
6-Carboxyfluorescein *N*-hydroxysuccinimide ester / Alzheimer's disease and Parkinson's disease / Derivatization / Memantine and amantadine / Micellar electrokinetic chromatography

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1 Introduction

Alzheimer's disease (AD) and Parkinson's disease (PD) are common neurodegenerative disorders resulting from an impaired cholinergic function with loss of cognitive activity and motor system disorder, respectively, in the brain. The progression of the diseases varies from individual to individual, so treatment is also individualized. AD and PD can impair the quality and length of life, so it devastates both the individual patient and his family. Strategies to reduce various burdens of the diseases by means of drug therapy are important.

Memantine, an amantadine derivative, is a novel class of AD medications acting on the glutamatergic system by blocking *N*-methyl-D-aspartate (NMDA) glutamate receptors. Long-term overactivation of NMDA receptors would lead to neuronal death; thus, it seems that this antagonism of NMDA receptors could provide neural protection and play a major role in the AD treatment. Targeting the NMDA receptors offers a novel approach for treatment in view of the limited efficacy of the existing acetylcholinesterase inhibitors (AChEI) (such as donepezil, rivastigmine, *etc.*) targeting the cholinergic system and resulting in symptomatic improvement in AD. The drug memantine is approved for the treatment of AD in moderate-to-severe dementia. Oral memantine has been evaluated as monotherapy or in addition to a stable dose of AChEI for moderate-to-severe AD patients [1–4].

Amantadine possesses pharmacological actions as both an antiparkinsonian and an antiviral agent [5, 6]. It also acts as an NMDA-receptors antagonist. The mechanism of anti-PD symptoms of amantadine is not precisely known. As a

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Abbreviations: AChEI, acetylcholinesterase inhibitors; **AD,** Alzheimer's disease; **CFSE,** 6-caroxyfluorescein *N*-hydroxysuccinimide ester; **EA,** ethyl acetate; **IS,** internal standard; **LLE,** liquid–liquid extraction; **NMDA,** *N*-methyl-D-aspartate; **PD,** Parkinson's disease

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monotherapy, amantadine is frequently used for the treatment of the early stage of PD and for weaker motor fluctuation and dyskinesias. Memantine and amantadine undergo hepatic metabolism by *N*-glucuronidation and *N*-acetylation, respectively; the parent drugs and their metabolites are eliminated primarily by the kidney. In older people and in the patients with a decreased kidney function, the dose of the drugs should be reduced.

The structures of amantadine (adamantan-1-amine) and memantine (3,5-dimethyladamantan-1-amine) are shown in Fig. 1; they are only different in two methyl groups. The p*K*a of memantine and amantadine are 10.3 and 10.1, respectively. The recommended initial dose for memantine in the treatment of moderate-to-severe dementia of AD is 5 mg once daily and the maintenance dose is 20 mg/day. It has been reported that following the oral administration of 10 mg memantine twice daily, the steady state of plasma concentration in healthy subjects ($n = 8$) was 82.4 ng/mL ± 17.8 ng/mL, whereas in renally impaired individuals, the peak concentration increased to 85.2 ng/mL ± 22.1 ng/mL (mild) and 128.1 ng/mL ± 27.2 ng/mL (moderate), respectively. The dosage of amantadine for the treatment of PD is 100–200 mg/day, and this can be increased to 200–400 mg/day. The plasma concentration of amantadine is individually variable, and the concentration would be affected by renal function.

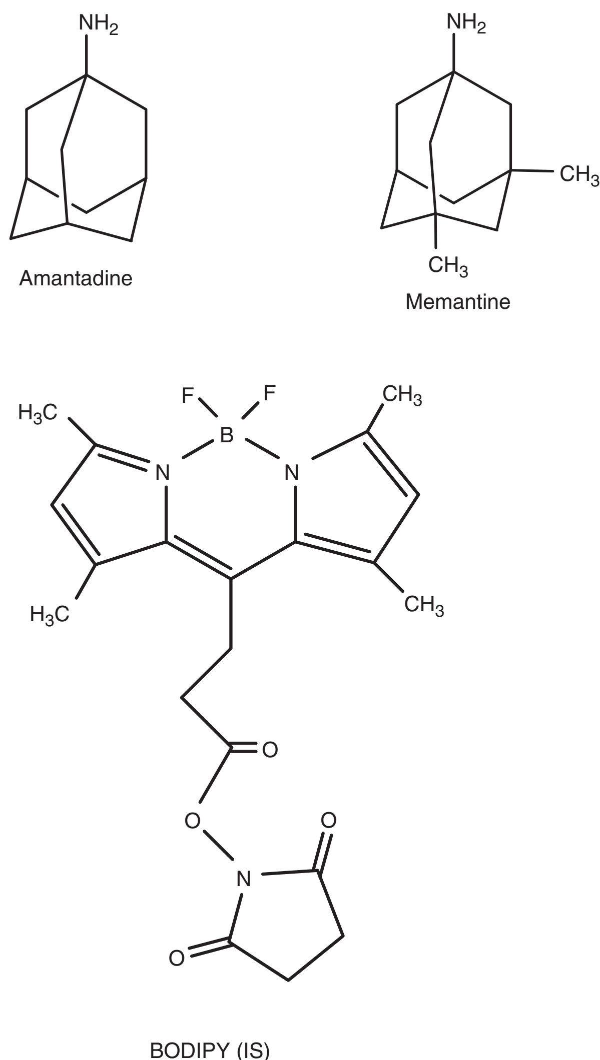

Figure 1. Chemical structures of memantine, amantadine, and BODIPY (IS)

The structures of the two adamantane derivatives (memantine and amantadine) are primary amines with aliphatic tricyclic moiety having neither UV absorption nor fluorescent properties. They are almost transparent in the UV–Vis range with low sensitivity. Therefore, a practical approach is required for CE with the LIF analysis of memantine and amantadine with labeling a fluorophore at a low level. The CE method with indirect detection using an absorbing background electrolyte was developed [7]. LC-MS [8–11] has been used for the direct determination of amantadine or memantine. To increase the sensitivity sufficiently to detect the concentration of biological samples, the samples were extracted with organic solvent and derivatized with labeling agents, then analyzed by GC-MS [12, 13] or HPLC [14–21]. A powerful mass spectrometry detector can improve detection sensitivity, but it is expensive and therefore not widely applied in laboratories. Compared with HPLC, CE has the advantages of short analysis time, small sample volume, high separation efficiency, and requires less organic solvent. Chemical derivatization can modify drugs to give efficient absorption in LC, and it is often also applicable to CE. Consequently, several detector-oriented chemical derivatization methods for labeling amino analytes with fluorophore have been developed. Nagaraj *et al.* [22] described a CE-LIF method for amantadine determination; the LOQ was 1.8 ng/mL after extraction from plasma into organic solvent, evaporation and then derivatized with Cy5.29OSu. We previously developed a sensitive CE combining on-column field-amplified sample stacking method for the determination of AChEIs (donepezil, galantamine, and rivastigmine) for the patients with AD [23–25]. However, donepezil, galantamine, and rivastigmine have strong UV absorption. So far, no CE method has been reported for the simultaneous determination of adamantane derivatives with neither UV absorption nor fluorescent property in human plasma for monitoring the drug concentrations in AD or PD patients. In this study, we aimed to develop a highly sensitive and efficient MEKC-LIF method using 6-caroxyfluorescein *N*-hydroxy-succinimide ester (CFSE) as a derivatizing reagent for the determination of the anti-AD agent, memantine, and the anti-PD agent, amantadine, in plasma. After liquid–liquid extraction (LLE), the primary amine group on memantine and amantadine reacts with the labeling agent CFSE to form strong fluorophore amide derivatives for the MEKC-LIF analysis. The memantine and amantadine derivatives from CFSE are relatively stable under the CE condition. The aim of the study was not only to develop a sensitive MEKC-LIF method for the simultaneous trace determination of memantine and amantadine in plasma, but also to evaluate the concentrations of memantine and amantadine in AD and PD patients at steady state. In this study, we used LLE with ethyl acetate (EA) for plasma

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pretreatment, then performed detection-oriented derivatization and combined with nonionic surfactant MEKC for separation. The concentrations of memantine and amantadine in six different AD and four different PD patients who were administered 5 or 10 mg of memantine (Witgen®) orally twice daily or 100 mg of amantadine (PK-Merz®) orally once daily at steady state were evaluated.

2 Materials and methods

2.1 Chemicals

Amantadine (1-adamantanamine hydrochloride) was purchased from Alfa Aesar (England). Memantine (3,5-dimethyladamantan-1-amine hydrochloride), CFSE, and polyoxyethylene lauryl ether (Brij-35®) were from Sigma (St. Louis, MO, USA). 4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-propionic acid succinimidyl ester (BODIPY® 493/503, SE) as internal standard (IS) was offered by Molecular Probes (Invitrogen, USA). Sodium hydroxide, SDS, disodium tetraborate (Borate), acetone, ACN, methanol, 1-propanol, *n*-hexane, toluene, dichloromethane, and phosphoric acid (H_3PO_4, 85%) were supplied by Merck (Darmstadt, Germany). EA was purchased from Tedia (Fairfield, OH, USA); dimethylformamide (DMF) was from J.T. Baker (Phillipsburg, NJ, USA). Milli-Q (Millipore, Bedford, MA, USA) treated water was used for the preparation of buffer and related aqueous solutions.

2.2 CE instrumentation

A Beckman P/ACE MDQ system (Fullerton, CA, USA) equipped with a LIF detector (λ_{ex}: 488 nm;λ_{em}: 520 nm) and a liquid-cooling device was used. CE was performed in an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 31.2 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 new capillary was conditioned with methanol for 10 min, 1 M HCl for 10 min, de-ionized water for 2 min, 1 M NaOH for 10 min, and de-ionized water for 2 min. The routine wash between runs was carried out every day using pressure with ACN (2 min), 1 M NaOH (3 min), de-ionized water (2 min), and rinse buffer (5 min) under positive pressure (30 psi) applied at the injection end. A Beckman P/ACE MDQ Microsoft Software system was used for the data processing.

2.3 Sample pretreatment and derivatization

Drug-free human plasma samples were obtained from two normal volunteers used as blanks. To prevent the effect of plasma protein on the separation efficiency and selectivity, LLE with organic solvent was used as a clean-up and preconcentration method for the biological samples. A 100-μL aliquot of patients' plasma or plasma spiked with memantine and amantadine was pipetted into a 1.5-mL Eppendorf vial. The plasma sample was alkalized with 10 μL NaOH (4 M), extracted with 300 μL EA, and then vortexed for 2 min. After mixing, the sample was centrifuged with 10 000*g* for 5 min, and an aliquot (270 μL) of supernatant was evaporated to dryness by centrifugal vaporizer (EYELA CVE-2000, Japan). The residue was reconstituted with 50 μL of ACN/borate buffer (5 mM, pH 9.0) (v/v, 40:60) with vortex (3 min), then derivatized with 2 μL CFSE solution (20 mM prepared in DMF) at 35°C for 3 h. After derivatization, 30 μL of the sample solution was mixed with 4 μL 800 ng/mL BODIPY as IS which was dissolved in DMF. Then, the sample was transferred to a 0.2-mL mini-vial, which was placed in the sample tray of a Beckman P/ACE MDQ system for CE analyses. Hydrodynamic injection (0.5 psi, 8 s) was used to introduce the derivatized sample, and the separation was performed in borate buffer (30 mM, pH 9.5) with nonionic surfactant Brij-35® (0.07% w/v); the separation voltage was 6 kV.

2.4 Sample preparation

Stock solutions of memantine and amantadine reference standard at 1.0 μg/mL were prepared in de-ionized water; BODIPY solution (IS) was dissolved in DMF and suitably diluted with borate buffer (5 mM, pH 9.0). Plasma sample standards were prepared by spiking 10 μL of various levels of reference standards (600.0, 300.0, 100.0, 50.0, and 20.0 ng/mL of memantine and amantadine) into 90 μL of drug-free plasma. The quantification ranges of plasma samples were the final memantine and amantadine concentrations over the range 2.0–60.0 ng/mL and mixed with 4 μL of 800.0 ng/mL of BODIPY (IS).

2.5 Application

The developed method is applied for the determination of tested drug concentrations at steady state in different patients with AD who had continuously received oral administration of daily doses of 20 or 10 mg of memantine (Witgen®), and patients with PD who had received 100 mg of amantadine (PK-Merz®) once a day to control their symptoms. All blood samples were centrifuged immediately and the plasma was separated and stored at −70°C until analysis. The study protocol was approved by the Ethics Committee of the Kaohsiung Medical University Hospital.

3 Results and discussion

Memantine and amantadine have neither a strong fluorophore nor a conjugated system in their structures. This

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means the direct CE of memantine or amantadine is not straightforward even when using sample stacking techniques. To ensure that memantine and amantadine have high fluorescence and enhanced CE characteristics, chemical derivatization is applicable. On the other hand, samples from biological origin, such as plasma, frequently contain significant amounts of protein or ions. These sample matrices can dramatically influence resolution and column when directly injected into the CE instrument. Thus, LLE with organic solvents was commonly tested as sample pretreatment for detectability enhancement. MEKC based on the partition and electrophoretic mobility was selected to separate and quantify the tested drugs after derivatization. Therefore, optimization of the extraction and derivatization conditions for memantine and amantadine and the MEKC method for the resulting derivatives were studied.

3.1 Liquid–liquid extraction

LLE with diethyl ether [8], diethyl ether-chloroform [10], *n*-hexane [13, 16], dichloromethane [15], toluene [17, 19, 20], and isoamyl alcohol in *n*-heptane [21] or the solid-phase extraction method [11] were used for the pretreatment of memantine or amantadine in the biological samples. In the study, we used LLE as the clean-up method and compared different organic solvents on the efficiency of extraction. The organic solvents, such as *n*-hexane, toluene, EA, and dichloromethane, were tested. Higher extraction recovery and a clean electropherogram was observed using EA as the extraction solvent. The order of extraction efficiency was EA>toluene>*n*-hexane>dichloromethane. EA was chosen as the sample pretreatment for the extraction of the analytes from human plasma.

3.2 Optimizing the derivatization for memantine or amantadine

3.2.1 Labeling reagent

In a preliminary study, different labeling agents were tested, including (2-naphthoxy)acetyl chloride (NAC), 1-naphthyl isothiocyanate (NITC), FITC, 5-([4,6-Dichlorotriazin-2-yl] amino) fluorescein hydrochloride (DTAF), and CFSE. The active hydrogen atom in the amino group of the tested drugs, which can react with NAC by nucleophilic reaction, reacted with isothiocyanate group of NITC and FITC or

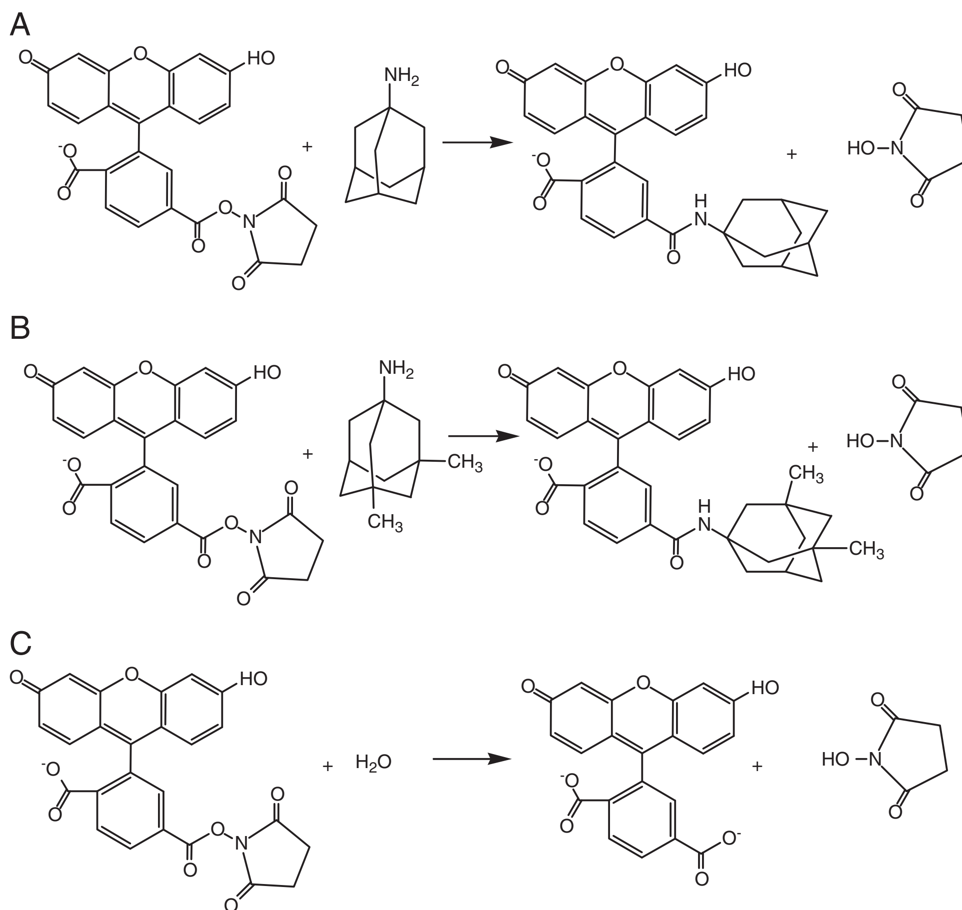

Figure 2. The derivatization scheme of CFSE with (A) memantine, (B) amantadine, and (C) hydrolysis.

removed succinimidyl moiety of CFSE to form the stable fluorescence corresponding derivatives. FITC is the most commonly used fluorescent probe, and the reaction was carried out at high pH. In the preliminary study, FITC reacted with a high concentration (100.0 μg/mL) of memantine, but it could not detect memantine derivatives at low concentration (1.0 μg/mL). It was inferred that the rate constant of derivatization (k_D) was similar to the rate constant of FITC hydrolysis (k_H). The reaction of DTAF or CFSE and tested drugs was also studied, and the results showed that the derivatization efficiency was better when CFSE was used as the labeling reagent. The sensitivity ratios of DTAF and CFSE as the labeling reagent reacted with memantine according to the peak height were about 0.3. Therefore, we chose CFSE for the derivatization of memantine and amantadine in this study.

To optimize the conditions to derivatize the tested drugs, several parameters were briefly studied, including the concentration of derivatizing reagent CFSE, borate buffer, and organic solvent. The effects of the parameters on the formation of the CFSE-memantine and CFSE-amantadine derivatives were evaluated by peak area ratios of memantine and amantadine derivatives to the BODIPY (IS). The derivatization reaction of CFSE with memantine and amantadine are shown in Fig. 2.

3.2.2 Derivatization medium

After extraction and then evaporation, the reconstructed solution was used as the derivatization medium. It is composed of borate buffer and organic solvents, so parameters influencing the derivatization efficiency, including borate buffer concentration, pH, and organic solvent, were investigated.

The pH of the derivatization medium is an important factor affecting reactivity of CFSE with amino compounds. Rapid reaction of succinimidyl reagents with amino acids under basic pH borate buffer was reported [25]. However, the labeling reagent tended to be hydrolyzed at higher pH value (see Fig. 2C). To optimize the derivatization conditions, different pH values ranging from 8.5 to 10.0 were investigated. From the results, the peak response is increased from pH 8.5 to 9.0 and then decreased as shown in Fig. 3A. The best peak response is obtained at pH 9.0 and the sensitivity ratios are 0.66, 1.0, 0.54, and 0.37 for memantine at pH 8.5, 9.0, 9.5, and 10.0, respectively; 0.58, 1.0, 0.63 and 0.42 for amantadine at pH 8.5, 9.0, 9.5, and 10.0, respectively. Therefore, pH 9.0 of borate buffer was selected for further study.

The effect of borate buffer (pH 9.0) concentration on the derivatization was investigated ranging from 2 to 30 mM. From the result, the fluorescence intensity of analyte derivatives was increased with increasing borate concentration from 2 to 5 mM. The optimal buffer concentration is 5 mM as shown in Fig. 3B; borate buffer at higher concentration (>5 mM) has a trend to decrease the peak area ratios but the reason for this is unclear.

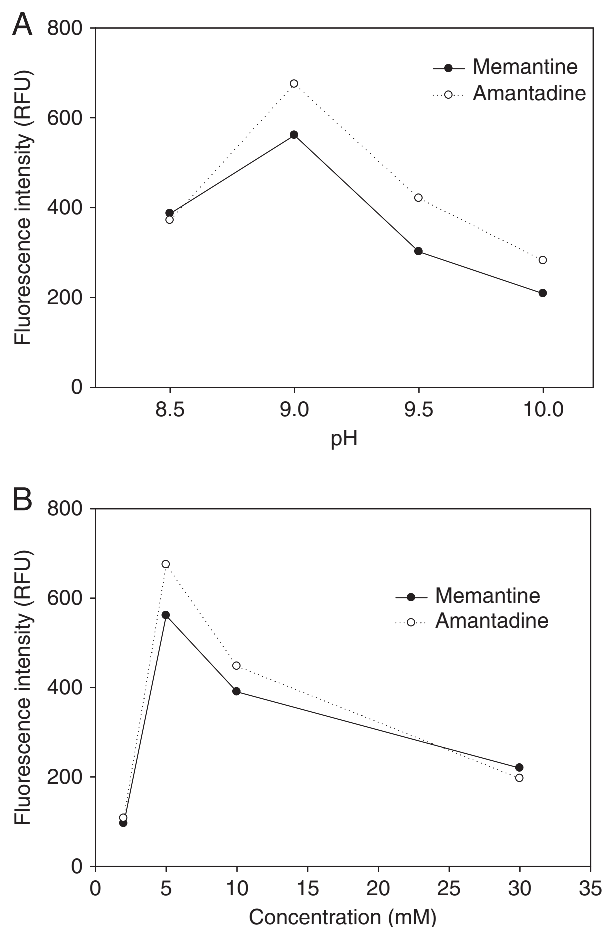

Figure 3. The effect of (A) pH values and (B) borate buffer concentrations in derivatization medium on the memantine and amantadine derivative formation and fluorescence intensity.

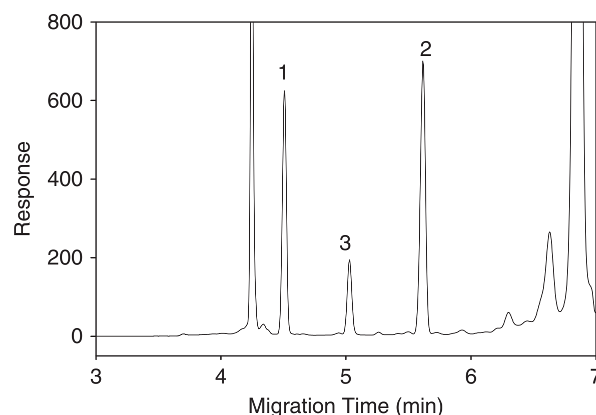

Figure 4. The effect of ACN in derivatization medium on the memantine and amantadine derivative formation. CE conditions: 30 mM Borate (pH 9.5) with 0.07% (w/v) Brij-35®. Peaks: 1, memantine (50.0 ng/mL); 2, amantadine (50.0 ng/mL); and 3, BODIPY (IS) (800.0 ng/mL). Applied voltage, 6 kV (detector at cathode side); uncoated fused-silica capillary, effective length 20 cm × 50 μm id; sample injection, 0.5 psi 8 s.

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The effects of 40% organic solvents in borate buffer (pH 9.0, 5 mM) on derivatization formation and fluorescence intensity were studied. We tested organic solvents, including methanol, 1-propanol, acetone, and ACN, as the additive in derivatization medium. ACN was used as the organic solvent in derivatization medium, which led to significant enhancement in the fluorescence intensity of CFSE-memantine and CFSE-amantadine derivatives as shown in Fig. 4. The influence of volume fraction of ACN in derivatization medium on derivatives formation and fluorescence intensity was studied. From the results, the volume fraction of ACN in derivatization medium has great influence on the sensitivity based on the peak height of CFSE-memantine and CFSE-amantadine derivatives. The peak response improved when the ACN content in derivatization medium increased from 20 to 40% v/v and then it decreased at 50% ACN, which may contribute to the increase in background noise. The peak heights of CFSE derivatives as the sensitivity ratios were 0.52, 0.71, 1.0, and 0.83 for memantine at 20, 30, 40, and 50% ACN, respectively; whereas they were 0.47, 0.64, 1.0, and 0.89 for amantadine at 20, 30, 40, and 50% ACN, respectively. Therefore, the highest sensitivity was obtained by employing the following: 60/40 (v/v) of borate buffer (5 mM pH 9.0) with ACN mixture as derivatization medium. On the other hand, ACN might decrease the conductivity of reaction matrix resulting in stacking the analyte derivatives. Therefore, 40% ACN in borate buffer for analyte derivatives formation was needed to increase the sensitivity of the method. The effects of temperatures (35, 50, and 60°C) on the derivatization of memantine and amantadine from spiked plasma (each 50.0 ng/mL) were studied. For derivatization at 50 and 60°C, the formation of the CFSE derivatives reached equilibrium in 1 h. However, the reaction at 35°C in 3 h resulted in a higher yield of the CFSE-memantine and CFSE-amantadine derivatives compared with the reactions at 50 and 60°C. The amount of CFSE required for the derivatization of the tested drugs was studied, and CFSE at 20 mM (2 μL; 40 nmol) at 35°C for 3 h seemed to be an optimum concentration for derivatizing memantine and amantadine.

The optimized derivatization procedure was as follows: patient plasma or spiked plasma samples were pretreated with EA for sample pretreatment and the residues after evaporation were reconstructed in 50-μL derivatization medium (5 mM, pH 9.0 borate buffer with 40% ACN), then 20 mM 2 μL CFSE was added to react at 35°C for 3 h. The stability of memantine and amantadine derivatives was tested under room temperature for 96 h and the peak response of the derivatives was found to be stable over the studied duration.

3.3 Optimizing the MEKC separation

In the study, we aimed to develop a CE method not only to separate memantine and amantadine derivatives, but also to investigate the interference of plasma or the excess derivatizing agent. Both memantine and amantadine are the adamantane derivatives, which differ by two methyl groups. The samples were hydrodynamically injected by 0.5 psi for 8 s, keeping the separation voltage at 6 kV (anode at the injection end). Thus, the CZE mode using 30 mM borate buffer (pH 9.5) as BGE was chosen in the preliminary study. The peaks of CFSE-memantine and CFSE-amantadine derivatives migrated closely and partial overlapped with poor resolution ($Rs = 0.8$).

The derivatives of CFSE-memantine and CFSE-amantadine are the ionizable species with varied hydrophobility resulted from two methyl groups in structures. MEKC mode was tested for efficient separation. The analyte derivatives also migrated closely and did not separate when anionic surfactant, SDS, was used; this seems to have been due to the weak interaction between the negative charges of analyte derivatives with anionic surfactant. On the other hand, neutral surfactants added in BGE have been reported to significantly increase the selectivity in CE separation of fluorescein-labeled compounds [26]. Thus non-ionic surfactant Brij-35® was investigated for the separation. Better separation efficiency without interference peaks was obtained, indicating that the nonionic surfactant Brij-35® as the micellar agent provided a suitable interaction with CFSE-memantine and CFSE-amantadine derivatives and it could shift the interfering peaks. Therefore, borate buffer with Brij-35® was selected for further investigation. As a consequence, simple parameters affecting the CE separation using Brij-35® as surfactant for memantine and amantadine determinations were studied, including concentrations of the buffer, Brij-35®, and pH. The effects of Brij-35® in a concentration range from 0.03 to 0.1% in borate buffer (30 mM, pH 9.5) were studied. Good resolution ($Rs \geqq 6.2$) of the tested drug at various concentrations was obtained. The CMC value of Brij-35® was about 91 μM and micelles were formed under these tested conditions. The micelles possess a hydrophilic surface and hydrophobic core, so the non-ionic micelle interacted with analytes *via* two mechanisms. The non-ionic surfactant consequently migrates with EOF, and thus the negative charge of CFSE derivatives will be in this zone. Analytes interacting with the micelles should be unequivocally reflected in migration times. For more hydrophobic compounds that interacted with Brij-35® more strongly, migration times were decreased. In its structure, memantine has two additional methyl groups compared with amantadine, which gives it a higher hydrophobic property, and memantine was observed to migrate faster than amantadine in the analysis. Migration time decreased with increasing Brij-35® concentration and a shorter migration time of the drug was obtained at higher concentration. The migration times of memantine and amantadine decreased from 5.29 min (0.03%) down to 4.3 min (0.1%) and 6.26 min (0.03%) down to 5.31 min (0.1%), respectively. The increase in concentration shortened the migration time and always decreased the resolution of the system if the interaction was not specific for the analyte with micelle. The memantine migrated

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closely with the front interference at 0.1% of Brij-35®. However, the Brij-35® concentration slightly influenced the peak height. Comparing the Brij-35® concentration from 0.03 to 0.1% on the effect of the peak height sensitivity for the memantine and amantadine derivatives, the 0.1% Brij-35® concentration gave a better response than any other. The sensitivity ratios of the memantine and amantadine derivatives according to the peak height were 0.86, 0.91, 0.96, and 1.0 for memantine, and 0.86, 0.87, 0.97, and 1.0 for amantadine at 0.03, 0.05, 0.07, and 0.1%, respectively. Therefore, 0.07% Brij-35® (equivalent to 0.58 mM) was chosen for the separation. The fluorescence intensity of CFSE-labeled compound is obviously affected by pH as higher fluorescent intensity was observed at basic condition. Therefore, the pH of the BGE could affect the fluorescence intensity and electrophoretic mobility. The effect of pH (8.5, 9.0, 9.5, and 10.0) of 30 mM borate buffer with 0.07% Brij-35® on the separation and sensitivity of analyte derivatives was studied. The CFSE-memantine and CFSE-amantadine derivatives dominated as the anionic species in electrolyte solution at the tested pH. Good resolution of the tested drug derivatives at various pH values was obtained. While the retention of CFSE-memantine, CFSE-amantadine, and the interference was increased with increasing pH value, a cleaner electropherogram was observed with higher pH. Considering the migration time and the separation efficiency, pH 9.5 (30 mM) borate buffer was chosen for separation.

The ionic strength of separation BGE would affect the conductivity of the solution and influence the migration velocity of analytes and EOF. We studied the influence of buffer concentration on the separation selectivity and fluorescence intensity in the range of 10–40 mM. The effects of concentration of borate buffer on the derivatives separation and fluorescence intensity are shown in Fig. 5. The migration time of CFSE-memantine and CFSE-amantadine increased with increasing concentration, which may have been caused by the decrease in EOF. The sensitivity ratios of the tested drug derivatives according to the peak height were 0.61, 0.61, 1.0, and 0.49 for memantine at 10, 20, 30, and 40 mM, respectively; and they were 0.63, 0.63, 1.0, and 0.46 for amantadine at 10, 20, 30, and 40 mM, respectively. The peak response became better from 10 to 30 mM; this may be due to the difference of electric field between sample and buffer zone, which made the analytes stack between the interfaces. The best result was found to be 30 mM borate buffer (pH 9.5). The 30 mM borate buffer (pH 9.5) with 0.07% Brij-35® was chosen as the optimal separation buffer to successfully separate CFSE-memantine, CFSE-amantadine, and excess CFSE labeling reagent. The typical electropherogram of the simultaneous determination of memantine and amantadine in plasma after LLE and derivatization with CFSE is shown in Fig. 6.

3.4 Method validation in human plasma

To evaluate the quantitative application of the proposed method, human plasma samples spiked with five different concentrations over the range 2.0–60.0 ng/mL of memantine and amantadine were pretreated by LLE, then derivatized with CFSE and mixed with a fixed concentration of BODIPY (800 ng/mL) (IS) for the analysis. The calibration graphs of memantine and amantadine spiked in human plasma were obtained with the corrected peak area ratio of memantine or amantadine to BODIPY (IS) as ordinate (y) *versus* the concentration of memantine or amanatdine in ng/mL as abscissa (x). The linear regression equations developed in plasma samples are listed in Table 1. The correlation coefficients of intra-day and inter-day regression equations were all 0.999. The intra-day and inter-day precision and accuracy examination were tested by analyzing three concentration levels of memantine and amantadine at 2.0, 10.0, and 60.0 ng/mL in plasma, and estimated from the back-calculated standard concentration. The intra-day precision and accuracy was defined by RSD and relative error (RE) from the analyses on the

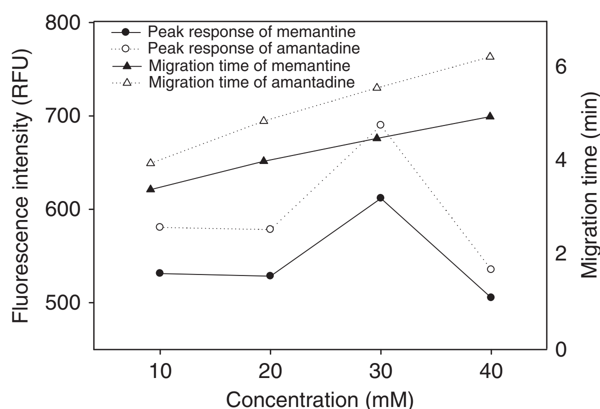

Figure 5. The effect of concentrations of borate buffer on the migration time and fluorescence intensity.

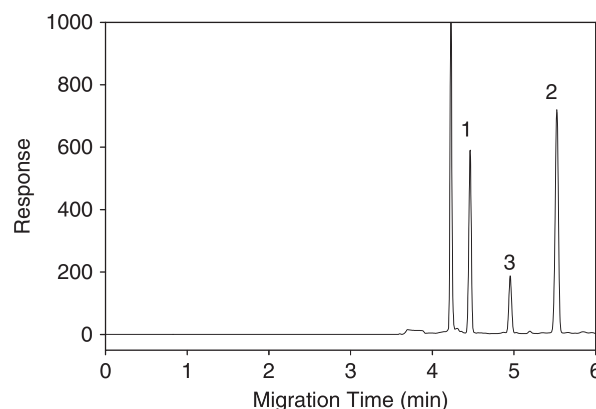

Figure 6. Electropherograms of the memantine and amantadine determinations in plasma. CE conditions as Fig. 4.

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same days; the inter-day precision and accuracy were calculated from repeated analyses of identical samples on consecutive days. The results (Table 2) show that the intra-day and inter-day RSD values of different concentrations were below 8.9% for memantine and 4.8% for amantadine. The RE values of memantine and amantadine at three concentrations were below 10.5 and 8.7%, respectively. Compared with the peak area ratio of standard memantine and amantadine dissolved in 50 μL derivatization medium, the absolute recoveries of memantine and amantadine in plasma at the concentration of 50.0 ng/mL are 52 and 62%, respectively, with the same derivatization condition. The LOD was determined by the signal-to-noise ratio of 3 ($S/N = 3$); the LOQ was determined by S/N ratio of 10. The LOQ of memantine and amantadine was both 2.0 ng/mL; LOD of the method was both 0.5 ng/mL. To evaluate the reproducibility of the method, the RSD values of the migration time of tested drug derivatives were both below 1.6%; RSD values of corrected peak area were both below 3.9% for memantine and amantadine.

Table 1. Regression analyses for the determination of memantine and amantadine spiked in plasma

Concentration range (ng/mL)	Regression equation[a)]	Correlation coefficient (*r*)
Memantine		
Intra-day ($n = 5$) 2.0–60.0	$y = (0.0526 \pm 0.0011)x + (0.0150 \pm 0.0079)$	0.999
Inter-day ($n = 5$) 2.0–60.0	$y = (0.0565 \pm 0.0004)x + (0.0137 \pm 0.0068)$	0.999
Amantadine		
Intra-day ($n = 5$) 2.0–60.0	$y = (0.0882 \pm 0.0013)x + (0.0228 \pm 0.0083)$	0.999
Inter-day ($n = 5$) 2.0–60.0	$y = (0.0825 \pm 0.0004)x + (0.0231 \pm 0.0053)$	0.999

a) Intra-day data were based on five replicate analyses and inter-day data were from four replications on five consecutive days.

Table 2. Precision and accuracy for the analysis of memantine and amantadine spiked in plasma

Concentration known (ng/mL)	Concentration found (ng/mL)	RSD (%)	RE (%)
Memantine			
Intra-day ($n = 5$)			
60.0	59.86 ± 1.43	2.4	0.2
10.0	10.74 ± 0.45	4.2	7.4
2.0	1.79 ± 0.10	5.7	10.5
Inter-day ($n = 5$)			
60.0	59.81 ± 1.93	3.2	0.3
10.0	10.05 ± 0.32	3.2	0.5
2.0	1.91 ± 0.17	8.9	4.5
Amantadine			
Intra-day ($n = 5$)			
60.0	59.89 ± 0.93	1.6	0.2
10.0	10.04 ± 0.24	2.4	0.4
2.0	2.17 ± 0.09	4.2	8.7
Inter-day ($n = 5$)			
60.0	59.98 ± 2.89	4.8	0.1
10.0	10.02 ± 0.48	4.8	0.2
2.0	2.12 ± 0.08	3.7	5.9

a) Intra-day data were based on five replicate analyses and inter-day data were from four replications on five consecutive days.

3.5 Application

The proposed method was applied to analyze six plasma samples from different AD patients who received oral administration of 10 or 5 mg of memantine (Witgen®) twice a day and four plasma samples from different PD patients who took 100 mg amantadine (PK-Merz®) daily orally, the observed steady-state concentrations are summarized in Table 3. The concentrations of memantine in AD patients were in the range 17.0–145.8 ng/mL; and the concentrations of amantadine in PD patients ranged from 66.9 to 1738.9 ng/mL. The observed concentrations of patients' samples were quite different at steady state. The times of peak plasma concentration are 6 and 2 h, and the times of elimination half-life are 63.6 and 16.7 h for memantine and amantadine, respectively [8, 27]. In healthy adults, the plasma concentrations at steady state of memantine and amantadine were about 82.4 ng/mL (10 mg twice daily) [28] and 636.2 ng/mL (100 mg twice daily) [29], respectively. The concentration of one patient with PD (No. 7) was significantly higher than other patients. The creatinine value of this patient was 4.36 mg/dL (reference standard 0.6–1.3 mg/dL) and the urea N was 70.8 mg/dL (reference standard 7.0–18.0 mg/dL), which were both out of the normal range. The literature confirms that the memantine concentration in plasma was affected by renal function [28] and the inter-individual variability of concentrations was associated with the apparent clearance of memantine [30]. On the other hand, it was also reported that

Table 3. Concentration of memantine and amantadine in AD patients and PD patients

Patient number	Age	Sex	Dosage	Memantine (ng/mL)	Amantadine (ng/mL)
No. 1	66	F	10 mg BID	17.0	
No. 2	56	M	10 mg BID	49.1	
No. 3	72	F	10 mg BID	30.0	
No. 4	77	F	10 mg BID	145.8	
No. 5	82	F	10 mg BID	59.2	
No. 6	85	F	5 mg BID	38.5	
No. 7	71	F	100 mg QD		1738.9
No. 8	74	F	100 mg QD		206.4
No. 9	81	F	100 mg QD		66.9
No. 10	81	M	100 mg QD		454.9

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the elimination half-life of amantadine was prolonged in patients with renal insufficiency [31]; thus, toxicity increased without monitoring the drug concentration and dose-adjustment. In fact, memantine and amantadine can be metabolized by hepatic cytochrome p450 isozymes. In an individualized efficacy therapy, which minimizes toxicity for patient safety, dose adjustment of memantine and amantadine in the elderly or in the renal failure patients can obtain better therapeutic efficacy. Therefore, physicians should take the concentration of medicine into consideration to make a decision regarding the treatment responses.

4 Concluding remarks

A sensitive and effective MEKC-LIF method was developed for the simultaneous determination of memantine and amantadine in human plasma. The plasma sample was pretreated with LLE using EA as the extraction solvent. The addition of nonionic surfactant Brij-35® was useful to increase separation selectivity. Validation of the method for the quantification of memantine and amantadine in plasma showed that the method had high sensitivity and accuracy with ng/mL level measurement. This method was successfully applied to monitor the concentration of tested drugs for the treatment of AD and PD patients. Therefore, the proposed method might be applicable to therapeutic drug concentration monitoring of memantine and amantadine in patient plasma and it could be used to investigate the relationship between the concentration of memantine and amantadine in plasma and the therapeutic effectiveness in AD and PD patients. Most often, AD and PD are common neurodegenerative disorders occurring in elder people between 50 and 80 years. It is possible that patients with AD also show PD symptoms; therefore, this method is particularly useful in that it can be applied to patients taking memantine and amantadine simultaneously.

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