

Research Article

HPLC-Fluorescent Analysis of Memantine: An Investigation on Fluorescent Derivative Formation

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Memantine is an N-methyl-D-aspartate receptor antagonist recommended for treatment of Alzheimer's disease. Due to the lack of chromophores/fluorophores in memantine molecule, this work focuses on a novel procedure for memantine-fluorescent derivative formation enabling it to be monitored by a fluorescence detector. 4-(N-Chloroformylmethyl-N-methyl)amino-7-N,N-dimethylaminosulphonyl 2,1,3-benzoxadiazole (DBD-COCl) was chosen as a fluorescent probe since the carbonyl chloride of DBD-COCl could easily react with the amine on memantine to form the fluorescent derivative. The derivatization was achieved at 5:1 of DBD-COCl and memantine, at 60°C for 50 min. Structure elucidation from mass spectrometry and infrared spectroscopy spectra confirmed the amide bond of memantine-DBD-COCl. The derivative was stable up to 24 h and could be monitored by high performance liquid chromatography (HPLC) coupled with a fluorescence detector using a VertiSep GES C18 column, acetonitrile and water (80:20) as the mobile phase with a flow rate of 1 mL/min using the excitation and emission wavelengths at 430 and 520 nm, respectively. The derivative was eluted at 4.50 min and was separated from the DBD-COCl. Preliminary validation data reveals good linearity ($r^2 \ge 0.99$), repeatability (%RSD < 0.54) and accuracy (%*R* between 94 and 119%) with a limit of detection of 0.79 µg/mL.

1. Introduction

Alzheimer disease (AD) was firstly identified by a German psychiatrist and neuropathologist, Alois Alzheimer, in 1906 [1]. AD is a common dementia found in elderly that affects more than 4.5 million Americans and this number is estimated to increase to 14 million by the year 2050 [2, 3]. Destruction of central nervous system, specifically cholinergic system, is a major cause of cognitive function impairments as well as behavioral and psychological symptoms in dementia. Neuropathology and neurotransmitter changes such as senile plaques, neurofibrillary tangles, amyloid angiopathy, and decreasing of neurotransmitter acetylcholine and glutamate in neuron cell are major causes of the disease [4, 5]. AD patients suffer from loss of memory, orientation and language skill, mobility, vision, reasoning, and decision making. These symptoms slowly progress but become devastating since they greatly affect daily life activities of patients [6, 7]. Eventually, complete care is needed at the late stage of AD and patients usually die within 6–12 years.

Currently, there is no curative treatment for AD; the available medication only slows down the progress of the disease. The drugs are classified into three major types, which include (1) acetylcholinesterase inhibitors (e.g., donepezil, rivastigmine, and galantamine), (2) N-methyl-D-aspartate receptor antagonists (e.g., memantine), and (3) supportive medications (e.g., vitamin E, Ginkgo biloba extract, etc.) [8]. Among these drugs, memantine (Figure 1) is recently approved, and the United State Pharmacopeia suggests gas chromatography with flame ionization detection for the assay of the drug substance and tablets [9]. However, memantine monograph is not yet available in the British Pharmacopeia [10]. It is evident that memantine does not absorb UV or fluorescence due to lack of chromophore and fluorophore in its molecules. Therefore, derivatization of memantine with some probes (e.g., anthraquinone-2-sulfonyl chloride,



FIGURE 1: Structures of memantine and DBD-COCl.

9-fluorenylmethyl chloroformate, dansyl chloride, 2-(napthoxy)acetyl chloride, *o*-phthaldialdehyde, and 4-(4,5diphenyl-1H-imidazol-2-yl) benzoyl) is required to enable the drug to be detected by UV or fluorescence detectors [11]. For example, precolumn derivatization followed by high performance liquid chromatography (HPLC) with UV or fluorescence detection has been demonstrated for analyses of memantine in rat plasma [12–14], human plasma [15], human urine [16], and active pharmaceutical ingredients [17]. Furthermore, HPLC coupled with mass spectrometer (MS) has been employed for quantitation of memantine in human plasma [18] and urine [19]. Other methods including gas chromatography [9, 20, 21], spectrofluorometry [22], and spectrophotometry [22, 23] are reported for quality control of memantine in raw material and tablet formulations.

This work aimed to develop a new and convenient derivatization procedure for memantine analysis using 4-(Nchloroformylmethyl-N-methyl)amino-7-N,N-dimethylaminosulphonyl 2,1,3-benzoxadiazole (DBD-COCl) as a fluorescent probe (Figure 1) [24]. The amine functional group of memantine could rapidly react with the carbonyl chloride of DBD-COCl under mild conditions to form a fluorescent derivative, which could be monitored by HPLC coupled with a fluorescence detector. Ratios of DBD-COCl and memantine, temperature, reaction time, and pH were investigated since they are key factors for the formation of the memantine fluorescent derivative. Formation of the derivative was achieved and it was characterized by HPLC, mass spectrometry, and infrared spectroscopy. Preliminary method validation supports the potential of the method for the analysis of memantine by HPLC coupled with fluorescence detection.

2. Materials and Method

2.1. Reagents and Equipment. Memantine hydrochloride was from Sigma-Aldrich (Munich, Germany). DBD-COCl was obtained from Santa Cruz Biotechnology (California, USA). Acetonitrile was of HPLC grade from RCI Labscan (Bangkok, Thailand). Pyridine was of reagent grade. Solid phase extraction (SPE) cartridges were Empore C_{18} -SD 4 mm/1 mL (Minnesota, USA). HPLC instrument was a Flexar-UHPLC from Perkin Elmer equipped with a UHPLC pump, column oven, and UV/Vis and FL (fluorescent) detectors (Waltham, Massachusetts, USA). HPLC column was a VertiSep GES C₁₈ (4.6 × 150 mm, 5 μ m) (Vertical, Bangkok, Thailand). Analytical balance was from Sartorius and pH meter was from Consort (Turnhout, Belgium). Mass spectrometer is LCQ Fleet ion trap from Thermo Scientific Waltham (Massachusetts, USA). Fourier transform infrared spectrometer was Nicolet 6700 from Thermo Scientific Waltham (Massachusetts, USA).

2.2. Solution Preparation. Memantine stock solution (1 mM) was prepared by dissolving 5.39 mg memantine with 25 mL acetonitrile. The working solution was prepared by diluting the stock solution with acetonitrile to obtain the concentration of 20 μ M. DBD-COCl stock solution (1 mM) was prepared by dissolving 8.32 mg DBD-COCl with 25 mL acetonitrile. DBD-COCl solution at concentrations of 20, 40, 60, 80, and 100 μ M was prepared by transferring appropriate amounts of DBD-COCl stock solution to 50 mL volumetric flasks and adjusting to volume with acetonitrile.

2.3. Memantine Fluorescent Derivative Formation. Memantine fluorescent derivative formation was studied by varying several factors such as ratios of DBD-COCl and memantine, reaction time, and temperature. One milliliter of the memantine working solution ($20 \ \mu$ M) was mixed with 1 mL of different concentrations of DBD-COCl ($20-100 \ \mu$ M) in amber glass vials, heated on a water bath at various temperature (60- 80° C) and reaction time ($0-90 \ min$). After every 10 min, 1 mL of water was added to the vials to stop the reaction and left to cool down at room temperature. One milliliter of the solution was filtered through a 0.22 μ m membrane filter prior to HPLC analyses.

HPLC analysis was performed on a C_{18} reversed phase column using acetonitrile and water (80:20) as the mobile phase at the flow rate of 1 mL/min. The injection volume was 20 μ L and the fluorescence detection was performed at the excitation and emission wavelengths of 430 and 520 nm, respectively.

Extraction of the memantine fluorescent derivative was carried out by SPE to ensure the purity of the derivative. The cartridge was preconditioned with 150 μ L of acetonitrile and 300 μ L of water. After loading the reaction solution to



FIGURE 2: Proposed mechanisms for the memantine fluorescent derivative formation.

the cartridge, the excess DBD-COCl was washed with 100 μ L of 40% acetonitrile three times and 100 μ L of 90% acetonitrile two times, respectively. The derivative was eluted with 100 μ L of methanol two times and left to dryness and the process was repeated five times. The residues were reconstituted with 1 mL methanol and injected into the HPLC and the LC fractions were collected and checked by mass spectrometry. The pure fractions were evaporated and subjected to infrared spectroscopy for structure elucidation.

3. Results and Discussion

Obviously, Figure 1 shows that memantine does not contain any chromophore or fluorophore in its molecule. Therefore, derivatization is necessary to enable it to be detected by UV or fluorescence detection. Presently, an approach for the formation of memantine fluorescent derivative was proposed (Figure 2). Reactions of DBD-COCl and memantine occurred via unimolecular nucleophilic substitution (S_N 1) due to the nucleophilic property of amine group on memantine that can easily react with –COCl on DBD-COCl to form amide bond. The reaction was considered to be stoichiometric and straightforward. However, factors affecting the derivative formation needed to be optimized to establish the derivatization protocol.

Effects of DBD-COCl and memantine ratios, reaction time, and temperature on the formation of memantine fluorescent derivative were optimized. DBD-COCl and memantine at ratios of 1:1, 2:1, 3:1, 4:1, and 5:1 were reacted



FIGURE 3: Effects of DBD-COCl and memantine ratios and time on the memantine fluorescent derivative formation.

for 10–90 min on a water bath at 60° C and the peak areas of the fluorescent derivative were monitored. At all ratios, peak area of the derivative increased as the reaction times lengthened and the area remained unchanged after 50 min (Figure 3). Except for the ratio 4:1, the peak area started to decrease dramatically after 50 min. After 80 min, the area declined independently on the DBD-COCl and memantine ratios. The maximum peak area was obtained at the ratio of 5:1 at the reaction time of 50 min. Next experiments were investigated by reacting DBD-COCl at the ratio of 5:1 for 10–50 min and varying the reaction temperature from 60 to 80° C. Elevating the temperature did not improve



FIGURE 4: Effects of temperature on the memantine fluorescent derivative formation.



FIGURE 5: Infrared spectra of (a) memantine, (b) DBD-COCl, and (c) memantine fluorescent derivative.

the peak area of the derivative. The maximum peak area was observed at 60° C after 50 min (Figure 4). This result was in good agreement with that obtained from the effects of DBD-COCl and memantine ratios. Thus, the optimal condition for memantine fluorescent derivative formation was by reacting DBD-COCl with memantine at the ratio of 5:1 for 50 min at 60° C.

The memantine fluorescent derivative was cleaned up by SPE using the condition described in Section 2. This procedure would be essential for the future work since the method will be applied to biological samples (e.g., serum or urine). The eluate was analyzed by HPLC and the pure fraction was preliminarily confirmed with the mass of 476.85 by MS. Structure elucidation of the derivative from the pure fraction was performed by IR and results are in Table 1 and Figure 5. The main difference among memantine, DBD-COCl, and the fluorescent derivative was the occurrence of the carbonyl amide at a wavenumber of 1644 cm⁻¹ indicating the amide bond of the derivative.

Figure 6 compares chromatograms of the memantine fluorescent derivative, underivatized memantine, mobile phase, and DBD-COCl. Under the reversed phase-HPLC, the derivative was eluted as a symmetric peak at 4.50 min and was well separated from DBD-COCl, which co-eluted as a solvent front. No interferences from the excess DBD-COCl, underivatized memantine, or mobile phase were observed. The method is selective for the fluorescent derivative.



FIGURE 6: Chromatograms of (a) memantine, (b) mobile phase, (c) DBD-COCl, and (d) memantine fluorescent derivative. HPLC condition: see Section 2.3.

 TABLE 1: Wavenumbers for the functional groups of memantine,

 DBD-COCl and the fluorescent derivative.

Functional group	Wavenumber (cm ⁻¹)		
	Memantine	DBD-COCl	Fluorescent derivative
C-N	1039	1039	1041
SO ₂		1375	1411
N–H bending	1640-1540		
C=O of amide			1644
C=O of ketone		1772	
sp ³ C–H stretching	<3000	<3000	<3000
sp ² C–H stretching		>3000	>3000
N–H stretching	3500-3000		

Stability of the memantine fluorescent derivative was evaluated at 0, 12, and 24 h after the formation, using

the HPLC condition described in Materials and Method. The derivative was stable during the investigated period and no significant changes in peak shape, resolution, retention time, and peak area were observed. The derivative was eluted at the same retention time of about 4.50 min (Figure 7) with the %RSDs for the peak area of 0.29%.

Preliminary experiments on method validation showed that the method was linear over a range of 1–5 μ g/mL with r^2 of 0.99 and the %RSDs for retention times and peak areas were less than 0.54% (n = 3). Accuracy of the method was represented by percent recovery (%*R*), which was between 94.8 and 119.4% (%RSD = 7.71%). Limits of detection and quantitations (calculated from the 3.3 SD/S and 10 SD/S, respectively, where SD was standard deviation of blank and S was slope from the calibration curve) were 0.79 and 2.42 μ g/mL, respectively. The data indicates the potential of the method for analysis of memantine, and sensitivity improvement is in progress for determination of the drug in biological samples.



FIGURE 7: Stability of memantine fluorescent derivative in 4 h. HPLC condition: see Section 2.3.

4. Conclusion

This work reports the first fluorescent derivative formation of memantine using DBD-COCl as the derivatizing reagent. The reaction was based on the amide bonding between carbonyl chloride of DBD-COCl and the amine group of memantine. The amide bond formation was confirmed by IR spectra. The fluorescent derivative was stable for 24 h and could be detected by reversed phase-HPLC-fluorescent using the excitation and emission wavelengths at 430 and 520 nm, respectively. In addition, preliminary results on the method validity show that the developed method could potentially be employed for memantine analysis because of its simplicity and reliability. Sensitivity enhancement and application of the method to real samples are under investigation.

Conflict of Interests

The authors confirm no conflict of interests to the paper contents.

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