H/D Exchange for 4-Aminopyridine: Application on MAO in Sera of Multiple Sclerosis Patients

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Abstract

Multiple sclerosis (MS) is a neurological disorder characterized by a complex array of symptoms affecting movement and the senses. It is an inflammatory disease of the central nervous system (CNS) causes an injury in the myelin sheaths, leading to demyelination and thus, consequently, a series of neurologically dysfunction known as relapses. 4-Aminopyridine is used in the treatment of MS patients as a selective voltage blocker channel. The study designed over two stages, first 4-aminopyridine went under H/D exchange, and then both deuterated and non-deuterated 4-AP was applied on the serum of MS patients and the activity of serum monoamine oxidase was estimated. The study included 120 subject which divided over two groups, control and MS patients, each of which contain 60 subject. The activity of MAO was significantly ($P<0.01$) higher in patients than that of control. The activity of MAO activity has decreased significantly ($P<0.01$) in the presence of 4-AP, as well as a further significant decrease in the presence of deuterated 4-AP. In conclusion, 4-AP has inhibitory effect on MAO activity, and the replacement of hydrogens with deuterium gives an enhancement on this inhibitory effect.

Keywords: *Multiple sclerosis, monoamine oxidase, H/D exchange, platinum catalyst.*

Introduction

Multiple sclerosis (MS) is a neurological disorder characterized by a complex array of symptoms affecting movement and the senses[1]. It is an inflammatory disease of the central nervous system (CNS) causes an injury in the myelin sheaths, leading to demyelination and thus, consequently, a series of neurologically dysfunction known as relapses[2]. Once it causes a relapses, it's called relapses-remitting multiple sclerosis (RRMS) and it is the most common subtype of MS. Another subtype is secondary progressive multiple sclerosis (SPMS) and is, often, follows RRMS when patient is no longer has no longer exacerbations and has continuous accumulation of disability with time. Primary progressive multiple sclerosis subtype (PPMS) is recognized by disease progression without remarkable exacerbations. Progressive relapsing multiple sclerosis (PRMS) is the rarest and most progressive subtype of MS[3].

Multiple sclerosis considered as the most epidemic of demyelinating diseases, as well as, it is the most common of the CNS disorders that causes a permanent disability in young adults[4, 5]. World health organization (WHO) have estimated the over 2 million people are suffering MS in the world[6]. The rate of the prevalence in the world is about 100 to 150 per 100000 populations, presented in the ages between 20 and 40 years with a higher percentage in women than men. It has been believed that the prevalence of MS increases with the increasing of distance away from equator[1].

Several studies were applying on the effect of 4-amonopyridine on the treatment of multiple sclerosis symptoms[7]. In myelinated neurons, under normal conditions, potassium (K+) voltage-gated channels Kv1.1 and Kv1.2 are gathering under the myelin sheath close to the nodes of Ranvier [8, 9]. Regarding to these channels location, when a demyelination occur it will be exposed, and migrate through the demyelinated fragment, also an increasing with several fold in expression will be concomitant[10-16]. This irregular redistribution of potassium channels reduces conduction of action potentials, and leads to neurological deficits[10,17-21]. 4-aminopyridine is a selective blocker of voltage-dependent channels[22-28]. 4-aminopyridine is using in a

clinical way to improve the neurological conduction in MS patients [29-32] by a mechanism in which it blocks the exposed potassium channels [23, 24, 26, 27, 33-36].

Deuterium labelled derivatives considered as an important materials for the development of drugs as becoming an internal standards of animal and human drug experiments, also they involved in the mechanical studies in the investigation of reaction pathway. Deuterated compounds share a similar characteristics of both physical and chemical properties, with other isotopologues and contain the same ionization properties. The whole process depend on the mass differences among the compound isotopologues, in which the analysis went more easily as the mass difference became greater than that of natural isotope due to the easing in separating signals[37].

Materials and Method

Chemicals: Deuterium oxide (TCI/Japan), 4-aminopyridine (TCI/Japan), potassium hexachloro palatinate (TCI/Japan), sodium hydroxide (MERCK/ USA), dichloromethane (Sigma-Aldrech/Germany), magnesium sulfate (Sigma-Aldrech/Germany), ethanol (Scharlau/Germany), NaH_2PO_4 (BDH/England), Na_2HPO_4 (BDH/England), benzylamin hydrochloride (BDH/England), perchloric acid (BDH/England), and cyclohexane (Sigma-Aldrech/Germany).

Subjects: Sixty patients whom already diagnosed with multiple sclerosis have been enrolled in this study, their ages were between 18 and 46 years old and the mean ± SD was (33.37 ± 8.36). The patients with multiple sclerosis who provided in the study were diagnosed acclimated at Neuroscience Hospital from January to September 2020. Sixty healthy individual have been volunteer as control group of the study, their ages were between 19 and 48 years old and the mean ± SD was (32.66 ± 8.66). Control group was selected from Mustansiriyah University.

Specimens Collection: A plastic syringe used to pull the blood from vein. The blood then transported to gel tube let few minutes to rest and clot then centrifuged at 1500 g for 10 min in order to collect the serum. The produced serum distributed over three Eppendorf tubes and stored in deep freezer at -20 ºC.

Method

H/D Exchange of 4-aminopyridine: In a microwave vial, a weight of 0.3g of 4-aminopyridine was added and followed by the addition of 5mL of D_2O. Next, 0.1g of K_2PtCl_6 were added to the vial mixture, then the vial was shacked gently and transferred into Antonpaar-Microwave synthesis reactor for 2 hours, under power equal to 300 W, pressure equal 150 Psi, and the temperature 190 °C.

After that, the vial permitted to stand at room temperature for 30 minutes. The mixture was filtered by using filter paper, and then the filter paper was washed with 10 mL (1M) hydrochloric acid. The filtered solution was neutralized with (1M) NaOH. The solution then transported into separation funnel, and followed by the addition of 10 mL from CH_2Cl_2, and the mixture shacked in order to extract the polar phase (The process repeated three times). A weight of 0.1g MgSO4 was added to the solution, and starred for 10 minutes at room temperature. And followed by filtration process. Then the solution was evaporated in a vacuum with evaporator devise (Rotavapor R-205). At the last step, the yield was analyzed for FT-IR and HNMR.

Determination of monoamine oxidase activity: The Activity of monoamine oxidase determined by using McEwen and Cohen method[38]. The method requires two test tubes (calibration tube and assay tube) for each sample. A volume of 600 µL of serum was added to the tubes and followed by the addition of 700 µL of sodium phosphate buffer (0.1M, pH=7.3). Then 200 µL of 5mM of benzylamine hydrochloride were added to assay tube only. Both of the tubes were incubated at 37 ºC for 3 h. After the end of the incubation 200 µL of benzylamine hydrochloride were added to calibration tube, and then volume of 200 µL of 50% perchloric acid was added to both tubes to stop the reaction. Next, volume of 1.5 mL of cyclohexane was added to each tube, and the tubes contents emulsified with super mixer. Then the tubes were allowed to stand for 15 minutes at room temperature, and a second emulsification was applied. The tubes were centrifuged at 1500 g for 5 minutes and the absorbance of benzaldehyde was read (in cyclohexane) at 242 nm. The activity of MAO calculated as the following equation:

$$MAO\ (U) = (calibration - assay) \times 100$$

The measurement of the activity of MAO in sera of patients was repeated but this time in the presence of 4-AP, and deuterated 4-AP. Three tubes were used this time, 4-AP, deuterated 4-AP, and ethanol (because ethanol was used as solvent). A volume of 200 µL of ethanol, 4-AP (0.1M), and deuterated 4-AP (0.1M) was added to corresponding tube, and tubes were incubated at 37 ºC for 5 min. after the incubation the instructions at the previous paragraph were repeated to determine the activity of MAO.

Results and Discussion

FT-IR Results: The peaks at the two charts are almost identical in position, which reflects the fact that the compound have no change in its functional groups. The extra peak 2974.56 cm^{-1} at assay chart could be attributed to C-D bond, which gaves initial impression on the H/D exchange in 4-aminopyridine, see Fig 1.

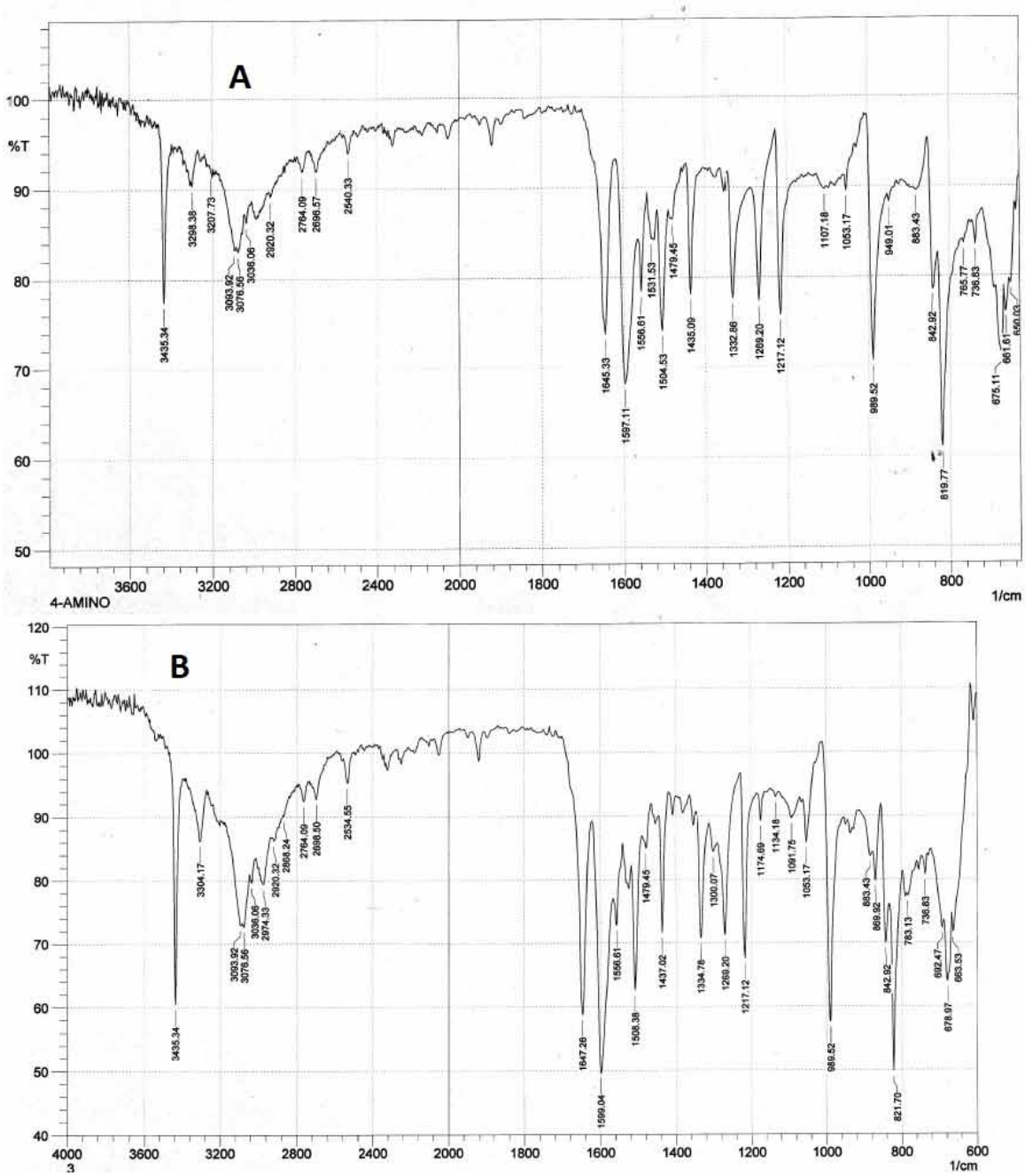

Figure 1: The FT-IR charts of 4-aminopyridine, a) before deuteration b) after deuteration.

HNMR Results: In the comparison of deuterated 4-AP chart with the initial 4-AP chart, it had been observed that the intensity of the aromatic peak at 8 ppm was reduced in deuterated 4-AP chart, which indicates the presumption that the protons at the difficult positions (2, and 6) were replaced by deuterium. It may attributed to platinum catalyst, K2PtCl6, which may underwent a reduction from Pt(IV) to Pt(II), and lose a chlorine into the reaction mixture that could react with the amine group of 4-AP in acid base reaction. The salts are deactivating groups that reduce the electron density of the ring and drive the replacement toward the difficult position.

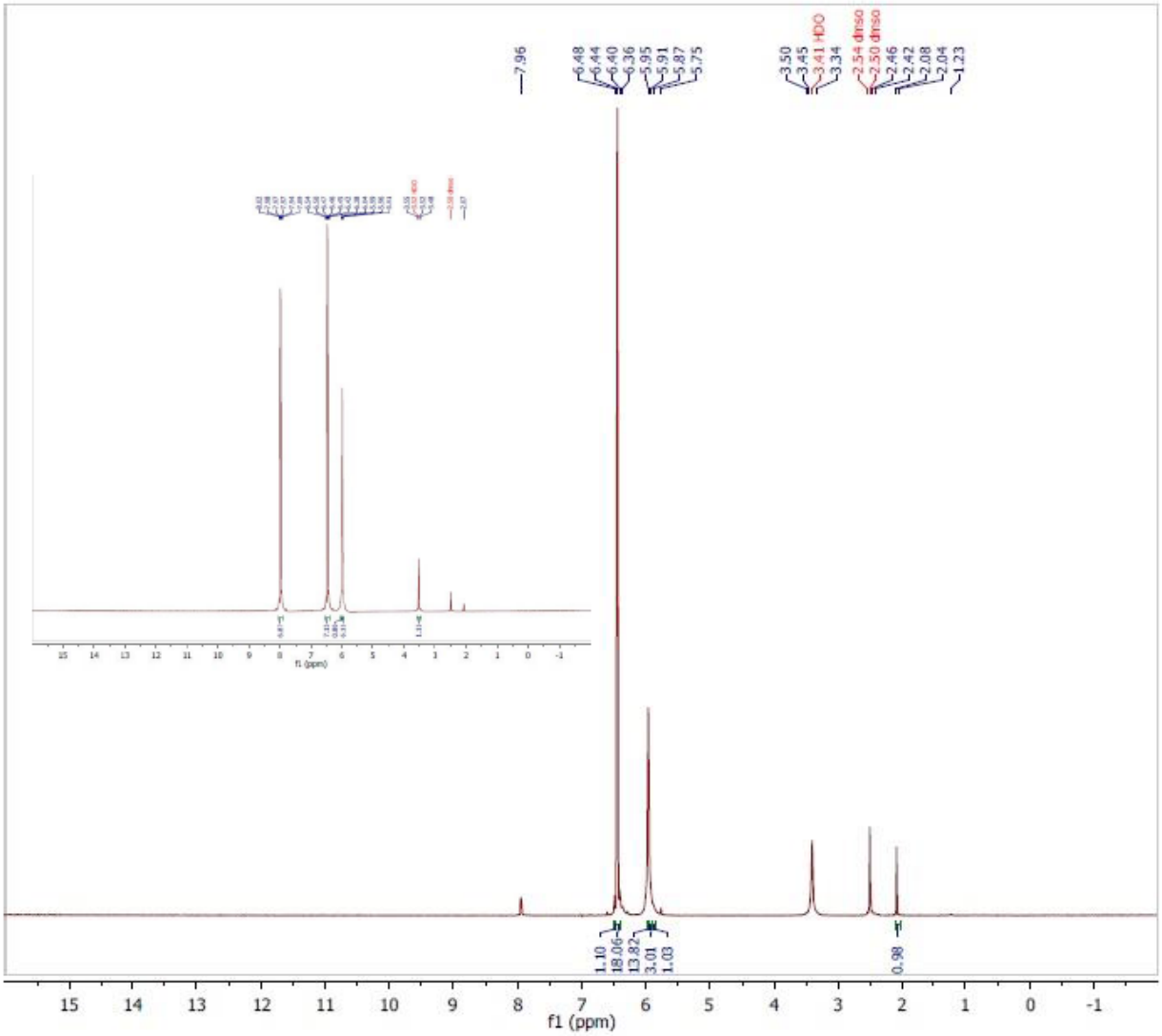

Figure 2: The HNMR charts of 4-aminopyridine.

MAO Activity Results: The activity of MAO was significantly (P<0.01) higher in MS patients group (44.99 ± 3.51 U) than control group (30.45 ± 2.1 U). The results are listed in Table 1.

Table 1: The means of MAO in MS patients and control groups.

Group	MAO activity (U) (Mean ± SD)		
	Male, N=24	Female, N=36	Total, N=60
Control	30.99 ± 1.61	30.1 ± 2.36	30.45 ± 2.1
MS	46.17 ± 1.6	44.7 ± 0.3	44.99 ± 3.51
P-value	< 0.01	< 0.01	< 0.01

The activity of MAO in MS group (44.99 ± 3.51 U) have decreased high significantly (P<0.01) in the presence of ethanol (37.03 ± 2.89 U), 4-AP (22.1 ± 1.74 U), and the deuterated 4-AP (22.1 ± 1.74 U), see Table 2.

Table 2: Means of MAO activity in MS group with and without the presence of ethanol, 4-AP, and the deuterated 4-AP.

Group	MAO (U) (Mean ± SD)	P-value
MS	44.99 ± 3.51	< 0.001
MS + Ethanol	37.03 ± 2.89	
MS + 4-AP	22.1 ± 1.74	
MS + DAP	20.37 ± 1.65	

Ethanol had been used as a solvent for both 4-AP and deuterated 4-AP. Thus, the activity of MAO in the presence of ethanol compared with 4-AP and DAP. The results declared high significant (P<0.01) difference in the activity of MAO between ethanol presence and 4-AP from one hand, and between ethanol presence and DAP from the other hand. Thus enable the assumption that both 4-AP and DAP have an inhibition effects on MAO enzyme. The inhibition percentage of DAP (54.72 ± 0.61) was the highest obtained value which is significantly (P<0.01) higher than ethanol inhibition percentage (17.69 ± 0.45) and 4-AP inhibition percentage (50.87 ± 0.83), see Table 3 and 4.

The presence of mono-amine group at 4-aminopyridine, drive a basic conclusion in which 4-AP is a competitive inhibitor which compete with the substrate on the active site of the enzyme, yet further information regarding Km and Vmax are required.

Table 3: The inhibition percentage of ethanol, 4-AP, and DAP in the activity of MAO for MS patients.

Inhibitor	% inhibition for MAO (Mean ± SD)	P-value
Ethanol	17.69 ± 0.45	< 0.001
4-AP	50.87 ± 0.83	
DAP	54.72 ± 0.61	

Table 4: Mean differences of MAO activity among ethanol, 4-AP, and DAP.

Inhibitor	Mean difference	P-value
Ethanol	33.17	< 0.001
4-AP		

Inhibitor	Mean difference	P-value
Ethanol	37.03	< 0.001
DAP		
4-AP	3.86	< 0.001
DAP		

Conclusion

The use of K_2PtCl_6 has shown to drive the electrophilic aromatic substitution to substitute at the difficult positions in the H/D exchange reaction. Further reactions are required with and without microwave assistant for more clarity on this type of catalysts. Also, 4-AP has shown a great inhibition effect on MAO enzyme, and the deuterium replacement gave enhancement to the inhibition effect.

Conflict of Interest: None

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Ethical Clearance: Not required

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