POTENTIAL ROLE OF METHYLGLYOXAL IN INDUCING PARKINSON'S DISEASE

by

SIYU LIU

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ABSTRACT OF THE THESIS

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By SIYU LIU

Thesis Director

Dr. Chi-Tang Ho

About one and a half million people are affected by Parkinson’s disease (PD) in the United States. It is a neurodegenerative disorder on dopaminergic neurons in the substantia nigra pars compacta, with primary effects on motion disorder. The causes for the Parkinsonism are generally divide into genetic factors like mutation and environmental factors like heavy metals as well as some endogenous or exogenous agents. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and other MPTP-like neurotoxins such as salsolinol are well known and considered to cause Parkinsonism due to their in vivo metabolic products’ specific toxicity for dopaminergic neurons.

Recent studies showed that the diabetic patient is more likely to develop the Parkinson's disease. It has been suspected that the methylglyoxal, one metabolic product of glycolysis, is associated with this phenomenon because the body concentration of methylglyoxal for diabetic patients will be three to six times higher than healthy people. The dopamine-derived tetrahydroisoquinoline (TIQ), 1-acetyl-6,7-dihydroxyl-1,2,3,4-tetrahydro- isoquinoline (ADTIQ), has been detected in frozen brain tissue of human with Parkinson's disease. It can be produced by the reaction of dopamine and methylglyoxal in physiological condition and has been regarded a novel endogenous neurotoxins.

In our study, in addition to ADTIQ, 6,7-dihydroxy-1,2,3,4-tetrahydro- isoquinoline or called norsalsolinol was also detected in the reaction system of dopamine and methylglyoxal by LC-MS/MS. And based on the Pictect-Spengler reaction, there are two regioselectivities for the products under neutral conditions. So there are two isomers for both ADTIQ and norsalsolinol. Additionally, according to the proposed reaction mechanism, the norsalsolinol was generated from ADTIQ by deacylation. On the other hand, with different ratios of dopamine and methylglyoxal, different levels of reaction under the physiological condition were also monitored by HPLC-UV. It revealed that when the ratio of dopamine and methylglyoxal is over 1:10, the reaction could almost be completed within 24 hours.

In conclusion, we showed for the first time that the potential neurotoxin norsalsolinol could be generated through the reaction of dopamine and methylglyoxal. And also can be one reason why the norsalsolinol was detected in the frozen brain of humans.

Acknowledgement

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Introduction

1.1 Methylglyoxal

1.1.1 Background:

Methylglyoxal (MGO), one of reactive carbonyl species (RCS), can be generated both from in vitro and in vivo. Exogenously, particularly from Maillard reaction, MGO can be derived from Schiff's base and Amadori compounds (Wang & Ho, 2012). Therefore, MGO commonly can be detected in various foods or beverages such as yogurt, wine, carbonated drinks, soy sauce, coffee and baked cookies. Significant concentrations of MGO can be found in high fructose corn syrup and manuka honey from New Zealand (Degen, Hellwig & Henle, 2012; Revel, Pripis-Nicolau, Barbe & Bertrand, 1999; Lo et al, 2007; Mavric, Wittmann, Barth & Henle, 2008; Hayashi & Shibamoto, 1985). On the other hand, endogenously, MGO is mainly generated from glycolysis pathway. It can be spontaneously degraded from triosephosphate, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Löbner, Degen & Henle, 2015; Dornadula, Elango, Balashanmugam, Palanisamy & Mohanram, 2015).

MGO, or is known as 2-oxopropanal, is one highly reactive carbonyl compound. The carbonyl group of MGO can actively react with amine groups from amino acids, proteins or even DNAs to form the advanced glycation end products (AGEs) and lead to carbonyl stress as well as oxidative stress or even the tissue damage. But MGO also

plays one important role in the formation of color and flavor from the Maillard reaction (Wang & Ho, 2012).

1.1.2 Reactive Carbonyl Species:

Reactive Carbonyl Species (RCS) are one class of byproducts or intermediates from both exogenous and endogenous oxidation. In general, nucleophilic compounds such as amino acids or proteins can be attacked by RCS covalently and then form the harmful adducts. The formed harmful adducts are named as advanced lipoxidation end products (ALEs) or advanced glycation end products (AGEs) depending on the attacking RCS originated from lipids or sugars respectively (Colzani et al, 2016). The adverse pathophysiological effects which can be induced by the RCS compounds accumulation as well as the formation of ALEs and AGEs has been described as the carbonyl stress in human body (Miyata, Striholj, Kurokawa & Baynes, 1999). Furthermore, RCS can be divided into three different classes based on their different chemical structures (figure 1.1): (1) α,β-unsaturated aldehydes (e.g. 4-hydroxy-trans-2-nonenal and acrolein); (2) di-aldehydes (e.g. glyoxal and malondialdehyde); (3) keto-aldehydes (e.g. MGO and 4-oxo-trans-2-nonenal) (Aldini, Dalle-Donne, Facino, Milzani & Carini, 2007).

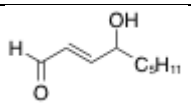
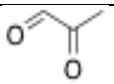
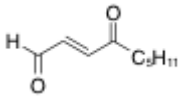
α, β-Unsaturated aldehydes:	 Acrolein	 4-hydroxy-trans-2-nonenal
Di-aldehydes:	 Malondialdehyde	 glyoxal
Keto-aldehydes:	 MGO	 4-oxo-trans-2-nonenal

Figure 1.1 RCSs: classification and structures

1.1.3 MGO Generation in vitro:

Exogenously, MGO are mainly generated from Maillard reaction and sugar autoxidation. In other word, MGO can be formed from the degradation of monosaccharide (e.g. glucose) and Schiff base adduct (Wang & Ho, 2012).

The beginning of monosaccharide autoxidation is the formation of ene-diol. This is caused by deprotonation of carbon-2 of glucose and then the re-distribution of electron density occurs between carbon-1 and carbon-2 or carbon-2 and carbon-3

respectively. So glucose can be transformed to 1,2-enol or 2,3-enol and following the formation of 1-deoxyglucone (1-DG) or 3-deoxyglucosone (3-DG) (Thornalley, 1985). And MGO can be yielded from the fragmentation of 3-DG (Wang & Ho, 2012) (Figure 1.2).

MGO can also be generated from Maillard reaction and the pathway is similar to glucose degradation but the presence of aldimine can be hydrolyzed into MGO directly (Wang & Ho, 2012). The condensation of the carbonyl group from the reducing sugar with the amine group will lead to the formation of Schiff base or aldimine. After the rearrangement, the Schiff base can be transformed to fructosamine or 3-DG. Both of fructosamine and 3-DG can degrade into MGO (Hayashi & Namiki, 1980) (Figure 1.3).

MGO can be generated from Maillard reaction in early stage, and it play important roles on the following color or flavor generation, especially for the aroma formation. For example, MGO can be transformed into 1-hydroxy-2-propanone through Cannizzaro reaction which can be recombined with another one MGO to generate 2,5-dimethyl-4-hydroxy-3*(2H)*-furanone (DMHF). And DMHF is the compound with intense caramel-like aroma as well as one typical Maillard reaction generated flavors. Therefore, as one flavor intermediate in the Maillard reaction, MGO can recombine or react with many other carbonyl compounds or amino acids to generate various flavors, such as pyridines, pyrrolines, thiazoles, thiazolines, alkypyrazines, oxazoles and oxazolines (Wang & Ho, 2012).

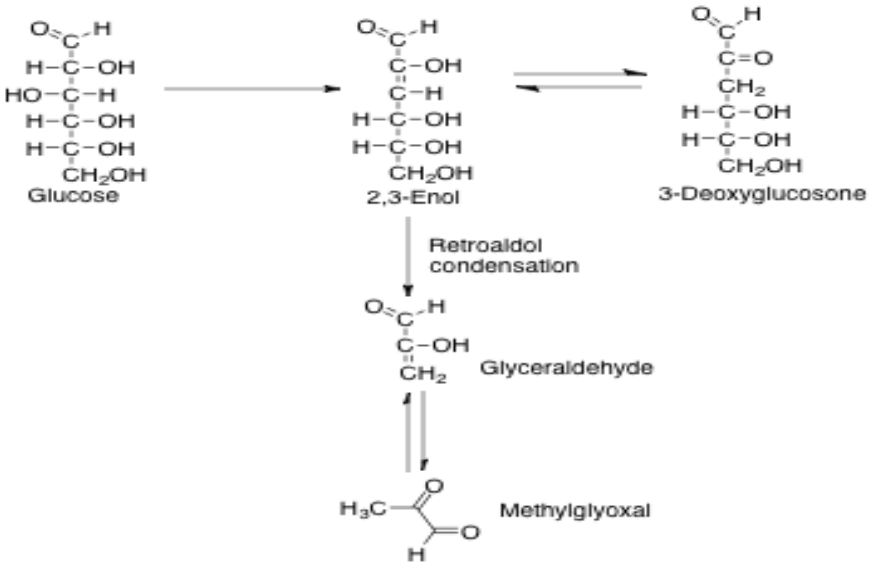


Figure 1.2 Oxidative formation of MGO from glucose

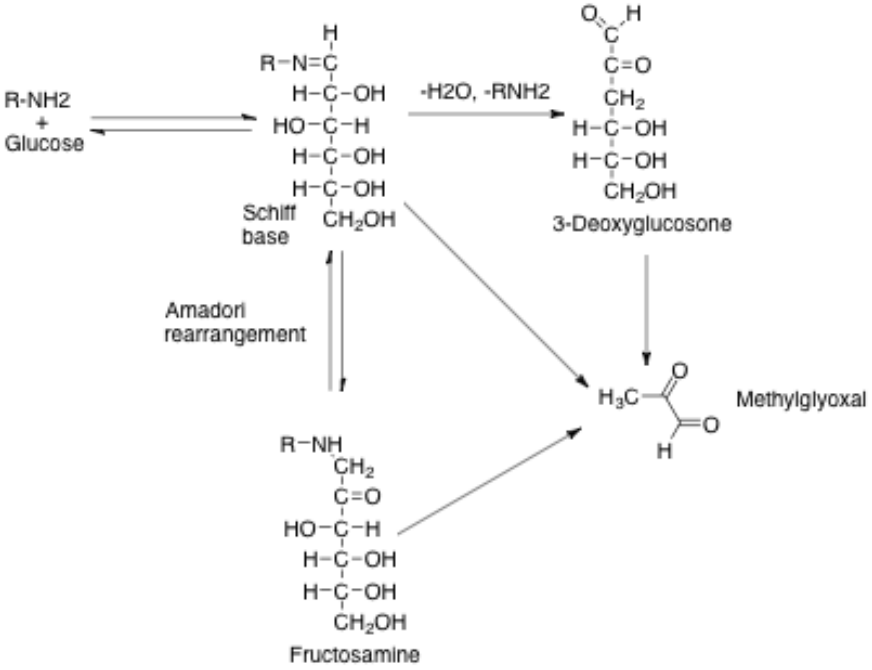


Figure 1.3 Formation of MGO in Maillard reaction

1.1.4 MGO Metabolism in vivo

MGO is ubiquitous in our body because it is one metabolic intermediate – it can be generated from the bypass of glycolysis (Saadat & Harrison, 1999) (Figure 1.4). The major pathway for MGO is non-enzymatic and/or enzymatic phosphate elimination from triose phosphate, glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), via the phosphate enediolate intermediate. Besides glycolysis, there is a small amount of MGO metabolized from the fats or proteins in vivo via the acetone, threonine or aminoacetone as the intermediate (Phillips & Thornalley, 1993, Dornadula et al, 2015). Although MGO has been confirmed as the cytotoxic compound, its formation still have the controversial role because this bypass pathway of glycolysis may have the regulatory effects. Normally, the G3P will be transformed to pyruvate with the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). But when the increase cellular uptake carbon-containing compounds, like glucose, or the abnormal glucose metabolism in diabetes, these will lead the GAPDH enzyme inhibition, NADH abundance and phosphate starvation. As the consequence, MGO pathway will be activated to relieve the glycolytic burden (Hopper & Cooper, 1971; Hopper & Cooper, 1972; Dornadula et al, 2015; Tötemeyer, Booth, Nichols, Dunbar & Booth, 1998; Saadat & Harrison, 1999). In this case, the triose phosphate isomerase will catalyze the G3P to DHAP and synthesize the MGO (Dornadula et al, 2015).

The synthesis of MGO in vivo is inevitable either under healthy or pathophysiologic conditions. However, many studies have proved the high toxicity of MGO in cells. Among many different detoxifying pathways, glyoxalase system is the most important

one as well as major detoxifying mechanism for MGO or even other reactive dicarbonyl compounds so that to protect our cells from glycation or other oxidative stress. Two enzymes, glyoxalase-1 (Glo-1) and glyoxalase-2 (Glo-2) and one additional necessary cofactor glutathione (GSH) are functional in this system. At first, GSH can react with MGO and yield hemithioacetal. Then, Glo-1 will catalyze hemithioacetal to S-D-lactoylglutathione. This compound will be transformed to D-lactate, which can be excreted into urine easily, with Glo-2 enzyme and recycle the GSH in the system (Figure 1.4). But if there is much more MGO than normal condition or the inefficiency of Glo-2, it may lead to the free GSH depleted and unable to recycle in the system. This will lead to the accumulation of MGO in cells and induce the glycation or increase the oxidative stress and promote the development of many degenerative conditions such as diabetic complications, cardiovascular disease and Alzheimer's disease (Allaman, Bélanger & Magistretti, 2015).

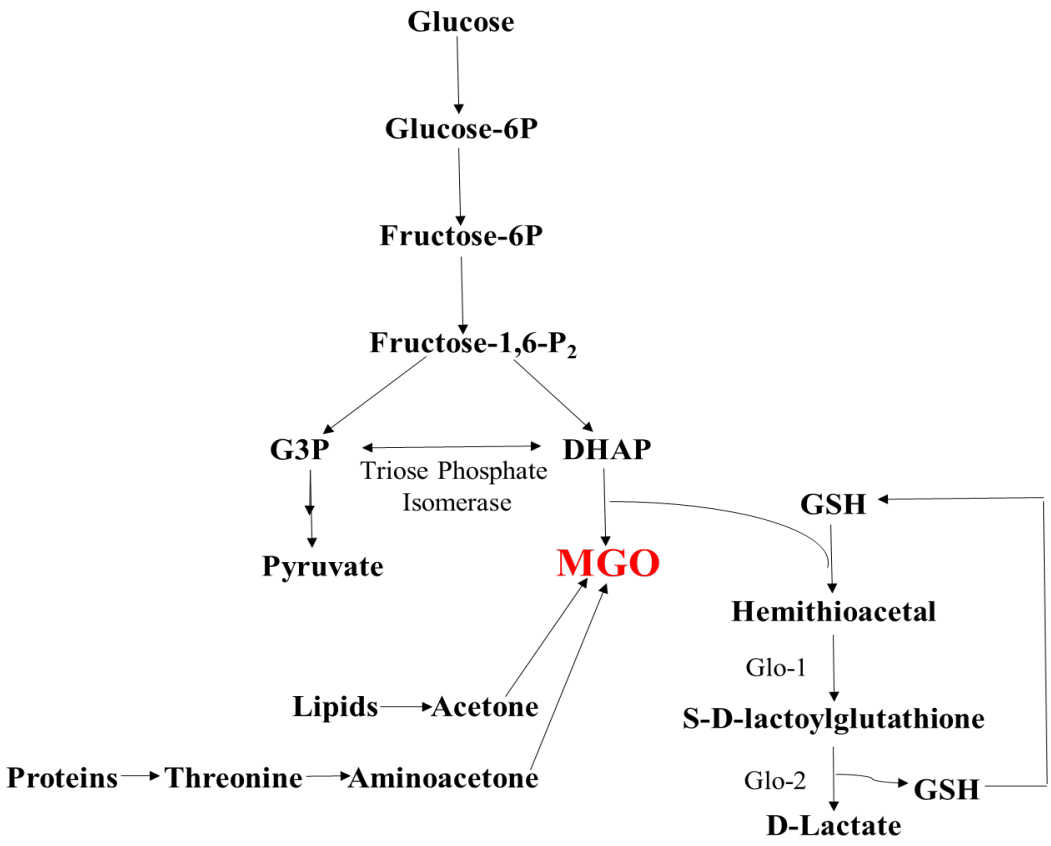


Figure 1.4 Metabolism of MGO *in vivo*

1.1.5 MGO and AGEs:

MGO is one typical RCS compound which can lead to the AGEs formation under physiological condition. Due to its high reactivity and ubiquitous generation, MGO has been regarded as the most important source of AGEs (Cristina, Zambonin & Hrelia, 2014). Compared with glucose in glycation reactions, MGO can be as much as 20,000 times more reactive (Thornalley, 2005). Particularly, MGO reacts with arginine residues is the most active glycation. And the following lesser extent reaction

is with lysine, cysteine and tryptophan (Angeloni, Zambonin & Hrelia, 2014). The reaction schemes are shown in figure 1.5.

The reaction between MGO and arginine will lead the cyclic imidazolone adducts formation. Depending on the nitrogen atoms involved in the cyclization as well as the different environmental pH value, there are three different structural isomers can be formed: N_{δ}-(5-methyl-4-imidazolon-2-yl)-L- ornithine (*MG-H1*), 2-amino-5-(2-amino-5-hydro-5- methyl-4-imidazolon-1-yl)pentanoic acid (*MG-H2*) and 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid (*MG-H3*). These three isomers are adducted in equilibrium. Because they can open and give the carboxyethylarginine (CEA) adduct as well as reverse back to re-cyclize so that the mutual interconversion can be occurred among the three isomer adducts. What is more, additional one methylglyoxal can be added and yield either THP (N^{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-L-

ornithine) or argpyrimidine (N^{δ}- (5-hydroxy-4,6-dimethylpyrimidine-2-yl)-l-ornithine) (Klöpfer, Spanneberg & Glomb, 2010; Vistoli et al, 2013; Angeloni et al, 2014; Oya et al, 1999; Shipanovaa, Glombb & Nagaraj, 1997).

MGO can react with lysine residues to form carboxylethyllysine (CEL) adduct with the aldimine as intermediate (Ahmed, Brinkmann, Degenhardt, Thorpe & Baynes, 1997). With the lysine dimer, MGO can be adducted to MOLD (6-{1-[(5S)-5-ammonio-6-oxido-6-oxohexyl]- 4-methyl- imidazolium-3-yl}-L-norleucine). With one lysine and one arginine residue, MGO can form MODIC adduct (2-ammonio-6-

({2–[4-ammonio-5-oxido-5- oxopently) amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene}amino)hexanoate) (Nasiri, Field, Zahedi & Moosavi-Movahedi, 2011; Nagaraj, Shipanova & Faust, 1996). Additionally, MGO can also react with cysteine residues to form the reversible hemithioacetal adducts and with tryptophane residue can generate β carboline derivatives (Lo, Westwood, McLellan, Selwood & Thornalley, 1994; Nemet & Varga-Defterdarovic, 2007).

Because of the abnormal glycation or crosslinkage, the amino acids and proteins will be dysfunctioned and lead the inflammation, oxidative stress, tissue injury or cell apoptosis (Ramasamy, Yan & Schmidt, 2006). As the result, these MGO-derived AGEs have been associated with the development of many degenerative healthy conditions, such as cataracts, cardiovascular disease, diabetic complications, nephrosis or brain health problems (de Arriba et al, 2006). For example, significant amount increases of CML in cerebrospinal fluid for the amyotrophic lateral sclerosis patients has been detected (Kaufmann et al, 2004) and CML amount level in cortical neurons and cerebral vessels has been reported to affect the severity of cognitive impairments among people with cerebrovascular disease (Southern, Williams & Esiri, 2007).

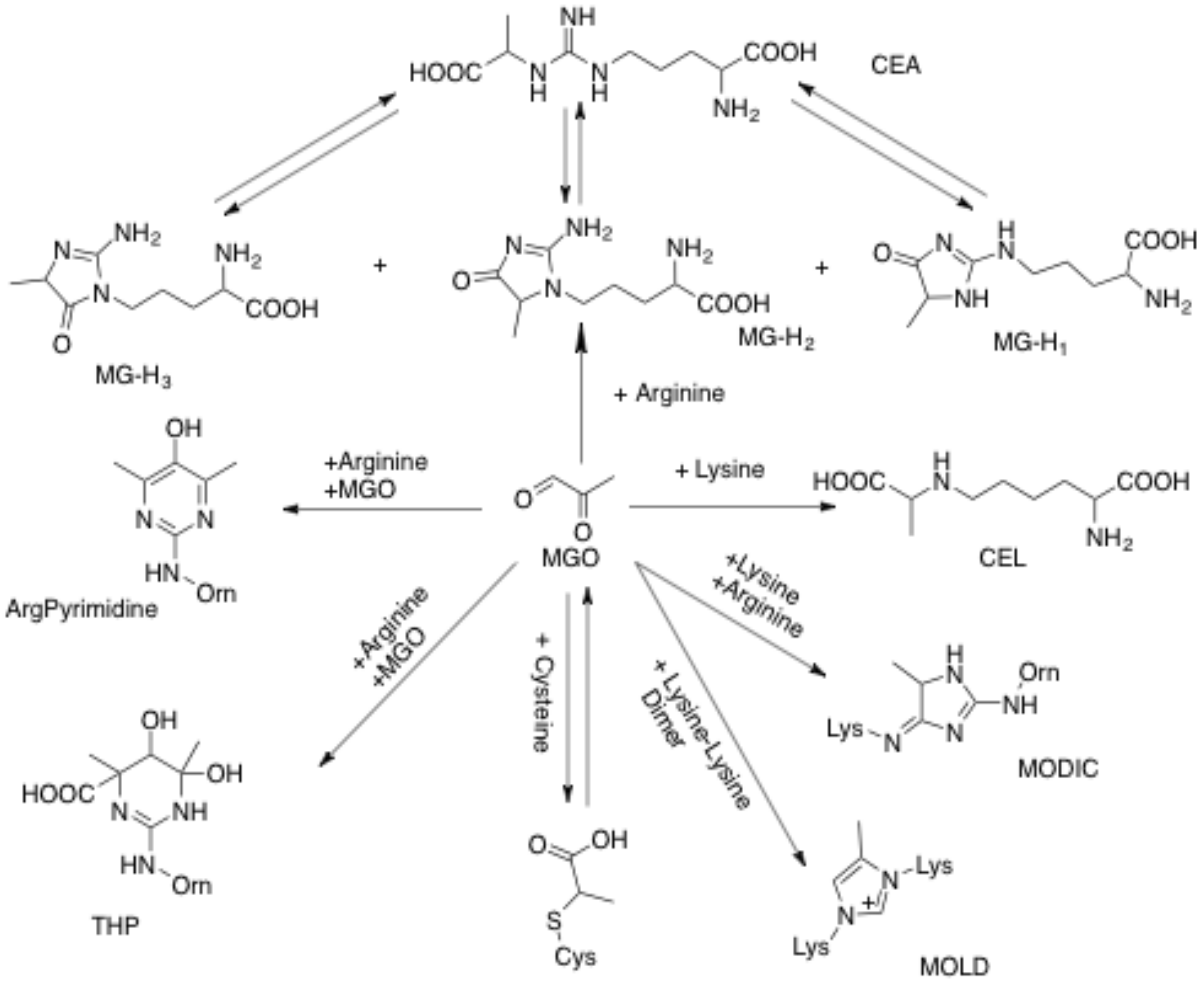


Figure 1.5 Major pathways for the formation of MGO-derived AGEs

1.1.6 MGO and Oxidative Stress

MGO is regarded as one toxic compound not only MGO itself can increase the oxidative stress but also the formed AGEs are potential to lead to the oxidative stress elevation (Matafome, Sena & Seiça, 2012). There is study showed the oxidative stress for healthy rats can be increased significantly with oral consumption of MGO (Sena

et al, 2012). And many other researches have revealed the MGO-induced oxidative stress pathways. For example: MGO can increase the formation of superoxide, hydrogen peroxide, peroxynitrite and proinflammatory cytokines in different cell types such as vascular smooth muscle cells, rat hepatocytes, neutrophils, platelets and so on (Chang, Wang & Wu, 2005; Dhar, Desai, Kazachmov, Yu & Wu, 2008; Ward & McLeish, 2004; Kalapos, Littauer & Groot, 1993). MGO can also promote the activity of several pro-oxidant enzymes such as NADPH oxidase (Chang et al, 2005). Additionally, MGO can amplify the oxidative stress by reducing several antioxidants in vivo such as GSH, glutathione peroxidase and glutathione reductase (Amicarelli et al, 2003; Paget, Lecomte, Ruggiero, Wiernsperger & Lagarde, 1998).

Furthermore, the formation of AGEs will not only affect the function of proteins or DNAs, it will also activate the membrane receptors, typically like RAGE, which can trigger specific intracellular signals (Matafome et al, 2012). This RAGE is soluble and has been proposed as the most important receptor for AGEs because it can recognized two major type AGEs, CML adducts and imidazolones, through specific regions and then activate NF-κB (Ramasamy, Yan & Schmidt, 2011). As the result, elevation of oxidative stress will occur and subsequently provoke positive inflammatory feedback, apoptosis, macrophage, platelet activation, thrombosis or develop the progression of vascular complications (Dornadula et al, 2015).

1.1.7 MGO and Type-2 Diabetes

As mentioned in 1.1.4, the increasing consumption of carbohydrates, like glucose, or abnormal glucose metabolism in diabetes will activate the MGO pathway in glycolysis and generate more MGO compared with normal condition. Therefore, the MGO concentration in diabetic patients' body will be 2-6 times higher than healthy people (Odani, Shinzato, Matsumoto, Usami & Maeda, 1999). This can be one reason to explain why the diabetic patients are normally suffered with diverse complications, such as cataracts, cardiovascular disease, nephrosis or neuro and brain health problems (Löbner et al, 2015).

However, on the other hands, overwhelming amount of MGO in vivo will also induce the type-2 diabetes or its complications. Nowadays, the resistance of insulin and β-cell loss are still the hallmarks of type-2 diabetes (Dornadula et al, 2015). There are many studies have focused on the pathological effects of MGO on diabetes. And the major effect is related to insulin. Insulin is the peptide hormone produced from pancreatic β-cell regulates the glucose homeostasis. The highly reactive carbonyl compound MGO can react with the N-terminus and arginine residue of human insulin. The formation of MGO-insulin adducts will affect the insulin–mediated glucose uptake, impair autocrine control of insulin release from β-cell and decrease hepatic clearance of insulin from liver cells (Jia, Olson, Ross & Wu, 2006; Oliveira et al, 2011). The glycation of insulin will also damage the ability of insulin to bind or activate its receptor and probably lead to insulin resistance (Matafome et al, 2013). Other possible pathological effects of MGO with type-2 diabetes will include: effects

on glucose transporter, pancreatic β-cell anion channel, hemoglobin, endothelial cells and so on (Dornadula et al, 2015).

1.1.8 MGO and Brain Health

MGO can be generated spontaneously and continuously in every mammalian cells. Due to its high reactivity and oxidative ability, the accumulated MGO can attack proteins and DNAs or other biomolecules to form the AGEs and cause the irreversible serious function loss or damage of organs (Tóth et al, 2014). However, on the other side, brain has the high energy needs and glucose is the major energy source for brain. As one by-product of glycolysis, metabolized MGO amount will be increased as the glycolysis rate elevation (Allaman et al, 2015). Therefore, it is not hard to understand that the amount of MGO in cerebrospinal fluid (CSF) will be five to seven times higher than in plasma. Briefly, MGO itself may be adverse to neurons directly because of depolarization, ROS production or even cell apoptosis as a result (Kuhla et al, 2005). What is more, the glycation or carbonyl stress have been involved in the neurological and neurodegenerative disorders (Münch, Westcott, Menini & Gugliucci, 2012). For example, MGO may promote the protein abnormal aggregation due to the crosslinkage and induce the oxidative stress. So, recently, the relationship between MGO and brain health problems is more and more eye-catching.

The role of MGO in Alzheimer's disease (AD) has been studied widely. The studies revealed the higher concentration MGO in CSF or for diabetic patients, the

pathogenesis of AD might be aggregated. Extracellular amyloid-β (Aβ) and intracellular neurofibrillary tangles (NFTs) are two distinct features of AD. These two protein aggregations are more stable and long-lived compared with other normal proteins, thus they are more ready to be glycated. After glycation, the AGE adducts, for example β-amyloid plaques, will be more insoluble and protease-resistance (Angeloni et al, 2014). And there is another study has showed the AGE adducts amount in AD brains are three times higher than in healthy brain and the AGE accumulation may also promote the aggregation of additional amyloids (Vitek et al, 1994). Additionally, the Aβ AGEs can also be recognized by RAGE. As the result, the oxidative stress, inflammation, amyloidosis and the neurotoxicity will be increased for microglia, the blood brain barrier and neurons (Naudí et al, 2013; Negre-Salvayre, Salvayre, Auge, Pamplona, & Portero-Otin, 2009).

There are also many studies focused on the glycation and amyotrophic lateral sclerosis (ALS). And the results showed the enzyme copper–zinc superoxide dismutase (SOD1), which can catalyze the quenching of superoxide radicals, is susceptible to be glycated because of several lysine and arginine residues in the primary structure of the enzyme. And the presence of CML and non-CML AGEs are found in the anterior horn motor neurons and microglia in the spinal cord of ALS patients (Takamiya et al, 2003; Kikuchi et al, 2002; Münch et al, 2012). Moreover, for multiple sclerosis (MS), the data showed there is no increase of AGEs in MS patients compared with healthy people. But the RAGE receptor in MS patients and animal models has been up-regulated (Kalousova, Zima, Tesar, Dusilova-Sulkova & Skrha, 2005; Andersson et al, 2008; Münch et al, 2012).

In this century, MGO and the glycation has been suspected to associate with Parkinson's disease (PD). Similar to Aβ and NFTs for AD, Lewy bodies are hallmark for the PD. And there are studies showed the Lewy bodies can be modified to AGEs and then alter the RAGE expression (Münch et al, 2000; Dalfo et al, 2005; Münch et al, 2012). Therefore, dopaminergic neurons might be destroyed.

1.1.9 MGO Trapping Agent

The accumulation of MGO in cells will lead the carbonyl stress and form the AGEs or even induce the degenerative healthy conditions especially for the diabetes. Up till now, there are several available pharmaceutical AGE inhibitors can be used to trap the MGO or prevent the AGE formation and relief the diabetic complications. For example: aminoguanidine, one nucleophilic hydrazine compound, which can react with MGO rapidly so that inhibit the AGEs formation and other related degenerative complications (Dukic-Stefanovic, Schinzel, Riederer & Münch, 2001; de Arriba et al, 2006).

Although the pharmaceutical compounds can trap the MGO or inhibit the AGEs formation significantly, the adverse side effects has limited them in clinical usage (Löbner et al, 2015). Whereas, many natural phenolic compounds showed the significant effect on trapping the MGO. And some flavonoids have shown the significant inhibitory effects on the AGE formation which is mediated by MGO. For

example: luteolin, rutin, (-)-epigallocatechin-3-gallate (EGCG) and quercetin can inhibit 82.2, 77.7, 69.1 and 65.5% AGE formation respectively based on former research results (Wu & Yen, 2005). From this perspective, some flavonoids have already been proven that they can trap the MGO efficiently and alleviate the AGE formation. Under physiological condition (pH 7.4, 37□C), EGCG from tea can trap over 90% MGO in 10 minutes and form the 8-mono-MGOEGCG, 6-mono-MGOEGCG and 6,8-di-MGOEGCG adducts as the products based on the different ratio of MGO and EGCG in the reaction condition (Sang et al, 2007). Similarly, genistein from soybean, quercetin from diverse plants and phloretin or phlorizin from apple, these flavonoids can also trap MGO effectively and form both mono-MGO adducts or di-MGO adducts based on different reaction reagents ration under controlled physical condition (pH 7.4, 37□C). More than 80.0% MGO can be trapped by genistein within 4 hours and the trapping efficiency can be as high as 97.7% after 24 hours (Lv, Shao, Chen, Ho & Sang, 2011). Quercetin can trap 80.1% of MGO after 1 hour (Li, Zheng, Sang & Lv, 2014). For the phloretin and its glucoside phloridzin, more than 80% MGO can be trapped by phloretin within 10 minutes and more than 70% MGO can be trapped by phloridzin within 24 hours (Shao, Bai, He, Ho, Yang & Sang, 2008). Noticeably, genistein and its MGO trapping research have been studied in vivo. Although there are some flavonoids or phenolic compounds has shown their significant ability in scavenging MGO, the studies are mainly focused and proceeded in vitro. In vivo, the MGO trapping condition will be much more complicated because of several factors: (1) systemic bioavailability of flavonoids; (2) the metabolism and their metabolites activities; (3) oxygen pressure; (4) the pH; (5) the presence of other endogenous and exogenous compounds and so on. Based on the result, only the mono-MGO adducts of genistein were found. Both the mono-MGO genistein adducts

and mono-MGO genistein metabolite adducts were found in the mice urines. Except for two genistein metabolites, 6-hydroxy-genistein and 8-hydroxy-genistein, all other form metabolites for genistein can be adducted by MGO and found in the mice urines. In the other words, genistein and its metabolites which have the same A ring structure can trap the MGO (Wang, Chen & Sang, 2016).

Besides on studying the MGO trapping ability for certain specific flavonoids or phenols, some researches have focused on the comparison of different flavonoids or parallel experiments among the similar structure compounds in order to study the MGO scavenging mechanism. There is one study focused on twenty different single benzene phenolic structure compounds. They incubated these different phenolic compounds with MGO respectively with the ratio of 1: 1 under physiological condition. The conclusion is mono-hydroxyl and di-hydroxyl benzene compounds are not efficient to trap MGO. For the benzenetriols, they can trap the MGO significantly. And the appropriate position for a carboxylic group on the benzenetriol structure can play a key role in the reaction (Lo, Hsiao & Chen, 2011). Similarly, another study more focused on the flavonoids (quercetin, luteoli, epicatechin, genistein, daidzein, apigenin and phloretin). Five typical sub-components of the flavonoids (gallic acid, phloroglucinol pyrogallol, pyrocatechol, and resorcinol) are involved in the research to simplify the influential factors. The results showed pyrogallol has the highest MGO-trapping ability that is 90% of MGO can be trapped in 24 hours. And then the MGO-trapping ability sequence is phloroglucinol (60.5%), resorcinol (31.6%), pyrocatechol (21.8%) and gallic acid (14.9%). It reveals that 1,2,3-trihydroxybenzen (pyrogallol) has higher MGO-trapping ability than other 1,2-dihyfroxybenzene and

1,3-dihydroxybenzene. And the substitution at position 5 of pyrogallol will decrease the trapping ability which means the position 5 is the active site of pyrogallol to trap MGO. According to phloroglucinol and resorcinol comparison as well as genistein (90%) and daidzein (54.5%) comparison, it shows that A ring is very crucial for the MGO trapping and the hydroxyl group on C-5 in A ring is very helpful for flavonoid about their MGO-trapping abilities. On the other hand, based on the comparison among quercetin (90% / 24 hours), luteolinand (90%/ 24 hours) and epicatechin (85% / 24 hours), it hints that the double bond between C-2 and C-3 can promote the MGO trapping ability for flavonoids. The last conclusion from this study is that the number of hydroxyl groups at B ring does not influence the MGO-trapping abilities significantly for flavonoids (Shao, Chen, Zhu, Sedighi, Ho & Sang, 2014).

1.2 Parkinson's Disease

1.2.1 Background

More than one million people in the United States are suffered with Parkinson's disease (PD) and about 60,000 Americans are newly diagnosed with PD every year. PD is the progressive neurodegenerative healthy problem. Clinically, the symptom of PD is called parkinsonism or Parkinson's syndrome, which is characterized as shaking or tremor at rest, rigidity and stiffness, bradykinesia (slowness of movement), postural abnormalities, and unbalance. Following, cognitive changes, mood disorders, hard to speak, swallow and sleep are the advanced parkinsonisms. The incidence of PD is highly related to age and the average onset age of PD is 55. In detail, the incidence of PD is 20/100,000 overall. But it increases sharply to 120/100,000 at age 70 (Dauer & Przedborski, 2003, Wolozin, Frasier, Synder, Choi, & Golts, 2005). Therefore, aging has been highly linked with PD incidence (Rodriguez, Rodriguez-Sabate, Morales, Sanchez & Sabate, 2015). Besides age, other studies have also revealed the incidence of PD is varied with gender, race or ethnicity (Van Den Eeden et al, 2003). For example, men are one and a half times more likely to be diagnosed with PD than women.

So far, the loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) of midbrain as well as the presence of intraneuronal proteinaceous cytoplasmic inclusions, Lewy Bodies, have been accepted as the pathological hallmarks of PD. About 5% incidence of PD is inherent or gene related, other 95% PD cases are sporadic or idiopathic (Dauer & Przedborski, 2003). Although there are a lot of research focusing on PD and its etiology, the pathogenesis or specific factors in causing PD are still not elucidated well. Besides the gene factor for inherent PD, environmental factors seem to significantly contribute to PD incidence. For example, heavy metals, pesticides, herbicides and fungicides all appear to induce PD. Some studies have pointed out that PD is more prevalent among rural farming people or factory workers. This phenomenon, perhaps, is due to they are more exposed to the heavy metals or pesticides compared with other people (Wolozin, Frasier, Synder, Choi, & Golts, 2005; Priyadarshi, Khuder, Schaub & Priyadarshi, 2001).

1.2.2 Dopamine and PD

Although James Parkinson has described the core clinical features of PD in 1817, the research about PD had little progress in the following century. Until 1958, Arvid Carlsson discovered the dopamine in mammalian brain, the pace of PD studies has been accelerated and the major pathological feature of PD, the selective loss of dopaminergic neurons in SNpc, was found. Now, dopamine is universally accepted as the neurotransmitter which is the most closely related to PD (Dauer & Przedborski, 2003; Murrin, 2005).

There are three different pathways for dopamine in our brain and each dopamine pathway has its particular role: (1) Mesolimbic and mesocortical pathways for reward systems, motivation, memory or addiction; (2) Nigrostriatal pathway for motor control; (3) Tuberoinfundibular pathway for hormonal regulation or other maternal and pregnant behaviors (figure 1.6). Different dopamine pathways are responsible for different roles of dopamine as one neurotransmitter. And the selective death of dopaminergic neurons in SNpc will lead to the damage of nigrostriatal pathways and then cause the PD. As in figure 1.7 showed, the nigrostriatal pathway transmits dopamine from SNpc to dorsal striatum which specifically is the caudate nucleus and putamen. And the neurons in SNpc are primarily projected to putamen. Figure 1.7 A showed the normal nigrostriatal pathway in thick solid red lines. But for the PD patients, their nigrostriatal pathways are weakened due to the death of dopaminergic neurons in SNpc so that the projection of dopamine to putamen is markedly reduced (dashed red line) but the projection to caudate is much less significant (thin red solid line). It is consistent with the other finding that depletion of dopamine is the most significant in the dorsolateral putamen of PD patients' brains (Bernheimer, Birkmayer, Hornykiewicz, Jellinger & Seitelberger, 1973). At the onset of PD or parkinsonism, putamen dopamine is depleted about 80% as well as about 60% SNpc dopaminergic neurons loss. On the other hand, neuromelanin can be regarded as one index of PD diagnosis. Neuromelanin is a dark pigment and found in a high concentration in specific brain sections, such as SNpc. Because neuromelanin is directly synthesized from L-dopa which is the precursor of dopamine *in vivo*, the selective death of dopaminergic neurons in SNpc will also lead to the loss of neuromelanin. For

example, PD patients just have 50% amount of neuromelanin in SNpc compared with other people without PD at the same age. Thus, in PD patients brain, SNpc depigmentation is also one characteristic feature as figure 1.7 showed.

Although the dopaminergic systems have been widely accepted as the neuropathology for PD, actually noradrenergic, serotonergic and cholinergic systems are also related with neurodegeneration for PD or other PD syndromes (Hornykiewicz & Kish, 1987). For example, some patients develop depression before the onset of PD motor symptoms; or the degeneration of hippocampal structures and cholinergic cortical inputs will lead the high rate of dementia accompanying with PD, especially for older patients. But the specific involvements for other neuronchemical systems in PD are still unclear (Dauer & Przedborski, 2003; Murrin, 2005).

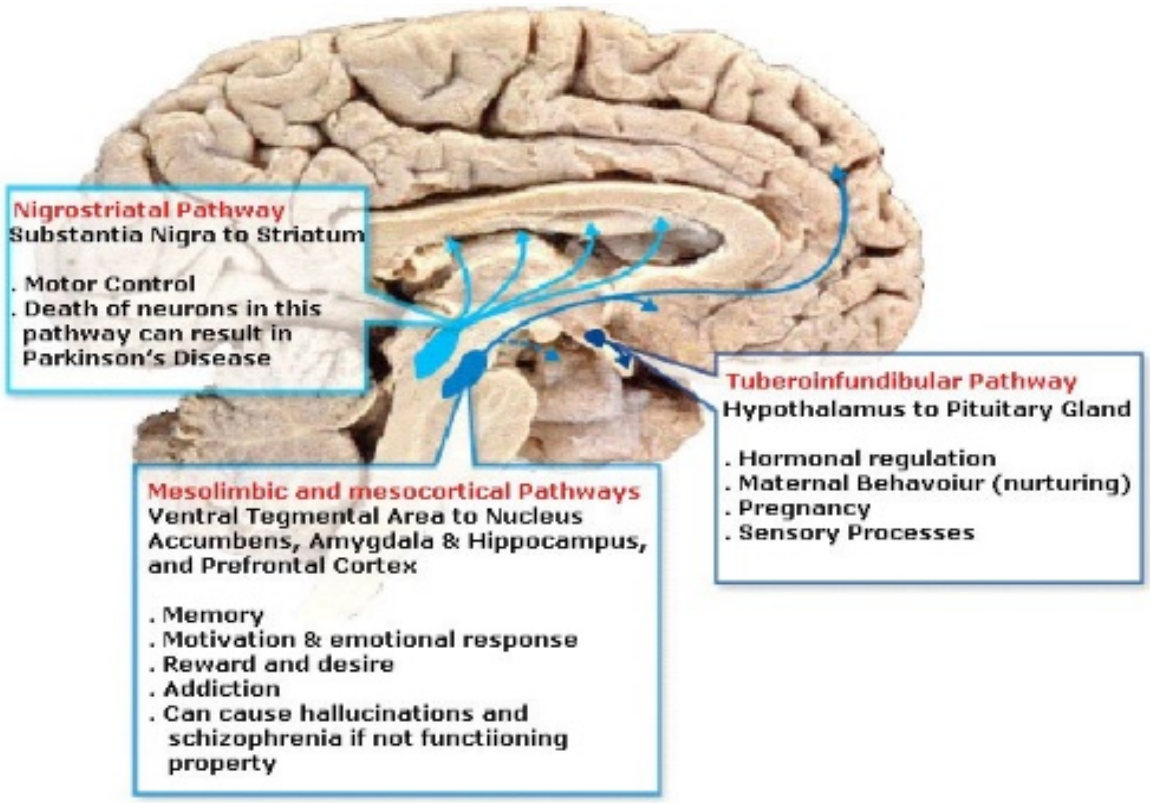


Figure 1.6 The Dopamine Pathways

1.2.3 Pathogenesis of PD

As mentioned in 1.2.1, the clinical diagnosis of PD is based on the identification of both the Lewy body formation and selective dopaminergic neurons loss in SNpc. This is originated from the two major pathogenesis concepts for PD: (1) abnormal protein

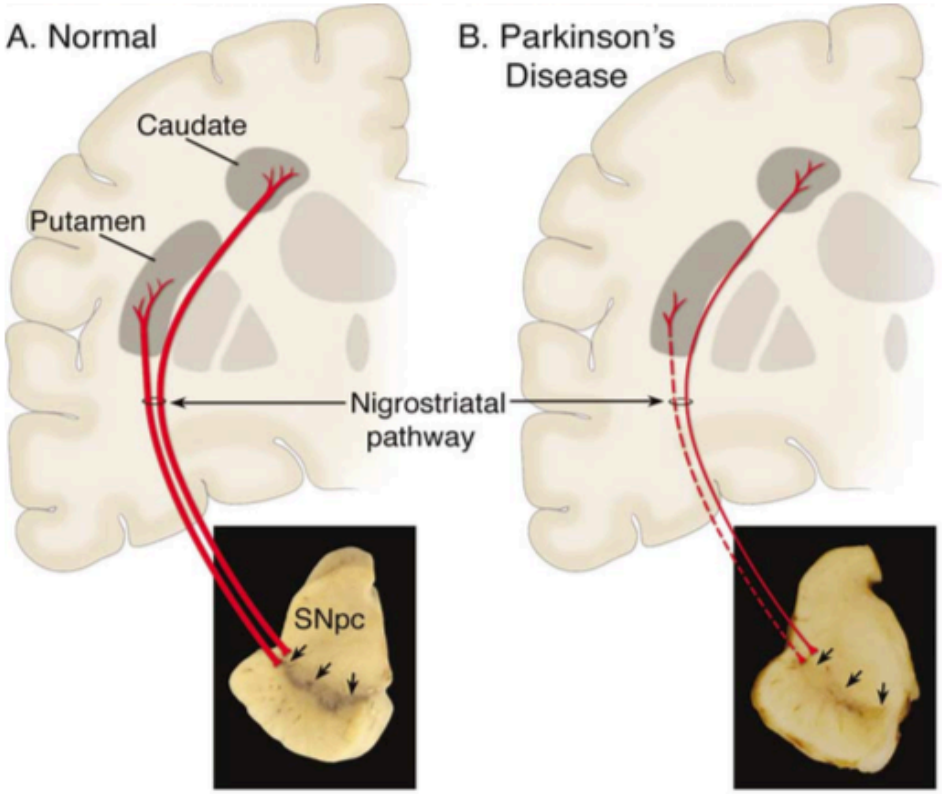


Figure 1.7 Neuropathology of PD

aggregation or misfolding; (2) mitochondria dysfunction and consequent oxidative stress. These two concepts are not mutually exclusive. Actually, these two concepts may be interacted (Dauer & Przedborski, 2003).

Abnormal protein aggregation has been regarded as one important factor in many neurodegenerative disease progress. For example: β-amyloid for AD; polyglutamine

mutation and expansions as well as aggregation in Huntington's disease; and Lewy body among PD. As different from other typical neurodegenerative disease hallmarks, Lewy body is not only specific for PD. It may also be found in AD and with the condition called "dementia with Lewy body disease". Lewy body is the spherical eosinophilic cytoplasmic protein aggregation and can be found in all affected brain regions (figure 1.8). The composition of Lewy body is various proteins including α-synuclein, parkin, ubiquitin and neurofilaments. In general, Lewy body is more than 15 μm in diameter and has an organized structure with a dense hyaline core surrounded by a clear halo of 10 nm radiating fibrils. Primarily, α-synuclein is the major component for Lewy body and the abnormal aggregation of α-synuclein is one important point for pathogenesis of PD. Especially for the inherent PD, the mutations which are A53T and A30P will increase the tendency of α-synuclein misfolding or abnormal aggregation. For the sporadic PD, several factors have also been pointed out that might increase the rate of protein aggregation. For example: increasing the concentration of α-synuclein will increase the intensity of its aggregation; some heavy metals accumulation, such as iron, shows the extensive α-synuclein aggregation; reactive oxygen species, such as hydrogen peroxide will also promote α-synuclein abnormal aggregation. Many studies give the conclusion that the abnormal protein aggregation, such as Lewy body, is toxic to neurons because these misfolding proteins may deform the cell or protein inclusions and may sequester other proteins that are vital for cell. But there is another opinion revealed the protein inclusion is probably one indicator to show the cell is under attack and provoke other mechanism to remove the toxic soluble misfolded proteins. For example, the ability of chaperones like Hsp-70 to protect against neurodegeneration provoked by these protein inclusions (Cummings et al, 1999; Cummings et al 2001; Auluck, Chan, Trojanowski, Lee &

Bonini, 2002; Warrick, Chan, Gray-Board, Chai, Paulson & Bonini, 1999). Therefore, the role of Lewy body is very controversial and there is no evidence powerful enough to prove its specific effect (Dauer & Przedborski, 2003).

For the mitochondrial dysfunction and its related oxidative stress, this pathogenesis do relate with abnormal protein misfolding or aggregation. Almost 100% oxygen molecule is consumed by mitochondrial respiration and the powerful oxidants are inevitable byproducts, such as hydrogen peroxide and superoxide radicals. It has been mentioned in the last paragraph that free radicals can promote the protein misfolding and aggregation, especially for α-synuclein in PD. On the other hand, these free radicals or oxidants will attack the electron transport chain in mitochondrial. As the result, mitochondrial will be damaged and further production of ROS. However, dopaminergic neuron is even a fertile environment to promote the generation of ROS. Because the metabolisms of dopamine will produce hydrogen peroxide and superoxide radicals. As this vicious cycle, the mitochondrial function may be suppressed, ATP synthesis may be depleted and the oxidative stress will be severe (Dauer & Przedborski, 2003; Wolozin et al, 2005). The relationship between mitochondrial dysfunction and PD pathogenesis will be discussed in more details in the neurotoxin section.

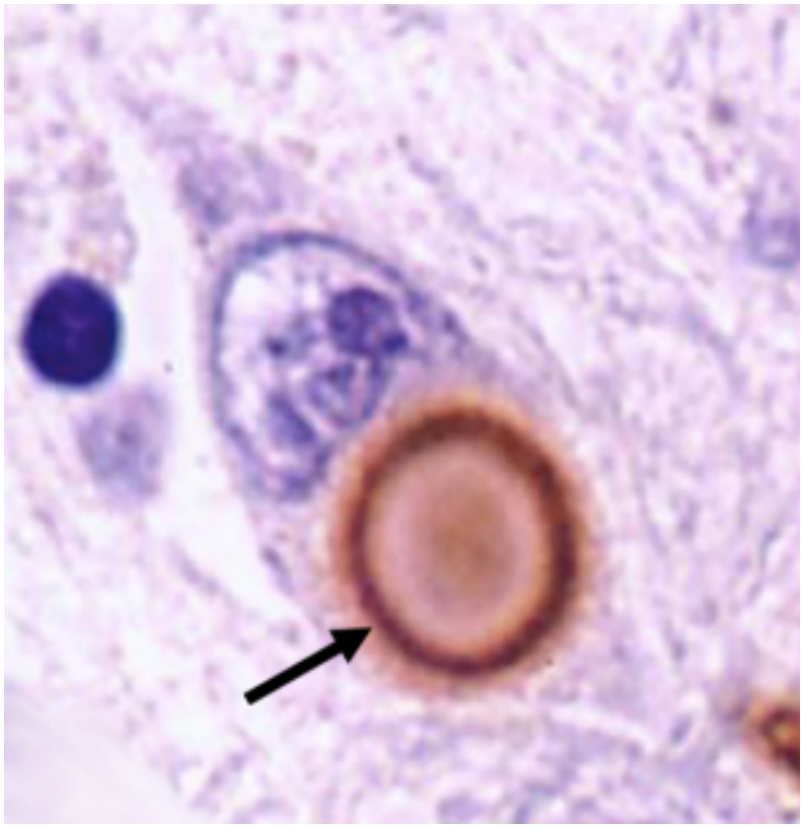


Figure 1.8 Immunostain of a Lewy body in SNpc neuron

1.2.4 Neurotoxins and PD

In 1976, one Maryland 23-year-old chemistry graduate student synthesized the illicit drug 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) in his lab and then injected himself with this impurity, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), contaminated MPPP drug. Within three days, the parkinsonism showed up. After he died, the destructive dopaminergic neurons in SNpc were found according to autopsy. And then, many studies have focused on the MPTP and its relationship with PD.

Nowadays, the MPTP metabolism and PD neurodegeneration selectivity can be elucidated clearly (figure 1.9). After administration, MPTP can cross the blood-brain barrier within minutes because of its high lipophilicity. And in glial cell, monoamine oxidase B (MAO-B) can oxidize the MPTP to 1-methyl-4-phenyl-pyridinium ion (MPP^+). And this MPP^+ is polar molecular and with high affinity for plasma membrane dopamine transporter (DAT). Therefore, MPP^+ can be actively and effectively transported into dopaminergic neurons. Inside neurons, MPP^+ can be accumulated in the mitochondria and inhibit multi-enzyme complex 1. Thus, the oxidative phosphorylation and mitochondrial electron transport chain will be impaired. Due to the damage for mitochondrial electron transport chain, the generation of ROS will increase, ATP synthesis will decrease, oxidative stress will be aggravated, and these downstream cellular events will lead to cell apoptosis eventually. In conclusion, the MPTP metabolized product MPP^+ will lead to the death of dopaminergic neurons. And some research showed that the striatum and ventral midbrain are particular MPTP-sensitive brain regions (Chan, DeLanney, Irwin, Langston & Monte, 1991; Fabre, Monserrat, Herrero, Barja & Leret, 1999). Besides MPTP, some other compounds have also been proved to induce the incidence of PD. For example, paraquat and rotenone and 6-hydroxydopamine. These neurotoxins all showed the specific toxicity to the midbrain region or SNpc part by promoting the Lewy body formation, increasing the oxidative stress and cell apoptosis (Dauer & Przedborski, 2003).

The MPTP has been regarded as a typical neurotoxin causing the acute parkinsonism. It leads to the search of MPTP analogue. Because, normally, the similar structural

compounds will lead to the similar effects. Tetrahydroisoquinolines (TIQs) are one family of MPTP analogues and can be derived from catecholamines (figure 1.10). For example, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol) can be synthesized through either enzymatic mechanism or Pictet-Spengler condensation reaction with acetaldehyde (figure 1.11) (DeCuypere, Lu, Miller & LeDoux, 2008; LeDoux, 2005). Thus, they are either endogenous in brain or exogenous in food and beverage (e.g. wine, cheese, cocoa, banana, broiled sardines, flour, eggs, beer and milk) (Makino, Ohta, Tachikawa & Hirobe, 1988; Niwa, Yoshizumi, Tatematsu, Matsuura & Nagatsu, 1989). Similar to MPTP, the toxicity for TIQs will be activated after metabolism in our body. As in figure 1.12 showed, TIQ will be converted to N-methyl-TIQ with N-methyl-transferase and then oxidized to N-methylisoquinolinium ion ($NMIQ^+$) under MAO-B enzyme catalysis (LeDoux, 2005; Musshoff, Schmidt, Dettmeyer, Priemer, Jachau & Madea, 2000). Compared with MPTP, TIQs have the lower affinity to DAT, limited MAO-B enzyme catalyzing activity and weaker inhibition of complex 1 in mitochondria. Therefore, in general, TIQs are a family of "weak" neurotoxins that may be significant for the pathogenesis of sporadic PD incidence because they can be synthesized *in vivo* spontaneously and continuously. And compared with MPTP acute toxic effect for dopaminergic neurons in SNpc, TIQs are regarded as to cause the neurodegeneration gradually in several years or decades (LeDoux, 2005). Some data suggested that *N*-methyl-(*R*)-salsolinol and (*R*)-salsolinol may be the most potent TIQ neurotoxins. *In vitro* tests showed they are associated with apoptosis after exposure to dopaminergic neurons (Maruyama, Benedetti, Takahashi & Naoi, 1997; Storch, Kaftan, Burkhardt & Schwarz, 2000). And the other study revealed that, in SNpc, both salsolinol/dopamine and N-methyl-salsolinol/dopamine ratios are significant higher in PD patient brains than normal

human brains (DeCuypere et al, 2008). In summary, endogenous or exogenous neurotoxins, TIQs, can be considered as a potential factor to induce the PD in a long term through the similar mechanism of MPTP.

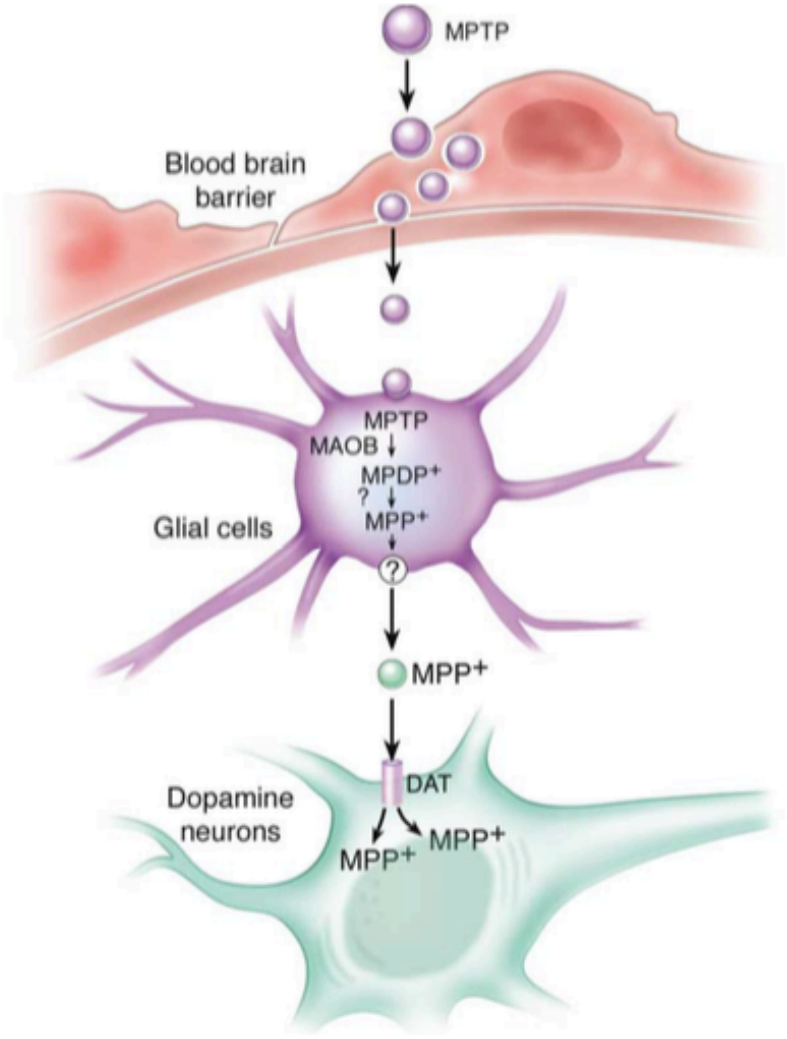


Figure 1.9 MPTP Metabolism Pathway in Brain

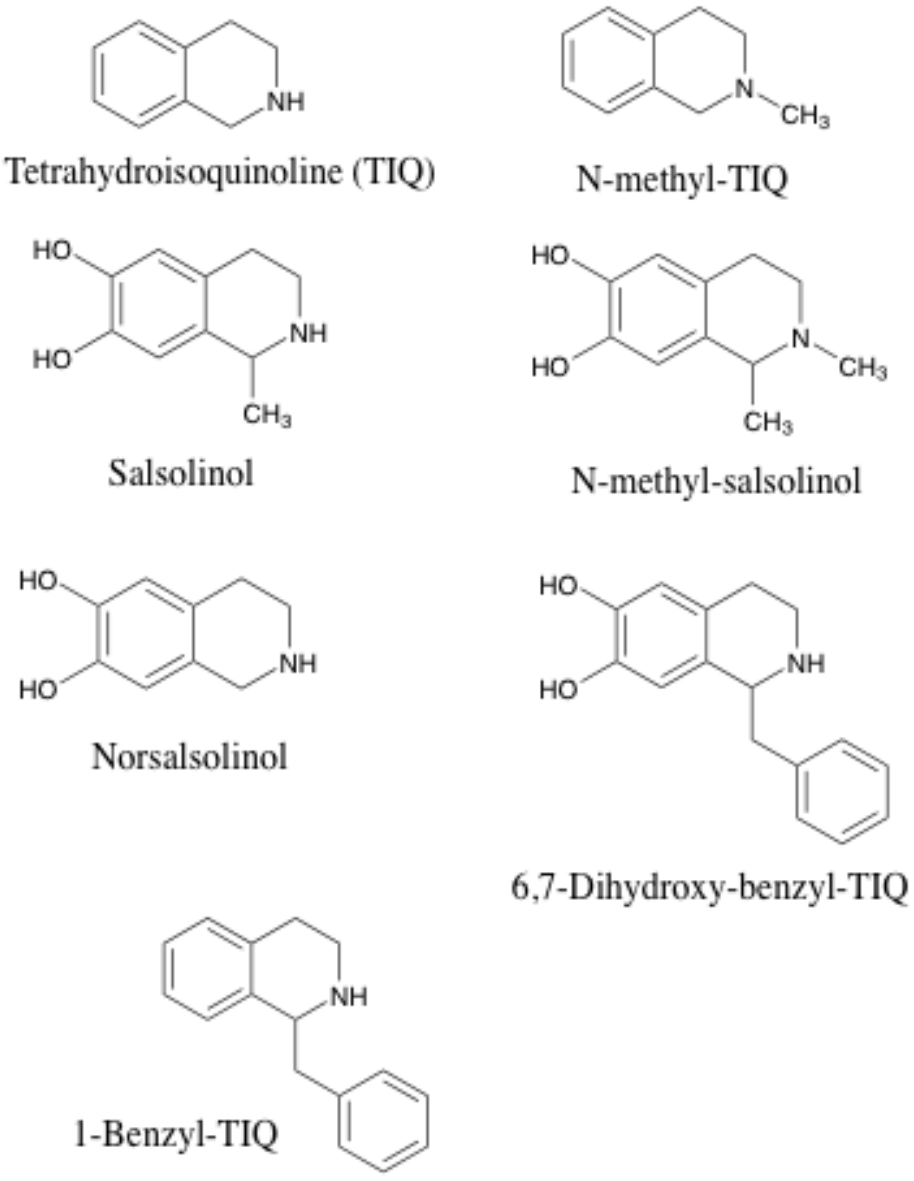


Figure 1.10 Chemical Structures for some TIQ compounds

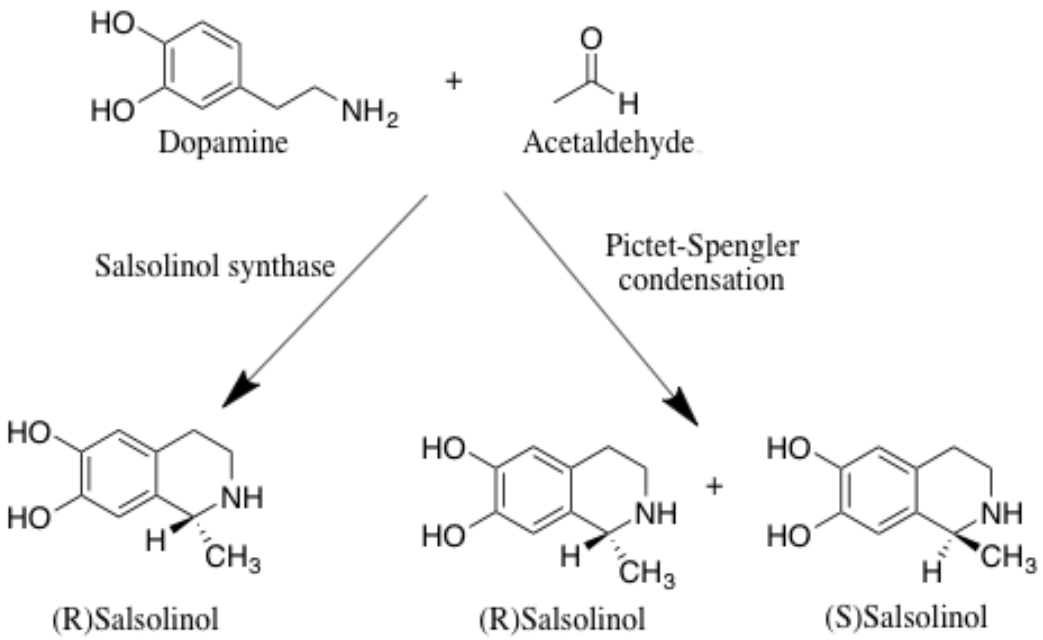


Figure 1.11 Salsolinol Synthesis Pathways

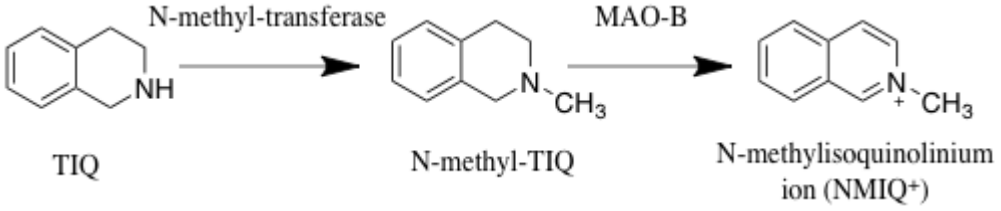


Figure 1.12 Conversion of TIQ to neurotoxic $NMIQ^+$ in vivo

Hypothesis and Objectives

2.1 Hypothesis

In the recent decade, some epidemiological studies showed that diabetic patients have the significant higher intensity to be diagnosed with PD (Driver et al., 2008; Xu et al., 2011). This phenomenon may be induced by the oxidative stress, inflammation or cerebrovascular disease that are linked with the diabetes. But the exact mechanism is still unclear.

On the other hand, neurotoxin is one of the environmental factors to induce the PD. And the neurotoxin is not only one exogenous source, it can also be generated endogenously like salsolinol. Therefore, some researchers are thinking whether the diabetic people can form certain endogenous neurotoxic compounds which can be derived from their abnormal glycolysis or other related metabolism products. For example, the MGO concentration in the diabetic patients' body is higher than the healthy people. Moreover, the MGO is one of the RCS and can actively attack other amine group compounds. Based on the pathway of salsolinol formation and the suspect of the relationship between MGO and neurotoxins, the reaction between dopamine and MGO to generate 1-acetyl-6,7-dihydroxyl-1,2,3,4-tetrahydroisoquinoline (ADTIQ) compound has been proposed (figure 2.1) (Xie et al., 2015). Theoretically, ADTIQ is also one of the TIQ family and can be metabolized to the MPP^+ structurally similar isoquinolinium ion (figure 2.2).

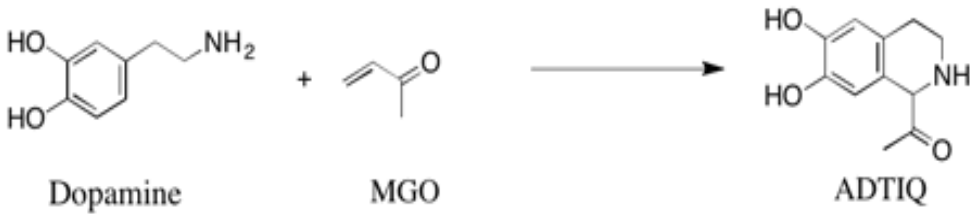
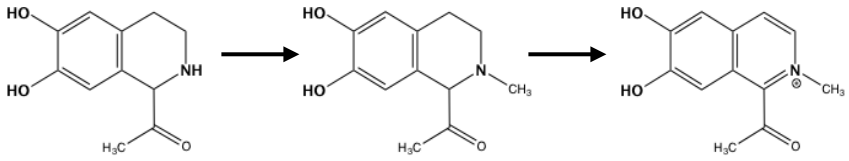


Figure 2.1 Synthesis of ADTIQ



ADTIQ N-methyl-ADTIQ N-methyl-1-acetlyl-6,7-dihydroxyl-isoquinolinium ion ($ADHIQ^{+)}$

Figure 2.2 Proposed metabolism mechanism of ADTIQ

One research group has focused on the reaction between dopamine and MGO. Both in vivo and in vitro tests, the ADTIQ compound has been detected. But there are several unreliable data in their results. So we want to prove the reaction between dopamine and MGO can generate the ADTIQ. Therefore, our first hypothesis is that the ADTIQ

is the product or one of the products from the reaction between dopamine and MGO. The second hypothesis is that the reaction rate for the dopamine and MGO may be increased with the concentration of MGO. And this may indicate why the diabetic patients are more risky to the PD.

2.2 Research objectives

1. To study the final products of the reaction between dopamine and MGO under physiological condition.
2. To study the reaction kinetics between dopamine and MGO.

Experimental

3.1 Materials

Dopamine hydrochloride, ammonium formate, 1,2,3,4-tetrahydroisoquinoline-6,7-diol hydrobromide, trizma base, sodium phosphate (monobasic) and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methylglyoxal (40% aqueous solution) were purchased from Fisher Scientific (Waltham, MA, USA). Acetonitrile (HPLC grade), water (HPLC grade), and methanol (HPLC grade), hydrochloric acid (ACS reagent grade) were purchased from Pharmco-AAPER (Brookfield, CT, USA).

3.2 Instruments

The high performance liquid chromatograph (HPLC) system consisted of a Dionex UltiMate 3000 HPLC series (Sunnyvale, CA, USA) including an UltiMate 3000 Pump, an UltiMate 3000 Variable Wavelength Detector, and an UltiMate 3000 Auto-sampler. Chromeleon software was used to perform instrument control and data analysis. Phenomenex Luna C18 HPLC column (25 cm × 4.6 mm, 3 μm) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Thermo Scientific Hypersil™

ODS C18 HPLC column (25 cm × 4.6 mm, 5 μm) was purchased from ThermoFisher Scientific (Waltham, MA, USA).

The Liquid Chromatograph - Mass Spectroscopy (LC-MS) is Agilent 1100 series LC/MSD System (Agilent Technologies, Waldbronn, Germany). It consisted of an auto-degasser, a quaternary pump system, a thermostatted column compartment, a diode-array detector (DAD), an ion trap analyzer and an electrospray ionization (ESI) source. The scan range is from 50 to 300 Da. The Thermo Scientific Hypersil™ ODS C18 HPLC column (25 cm × 4.6 mm, 5 μm) (Waltham, MA, USA) was used for chromatographic separation.

For the Liquid Chromatogragh - Mass Spectrometer / Mass Spectrometer (LC-MS/MS), the Phenomenex Gemini C18 column (5 μm, 3.0 mm i.d. ×150 mm) (Torrance, CA, USA) was used for chromatographic separation. The LC-MS/MS system is the Thermo-Finnigan Spectra System consisting of an Ultimate 3000 degasser, an Ultimate 3000 RS pump, an Ultimate 3000 RS autosampler, an Ultimate 3000 RS column compartment, and an LTQ Velos Pro ion trap mass spectrometer (Thermo Electrom) equipped with an electrospray ionization (ESI) interface. The full scan range is from 50 to 300 Da and the selected reaction monitoring mode is with target ions at 166, 175 and 208 Da.

3.3 Method

3.3.1 HPLC Analysis of Dopamine

The dopamine hydrochloride (0.0190 g) was dissolved in distilled water within a 10 mL volumetric flask to make the dopamine standard solution which concentration is 0.01 mol/L. After filtration with 0.45 μm PTFE filter, the samples were ready for HPLC injection.

The HPLC analysis for dopamine is with Thermo Scientific Hypersil™ ODS C18 HPLC column (25 cm × 4.6 mm, 5 μm). The mobile phase is the mixture of 25% methanol and 75% 10 mM ammonium formate water buffer (B). The pH value for the mobile phase B is 3.5. If necessary, the formic acid can be used to adjust the pH value to 3.5. The HPLC program for the dopamine analysis is isocratic with the flow rate as 0.5 ml/min. The injection volume was set at 5 μL. From 0 min to 15 min, 100% mobile phase B is used to elute. From 15 min to 16 min, the mobile phase is changed to 100% acetonitrile (C) and kept for 1 min to wash the column. From 17 min to 18 min, the mobile phase is changed back to 100% B for equilibrium and the equilibrium lasts for 2 mins. Therefore, one program for the analyzing the dopamine lasts for 20 minutes totally. Data was collected at wavelength of 214 nm, 280 nm, 326 nm.

3.3.2 Incubation the Reaction under Physiological Condition

The reaction is carried out in 10 mmol/L Tris-HCL buffer to maintain the physiological pH condition which is around 7.4. After dissolving the Trizma base in the distilled water accordingly, hydrochloric acid is used to adjust the pH to 7.4. Then, 0.26 mol/L MGO is added to react with 0.00523 mol/L dopamine hydrochloride in the prepared buffer so that the reaction reagent ration for dopamine : MGO is 1 : 50. The reaction or incubation temperature is equal to 37□C which is healthy people's physiological temperature. Due to instability of dopamine, the reaction is proceeded under the nitrogen protection. And this reaction should be completed after overnight incubation(about 16 hours).

After the reaction completed, the crude reaction liquid is filtered by 0.45 μm PTFE filter and it is ready to go in the HPLC to test. The HPLC program is the same one for the dopamine standard analysis. The column used is Thermo Scientific Hypersil™ ODS C18 HPLC column (25 cm × 4.6 mm, 5 μm). The injection volume was set at 5 μL and the flow rate is 0.5 mL/min.

3.3.3 LC-MS Analysis of Crude Reaction Liquid

There is one research group has focused on the reaction between dopamine and MGO before. And they have published several papers (Xie et al., 2015; Deng et al., 2011) to study the reaction product by LC-MS/MS and nuclear magnetic resonance (NMR). But we think the data they presented is not very reliable. Therefore, I follow their experimental procedures at first and use the LC-MS to detect the reaction product.

The LC-MS analysis with the electrospray ionization (ESI) interface. The dry gas flow rate is 9 L/min and the dry temperature is 325□C. The nebulizer is with 40.0 psi. The set compound stability is 80%. The LC/MS is under the positive polarity and the scan range is from 50 to 300 Da. For HPLC separation part, crude reaction liquid was run with the Thermo Scientific Hypersil™ ODS C18 HPLC column (25 cm × 4.6 mm, 5 μm). The mobile phase is the mixture of 25% methanol and 75% 10 mM ammonium formate water buffer (B). The pH value for the mobile phase B is 3.5. If necessary, the formic acid can be used to adjust the pH value to 3.5. The LC program is isocratic with the flow rate of 0.5 ml/min. The injection volume was set at 5 μL. From 0 min to 15 min, 100% mobile phase B is used to elute. From 15 min to 16 min, the mobile phase is changed to 100% acetonitrile (C) and kept for 1 min to wash the column. From 17 min to 18 min, the mobile phase is changed back to 100% B for equilibrium and the equilibrium lasts for 2 mins. Data was collected at wavelength of 214 nm, 280 nm, 326 nm.

3.3.4 HPLC and LC-MS Analysis of Freeze Dried Reaction Products

In order to detect the reaction products better, freeze dry system is used to remove the liquid and control the extent of reaction. After overnight freeze dried, the remaining solids should be waterless. Then, the solids are dissolved in methanol and run both HPLC and LC-MS to confirm the stability of reaction products. After filtration with 0.45 μm PTFE filter, the samples were ready for HPLC and LC-MS injection. The method for the HPLC and LC-MS to analyze the freeze dried product are the same as the ones used to test the crude reaction liquid in 3.3.2 and 3.3.3.

3.3.5 HPLC and LC-MS Analysis of Norsalsolinol Standard

Since the ADTIQ compounds may not be the reaction final products as well as the major peak which with the molecular ion mass equal to 166 may be the products between the reaction of dopamine and MGO, we need to study what compound it is for the major peak. If 166 was subtracted by 208, it is equal to 42 which is the mass of one acetyl group (-$COCH_3$). If the acetyl group in ADTIQ was missed, it will turn to the compound named as 6,7-dihydroxy-1,2,3,4,-tetrahydroisoquinoline or norsalsolinol. In order to prove this hypothesis, the norsalsolinol bromide was purchased from Sigma to make the comparison. The norsalsolinol bromide was dissolved in the methanol/water solvent and ready to run the HPLC analysis after

filtered by 0.45 μm PTFE filter. The HPLC method is the same as the one for dopamine in 3.3.1.

After the HPLC analysis, the LC-MS was carried out to identify the retention time of norsalsolinol standard and molecular ion mass. After the filtration with 0.45 μm PTFE filter, the LC-MS was run with the same program in 3.3.3.

3.3.6 Comparison of Norsalsolinol and Reaction Products on LC-MS/MS

Besides the retention time and molecular ion mass, the ion fragmentation from the LC-MS/MS can be used to compare two products and confirm whether they are the same compound. Because for the same compound, no matter what matrix that is, they should be eluted out at very similar time and with the same ion fragmentation detected in the tandem mass spectrometry.

LC-MS/MS condition is with ESI interface, full scan range from 50 to 300 Da; Selected reaction monitoring mode with target ions at 166, 175, and 208 Da. HPLC condition for LC-MS/MS: Chromatographic separation was performed using a Gemini C18 column (5 μm, 3.0 mm i.d.× 150 mm) which was purchased from Phenomenex (Torrance, CA, USA). The mobile phase consisted of 5% aqueous methanol with 0.1% formic acid (mobile phase A) and 95% aqueous methanol with

0.1% formic acid (mobile phase B). The chromatographic separation was performed for 15 min at a flow rate of 0.3 mL/min with the following gradient program: 0% B from 0 to 3 min, 0% - 10% from 3 to 10 min, and 0% B from 10 to 15 min. Injection volume was 10 μL.

3.3.7 Dopamine Standard Curve

The other part of this study is to test the reaction dynamics between dopamine and MGO. In this experiment, I focused on tracking the dopamine remaining concentration to express the reaction speed. Therefore, the dopamine standard curve is used to calculate back the amount of dopamine during the reaction. The concentration of dopamine to make the standard curve is 0.00001 mol/L, 0.0001 mol/L, 0.001 mol/L, 0.005 mol/L and 0.01 mol/L. The dopamine standard solution is ready to be injected into HPLC after filtered by 0.45 μm PTFE filter.

The isocratic program of HPLC which is used for detecting the reaction products is not good enough to track the dopamine during the reaction progress, because the peak of dopamine overlaps the peak of norsalsolinol. Thus, the other HPLC program method needs to be introduced here. The HPLC analysis for dopamine this time is with Phenomenex Luna 5u C18 HPLC column (25 cm X 4.6 mm, 5 um). The mobile phase is 0.2 mol/L phosphate buffer (B) and methanol (D). The pH value for the mobile phase B is 5.0. If necessary, the sodium hydroxide solution can be used to adjust the pH value to 5.0. The HPLC program for the dopamine analysis is isocratic with the flow rate as 0.8 ml/min. The injection volume was set at 5 μL. At 0 min, the

initial mobile phase is 95% B and 5% D. At 10 min, the mobile phase changed to 90% B and 10% D. This gradient of mobile phase maintain for 2 mins. From 12 min to 14 min, the mobile phase changed from 90% B and 10% D to 100% water (A). Water is used to wash the column for 2 mins till 16 min. And at 18 min, the mobile phase is changed back to initial gradient of 95% B and 5% D. This initial gradient is maintained till 20 min for the equilibrium of column. Therefore, one program for the analyzing the dopamine lasts for 20 minutes totally. Data was collected at wavelength of 214nm, 280nm, 326nm.

3.3.8 Reaction Kinetics of Reaction between Dopamine and MGO

To confirm whether the reaction speed as well as the extent of reaction will change with the alteration for dopamine and MGO concentration ratio, the reaction kinetics between the dopamine and MGO need to be studied.

There are four different ratios of dopamine and MGO involved in this experiment: 1 : 3, 1 : 10, 1 : 20 and 1 : 50. The reactions were all incubated under nitrogen protected and physiological condition (pH= 7.4 and 37□C) which has been described in the 3.3.2. Each different reaction ratio of dopamine and MGO trials were triplicate. Each reaction was monitored for 24 hours and 12 time points: 0 min, 5 min, 10 min, 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, 420 min and 1440 min. At every time point, about 1 mL reaction liquid was taken out by syringe. After filtration with 0.45 μm PTFE filter, the collected sample is ready to be detected.

The reaction kinetics or reaction extent is based on the consumption percentage of dopamine versus the time under the different reaction ratio for dopamine and MGO. HPLC is used to detect the remaining dopamine in the reaction liquid by running the program described in 3.3.7. The unreacted dopamine amount can be calculated based on the dopamine standard curve. If the collected sample cannot be detected immediately, the filtered sample was placed in the freezer to stop the reaction.

Results

4.1 HPLC Analysis of Dopamine

The HPLC chromatogram is shown below in figure 4.1. Based on the peak areas, the purity of dopamine is above 98%.

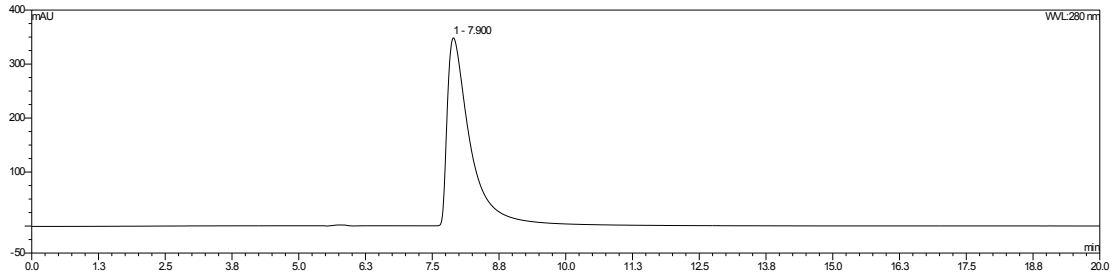


Figure 4.1 HPLC chromatogram of dopamine

4.2 HPLC Result for the Crude Reaction Liquid:

The HPLC chromatogram is shown below in figure 4.2. After the overnight reaction, there was still some unreacted dopamine left. But due to the major peak was still around 8 min, there is another possibility that one of the reaction products or intermediates has the similar retention time as dopamine under this HPLC program. Besides the major peak which was at 8 min, some impurities came out at 5 – 7 min,

and there were other detectable major peaks at 10.5 min. It might be the products from this reaction. Under the principle Pictet-Spengler reaction, the formed reaction product might be the tetrahydroisoquinoline. To confirm the hypothesis, the LC-MS is needed to detect the responding molecular mass for each possible product peak.

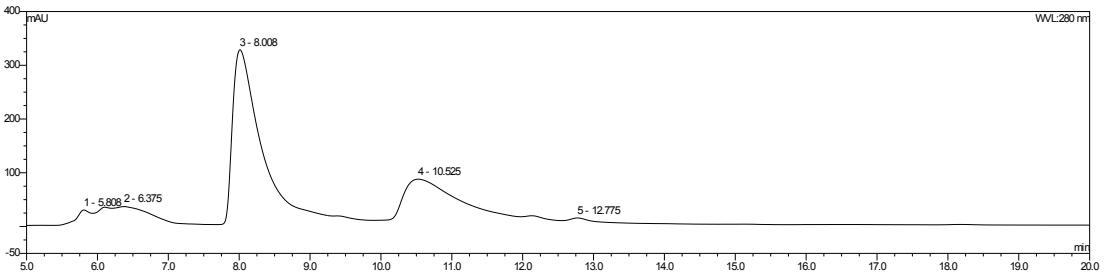


Figure 4.2 HPLC chromatogram for the crude reaction liquid

4.3 LC-MS Result for the Crude Reaction Liquid:

Figure 4.3 is the LC-MS result. We can find that the LC chromatogram under 280 nm absorbance from LC-MS is matched with HPLC chromatogram in 4.2. Around 5 min to 7 min, some impurities were eluted. The major peak was eluted at 8 min and other peak could be detected around 10 - 11 min under the UV absorbance. The major peak in the LC chromatogram was confusing and I wanted to know whether it was solely the unreacted dopamine or the other reaction intermediate from the reaction. According to the mass spectrum, we could know that the major peak was the compound with molecular ion mass of 166. As mentioned in the 3.3.3, one research

group has focused on studying the reaction product of MGO and dopamine. Based on their data from LC-MS/MS, no peak or compound with the molecular ion mass of 166 was found. Their major product is named as N-methyl-1-acetlyl-6,7-dihydroxyl-isoquinolinium ion (ADTIQ) and the reaction pathway is shown in figure 2.2. Because the molecular ion mass of ADTIQ compound is 208, the extracted ion chromatography (EIC) technique is used to help us determine the target compounds in this mixture. We can find two peaks with the molecular ion mass of 208 eluted from the crude reaction liquid at around 12.5 min and 18.5 min. It means there are two different compounds with the same molecular ion mass eluted from the crude reaction liquid at different time. Based on the Pictet-Spengler reaction, when the reaction condition is neutral, two structural isomers may be formed (figure 4.4). Therefore, two isomers can be formed from the reaction of dopamine and MGO under the physiological condition (pH = 7.4).

In this step, we know that the major peak from the crude reaction liquid is not the unreacted dopamine itself. It should be other compounds formed from the reaction of dopamine and MGO. But we need to do the further study to prove whether it is the intermediate in the reaction or even another product formed from this reaction. And also, we should know what the exact compound for this peak.

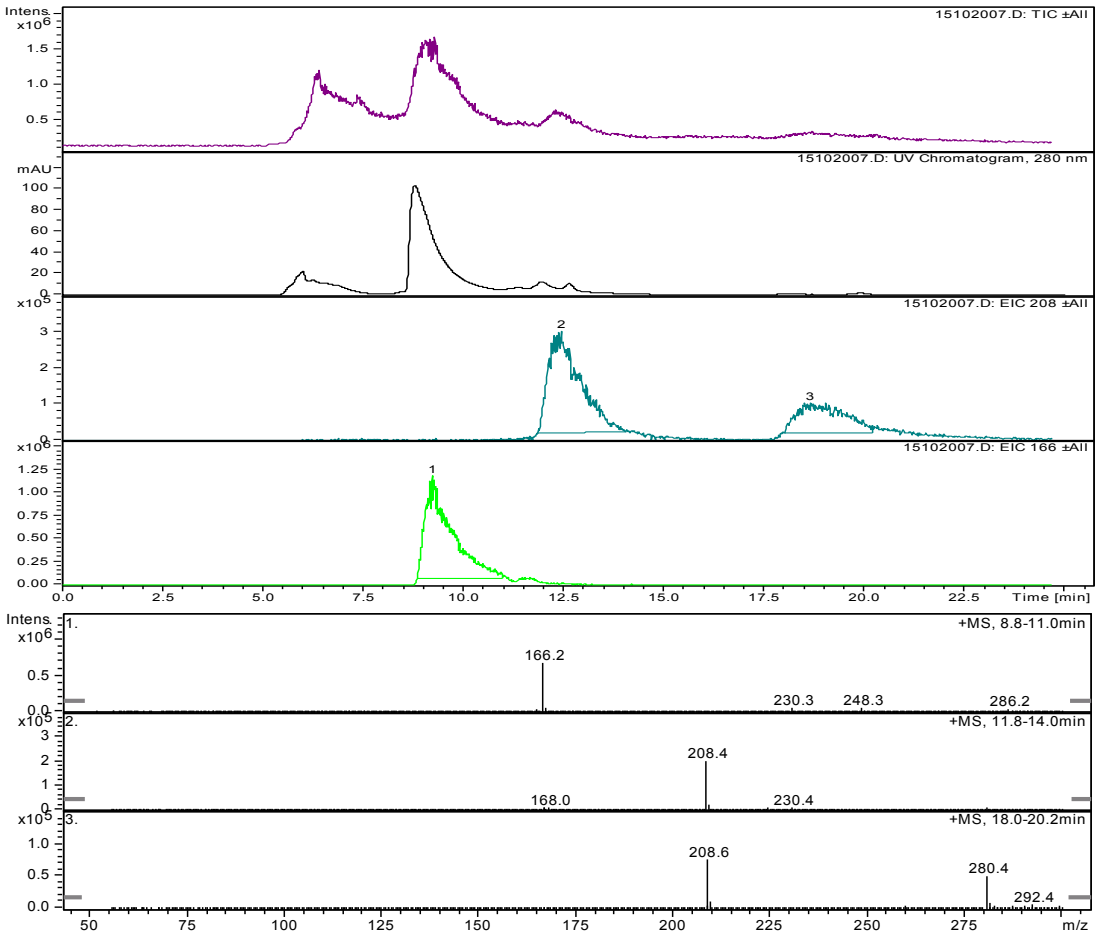


Figure 4.3 LC-MS result for the crude reaction liquid

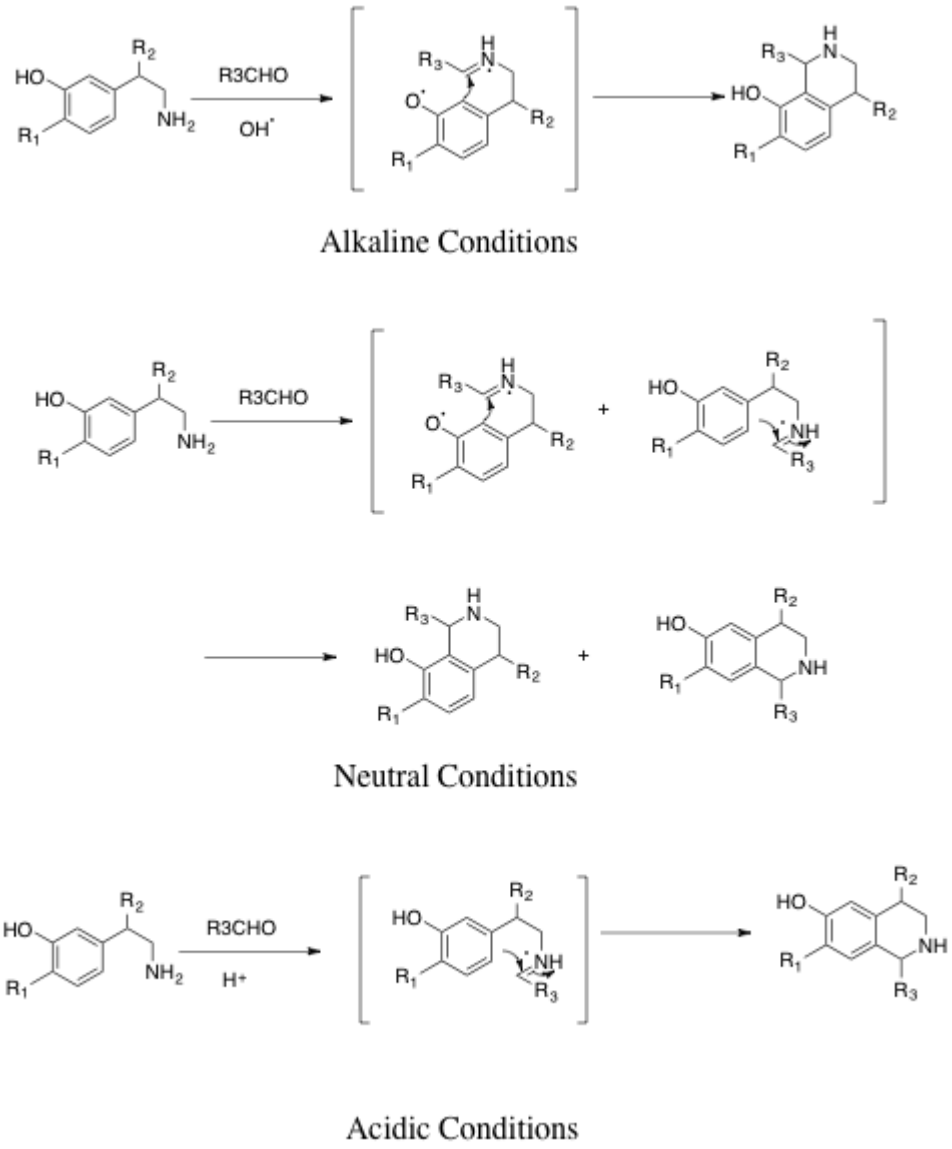


Figure 4.4 Different pathways for the Pictet-Spengler Reaction under different pH value

4.4 LC-MS Result for the Freeze-Dried Reaction Products:

The HPLC chromatogram and LC-MS result for the freeze-dried reaction products are presented in figure 4.5. The major peak was the same as the one in crude reaction liquid and was eluted out around 8-9 min. There is about 1-2 minutes shift which may

be caused by the instrumental condition or the pressure variation. The mass spectrum for this major peak showed the major molecular mass of 166. Surprisingly, the other peak which was eluted after the major peak was almost disappeared in the freeze-dried reaction products based on both HPLC chromatogram and LC-MS data. And the EIC technique in LC-MS was used to search the compounds with molecular ion mass equal to 208, and we found no peak. Therefore, it seems that the reaction product, ADTIQ, which has been confirmed from former research group is not stable or they are only the reaction intermediates instead of the real reaction products. And for the stable major peak compound with the molecular ion mass of 166 may be the real final product from the reaction between dopamine and MGO.

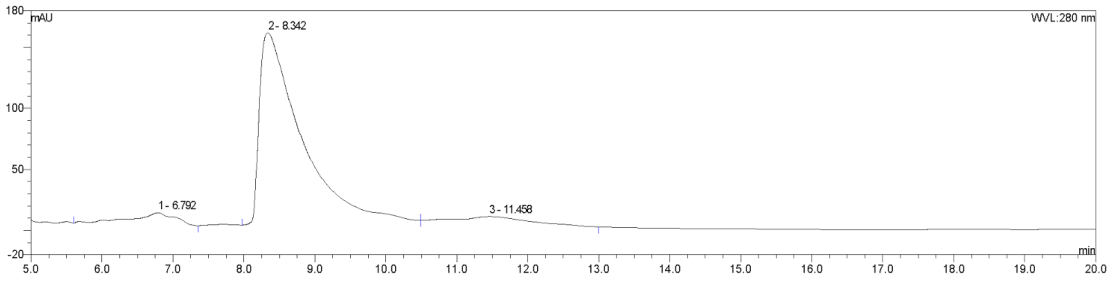


Figure 4.5 HPLC chromatogram for the freeze-dried reaction products

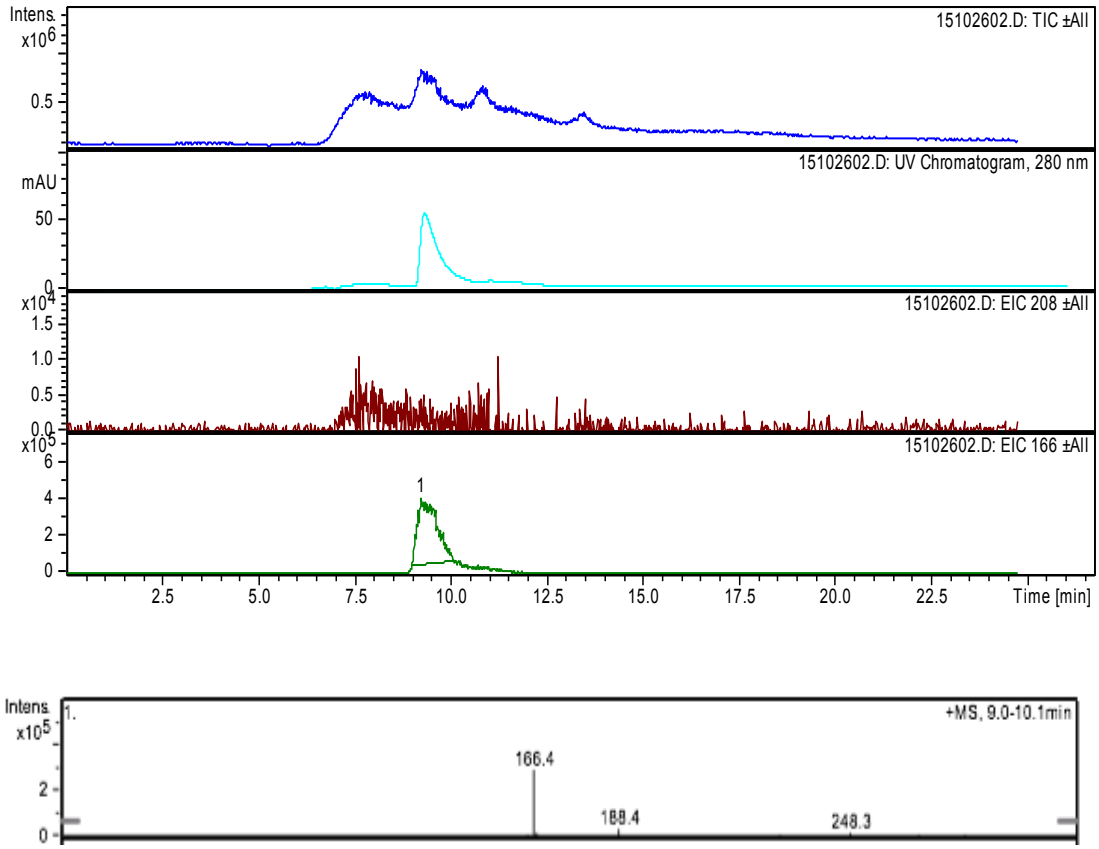


Figure 4.5 LC-MS result for the freeze-dried reaction products

4.5 HPLC and LC-MS Result for Norsalsolinol Standard

The HPLC chromatogram for the norsalsolinol standard is presented in figure 4.6. With the same HPLC program and method as the dopamine standard, the retention time for the norsalsolinol is very similar with the dopamine which is around 8 min.

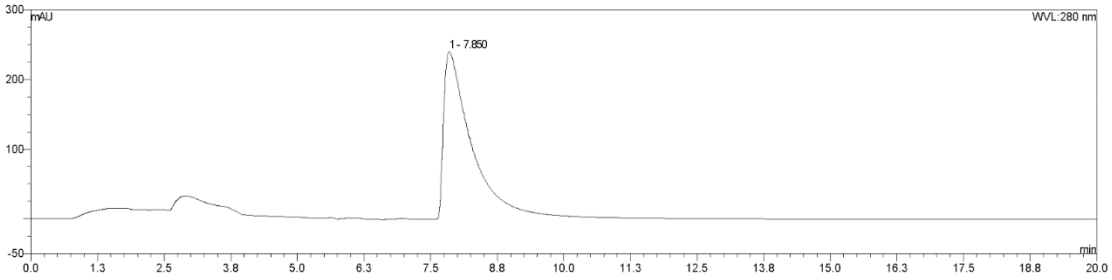


Figure 4.6 HPLC chromatogram for norsalsolinol standard

Figure 4.7 shows the LC-MS data for norsalsolinol standard. The retention time for the norsalsolinol standard is consistent with the major peak from both crude reaction liquid and freeze-dried reaction products. And the mass spectrum indicates they have the same molecular ion mass which is equal to 166.

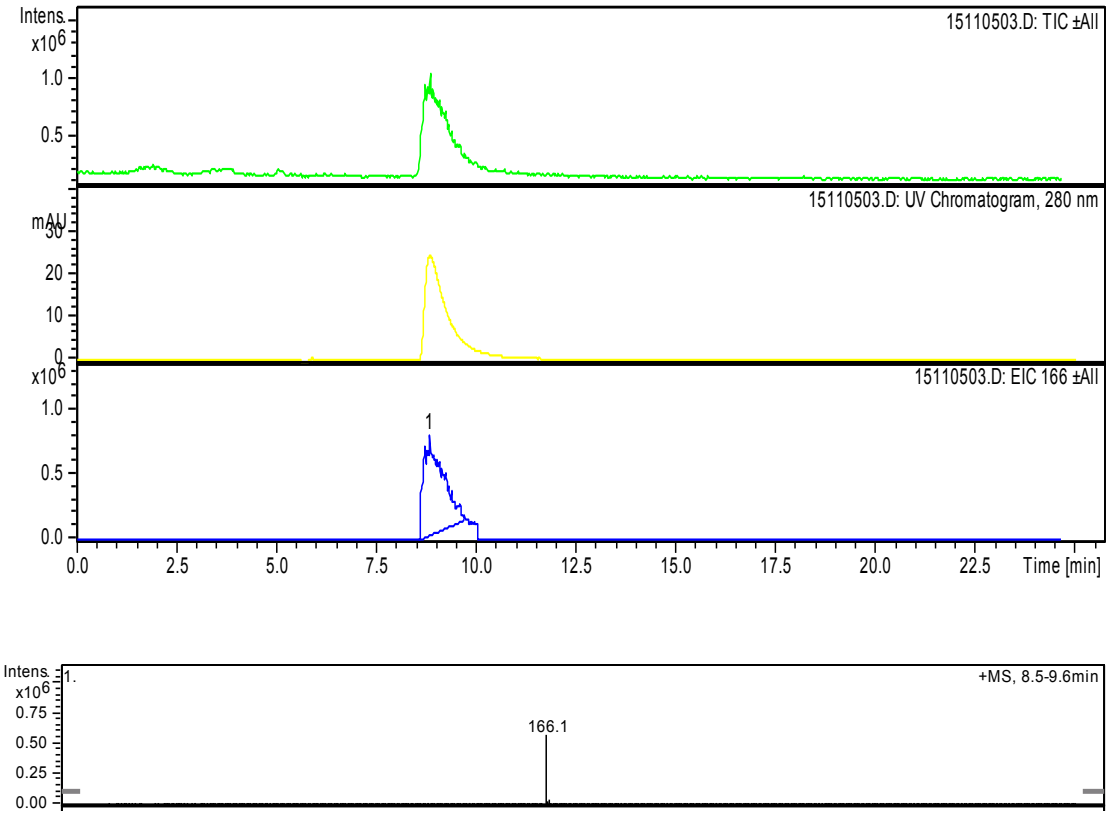
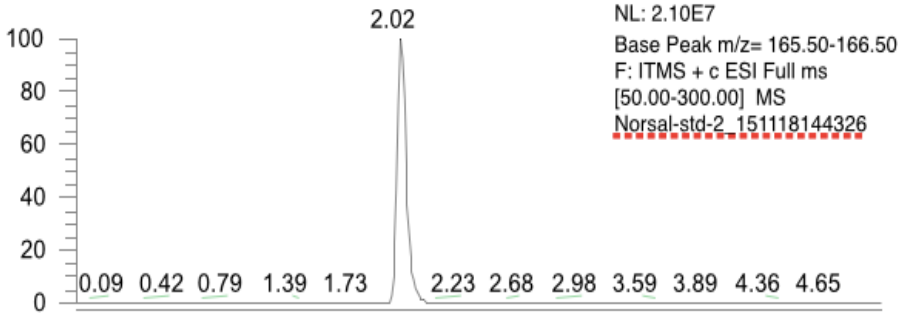


Figure 4.7 LC-MS result for the norsalsolinol standard

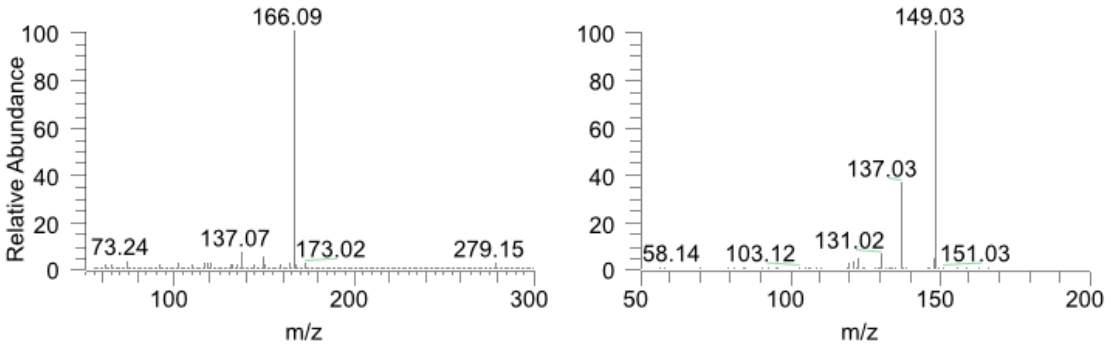
4.6 Comparison of Norsalsolinol and Reaction Products on LC-MS/MS

Figure 4.8 (a) shows the HPLC chromatogram from LC-MS/MS for norsalsolinol standard. The major peak at 2.02 min stands for the norsalsolinol compound. Figure 4.8 (b) shows that the LC-MS data and figure 4.8 (c) present the tandem MS data from the LC-MS/MS.

Figure 4.9 (a) is the HPLC chromatogram for the reaction products. It is obvious that there are three peaks (peak A, peak B and peak C). According to the LC-MS data in figure 4.9 (b), the peak A and peak B have the same molecular ion mass which of 166. And the molecular ion mass for peak C is 208. It seems that peak A and peak B are isomers of norsalsolinol and peak C is the ADTIQ compound. But compared with peak A and peak B, peak C has much lower content in the products. And figure 4.9 (c) shows the tandem MS data for both peak A and peak B. Peak A and peak B as well as the norsalsolinol have the same ion fragmentation. It implies that both peak A and peak B are the norsalsolinol but with different regioselective chemical structure.

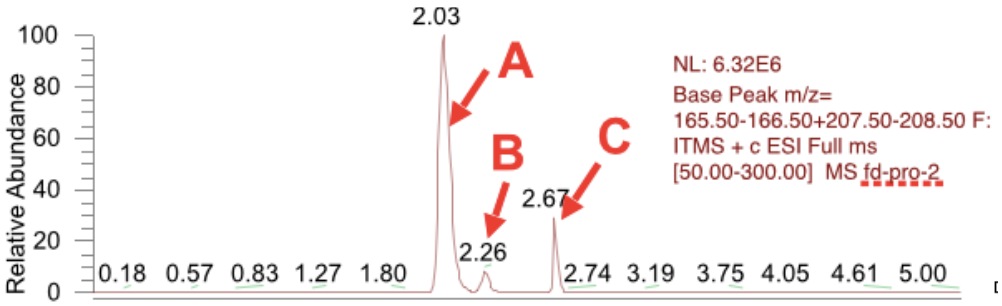


(a)

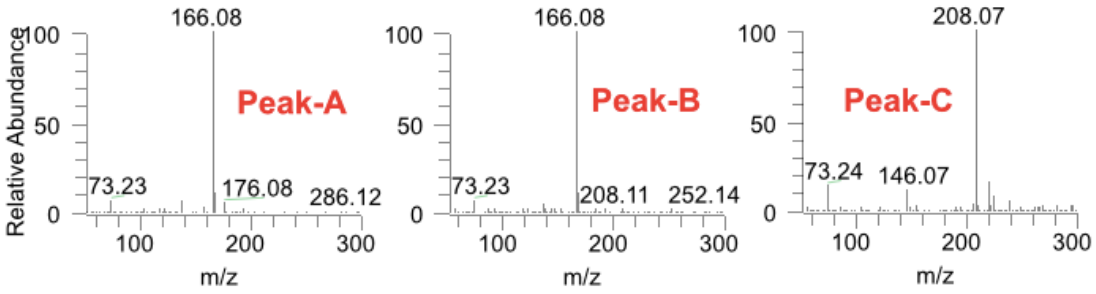


(b) (c)

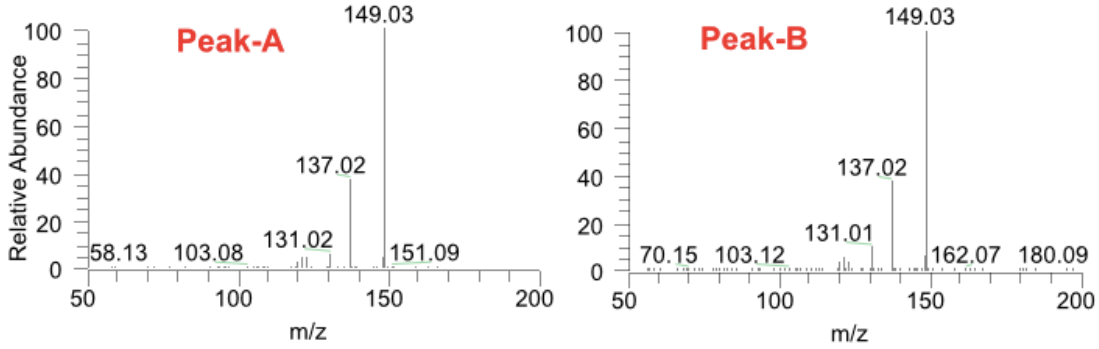
Figure 4.8 LC-MS/MS data for norsalsolinol standard. (a) HPLC chromatogram for norsalsolinol. (b) The molecular ion for norsalsolinol based on LC-MS data. (c) The ion fragmentation for norsalsolinol based on LC-MS/MS data.



(a)



(b)



(c)

Figure 4.9 LC-MS/MS data for reaction products. (a) HPLC chromatogram for reaction products. (b) The molecular ions for peak A, peak B and peak C based on LC-MS data. (c) The ion fragmentation for peak A and peak B based on LC-MS/MS data.

4.7 Dopamine Standard Curve

Figure 4.10 shows the dopamine standard curve varied from 0.00001 mol/L, 0.0001 mol/L, 0.001 mol/L, 0.005 mol/L to 0.01 mol/L. The absorbance area is based on 280 nm absorbance and R^2 value is equal to 0.9992.

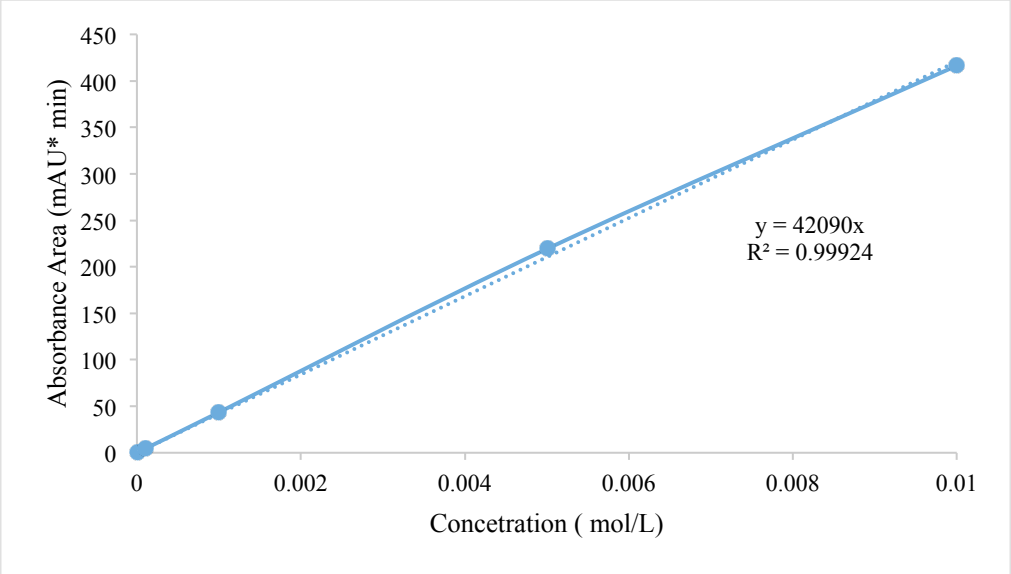
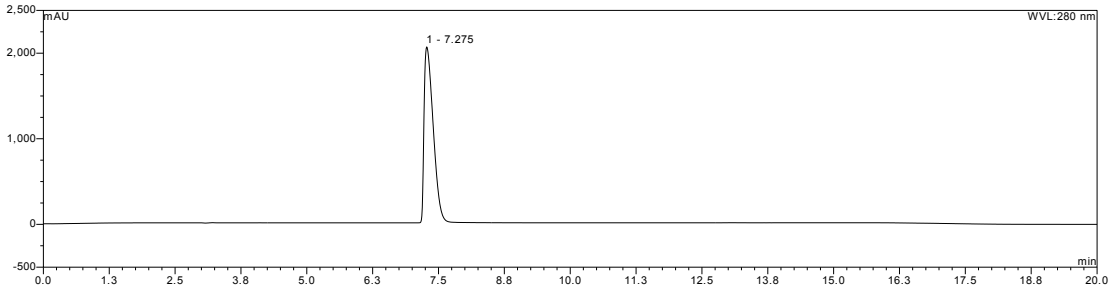


Figure 4.10 Dopamine standard curve

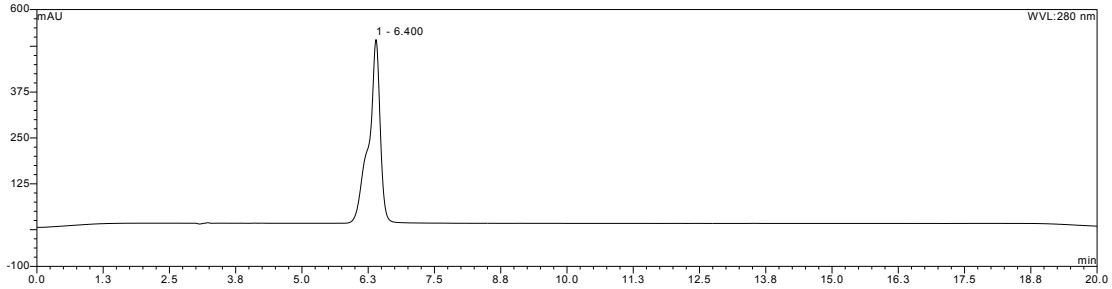
4.8 Reaction Kinetics of Reaction between Dopamine and MGO

Based on the HPLC program with the 0.2 mol/L phosphate buffer (pH=5) as mobile phase B, the chromatograms for dopamine and norsalsolinol have changed. Figure 4.11 (a) shows the dopamine standard chromatogram and figure 4.11 (b) is for norsalsolinol standard. Because the dopamine and norsalsolinol have the different retention time now and these two peaks will be not be overlapped anymore, dopamine concentration can be calculated back from the absorbance area from the chromatogram by the equation generated from the dopamine standard curve. The initial HPLC chromatogram (0 min) and the final HPLC chromatogram (24 hours) for the reaction between dopamine and MGO which concentration ratio varied from 1 ： 3、 1 : 10、 1 ： 20 and 1 : 50 can be found in the appendix.

Each different concentration ratio for the reaction has been triplicated and the data are concluded in figure 4.12. As the data showed, when the concentration ratio of dopamine and MGO was 1 : 3, there was still about 37.23% dopamine remained or unreacted after 24 hours. When the reagents concentration ratio was 1 : 10, there was about 2.98% dopamine remained after 24 hours. For 1 : 20, about 1.14% dopamine remained after 24 hours. And when the ratio of reagents was 1 : 50, there was 0.11% dopamine unreacted after 24 hours. Obviously, the extent of reaction between dopamine and MGO would be changed with the different reagents concentration ratios. When the MGO concentration ratio increased in this reaction, the reaction extent and speed would also rise up.



(a)



(b)

Figure 4.11 HPLC chromatogram for the dopamine standard (a) and norsalsolinol standard (b)

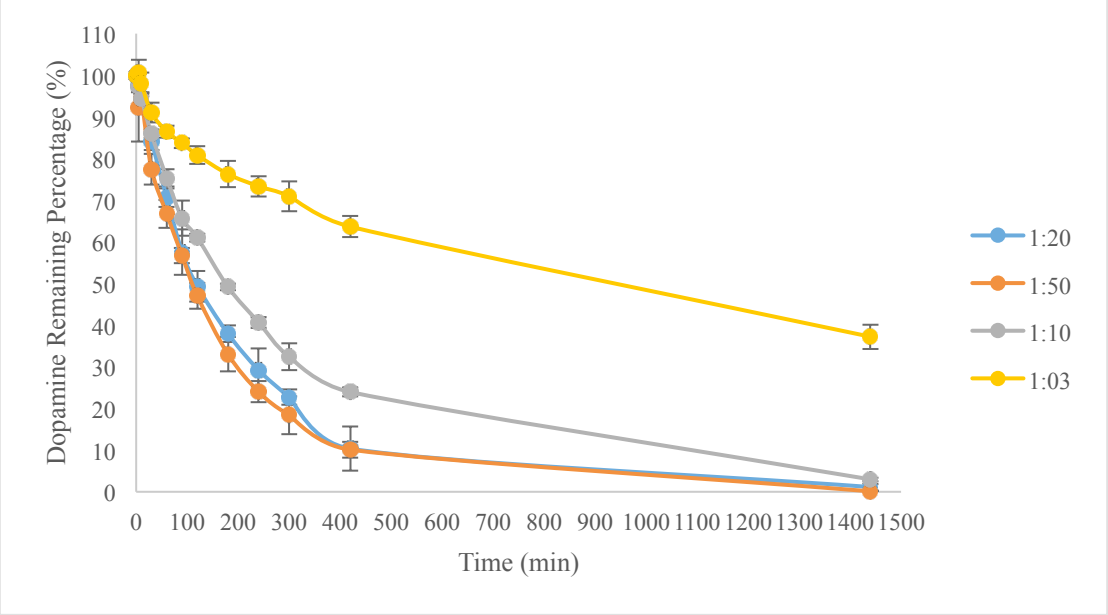


Figure 4.12 Reaction Kinetics between Dopamine and MGO with Different Concentration Ratio

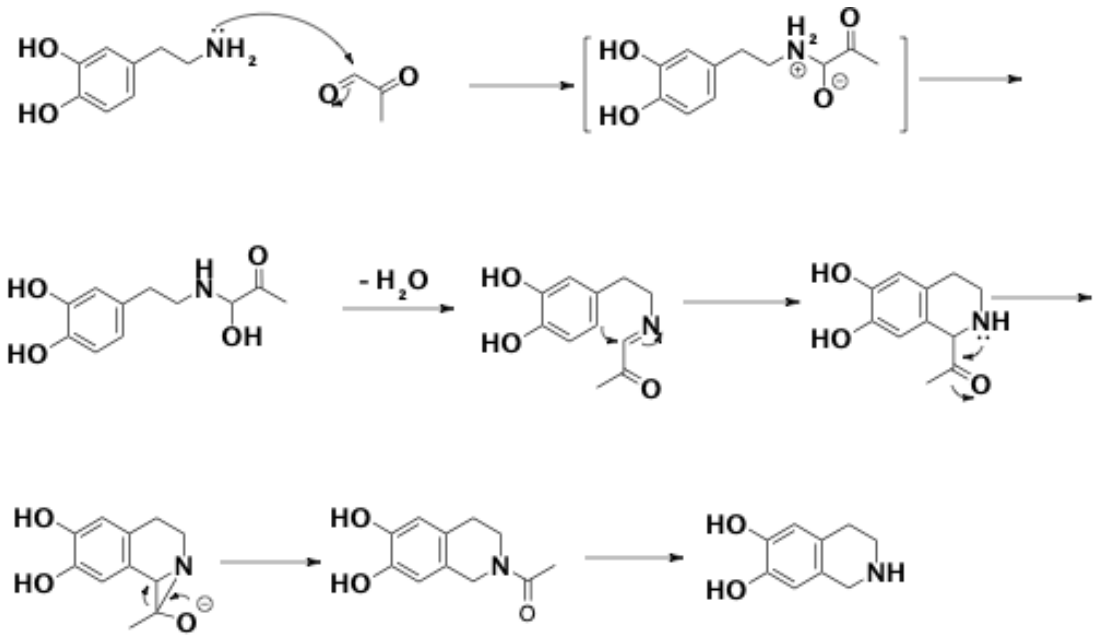
Discussion

5.1 Reaction between Dopamine and MGO

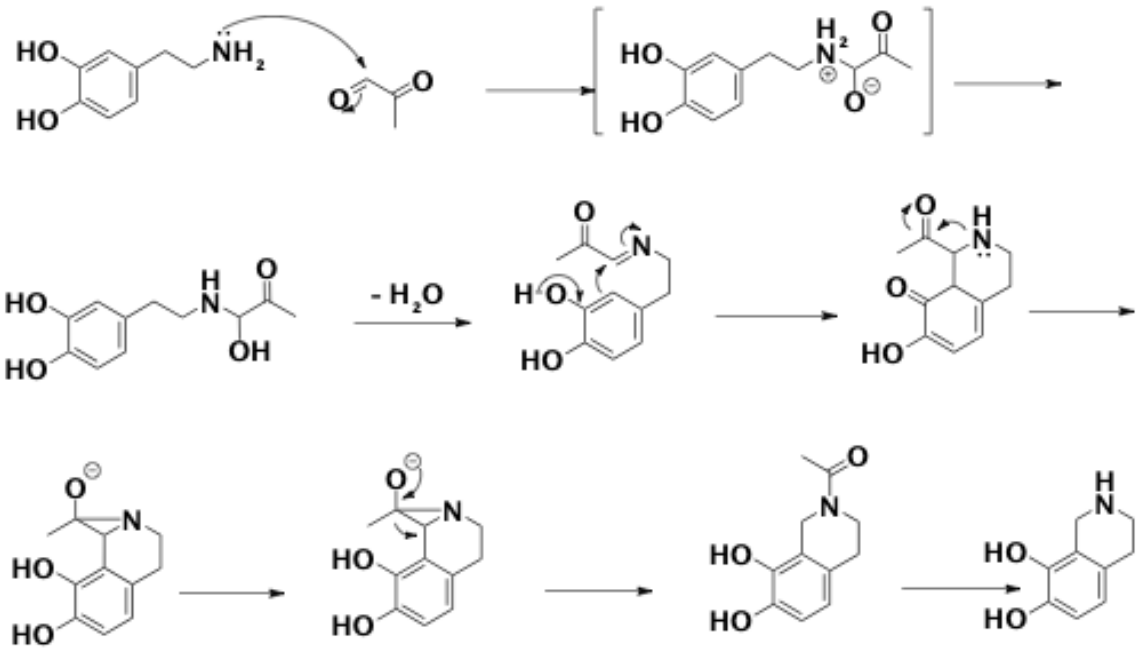
5.1 Reaction between Dopamine and MGO

The former researches detected the ADTIQ compound as the product for the reaction between dopamine and MGO from both in vivo and in vitro trials. Since ADTIQ is structurally homogenous of TIQ, it has been concluded that the reaction between dopamine and MGO can generate the neurotoxin, ADTIQ, which is related with the PD (Xie et al., 2015). However, based on the HPLC chromatogram, LC-MS as well as LC-MS/MS data from this experiment, it showed that the ADTIQ should not be the final products and the ADTIQ should have two different isomers under this reaction condition. The norsalsolinol should be the final product for the reaction and the ADTIQ might be the reaction intermediates. Also, norsalsolinol have two different isomers under this reaction condition. The ADTIQ disappeared after freeze dried processing or the crude reaction liquid was stored under room temperature for two days. The norsalsolinol is stable in the matrix. Thus, based on the data detected in this experiment and the Picted-Spengler reaction principles, the proposed reaction pathways are presented in figure 5.1. Figure 5.1 (a) and figure 5.1 (b) show the different reaction region-selectivity pathways.

Dopamine is nucleophile and MGO is electrophile. Therefore, the long pair electron of amine group in dopamine will attack the aldehyde group in MGO due to its high electronegativity. After dehydration, the imine compound will be formed. Then, intramolecular nucleophilic addition will occur and form the TIQ structure. After that, the intra-rearrangement will produce the ADTIQ compound as the intermediate. In the last step, the acetyl group will be cut and yield the norsalsolinol as the final product. There are two isomers for both ADTIQ and norsalsolinol because two regioselectivities of principle Pictet-Spengler reaction under the neutral condition.



(a)



(b)

Figure 5.1 Proposed reaction pathway between dopamine and MGO

5.2 Reaction Kinetics between Dopamine and MGO

The result in 4.8 shows that the reaction speed between dopamine and MGO will be varied with the reaction reagents ratio. If the ratio of MGO in the reaction condition increased, the reaction speed raised up. When dopamine : MGO = 1 : 3, there was still part of the dopamine unreacted or remained after 24 hours. But once the ratio increased to 1 : 10, the reaction almost completed after 24 hours and the reaction rate is much faster than 1 : 3 ratio reaction. And with the ratio of dopamine and MGO increasing, the reaction will be completed more thoroughly and the reaction speed

will be faster. Therefore, when the amount or concentration of MGO in the reaction matrix is higher, the reaction will be more drastic and faster.

Table 5.1 reflects the level of dopamine in various human brains areas (Musshoff, Schmidt, Dettmeyer, Priemer, Wittig and Madea, 1999). Dopamine concentration is highest in the basal ganglia or striatum (nucleus cadatus, putamen and nucleus accumbens). And the following is substantia nigra pars compacta where dopaminergic neurons located and generate the motor-control dopamine. In this study, we mainly discuss the dopamine concentration in the substantial nigra pars compacta which is 383 ± 131.6 ng/ g and equal to 2.51 ± 0.88 nmol/ g. From the other research paper, the mean concentration of MGO in cerebrospinal fluid (CSF) is 10.1 ± 5.2 nmol/ ml from healthy people (Kuhla, LÜTH, Haferburg, Boeck, Arendt and Münch, 2005). Because the density of CSF is about 1 g/ml, the mean concentration of MGO in healthy people's CSF can be calculated to 10.1 ± 5.2 nmol/ml. And as introduced in the 1.1.7, the MGO concentration of diabetic patients' body will be 2 – 6 times might be much higher than healthy people, thus the concentration of MGO in diabetic patients' CFS might be higher than healthy people. As the result, the concentration ratio of dopamine and MGO might be close to 1 : 10 or even higher. And then based on the reaction kinetics results showed, the reaction between dopamine and MGO in substantia nigra pars compacta, especially for the diabetic patients, may be drastic and fast. The result will have two impacts: (1) due to the reaction, dopamine as one reaction reagent will be depleted so that the dopamine pathway from substantia nigra pars compacta to dorsal striatum (caudate nucleus and putamen) which controls the body movement will be impaired; (2) the reaction product, norsalsolinol, is one of the TIQ structural compounds and can be methylated and oxidized in the brain to

generate the isoquinolinium ion which is neurotoxic. In conclusion, the reaction between dopamine and MGO in brain, specifically in the substantia nigra pars compacta will not only deplete the dopamine itself, but it can also generate one potential endogenous neurotoxin product which can kill or deteriorate the dopaminergic neurons.

n = 32	Dopamine (ng/ g ± S. D.)
Nucleus accumbens	983 ± 435.3
Nucleus cadatus	2470 ± 828.6
Putamen	1170 ± 450.8
Substantia nigra	383 ± 131.6
Hypothalamus	186 ± 134.9
Medulla oblongata	102 ± 80.5
Thalamus	43 ± 18.9
Nucleus amygdalis	20 ± 7.7
Frontal cortex	19 ± 10.0
Gyrus precentralis	15 ± 12.1
Nucleus tegment	12 ± 9.9
Hippocampus	11 ± 3.9
White matter	10 ± 10.1
Cerebellum	8 ± 6.0

Table 5.1 Levels of dopamine in various brain areas

Conclusion and Future Work

MGO, one of the reactive carbonyl compounds, has attracted more and more attention because of its potential relationship with many degenerative healthy problems. MGO can actively react with the amine group compounds and form the AGEs as the products. What's more, MGO amount is about 2-6 times higher in diabetic patients' body than the healthy people's body. Therefore, it is one reason why the diabetic patients suffer with different complications such as cataracts, cardiovascular disease and nephrosis. In this decade, there are more and more researches studied on the relationship between MGO and brain healthy problems like AD and PD. For the AD, many studies showed the higher concentration of MGO in CSF for diabetic patients may deteriorate the AD progress. For example, MGO may actively glycate extracellular amyloid-β (Aβ) to β-amyloid plaques which is more insoluble and protease-resistance (Angeloni et al, 2014). For the PD, the epidemiological studies also showed the diabetic patients are more easily diagnosed with PD compared with healthy people. Thus, the suspension about whether the higher amount of MGO may also induce the parkinsonism has been raised up.

In this study, I focused on the relationship between MGO and PD. In detail, it is the reaction between the dopamine and MGO under physiological condition. The result from this study showed (1) dopamine can react with MGO to form one type of potential endogenous neurotoxin norsalsolinol as the final product under the physiological condition; (2) There are two different isomers for the formed

norsasolinol because the different reaction regioselectivities based on the principle Pictet-Spengler reaction mechanism; (3) The reaction kinetics between dopamine and MGO were performed. Based on the concentration ratio of MGO and dopamine in human brain, or especially for the diabetic patients brain, the reaction rate should be relative drastic and fast.

There are still some work are worthy to do in the future and prove the relationship between MGO and PD derived from the reaction of MGO and dopamine in human brain.

(1) Isolation and purification for both ADTIQ and norsalsolinol compound.

Based on some former studies, they showed the ADTIQ or norsalsolinol can be isolated and purified with the preparative HPLC. In this study, I mainly focused on the reaction progress and pathway between dopamine and MGO. After this, the reaction intermediates or final reaction products are valuable to delve into.

(2) Determine the structure of isomers for norsalsolinol and ADTIQ.

Although the LC-MS/MS has been used in this experiment to determine the norsalsolinol as the final product from the reaction between dopamine and MGO through the comparison with norsalsolinol standard, the NMR data for the product of norsalsolinol from this reaction is still needed.

On the other hand, the reaction mechanism between dopamine and MGO is proposed. The ADTIQ, which is regarded as the intermediates in this reaction, also needs to be confirmed their isomers chemical structures in order to prove the pathway of the ADTIQ transferring to the norsasolinol in the last step.

(3) The toxicity comparison of potential endogenous neurotoxins ADTIQ, norsalsolino and salsolinol by in vivo and in vitro tests.

The significance of the reaction between dopamine and MGO in brain is not only the depletion of dopamine, but it can form the potential endogenous neurotoxin norsalsolinol as the final product. The norsalsolinol is one of the TIQ compounds and structurally homologous to MPTP. It has been confirmed that MPTP can metabolize to MPP^+ ion in the brain and this ion is very toxic to dopaminergic neurons. Therefore, according to many studies, MPTP has been regarded as the exogenous neurotoxin that can lead the PD. The ADTIQ, norsalsolinol and salsolinol are all the TIQ-structure compounds and they can be methylated and then oxidized to N-methylisoquinolinium ion ($NMIQ^+$). In theory, the $NMIQ^+$ is also toxic to dopaminergic neurons so that may also induce the PD. In fact, the toxicity of ADTIQ, norsalsolinol and salsolinol is lower than MPTP. This may be caused by their

methylation activity or less oxidation ability due to the particular enzymes. But the ADTIQ or norsalsolinol is regarded as the endogenous neurotoxins which can be formed continuously and spontaneously in our brain. Thus, in a long term, these neurotoxins may pose the significant threat to the neurodegenerative problems. And the salsolinol is the other suspected PD-related neurotoxin which has been studied for many years. It will be very valuable if the toxicity among salsolinol, ADTIQ and norsalsolinol can be compared both in vitro and in vivo studies.

Reference

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Appendix

1. Dopamine : MGO = 1 : 50

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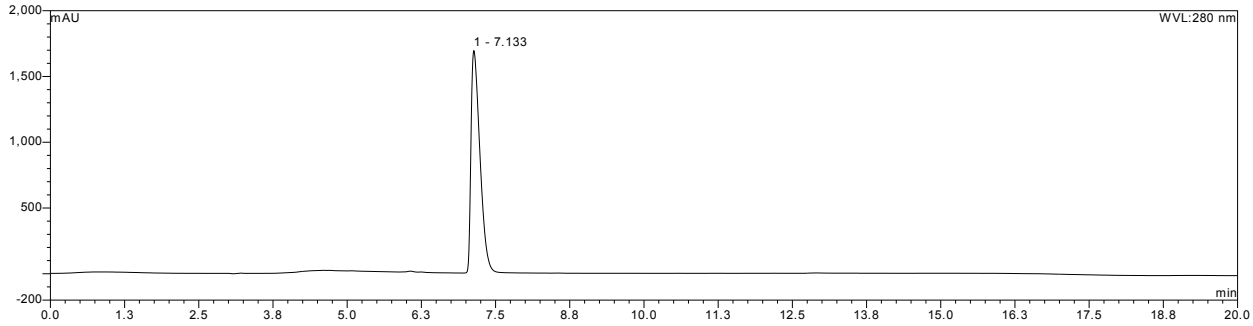

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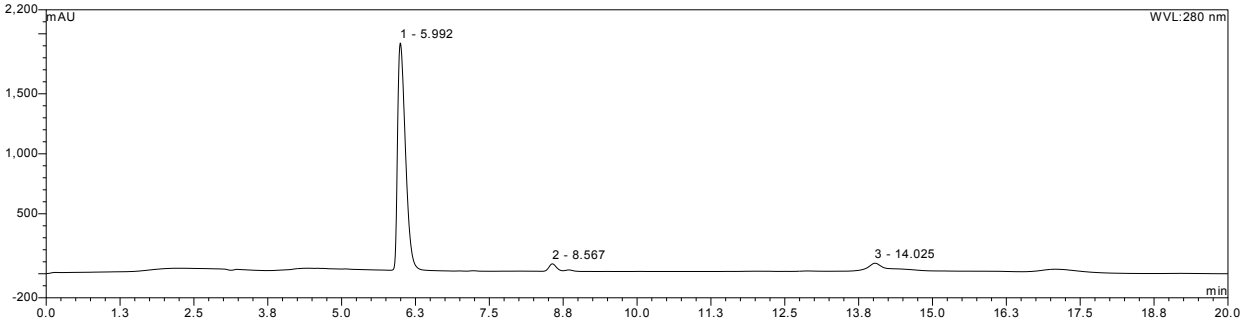

2. Dopamien : MGO = 1 : 20

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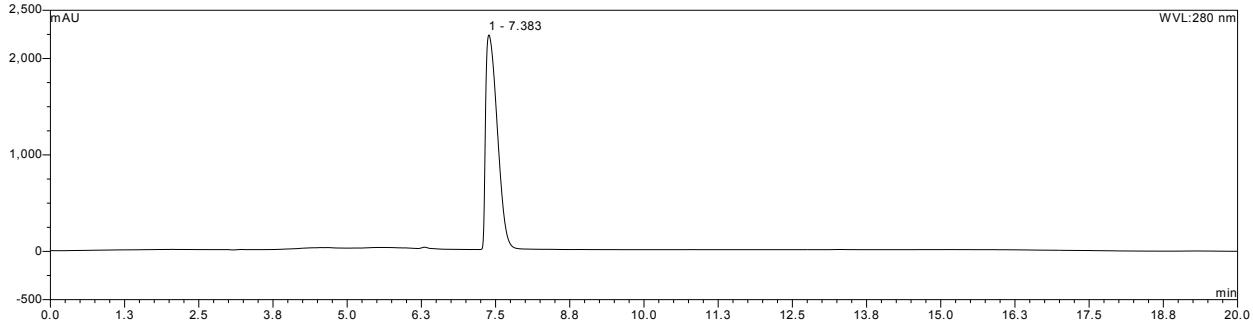

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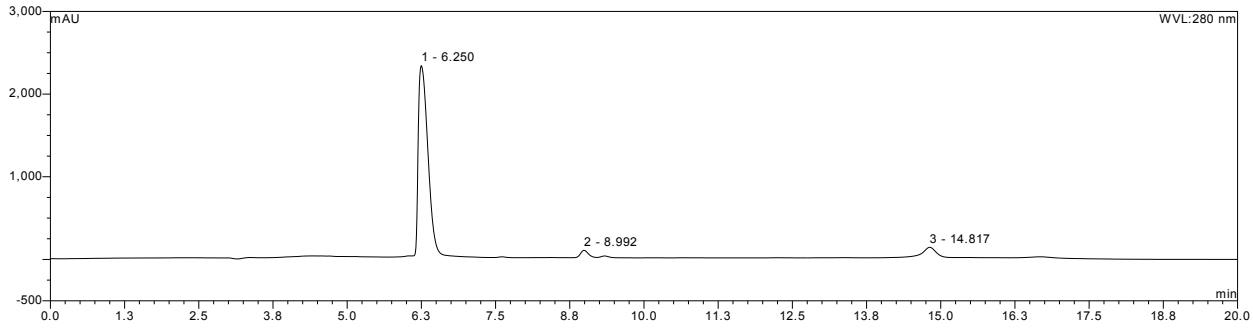

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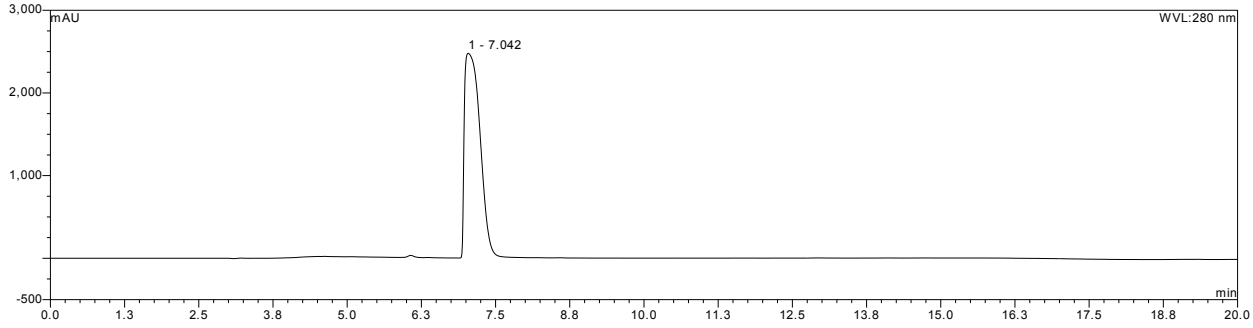

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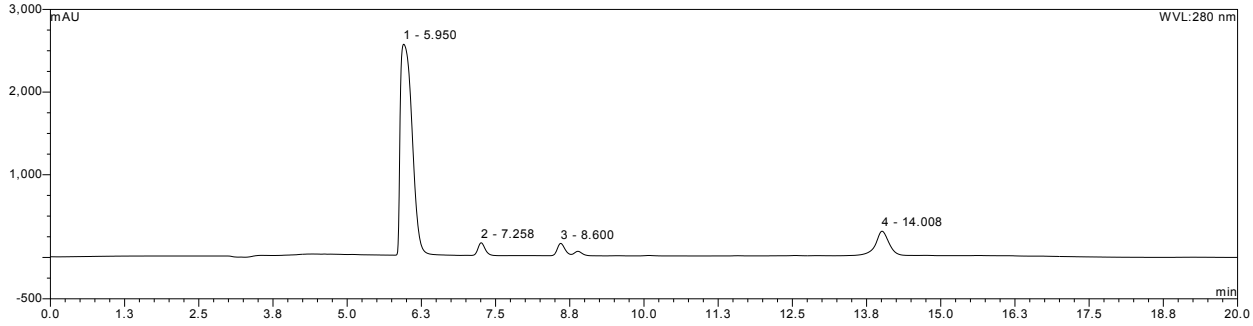

4. Dopamien : MGO = 1 : 3

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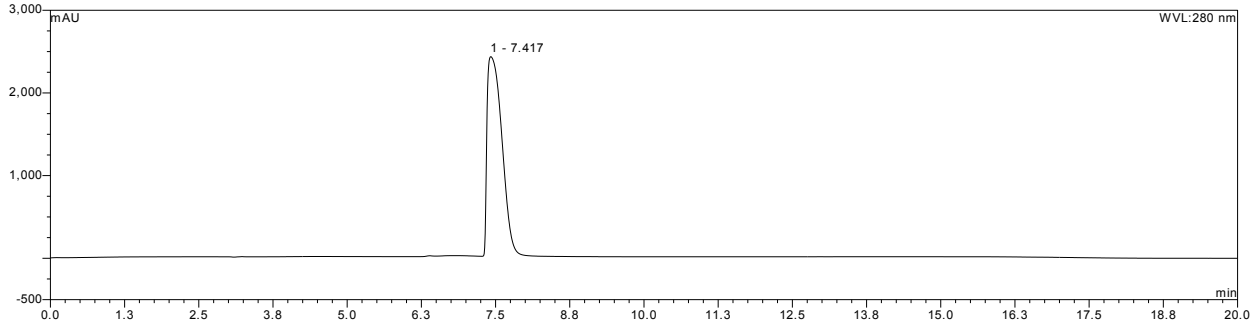

(2) 1440 min