IN VITRO STABILITY AND ANTIOXIDANT POTENTIAL OF THE NEUPROTECTIVE METABOLITE 6-HYDROXY-NICOTINE

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Abstract: Alzheimer's disease is complex, and it is unlikely that any one single drug or intervention can successfully treat it. The implication of nicotinic acetylcholine receptors (nAChR) in Alzheimer's disease pathogenesis has opened a new perspective on finding drugs usable for ameliorating the memory problems associated with AD. By using nAchR modulators, the availability of nAChRs for acetylcholine will be increased and the loss of forebrain cholinergic neurons associated with AD might be overcome. The microbial metabolite 6-hydroxy-D-nicotine has the ability to sustain spatial memory in rats by lowering the levels of oxidative stress in the brain and modulating the nAChRs function probably by binding to a specific site. Its success from a lab chemical to drug depends highly on its stability or shelf life. We have shown that 6-hydroxy-D-nicotine have a more potent antioxidant effect than nicotine when measured as FRAP units. Also, the pH stability of 6-hydroxy-D-nicotine is similar to nicotine, the compounds withstanding 48h exposure to pH above 3 up to 9 at room temperature.

INTRODUCTION

Alzheimer's disease (AD) is an irreversible, progressive brain disorder that slowly affects the ability to carry out the simplest tasks by destroying memory and thinking skills. The Alzheimer's association reports in its March 2018 fact sheet that this disease is the most expensive one in America, costing more than cancer and heart disease. With an estimated direct cost to American society of caring for those with AD of $277 bilion (Alzheimer's disease Association, 2018), the impact of the Alzheimer's disease on the society is undeniable not negligible.

Alzheimer's disease is complex, and it is unlikely that any one single drug or intervention can successfully treat it. Current approaches focus on helping people maintain mental function and slow down memory loss. The implication of nicotinic acetylcholine receptors (nAChR) in AD pathogenesis (Bencherif and Lippiello, 2010) has opened a new perspective on finding drugs usable for ameliorating the memory problems associated with AD. By using nAchR modulators, the availability of nAChRs for acetylcholine will be increased and the loss of forebrain cholinergic neurons associated with AD might be overcome (Russo *et al.*, 2014).

As the nicotine alkaloid is the archetypal nAChR agonist, we have focused on identifying novel derivatives of this compound that might have the same nAChRs binding properties but without the toxic effects of nicotine. In previous *in silico* and *in vivo* studies, we have shown that the microbial metabolite 6-hydroxy-D-nicotine (6HNic) has the ability to sustain spatial memory in rats (Hritcu et al., 2015; Hritcu et al., 2017) by lowering the levels of oxidative stress in the brain (Hritcu *et al.*, 2013) and probably by binding to nAChRs and modulating their function (Mihasan *et al.*, 2013). Moreover, as the 6-hydroxy-D-nicotine is a metabolic intermediate found to be accumulating in the growth media of *Paenarthrobacter nicotinovorans* pAO1+ when grown on nicotine (Boiangiu *et al.*, 2014), a working methodology for production of 6HNic with higher yield has been developed (Mihalache *et al.*, 2016). Not only that 6HNic has some potential as a neuroprotective drug, but also a feasible biotechnology for its production has been established making the compound highly attractive. Still, two key issues with high impact on 6HNic practical applications remain unsolved. First is the half time and toxicology of the compound in plasma and second is its shelf live. The current work tackles on the second issue and uses nicotine as a reference substance to compare the *in vitro* stability of 6HNic at various pHs in different aqueous buffers. Supplementary, the antioxidant potential of 6HNic was also assayed and compared with the reference compounds nicotine and ascorbic acid in an attempt to understand the underlying mechanisms of actions of 6HNic.

MATERIAL AND METHODS

Chemicals. All chemicals were purchased from well-known suppliers and were of greatest purity available unless otherwise stated 6HNic was a kind gift from Prof. Dr. Roderich Brandsch and was obtained by chemical synthesis. Pure 6HNic was kept at 4ºC.

Stability assay. 0.05% nicotine and 6HNic were prepared in buffer solutions with the following pHs: 3, 5, 7, 9, 13. For pH 3, 5, 7; 50mM citric acid/ citrate buffer was used. For pH 9 a 50 mM TRIS was employed and for pH 13 a 50mM boric acid/borate buffer was prepared. All samples were prepared simultaneously and incubated at room temperature for 48 h. The pH of the samples was periodically monitored and no significant changes were observed. At precise time intervals, samples were taken and frozen at -20ºC until further analysis.

Analytical methods. Nicotine and 6HNic levels were assayed using an RP-HPLC based method described previously (Boiangiu *et al.*, 2014). Briefly, an Shimadzu Prominence HPLC system equipped with an auto-sampler, an DAD detector and a Machery-Nagel Nucleodur RP C18 ec column (150x4.6 mm, particle size 3µm) incubated at 30ºC was used. 20 µL of sample was injected and isocratic elution for 10 min with 1 mM H_2SO_4 : methanol 75:25 at room temperature was used for separation. Nicotine levels were monitored at 250 nm and 6HNic levels at 290 nm. Peak hight and peak area were used as indications for concentration.

Antioxidant power of nicotine and 6HNic was compared using the ferric reducing ability of plasma or the FRAP Assay as described by (Benzie and Strain, 1996). Unlike the original method that uses 10 mM TPTZ as Fe^{3+} chelator, we used 10 mM ferrozine (Sodium 4-[3-pyridin-2-yl-5-(4-sulfophenyl)-1,2,4-triazin-6-yl]benzenesulfonate) and the complex was measured at 562 nm. OD readings were calibrated using $FeSO_4$ (range 0-75 microM) and FRAP values were expressed as microM ascorbic acid.

Samples were measured in triplicate and standard error was calculated. Statistical significance was assayed using the Student-test ($p<0,05$) performed with Microsoft Excel, Data Analysis Package.

RESULTS AND DISCUSSIONS

6HNic is a better antioxidant then nicotine. By using a round of *in-silico* docking experiments, we have identified the nicotine metabolite 6HNic as a putative ligand for nAChRs (Mihasan *et al.*, 2013). The *in-vivo* tests performed on rats showed that indeed the compound is able to improve memory, having the same beneficial effects as nicotine but at a larger extent (Hritcu *et al.*, 2017b). Surprisingly, the presence of 6HNic in the brain was associated with an decrease of the oxidative stress in rat hippocampus (Hritcu *et al.*, 2013; Hritcu *et al.*, 2015). This could be due to the 6HNic ability to modulate the nAChRs or might be a more direct action of 6HNic by directly scavenging ROS. The FRAP test is an accepted assay for testing the ability of various compounds to function as reductants and to delay or inhibit ROS action on biological substrates. Both nicotine and 6HNic were tested for their ability to inhibit the formation of Fe^{2+} from Fe^{3+} at 3 different concentrations: 15, 30 and 45 microM. In all the conditions tested, 6HNic had an better antioxidant potential then nicotine (figure 1). This is actually in good accordance with the fact that the antioxidant activity related to the compound structure is dependable on the number of the included active OH or NH_2 groups (Bendary *et al.*, 2013). The more active groups in the molecule, the more active the compound is.

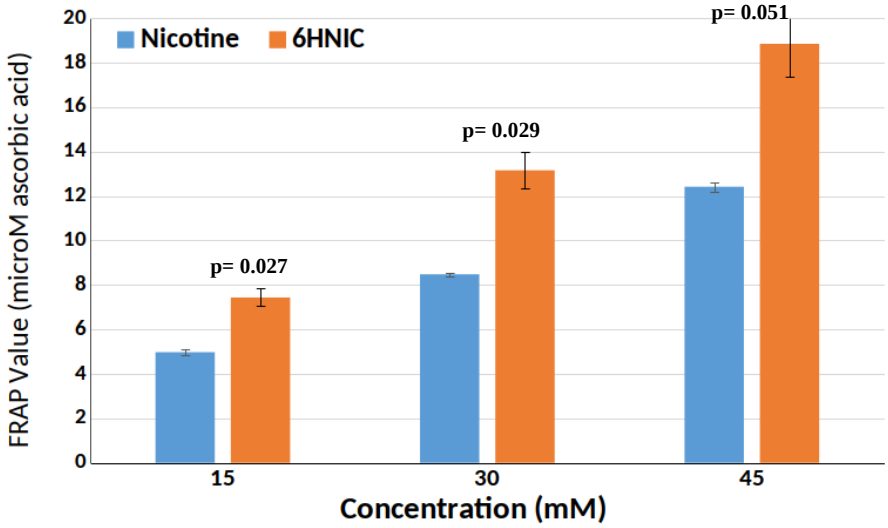

Figure 1. 6HNic is a better antioxidant then nicotine as shown by the FRAP assay.

Nicotine is rather stable at room temperature at pH up to 9. The observed neuroprotective effects of 6HNic make this compound very attractive. Moreover, a biotechnology is currently developed to produce 6HNic starting from nicotine by using biological conversion performed with the help of *Paenarthrobacter nicotinovorans* cells. The biotechnology developed in our lab is using pure nicotine, but several groups from China are working on using toxic byproducts and waste water contaminated with nicotine deriving from tobacco industry (Seckar *et al.*, 2008) as a nicotine source. Their efforts were successful and two studies report the successful production of 6-hydroxy-3-succinoyl-pyridine (Wang *et al.*, 2005) and also 6HNic (Yu *et al.*, 2017) from nicotine waste.

So not only that 6HNic has some potential as a neuroprotective drug, but it also promises a biotechnology that might convert toxic waste in green chemicals. Its success from a lab chemical to drug depends highly on its stability or shelf life. In order to gain some information on this issue, we have incubated nicotine and 6HNic for up to 48 hours at room temperature and various pHs. At precise time intervals, samples were taken and the levels of nicotine and 6HNic were determined using reverse-phase HPLC on a C18 column. No mater which buffer was used to control the pH, the obtained chromagrams (Figure 2) allowed us to quantify the levels of nicotine and 6HNic with high precision.

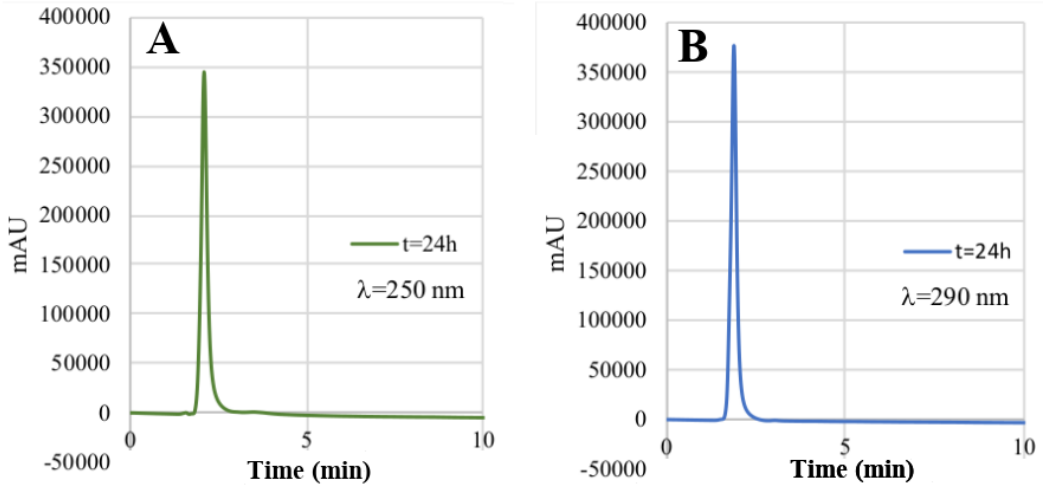

Figure 2. Typical chromatograms used to quantify A. nicotine and B. 6HNic.

Apparently, both nicotine and 6HNic are highly stable at pHs ranging from 5 to 9. At pH 12, both compounds are found at low levels by the HPLC method used (Figure 3). It is not clear if the two compounds were affected in terms of chemical modifications or chemical bonds lysis, or at pH 12 the compounds are ionized and do not have affinity to the C18 column.
At pH 3, nicotine appears to be stable for 5 samples, but 6HNic could be detected at low concentrations only for the first 2 samples.

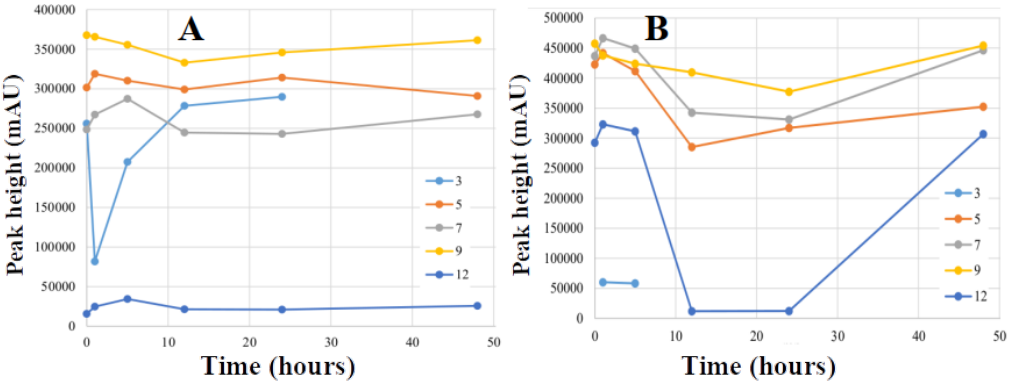

Figure 3. Stability of A. nicotine and B. 6HNic at various pH and room temperature.

CONCLUSIONS

The neuprotective metabolite 6-hyroxy-nicotine is a promising chemical that could be obtained by biological transformation of nicotine containing toxic waste. 6HNic proved to have a more potent antioxidant effect than nicotine when measured as FRAP units. The pH stability of 6HNic is similar to nicotine, the compounds withstanding 48h exposure to pH above 3 up to 9 at room temperature.

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