In vitro kinetic study of donepezil N- oxide metabolites

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ABSTRACT

Objective: The aim of this study was to examine the metabolism of donepezil via N – oxidation of donepezil HCl in vitro, and the kinetic studies at a temperature similar to that of the human body.

Methods: Direct reaction of donepezil with hydrogen peroxide and formic acid at room temperature, the N – oxide undergoes a series of rearrangements, the reaction was followed by ultraviolet at λ_{max}= 268 nm at 37 ˚C. The donepezil N- oxide was also tested as potential cholinesterase inhibitor using electrometric for cholinesterase determination.

Results: According to the present work , the structure of donepzil N- oxide was elucidated using spectroscopic analysis , also , in vitro kinetic study at 37 ˚C using ultraviolet spectrophotometer at λ_{max} = 268 nm showed that the reaction proceeded at a first order reaction . Biologically, the compound was found to inhibit both plasma and erythrocyte cholinesterase activities in vitro ..

Conclusion: We concluded that the N- oxide of denepezil undergoes a series of rearrangements, the reaction followed first order at 37 ˚C. It has a potential anticholinesterase activity

ألخلاصة

أهداف البحث: إن الغاية من هذه الدراسة هو تفسير ايض عقار الدونيبيزيل الذي يستخدم لعلاج خرف الشيخوخة عن طريق أكسدة ذرة النتروجين ، ودراسة حركية الدواء في نفس درجة حرارة جسم الإنسان.

طريقة العمل: يتم أكسدة الدونيبيزيل بتفاعل مباشر مع حامض الفورميك وبيروكسيد الهيدروجين في درجة حرارة المختبر، وان جزئية الاوكسيد الناتج تعاني من إعادة ترتيب ، تم تتبع هذا الترتيب باستخدام جهاز طيف الأشعة تحت البنفسجية عند طول موجي = ٢٦٨ نانوميتر ، وان التفاعل من المرتبة الأولى ، بالإضافة إلى البيولوجية خارج جسم الكائن الحي بإجراء فحص مثبط إنزيم اسيتايل كولين استراز في البلازما وكريات الدم الحمراء وذلك بتغير درجة ألبها.

النتائج: نستنتج من هذه الدراسة بان ايض الدونيبيزيل عن طريق أكسدة ذرة النتروجين ، ويعاني إعادة ترتيب جزئي ومتابعة حركية التفاعل عند درجة حرارة ٣٧ °م، وتم اختبار اوكسيد النتروجين خارج جسم الكائن الحي كمثبط إنزيم كولين استراز وذلك عن طريق متابعة تغيير درجة ألبها.

الاستنتاج: إن اكسدة النتروجين للدونيبيزيل يعاني من اعادة ترتيب جزئي وأن التفاعل من الرتبة الأولى في درجة حرارة ٣٧ °م. وكانت فعالية المركب الناتج من الاكسده مثبطة لأنزيم كولين استراز خارج جسم الكائن الحي .

Alzeheimer disease (AD) is a neurodegenerative, irreversible disorder involving a progressive loss of cognitive functions and motor abilities, accompanied by behavioral disturbances that invariably lead to premature death[1]. The hallmarks of the disease are the presence of senile plaques, neurofibrillary tangles and prominent cortical neuron loss. In addition, the levels of many neurotransmitters are greatly reduced[2]. Depletion of acetylcholine (Ach) represents the most important event but other neurotransmitters such as serotonin (5HT), noradrenalin (NA), dopamine (DA), glutamate and substance P are also involved[3-5].

Cholinergic therapy for AD initially concentrated on

acetylcholinesterase (AchE) inhibition since this is the main enzyme involved in the hydrolysis of Ach in the normal brain[6]. Recent research has shown that in the cortex of patients affected by AD, AchE decreases progressively while butyrylcholinesterase (BuChE) activity unchanged or even increased[5].With progression of the disease, both AchE and BuChE accumulate with amyloid-beta (Ab) peptide in plaques intensifying Ab neurotoxicity[6].

Donepezil is a potent acetylcholinesterase inhibitor used in patients with AD to help stave off the loss of cognitive abilities[7]. The biological donepezil N- oxidation of piperidine ring form a pharmacologically active biogenic amine of mammals[8,9].

In this paper, donepezil N- oxide was prepared in vitro as route of metabolism via N-oxidation, and the structure was identified by using physical and spectroscopic techniques. The order of this reaction can be determined by using kinetic measurements at 37 °C. The biological study of donepezil N- oxide as a cholinesterase inhibitor in plasma and erythrocyte was also tested .

Materials and Method

Chemicals

Donepezil Hydrochloride, (±) -2-((1-benzylpiperidin-4-yl)-methyl) 5,6 – dimethoxy indan- 1- one mono hydrochloride was purchased from (Atomax Chemical Co., L.t.d. China). All chemical and solvents were purchased from Merck (Germany) and BDH (England).

Ultraviolet and kinetic studies were taken in watery solution using Carrywinn varian UV (Australia). Infrared spectroscopy was obtained on Brucker FT-IR Spectrometer 14181 in range 400–4000 cm^{-1} (resolution 4 cm^{-1}) as KBr pellets.

The ^{1}H experiments were performed on Brucker 400 MHz (France) and the ^{13}C and DEPT NMR experiments were performed on a 200 MHz instrument model 2000 at 25 ˚C in CDCl3. The ^{1}H chemical shift values were reported on the δ scale in ppm, relative to TMS (δ = 0.00 ppm) as internal reference and the ^{13}C chemical shift values were reported.

In nmr description, s = singlet, d = doublet, t = triplet, m = multiplets, $\Delta\delta$ in ppm represent as a change in chemical shift between donepezil N-oxide and the donepezil.

Methodology

Chemistry

Preparation of donepezil N- Oxide[10]

To an ice cooled solution of donepezil (4.743g , 12.5 mmole) in 98% formic acid (19.4g , 9 mmole), 30 % hydrogen peroxide (H_2O_2) about 8.5 ml was added drop wise with stirring for 24hr at room temperature, the excess of the formic acid was neutralized with anhydrous sodium carbonate with cooling and stirring . The paste was extracted with (3 x 50 ml) of dry chloroform , then combining the chloroform extract portions and evaporate under vacuum. Donepezil N-oxide was dissolved in dry ether then HCl gas (Sodium Cholride with sulphuric acid) was passed for 30 minutes , a white precipitate was formed, evaporated the ethereal layer and keeping the N-oxide in dry closed container in refrigerator .The m.p = 166 ˚C (Decomposed), the yield percent 63 % , the uv wave length λ_{max} = 268nm , the IR spectra shoes the following bands: 3019 (aromatic C–H stretching), 1717 (C=O stretching), 1591 (aromatic C=C stretching), 1500, 1457 (aliphatic C–H bending), 1314 (C–N stretching), 1265, 1034 (C–O stretching), 748, 700 (aromatic C–H bending), 942 (N-O bond) (Tables 1 & 2)

Thermal rearrangement of donepezil N- Oxide[10]

A (0.5 g, 1.157 X 10^{-5} mole) of donepezil N-oxide was heated at 37 ^{0}C in dry ethanol (35ml) with stirring for different period times, the reaction mixture was monitored by measuring the wavelength at ultraviolet until new wavelength will be appeared. The brown semisolid product as unknown rearrangement product was formed.

Kinetic measurement for the donepezil N-oxide metabolite:

A stock solution was prepared by dissolving about 0.01g of donepezil N-oxide in 10 ml of dry ethanol and kept in constant temperature in water bath 37 ˚C.

Runs were carried out to 70-95% completion. Each run was repeated three times.The rate of the rearrangement of donepezil N- oxide (K) was obtained from the plot of time (min) Vs ln (a-x), at the wave length λ_{max} = 268 nm.

Biological studies

Collection and preparation of blood samples[11]

Human venous blood samples (2-5 ml) were obtained and transferred into dry clean test tubes containing anticoagulant (EDTA). Blood samples were immediately kept on an ice bath. Plasma was separated from erythrocytes by centrifugation at 3000 rpm for 15 minutes, and both transferred into dry test tubes and placed in ice bath until they were used within one hour or next day.

Measurement of cholinesterase activities[11].

Three ml of distilled water were placed in 10 ml glass container, 0.1 ml was added of plasma or erythrocyte and mixed. Then 3 ml of barbital–phosphate buffer pH= 8.1 were added and mixed. PH of the mixture was measured (pH_{Blank}, pH_1). Then 0.1 ml of the substrate (acetylcholine iodide, 7.5 %) was and mixed. The reaction mixture was incubate in a water bath at 37 ˚C for 20 minutes, then measureed for pH (pH_2) of the reaction mixture. The change in pH represented the ChE activity per 20 min.

ChE activity (Δ pH/20min) = (pH_1- pH_2) - pH_{blank}

The blank contained all the above substances except the enzyme sources (plasma or erythrocyte sample).

In vitro ChE inhibition by donepezil N-oxide

Plasma and erythrocyte samples (N=2-8) were collected from healthy volunteers. Donepezil hydrochloride was diluted with distilled water and added in a volume of 0.1 ml to the reaction mixture of plasma or erythrocytes to obtain a final solution incubated at 37˚C for 10 minutes to inhibit ChE activity in the blood sample (prior the measurement of the pH_1). Therefore, the residual ChE activity in the reaction mixture was measured as before and the percent of enzyme inhibition was as follows:

% ChE inhibition = ChE2 - ChE1/ChE2 * 100

Table 1. ^{1}H NMR Assignments for donepezil and its N-Oxide

position	^{1}H donepezil δ ppm	^{1}H donepezil N-Oxide Δ ppm
2	3.28 (m,1H) CH	3.27(m ,1H) CH
3	2.65(d,2H) CH_2	2.60(m, 2H) CH_2
6	6.82(s, 1H)CH aromatic	6.79(s,1H) CH aromatic
9	7.09(s,1H)CH aromatic	6.88 (s,1H) CH aromatic
10	1.53(m,1H)and 1.84(m,1H)	1.53(m ,1H , s) and 1.82(m ,1H)
11	1.84 (m, 1H) CH*	1.82 (m , 1H) CH*
12 & 16	2.06 (m, 4H) $2CH_2$	1.3-1.9(m, 4H) $2CH_2$
13 &15	3.47(m, 4 H) $2CH_2$	2.65-3.03(m, 4H) $2CH_2$
17	4.17 (S ,2H) CH_2	4.10 (s , 2H)CH_2
19-23	7.61 (m, 5H) 5CH aromatic	7.60(m,5H) 5 CH aromtic
24 & 25	3.95 (s,6H)2 OCH_3 methoxy groups	3.93(s, 6H) 2 OCH_3 methoxy groups

*= chiral carbon atom.

Table 2. ^{13}C- NMR assignments for donepezil and its N-Oxide

position	^{13}C donepezil ppm	^{13}C donepezil N-Oxid ppm	Δδ ppm
1	206.4 for C=O	- 207.84 for C=O	-0
2	45.21 for CH	45.47 for CH	+0.53
3	38.72 for CH_2	38.55 for CH_2	-0.17
4	129.3 for =C aromatic	129.25 for =C aromat	-0.05
5	148.79 for =Caromatic	148.51 for =aromatic	-0.28
6	104.52 for CH	104.34 for CH	-0.18
7	155.45for O-CH_3	155.27 for O-CH_3	-0.18
8	144.43 for O-CH_3	144.25 for O-CH_3	-0.18
9	107.49 for CH	107.21 for CH	0.28-
10	33.16 for CH_2	33.03 for CH_2	-0.13
11	34.45 for CH	34.21 for CH	-0.24
12	32.89 for CH_2	31.78 for CH_2	-1.11
13	53.79 for CH_2	53.79 for CH_2	0
15	53.79 for CH_2	53.79 for CH_2	٠
16	31.78 for CH_2	31.66 for CH_2	-0.12
17	63.48 for CH_2	63.29 for CH_2	-0.19
18	138.46 for =C aromatic	138.34 for =aromatic	-.12
19	129.31 for CH aromatic	129.02 CH aromatic	-0.29
20	128.42 for CH aromatic	127.96 CH aromatic	-0.46
21	126.89 for CH aromatic	126.34 for CH aromatic	-0.55
22	128. 32 for CH aromatic	127.96 f CH aromatic	-0.36
23	129.33 for CH aromatic	129.02 for CH aromatic	-0.31
24	55.91 for O-CH_3	55.88 for O-CH_3	-0.03
25	56.16 for O-CH_3	56.03 for O-CH_3	-0.13

Table 3. Run of rearrangement of the donepezil N- oxide in water at 37 ˚C in ethanol using UV spectrophotometer at λ_{max} = 268 nm

Absorbance	Time (min)	a-x X 10^{-3}	In a-x
0.25	0	2.265	-6.090
0.213	15	1.297	-6.651
0.211	30	1.291	-6.653
0.266	45	1.4	-6.571
0.247	60	1.19	6.733 -
0.246	75	1.04	-6.812
0.257	90	0.08	-9.433
0.312	105	0.06	-
0.262	120	-	-

Table 4. In vitro inhibition of plasma and erythrocyte cholinesterase activities by donepezil N- oxide .

Concentration of Donepezil N-oxide M)(μ	Plasma ChE		Erythrocyte ChE		P values
	ΔpH / 20min.	% inhibition	ΔpH / 20 min.	% inhibition	
0	0.145±0.005	---	0.56±0.010	---	--
5	1.335±0.042	---	0.45±0.015	19.64	p≤0.05
10	1.285±0.23	1.53	0.35±0.015	37	p≤0.05
20	1.563±0.005	---	0.56±.012	45.54	p≤0.05

Results and Discussion

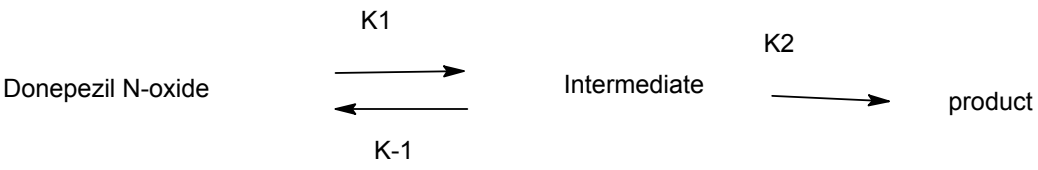
A number of drugs with a piperidine ring has been found to possess a high degree of specificity as central nervous system agents[9]. Among these donepezil play an Alzheimer's disease is one of the most common causes of mental deterioration of elderly people. It is frequently prescribed drugs for the treatment of Alzheimer's disease characterized by degeneration of various structures in the brain. Acetylcholinesterase inhibitors are the most frequently prescribed drugs for the treatment of Alzheimer's disease[4,5]. Donepezil is a specific, selective and potent inhibitor of acetylcholinesterase[6,7] has been approved for the treatment of Alzheimer's disease.

During metabolism donepezil to donepezil N- oxide appears as a distinct but minor metabolite[12]. This N-oxide is unstable, and can undergoes series of rearrangements (scheme 1). The main oxidation occurs on nitrogen atom of piperidine ring , this N-oxide is expected to exist in a chair conformation in which the N-O substituent isomer occupies axial position.

The nmr , proton of the piperidine moiety showed down field, and lower alpha (α) axial than beta (β) equatorial .This deshielded effect of the axial N-oxide substituent polar group[12],[13]C-nmr showed the change in chemical shift as Δδ in ppm as shown in table 3. The ir spectra support the structure of N-oxide, the N-O stretching band appear at 955 cm^{-1}. This N – oxide was hygroscopic and unstable at higher temperature . The aim of this work to study the rate of rearrangement of donepezil N–oxide in vitro using spectrophotometer at 37 ˚C.

Table 3 shows the rate of rearengment of donepezil N-oxide increased with time, in order to obtain the rate constant (K) at 37 ˚C for the experimental rate data, it is necessary to know the power of the concentration term in the rate expression:

Treatment of the experimental data according to integration and half life method, the donepezil N-oxide follow first order reaction. The kinetic measurement give a picture for this rearrangement while went from polar (N^+ - O^-) to non polar product. This proposed mechanism undergoes from



$$-\frac{d(\text{donepezil N-oxide})}{dt} = \frac{K1K2}{K-1 + K2}$$

rate = K obs (donepezil N-oxide)

$$\text{where } k_{obs} = \frac{K1K2}{K-1 + K2}$$

the migration and can be explained as intramolecular cyclic process scheme 1. However, upon careful examination of the crude product mixtures, benzaldehyde was found. Furthermore, trapping and isolated as 2,4 dinitrophenylhydrazone.

The biological studied showed that both donepezil N- oxide metabolite have a potent activity for inhibition ChE in erythrocyte at concentrations of 20,10 and 5 µM in the reaction mixture, there is no specific activity in plasma, but the percentages of inhibition in erythrocyte ChE activity were 45.54, 37.50 and 19.64% as shown in table 4. Accordingly, both donepezil and its N- oxide had a specific activities on erythrocytes while the base donepezil showed activities on both plasma and erythrocyte cholinesterases[11].

Acknowledgement

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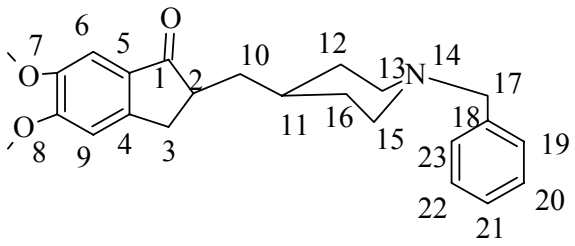
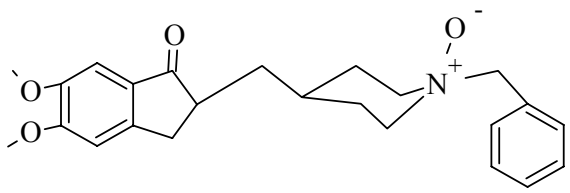


. HCl

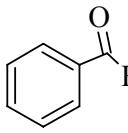
Donepezil Hydrochloride

Oxidation ↓ 1- $NaHCO_3$
2- H_2O_2 / HCOOH



Donepezil N - oxide

Rearrangement ↓ C_2H_5OH at 37 ^0C

Product(s) + () Benzaldehyde

Scheme 1 : Synthesis od donepezil N- oxide and proposed rearrangement product (s) .