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Free radicals detection by ESR PBN spin-trap technique

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The formation and reaction of hydroxyl radicals in γ-irradiated Metoclopramide (4-amino-5-chloro-N-[2-(diethylamino)ethyl]-2-methoxy benzamide monohydrochloride monohydrate) and complex Fenton reaction was studied by Electron Spin Resonance spectroscopy (ESR). Specific radicals derived from purely chemical structures and reactions were detected using N-t-Butyl- α-phenylnitrone (PBN) as spin trap. Some spectroscopic properties and suggestions concerning possible nature of the radicals are discussed.

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1. Introduction

Free-radicals are highly reactive molecules species, that possess an unpaired electron in the outer shell of the molecule and it can react with a large number of other molecular targets, generating different radical species as intermediate products. There exist a large family of free radicals involved in biopharmaceutical systems, but oxyradicals which include superoxide oxygen radical ($\cdot O_{2-}$) and hydroxyl radical (·OH), have received a considerable attention [1]. Among the available methods for detecting free radicals, spin trapping ESR spectroscopy, has been extensively used to detect these transients molecular species. This technique, involves the indirect detection by addition of a primary free radical across the double bond of a diamagnetic compound named spin trap to form a more stable radical than the primary free radical named spin aducts [2, 3]. The main types of spin traps, which find use in studies on free radicals in biopharmaceutical systems, are nitroso and nitrone derivates. In both cases the spin aducts are nitroxide radicals which are relatively stable, because the unpaired electron is sterically protected. However, there exist some difference concerning their stability and identity of radical trapped. Such, the nitrones and their adducts are more thermally and photolyticaly stable and adducts are stable to 140^{o}C, but the radical types can be distinguished only from the coupling constants of nitrogen and single β-proton, so that the identity of radical can be uncertain. The nitroso traps although less stable allow more definitive assignment of the trapped radical since their adducts show couplings to nuclei in the R group which is bound to the nitrogen spin centre. Anyway, they can also be used to detect and monitor radicals located in regions inaccessible to direct ESR observation such as in organs of experimental animals or in human patients.

The most commonly used spin traps are DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) and PBN (N-t-Butyl- α-phenylnitrone), both having a β-proton that can give considerable information about the radical trapped [4]. Our contribution concern the application of ESR spectroscopy in the study of hydroxyl free radical (•OH) generated in biopharmaceutical systems by ionizing radiations and in complex Fenton reaction. The spin trapping of hydroxyl radical scavenger derived radicals is the most reliable method of detecting hydroxyl radical in complex biological systems [5-7].

2. Experimental

Metoclopramide (4-amino-5-chloro-N-[(diethylamino)ethyl]-2-methoxy benzamide mono-hydrochloride monohydrate) was purchased from Medicine Research Center, Beijing Shuangziao Pharmaceutical Corporation, China. Fresh Metoclopramide in the form of microcrystalline powder was exposed to γ-radiation from a ^{60}Co source (GAMMA CHAMBER 900) in ambient conditions. The ^{60}Co source gives a compact and uniform density of radiations and a moderate dose debit of 35 Gy/h evaluated by ferrous sulfate dosimetry. The absorbed dose of drugs was in the range from 0 to 17 kGy. Powder samples (non-irradiated, and irradiated) were placed in a 20 mm length, 1 mm inside diameter quartz capillary. The mixture of irradiated Metoclopramide and spin trap N-t-Butyl- α-phenylnitrone (PBN) was solved in acetone.

A solution of 1 mM ferrous sulfate in water with molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm, has been deoxygenated using nitrogen bubbling. The hydroxyl radical was generated by addition of 1 mM Fe^{2+} to the reaction mixture (Fenton reaction). The spin trap N-t-Butyl- α-phenylnitrone (PBN) was solved in acetone. The hydrogen peroxide was conditioned as a 6% oxi cream with EDTA (ethylenediaminetetraacetic acid), cetearyl alcohol and lauric acid ($CH_3(CH_2)_{10}COOH$). Cetearyl alcohol is a mixture of fatty alcohols consisting predominantly of cetyl alcohol ($CH_3(CH_2)_{14}CH_2OH$) and stearyl alcohol ($CH_3(CH_2)_{16}CH_2OH$). Samples were measured using 20 mm length, 1 mm inside diameter quartz capillary.

EPR spectra were recorded at room temperature with a PORTABLE EPR SPECTROMETER, PS 8400, Resonance Instruments Inc., operating in the X-band (~9.5 MHz) and equipped with a computer acquisition system. The spectrometer settings used for the experiments were as follows: modulation frequency, 100 kHz; sweep width, 100 G; sweep time, 300 s; number of data points, 4096. The computer simulation and analysis of the spectra was made by using WINSIM program that is available to the public through the internet [8].

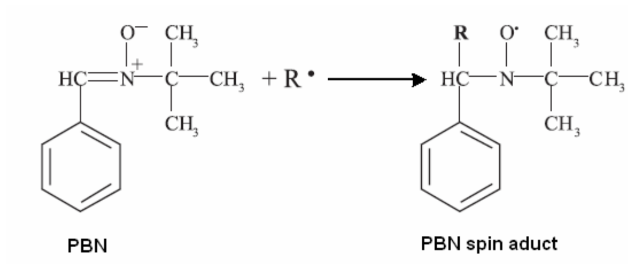
3. Results and discussion

The EPR spectrum of γ-irradiated Metoclopramide in solid state, represent a sum of individual spectra corresponding to all free radicals simultaneously present in the samples or the same free radicals localized in the various local environments. The spectrum samples is dominated by a broad central signal with specific characteristics given by chemical structures, centered on *g*=2.0047 an peak-to-peak line widths of 11 G. The values of the *g*-factor are characteristic for carbon- or nitrogen-centered radicals. The analysis of radicals nature of irradiated Metroclopramide was presented elsewhere [9].

The experimental and simulated ESR spectrum of mixture of irradiated Metoclopramide and PBN in acetone is presented in (Fig. 1). A good agreement between experimental and simulated spectrum, was obtained by simulation with three radicals species. First species (subspectrum 1) is typical PBN/$OH^{\bullet}$ radical. The characteristic features of this species having *A*(N)=14.6 G, and *A*(H)=3.1 G, are typical PBN/$OH^{\bullet}$ spin adduct [2]. The presence of $OH^{\bullet}$ radical was not properly observed by classical methods, but their presence is motivated by the fact that Metoclopramide is monohydrated and have pronounced hygroscopic characteristics and therefore, ionizing radiation generates hydroxyl radicals from absorbed water molecules through a commonly process characterized as follows: [10]

$$H_2O + h\nu \rightarrow H_2O^{+}_{\bullet} + e^{-}(\rightarrow e^{-}_{aq})$$
$$H_2O^{+}_{\bullet} + H_2O \rightarrow OH^{\bullet} + H_3O^{+}$$

To detect $OH^{\bullet}$ radical by spin trapping method, we used the acetone as neutral environment, which prevent the oxidation processes. The nitrones used as spin traps, N-t-Butyl- α-phenylnitrone (PBN) is a stable compound and forms relatively long-lived spin adducts with various types of radicals as in Scheme 1.



Scheme 1. Formation of spin adduct with PBN.

The second radical species (subspectrum 2) generated in the irradiated Metroclopramide, seems to be a radical of type $R-\overset{}{\underset{\bullet}{C}}OO^{-}$, formed by breaking chemical bond between amidic carbon and amidic nitrogen in presence of some hydroxyl radicals from irradiated water molecules.

The hyperfine structure of the third radical of 5.8 G (subspectrum 3) centered on *g*=2.0035 and peak to peak line-width of 3.7 G, is compatible with a radical produced by breaking the bond between carbon and nitrogen from imidazolic group and addition of an hydrogen atom at one of the carbon atoms of the aromatic ring and thus, the unpaired electron occupies a highly delocalized orbital [8].

Other investigation using the same nitrone spin trap, N-t-Butyl- α-phenylnitrone (PBN), is the formation and reaction of some radicals, in Fenton reaction (Scheme 2) in the presence of cetearyl alcohol and lauric acid. The classical Fenton mechanism predicts that hydrogen peroxide is reduced at the iron center with generation of free hydroxyl radical [11]. The reaction has the same pattern when even ligands are included:

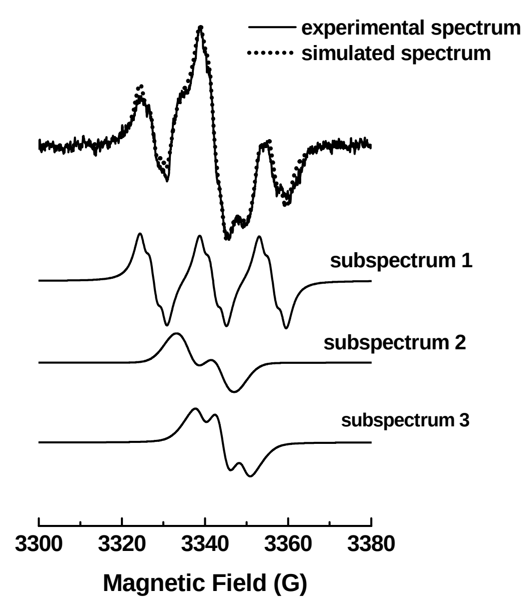


Fig. 1. Experimental and simulated ESR spectrum of PBN/irradiated metroclopramide in acetone.

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^- + {}^\bullet OH$$
$$Fe(H_2O)_6^{+2} + H_2O_2 \longrightarrow [Fe(H_2O)_5OH]^{+2} + OH^- + {}^\bullet OH$$

Scheme 2.

ESR measurements of sample in the presence of the spin trap gave complex results (Fig. 2). From spectra analysis by computer simulation, it can be concluded that HO radicals are not the dominant reactant at all. Possibly hydroxyalchyl radicals were generated in situ from acetone. We assume that an attack of hydroxyl radical on the long chains alcohols was also employed. One of the most important drawbacks of this nitrone is that the EPR spectra of the various amin-oxyl spin adducts are not very characteristic of the radical trapped.

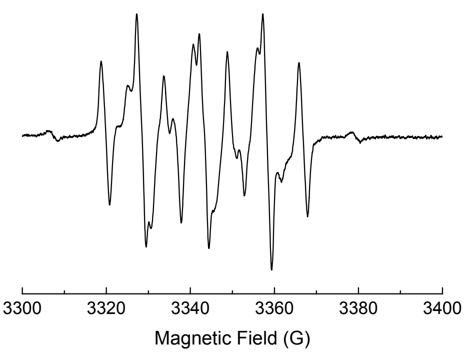


Fig. 2. EPR spectrum of spin-aduct-PBN in Fenton reaction was solved in acetone and conditioned with 6% oxi cream with EDTA, cetearyl alcohol and lauric acid.

The difference in the EPR signal total width of methyl, hydroxyethyl and acetyl radical spin adducts of PBN (PBN-CH_3**,** PBN-$CH(OH)CH_3$•, PBN-$COCH_3$• respectively) can be evaluated only by computer simulation. A direct evidence of characteristic different spin adducts can be, probably, observed by NMR spectroscopy after the reduction of the species to the corresponding hydroxylamines. The Fe^{2+}/EDTA/$H_2 0_2$ hydroxyl radical generating system produced the hydroxyl radical spin trap PBN-OH (a_N = 15.2 G, a_H = 3.2 G). The PBN-OH adduct signal was less intense after few days (Fig. 3). A relatively stable PBN-trapped species PBN-R, where R denotes an unknown radical, was observed. The EPR signal from PBN- R decays slowly over the course of several days. The spin-adduct had hyperfine splitting constants of a_N = 15.8 G and a_H=2.9 G corresponding to neither PBN-OOH nor PBN-OH most probably due to PBN-CH_3 trapped in the solid matrix. During evaporation of the acetone the EPR spectra resembles more to a spectra of stable nitroxide radicals (where R=CH_3 in the Scheme 1).

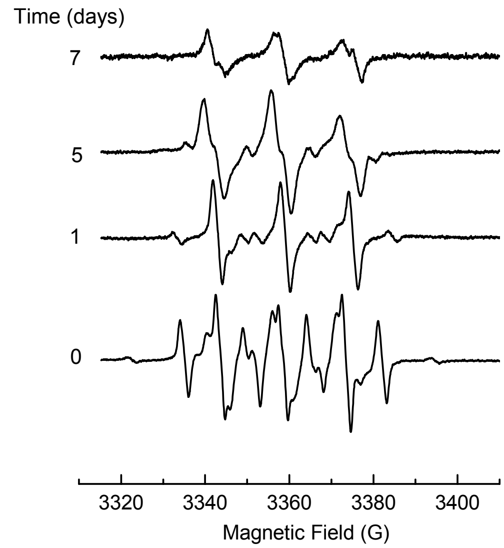


Fig. 3. EPR spectra of free radicals generated in complex Fenton reaction using spin trapping PBN during acetone evaporation.

4. Conclusions

The nonspecific spin trap N-t-Butyl- α-phenylnitrone (PBN) is able to trap some radical species. The free radicals generated in the solid drugs in different stress conditions, can be detected by spin trapping method using adequate solvents to obtain specific spin adduct, detectable by EPR spectroscopy.

Beside the well-known OH• radical, ESR spectra shown the existence of some carbon centered radicals, such CH_3•**,** $CH(OH)CH_3$•, $COCH_3$• giving the related spin adducts. The obtained spin adducts have specific features expressed by different living-times. Thus, during the ESR measurements, we observed a time dependence of the spectral characteristics and signal intensities.

Acknowledgements

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