

The Use of Multi-frequency EPR Techniques to Identify the Radicals Produced in Irradiated β -Blockers

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The identification of radicals trapped in irradiated drugs can be very intricate. A multi-frequency electron paramagnetic resonance (EPR) study is proposed to resolve this problem. The Q-band (ca. 34 GHz) comparison with X-band (ca. 9 GHz) did not show significant differences for the four β -blockers studied (atenolol, esmolol, nadolol and propranolol). The use of a higher frequency (285 GHz) was required. It enabled us to determine the g -tensor values of the radicals present in atenolol and esmolol, respectively, $g_1 = 2.0086$, $g_2 = 2.0059$ and $g_3 = 2.0021$ and $g_1 = 2.0066$, $g_2 = 2.0044$ and $g_3 = 2.0021$. The latter was assigned as a phenoxyl radical, which can not be the case for the former. Therefore, radicals produced in esmolol may result from a more complex mechanism than the abstraction followed by the diffusion of an H atom inside the solid. In addition, two molecules as similar as atenolol and esmolol hydrochloride do not contain the same radicals after irradiation. These two conclusions drawn from the EPR results on β -blockers show clearly the importance of continuing the investigations on radiolytic mechanisms in solid-state drugs.

Keywords: X-band / Q-band EPR; HF-EPR; Irradiation; Solid-state drugs; Phenoxyl radical

INTRODUCTION

Ionizing irradiation (gamma-, X-rays, UV) of solids produces radicals: some of them stay trapped in the matrix for years. This occurs as well in solid-state drugs that are irradiated to be sterilized.^[1,2] This mean of sterilization is called radiosterilization and is part of the techniques recommended by the pharmaceutical reference books, the US and

European pharmacopoeias.^[3,4] Its efficiency is as great as the sterilization technique of reference (autoclaving). Moreover, low dose-rate irradiation does not induce a significant rise of temperature in the solid,^[5] that could denature drugs such as thermosensitive ones or proteins. It would be, therefore, very useful for these cases.

The study of the radicals produced in the drugs after irradiation can be performed by electron paramagnetic resonance (EPR). This very sensitive method has already been widely used in X-band (9 GHz) to compare non-irradiated samples to irradiate ones,^[6] to make post-dosimetry or to follow the decay of the radicals.^[7] Up to now, few works have been focused on the identification of the radicals in irradiated drugs.^[8,9]

This identification is necessary in order to study the radiolytic mechanisms. There is indeed no clear knowledge concerning these mechanisms, which prevents the wide-spread use of radiosterilization. If the nature of the radicals in a drug was determined, it could be compared to the radiolytic products (obtained after dissolution of the drug into water) and it would help resolving the radical part of the mechanisms.

Two major restraints make this identification difficult. First, the X-band EPR spectrum of an irradiated drug is often composite: several types of radicals can be trapped within the matrix. The different spectra must therefore be separated, which can be achieved by playing on certain parameters such as temperature^[10] or the microwave power at the EPR measurement.^[1] Secondly, the EPR spectrum obtained is a typical powder one where

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radicals are randomly orientated. The spectrum is broad with little information, only some EPR parameters can be assigned.^[8]

To overcome these problems of resolution an investigation at higher frequency can be done. EPR techniques at high frequency and high fields named HF-EPR is not yet very commonly used, but has already permitted the identification of radicals in cases as intricate as in irradiated drugs.^[11,12] The resolution enhancement at high magnetic fields is based on the fact that electron Zeeman splitting of unpaired electrons scale with the magnetic field, and therefore anisotropic *g*-tensor components will be distributed over a larger magnetic field range, whereas the magnitude of hyperfine splittings remain largely unchanged. In practical terms, the nuclear Zeeman splittings from interacting nuclei, which also scale with the magnetic field, are too small to be observed by HF-EPR.^[11]

This article deals with antihypertensive drugs. Four β -blockers were chosen: atenolol, esmolol hydrochloride, nadolol and propranolol hydrochloride. A quantitative X-band EPR study has previously showed that these drugs have a radioresistant tendency.^[13] their radical yields are low. Unfortunately, the poorly resolved spectra did not allow for identification of the radicals involved. We have, therefore, studied for the first time the contribution of HF-EPR to the identification of the radicals in irradiated drugs.

MATERIAL AND METHODS

Drugs

Nadolol and atenolol were purchased from Sigma and propranolol hydrochloride from Fluka whereas esmolol hydrochloride was kindly provided by Baxter.

Irradiation

β -blockers were all irradiated with gamma rays from a ⁶⁰Cobalt panoramic chamber (UCL, Louvain-La-Neuve, Belgium) at 30 kGy. This source was calibrated with an alanine dosimetry: alanine pellets were supplied and analyzed by Risø National Laboratory (Denmark). The calibration determined a dose rate of 417 Gy h⁻¹.

X-band EPR Experiments

The X-band EPR (9.3 GHz) spectra at room temperature have been taken with a Bruker EMX-8/2.7 spectrometer (UCL, Brussels, Belgium). The magnetic field was measured by a Bruker ER 036 TM NMR gaussmeter and the microwave frequency by a Bruker EMX 040-1161.8A frequency counter.

For X-band EPR (9.66 GHz) spectra at low temperature (30 K), a Bruker E300e was used with

a Bruker ER 4116DM dual mode resonator equipped with an Oxford Instruments ESR900 Helium flow cryostat (UiO, Oslo, Norway).

The low temperature X-band spectra were simulated using the second order algorithm of the software program Simfonia version 1.25 by Bruker.

Q-band (34 GHz) EPR Experiments

The Q-band EPR spectra have been taken with a Bruker Eleksys E500 spectrometer equipped with a rectangular ER 5106 QTE ENDOR cavity (UGent, Ghent, Belgium). The magnetic field was given by a Bruker ER035M NMR gaussmeter and the microwave frequency by an EIP 548B frequency counter. All experiments of comparison with 9.3 GHz were performed at room temperature. For ENDOR studies, an Oxford CF 935 cryostat was used, which enables to reach temperature down to 4 K. The drugs were irradiated using a higher dose (120 kGy) and the measurement temperature was cooled down to 100 K in order to increase the sensitivity of the technique.

285 GHz Experiments

The apparatus at GHMFL belongs to the class of single-pass transmission spectrometers (CNRS/MPI, Grenoble, France),^[11] which can work in a very broad frequency range. The applied frequency was 285 GHz, using a Gunn diode (Radiometer Physics) and a multiplier (3 × 95 GHz). The main magnetic field is provided by a superconducting magnet with a maximum field of 12 T at 4.2 K (Cryogenics Consultant). The detection of the light transmitted through the sample is performed with an InSb bolometer (QMC Instruments); a Variable Temperature Insert (Oxford Instrument) enables to vary the sample temperature from 5 to 300 K.

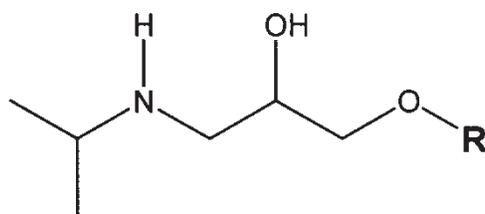
For X- and Q-band spectra, the absolute *g*-values have been determined by comparison with a Bruker reference: a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) sample (*g* = 2.0036).

The *g*-values from HF-EPR were obtained by comparison with a sample of protein R2 of mouse ribonucleotide reductase which contains a tyrosyl radical with known *g*-tensor components,^[14,15] run under identical spectrometer conditions.

RESULTS AND DISCUSSION

Comparison between Room Temperature Spectra from X- and Q-band

The molecular structures of the four β -blockers studied in this article are given in Fig. 1. Atenolol, esmolol hydrochloride, nadolol and propranolol



	R
Atenolol	
Esmolol hydrochloride	
Nadolol	
Propranolol hydrochloride	

FIGURE 1 Molecular structures of the β -blockers studied.

hydrochloride all have a common lateral chain and possess at least one aromatic ring. They were submitted to gamma rays and irradiated to 30 kGy. This rather high dose of irradiation in comparison to the 25 kGy of reference in the pharmacopoeias^[3,4] was necessary in order to obtain a good signal-to-noise ratio. This family of drugs is indeed radio-resistant, and few radicals are produced and trapped after irradiation.^[13] Figure 2 shows the study on the four drugs at two different frequencies. The first analyses (left panel) were performed with a X-band spectrometer. Powder spectra are obtained, which indicate

that the spins are randomly orientated and, therefore, the lines are broadened. The spectra do not allow for drawing any conclusions on the nature of the radicals present. In such cases, it is difficult to attribute lines to the g -anisotropy or to the hyperfine couplings. Q-band EPR has the advantage of being at a higher frequency (ca. 34 GHz) which means that anisotropic g -tensor components will be distributed over a larger absolute magnetic field. The Q-band EPR spectra are given on the right panel of Fig. 2. However, even at Q-band, the g -anisotropies of all the four samples are too low for the g -components to be separated. Furthermore,

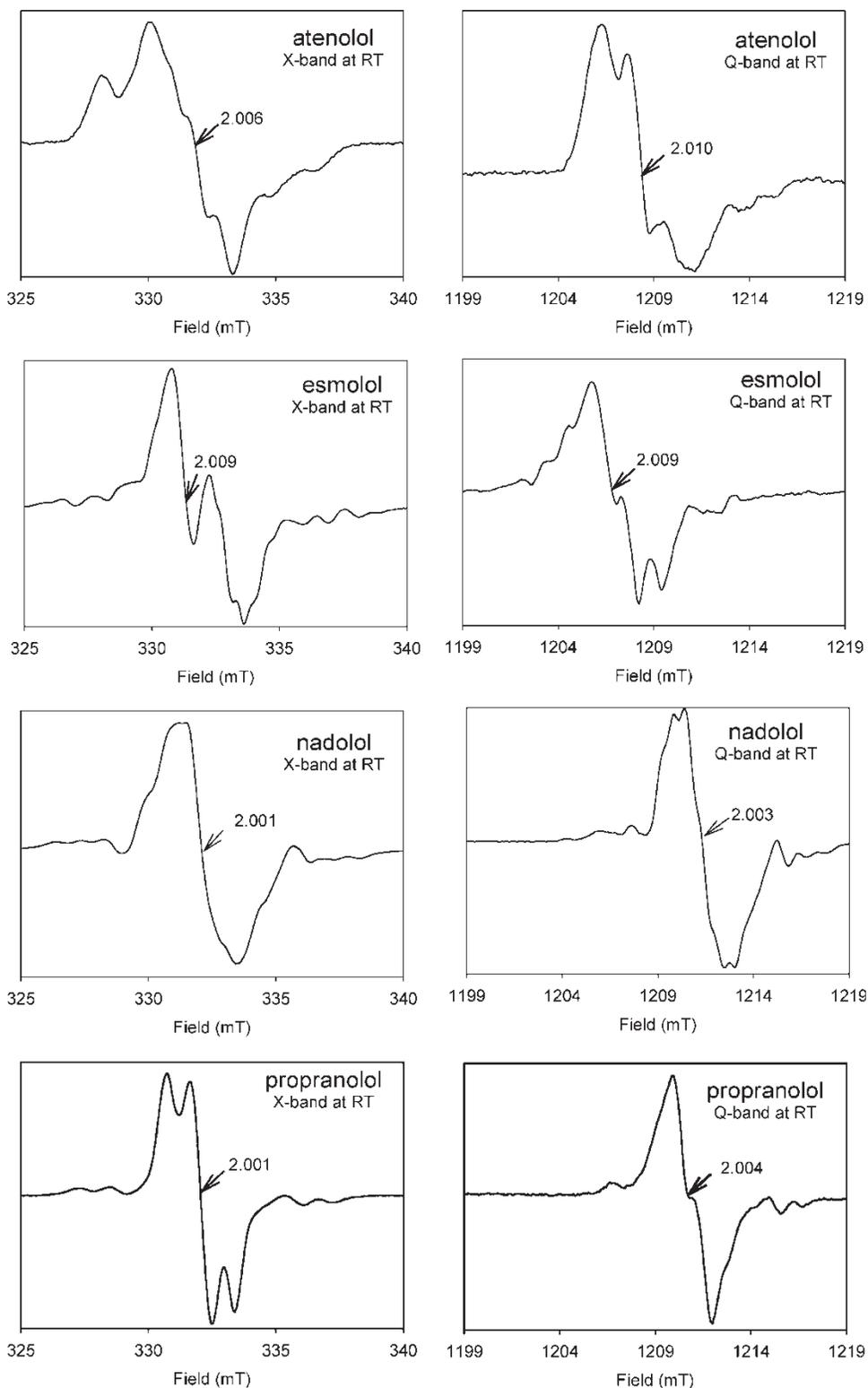


FIGURE 2 Comparison of EPR spectra in X- (left) and Q-band (right) at room temperature for four different γ -irradiated drugs: atenolol, esmolol hydrochloride, nadolol and propranolol hydrochloride. Modulation amplitude, 0.3 mT; microwave power, 0.250 mW.

the hyperfine splittings are even less resolved than at the X-band, as expected due to the line broadening which increases with increasing fields. The differences in resolution between the X- and Q-band spectra are very slight. It seems that the spectral features are

essentially due to hyperfine splittings and a slight g -anisotropy could also be involved. Hence, from these four comparisons, no exploitable data are found. The Q-band frequency is indeed not far higher than the X-band frequency and in the case of β -blockers, it

seems that it is not high enough to obtain important changes in the spectra.

The drugs were also studied by electron nuclear double resonance (ENDOR) in Q-band in order to observe hyperfine couplings with proton(s) or possibly nitrogen. The ENDOR spectra were poor (only the matrix protons were observed) and these analyses were considered as not conclusive.

Use of a Very High Frequency EPR Spectrometer

A major established advantage of high-field EPR spectroscopy (HF-EPR) is in enhancing g -factor resolution in radicals.^[11,14,15] HF-EPR can help resolve overlapping spectra and thereby gain information about the identity of the radicals. The measurements of β -blockers were performed at 285 GHz. For the irradiated nadolol and propranolol samples, the g -anisotropy was not solved even at this high field (spectra not shown). For atenolol and esmolol, however, the HF-EPR spectra (Fig. 3) are dominated by g -anisotropy, and the hyperfine couplings are no longer observable due to line broadening. Hence, it allows us to measure the g -tensor components with a high degree of accuracy.

For atenolol, the values observed are $g_1 = 2.0086(2)$, $g_2 = 2.0059(2)$ and $g_3 = 2.0021(2)$ and for esmolol, $g_1 = 2.0066(2)$, $g_2 = 2.0044(2)$ and $g_3 = 2.0021(2)$, errors in the last digit are given in parenthesis. They are presented in Table I together with values of radicals with similar g -tensor components taken from the literature.

The g -anisotropy defined by $\Delta g(g_1 - g_e)$ where g_e is the g -value for a free electron (2.002319), is partly depending on the atomic spin orbit coupling constant.^[16] For both atenolol and esmolol, Δg resembles oxygen centered radicals, whereas

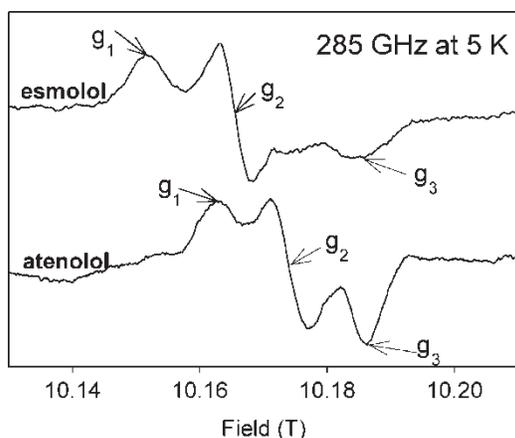


FIGURE 3 HF-EPR (microwave frequency, 285 GHz) spectra of γ -irradiated atenolol and esmolol hydrochloride measured at 5 K. It enables to determine the g -tensor values. For atenolol, $g_1 = 2.0086(2)$, $g_2 = 2.0059(2)$ and $g_3 = 2.0021(2)$; for esmolol, $g_1 = 2.0066(2)$, $g_2 = 2.0044(2)$ and $g_3 = 2.0021(2)$. Modulation amplitude, 0.8 mT; scan rate, 0.5 mT/s.

TABLE I g -Tensor components of tyrosyl and quinone radicals

Species	g_1	g_2	g_3	References
Irradiated Tyr	2.0067	2.0045	2.0023	[25]
BQ*	2.00645	2.00526	2.00229	[27]
Mouse R2 [†]	2.0076	2.0043	2.0021	[20,21]
<i>E. coli</i> R2 [†]	2.0091	2.0046	2.0023	[23]
Esmolol [‡]	2.0066(2)	2.0044(2)	2.0021(2)	This work
Atenolol [‡]	2.0089(2)	2.0059(2)	2.0021(2)	This work

* Benzoquinone radical anion. [†] Tyrosyl radical in ribonucleotide reductase protein R2. [‡] Error in last digit given in parenthesis.

the smaller g -anisotropy of nadolol and propranolol is more typical for carbon centered radicals, since carbon has a smaller spin orbit coupling constant than oxygen.

The g -tensor components found by the HF-EPR enable us to make simulations in order to understand the spectra in X-band. Spectra have been taken at 9.66 GHz at 30 K, which gives less line broadening than the room temperature spectra, and thus, better resolved hyperfine couplings. They are given in Fig. 4A for the esmolol together with the simulation. The hyperfine interactions come from four protons

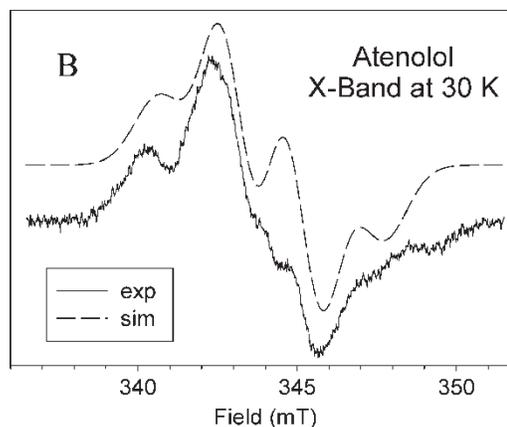
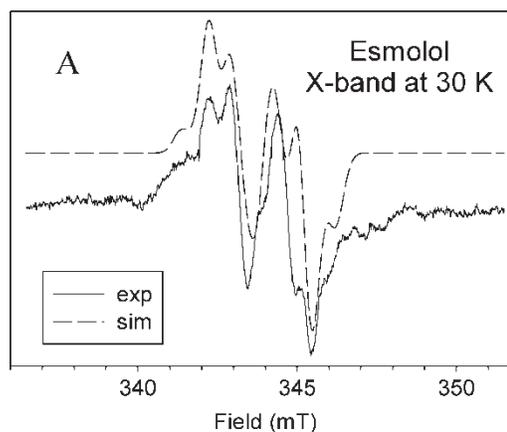


FIGURE 4 X-band EPR experimental (solid lines) and simulated spectra (dashed lines) of γ -irradiated esmolol hydrochloride (A) and atenolol (B) measured at 30 K. Modulation amplitude, 0.2 mT; microwave power, 10 μ W; microwave frequency, 9.67 GHz. Simulation parameters are listed in Tables I and II.

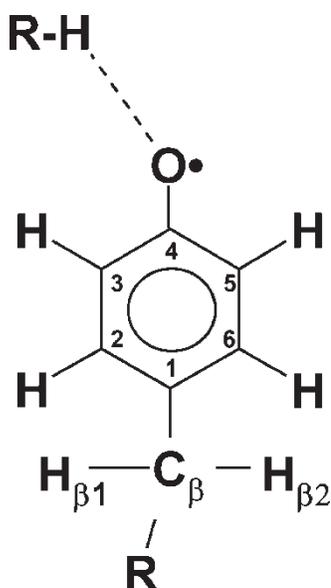


FIGURE 5 Molecular diagram of a phenoxyl radical possibly induced in esmolol.

with hyperfine tensor components, A_1 , A_2 and A_3 . One proton has a nearly axial hyperfine tensor, 2.0, 1.7 and 1.75 mT. In addition, there seems to be three almost equivalent protons with one hyperfine component of ca. 0.9 mT.

The g -tensor components observed for esmolol hydrochloride are actually corresponding to a radical well described in the literature, the phenoxyl radical (Fig. 5) (see Table I).^[17–19] Interestingly, this type of radical is present in many proteins such as photosystem II^[20,21] and ribonucleotide reductase^[22,23] and has, therefore, been analyzed by numerous detailed multi-frequency EPR studies. This type of radical is now well characterized. The g -tensor components are depending on the local environment and have been shown to vary in different systems.^[22] g_1 is the most sensitive to electrostatic changes, depending largely on the strength of hydrogen bonding to the phenoxyl oxygen, whereas g_2 and g_3 are more constant.^[24] The g_1 -value found for esmolol corresponds to a strong hydrogen bond to the phenoxyl oxygen, as observed for irradiated tyrosine HCl.^[25] The proton with an axial hyperfine tensor resembles a β -proton on a phenoxyl radical with a dihedral angle of $10 (\pm 5)$ degrees off the phenol ring plane normal. Concerning the three almost equivalent protons with an isotropic tensor, two of them could be two of the α -protons on the phenoxyl ring which have an expected rhombic hyperfine tensor of about -0.9 , -0.3 and -0.7 mT (the negative signs do not play any role in the EPR spectrum). The third 0.9 mT-proton could possibly be the second β -proton of the β -methylene group; its dihedral angle should be either 110 or 130, in order to be 120° off the other β -proton that was $\pm 10^\circ$. For 130° , this gives a hyperfine tensor of approximately 0.8, 0.6, 0.6 mT.^[26] If the angle is 110, the hyperfine components would be smaller

than 0.5 mT, and therefore, not resolved in the X-band EPR spectrum due to its linewidth. The best fit is, therefore, obtained with dihedral angles for the two β -protons of $\theta(H_{\beta 1}) = 10 \pm 5^\circ$ and $\theta(H_{\beta 2}) = 130 \pm 5^\circ$.

Up to now, identification of radicals in irradiated drugs showed that main radicals came from the rupture of a C–H bond.^[9,13] The abstracted hydrogen atom is small and is likely to move. Therefore, radical recombination is not facilitated. In the case of breakage of internal bonds, two parts of the molecules are dissociated. As they are large, they should be immobilized by the environmental structure (cage effect) and hence, recombine. *In fine*, the radicals observed are the ones that do not recombine. The presence of a phenoxyl type radical in esmolol is therefore interesting. It implies a more complex mechanism. The radical could arise from a C–H bond rupture followed by an inter- or intramolecular radical transfer, possibly across the phenol ring, leading to a breakage of the ether bond. The homolytic breakage of the internal ether bond is another possibility but seems less probable because of the cage effect.

The g -values of atenolol do not match with the one of a phenoxyl radical; g_2 value of 2.0059 is indeed too high. g_2 -Values approaching this value have been observed for semiquinone radical anions, with $g_2 = 2.0052$,^[27] but in those cases, the g_1 -values are significantly lower than what is observed for atenolol (see Table I). Furthermore, it is difficult to comprehend how a semiquinone radical could be formed upon irradiation of atenolol. Considering the hyperfine couplings, due to the large linewidth of the atenolol EPR spectrum, only components that are larger than 1.6 mT can be detected. An X-band EPR spectrum of irradiated atenolol recorded at 30 K along with a simulation based on parameters given in Table II is presented in Fig. 4B. The suggested simulation uses three equivalent protons with 2.1, 1.7 and 1.75 mT. It is not possible to simulate the spectrum of atenolol using phenoxyl radical type hyperfine interactions. Nevertheless, the g -anisotropy suggests that this is also an oxygen centered radical, which could indicate that the internal ether bond is also broken in atenolol.

Even though an unambiguous identification of the main radical species contributing to the EPR spectrum of irradiated atenolol was not possible, it is very interesting to note that the EPR spectrum is clearly different to the one observed for irradiated esmolol, a structurally very closely related drug, indicating that these samples contain two different radical species. Up to now, drugs were collected under families concerning their behavior to irradiation. For instance, cephalosporines were considered as radiosensitive, on the contrary to tetracyclines. In the case of β -blockers, it seems that radiolytic pathways are not exactly the same and can lead to different radicals.

TABLE II Hyperfine coupling tensor components (A) and dihedral angles (θ) of tyrosyl radicals

Species	$A(H_{\beta 1})$	$A(H_{\beta 2})$	$A(H_{2,6})$	$A(H_{3,5})$	$\theta(H_{\beta})$	$\theta(H_{\beta 2})$	References
Irradiated tyrosine	0.15	0.95	0.17	-0.96	75	45	[17,18,25]
	0.11	0.92	0.27	-0.28			
	0.11	0.92	0.04	-0.70			
Tyrosyl radical in <i>E. coli</i> RNR R2*	2.18	0.08	0.18	-0.95	30	90	[19,23]
	1.91	-0.18	0.27	-0.30			
	1.91	-0.14	0.08	-0.70			
Esmolol [†]	2.1(\pm 0.1)	-0.8(\pm 0.1)		-0.95 (\pm 0.1)	10(\pm 5)	130(\pm 10)	This work
	1.7(\pm 0.1)	-0.6(\pm 0.1)		-0.3 (\pm 0.2)			
	1.75(\pm 0.1)	-0.6(\pm 0.1)		-0.7 (\pm 0.1)			
Atenolol ^{†,‡}	2.1(\pm 0.2)						This work
	1.7(\pm 0.2)						
	1.75(\pm 0.2)						

All hyperfine values in mT. For proton numbering, see Fig. 5. *Ribonucleotide reductase protein R2. [†]Errors are given in parenthesis. [‡]Atenolol is simulated using 3 equivalent protons.

In addition to the main spectral features, satellite lines were observed in the X-band EPR spectra at room temperature that saturate differently compared to central ones with regards to the microwave power. This indicates the presence of other radical species underlying the main spectra, which would have stronger hyperfine splittings. They are in very small amount compared to the main radical.

CONCLUSION

Even though the identification of the radicals contained in the gamma-irradiated β -blockers was not achieved for all the drugs with the methods used, these first EPR measurements highlight important facts. On one hand, the irradiation of esmolol hydrochloride seems to lead to the production of phenoxyl radicals. Though the mechanisms have not been elucidated, two major hypotheses can be advanced. The radicals could come from a C-H bond rupture followed by an inter- or intra molecular radical transfer leading to a breakage of the ether bond or from the homolytic cleavage of the internal ether bond.

On the other hand, from these results, the g -values and the hyperfine couplings obtained for atenolol seem to indicate that the main radical(s) are not compatible with the presence of a phenoxyl radical, although it probably is also oxygen centered.

This implies that drugs coming from a single therapeutic family and very similar in structures do not present the same radicals. Therefore, the radiolytic scheme if involving the radicals will not be identical.

In the case of the β -blockers, Q-band does not permit to comprehend the spectra. It is necessary to go to frequencies as high as 285 GHz to resolve the g -anisotropy and thereby reveal the individual g -tensor components.

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References

- [1] Gibella, M., Crucq, A.-S., Tilquin, B., Stocker, P., Lesgards, G. and Raffi, J. (2000) "Electron spin resonance of some irradiated pharmaceuticals", *Radiat. Phys. Chem.* **58**, 69-76.
- [2] Basly, J.-P. and Bernard, M. (1997) "Radiosterilization dosimetry by EPR spectroscopy: ritodrine HCl and comparison with other sympaticomimetics", *Int. J. Pharm.* **149**, 85-91.
- [3] Textes généraux sur la stérilité (2000) In: *Pharmacopée Européenne 4ème Edition* (Direction Européenne de la Qualité du Médicament, Strasbourg) pp. 439-444.
- [4] Sterilization and sterility assurance of compendial articles (2000) In: *The United States Pharmacopoeia USP 24* (United States Pharmacopoeial Convention, Rockville) pp. 2143-2147.
- [5] Reid, B.D. and Fairand, B.P. (1998) "Gamma radiation sterilization of pharmaceuticals" In: Nordhauser, F.M. and Olson, W.P., eds, *Sterilization of Drugs and Devices Technologies for the 21st Century* (Interpharm Press, Buffalo Grove) pp 311-392.
- [6] Raffi, J., Gelly, S., Barral, L., Burger, F., Piccerelle, P., Prinderre, P., Baron, M. and Chamayou, A. (2002) "Electron paramagnetic resonance of radicals induced in drugs and excipients by radiation or mechanical treatments", *Spectrochim. Acta A* **58**, 1313-1320.
- [7] Basly, J.-P., Basly, I. and Bernard, M. (1998) "Electron spin resonance identification of irradiated ascorbic acid: dosimetry and influence of powder fineness", *Anal. Chim. Acta* **372**, 373-378.
- [8] Ambroz, H.B., Kornacka, E.M., Marciniak, B., Ogródowczyk, M. and Przybytniak, G.K. (2000) "EPR study of free radicals in some drugs γ -irradiated in the solid-state", *Radiat. Phys. Chem.* **58**, 357-366.

- [9] Köseoglu, R., Köseoglu, E. and Köksal, F. (2003) "Electron paramagnetic resonance of some γ -irradiated drugs", *Appl. Radiat. Isot.* **58**, 63–68.
- [10] Vanhaelewyn, G.C.A.M., Mondelaers, W.K.P.G. and Callens, F.J. (1999) "Effect of temperature on the electron paramagnetic resonance spectrum of irradiated alanine", *Radiat. Res.* **151**, 590–594.
- [11] Andersson, K.K. and Barra, A.-L. (2002) "The use of high field/frequency EPR in studies of radical and metal sites in proteins and small inorganic models", *Spectrochim. Acta A* **58**, 1101–1112.
- [12] Callens, F., Vanhaelewyn, G. and Matthys, P. (2002) "Some recent multi-frequency electron paramagnetic resonance results on systems relevant for dosimetry and dating", *Spectrochim. Acta A* **58**, 1321–1328.
- [13] Engalytcheff, A., Deridder, V., Debuyst, R. and Tilquin, B. (2003) "Determination of radical yields in solid-state drugs: radioresistance of beta-blockers", *Radiat. Res.* **160**, 103–109.
- [14] Andersson, K.K., Schmidt, P.P., Katterle, B., Stand, K.R., Palmer, A., Lee, S.-K., Solomon, E.I., Gräslund, A. and Barra, A.-L. (2003) "Examples of high frequency EPR studies in bioinorganic chemistry", *J. Biol. Inorg. Chem.* **8**, 235–247.
- [15] Barra, A.-L.A., Gräslund, A. and Andersson, K.K. (2004) "The use of very high frequency EPR (VHF-EPR) in studies of radicals and metal sites in proteins and small inorganic models", *Biol. Magn. Reson.* **22**, Chapter 5.
- [16] Stone, A.J. (1963) "Gauge invariance of the g -tensor", *Proc. R. Soc. Lond. Ser. A*, 271–424.
- [17] Hulsebosch, R.J., van den Brink, J.S., Nieuwenhuis, S.A.M., Gast, P., Raap, J., Lugtenburg, J. and Hoff, A.J. (1997) "Electronic structure of the neutral tyrosine radical in frozen solution. Selective 2H-, 13C- and 17O-isotope labeling and EPR spectroscopy at 9 and 35 GHz", *J. Am. Chem. Soc.* **119**, 8685–8694.
- [18] Nieuwenhuis, S.A.M., Hulsebosch, R.J., Raap, J., Gast, P., Lugtenburg, J. and Hoff, A.J. (1998) "Structure of the YD tyrosine radical in photosystem II. Determination of the orientation of the phenoxyl ring by enantioselective deuteration of the methylene group", *J. Am. Chem. Soc.* **120**, 829–830.
- [19] Hoganson, C.W., Sahlin, M., Sjöberg, B.-M. and Babcock, G.T. (1996) "Electron magnetic resonance of the tyrosyl radical in ribonucleotide reductase from *Escherichia coli*", *J. Am. Chem. Soc.* **118**, 4672–4679.
- [20] Schmidt, P.P., Andersson, K.K., Barra, A.-L., Thelander, L. and Gräslund, A. (1996) "High field EPR studies of mouse ribonucleotide reductase indicate hydrogen bonding of the tyrosyl radical", *J. Biol. Chem.* **271**, 23615–23618.
- [21] Bleifuss, G., Kolberg, M., Pötsch, S., Hofbauer, W., Bittl, R., Lubitz, W., Gräslund, A., Lassmann, G. and Lenzian, F. (2001) "Tryptophan and tyrosine radicals in ribonucleotide reductase: a comparative high-field EPR study at 94 GHz", *Biochemistry* **40**, 15362–15368.
- [22] Un, S., Dorlet, P. and Rutherford, A.W. (2001) "A high-field EPR tour of radicals in photosystems I and II", *Appl. Magn. Reson.* **21**, 341–361.
- [23] Gerfen, G.J., Bellew, B.F., Un, S., Bollinger, J.M., Jr, Stubbe, J., Griffin, R.G. and Singel, D.J. (1993) "High-frequency (139.5 GHz) EPR spectroscopy of the tyrosyl radical in *Escherichia coli* ribonucleotide reductase", *J. Am. Chem. Soc.* **115**, 6420–6421.
- [24] Un, S., Gerez, C., Elleingand, E. and Fontecave, M. (2001) "Sensitivity of tyrosyl radical g -values to changes in protein structure: a high-field EPR study of mutants of ribonucleotide reductase", *J. Am. Chem. Soc.* **123**, 3048–3054.
- [25] Fasanella, E.L. and Gordy, W. (1969) "Electron spin resonance of an irradiated single crystal of L-tyrosine-HCl", *Proc. Natl Acad. Sci. USA* **62**, 299–304.
- [26] Himo, F., Gräslund, A. and Eriksson, L.A. (1997) "Density functional calculations on model tyrosyl radicals", *Biophys. J.* **72**, 1556–1567.
- [27] Burghaus, O., Plato, M., Rohrer, M., Möbius, K., MacMillan, F. and Lubitz, W. (1993) "3-mm High-field EPR on semiquinone radical anions Q—related to photosynthesis and on the primary donor P⁺ and acceptor QA—in reaction centers of *Rhodobacter sphaeroides* R-26", *J. Phys. Chem.* **97**, 7639–7647.