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Original Citation

Vallotto, C., Williams, H.E., Murphy, D.M., Ayres, Z.J., Edge, R., Newton, M.E. and Wedge, C.J. (2017) An Electron Paramagnetic Resonance (EPR) spectroscopy study on the γ-irradiation sterilization of the pharmaceutical excipient l-histidine: Regeneration of the radicals in solution. International Journal of Pharmaceutics, 533 (1). pp. 315-319. ISSN 0378-5173

An Electron Paramagnetic Resonance (EPR) spectroscopy study on the γ-irradiation sterilization of the pharmaceutical excipient L-histidine: regeneration of the radicals in solution

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Supplementary Material

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1) Additional Experimental Details

Preparation of MNP stock solutions. A solution of the spin trap 2-methyl-2-nitrosopropane (MNP) was prepared by solubilising the solid dimer (*Sigma Aldrich*) in acetonitrile at a concentration of either 0.8 M or 1.6 M and subsequently diluting it by 1:20 into the aqueous buffered solutions to give a final concentration of 40 or 80 mM.

Sample preparation for spin-trapping experiments. To perform the spin-trapping experiments on L-histidine (L-his), an excess of the powder was added to a solution of MNP (40 or 80 mM) in order to obtain the maximum concentration of L-his in solution (typically *ca*. 10 – 12 mg in 0.2 ml). The solution was then vortexed for 1 min, the supernatant was separated from the undissolved powder by centrifugation and analysed. The concentration of L-his in solution was therefore considered to be close to saturation (≈ 0.27 M) (Brown, 1991).

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When testing the regeneration of the radical, the excess of powder was added to a solution lacking the MNP spin-trap, and the supernatant was separated from the undissolved powder before the addition of the 0.8 M stock solution of MNP to give a final MNP concentration of 40 mM with minimal dilution of the L-his. This lower concentration of MNP was used in all delayed MNP addition experiments, and for all quantitative measures of the concentration of the MNP-his spin-adduct, in order to minimize the concentration of di-*tert*-butyl nitroxide (DTBN) which can interfere with the analysis

X-ray fluorescence (XRF) analysis. XRF was conducted using an Energy Dispersive (ED)-XRF system (*NEX CG, Rigaku*, Japan) operating an X-ray tube with Pd anode at 50 kV with a 1 mA current and utilising a Cu secondary target (Kα = 8.046). All spectra were measured under moderate vacuum (12 Pa) for a sampling time of 150 s, and background subtracted. The interrogation area of the X-ray source on the sample is elliptical and *ca.* 1.2 cm in diameter, thus in order to make the measurements, 3 of the 12 cm needles were cut into smaller (1.2 cm) segments and placed into the centre of a sample holder to ensure they came into contact with the X-ray beam.

2) EPR simulations

EPR spectral simulations were performed in *Matlab* using the *EasySpin* package (Stoll and Schweiger, 2006). The routine *pepper* was used for the solid state simulation, while the *garlic* routine (appropriate for the fast-motional regime) was used for all the liquid phase solution experiments. Simulation of the MNP-histidine spin-adduct signal was performed by considering axial hyperfine couplings and **g**-tensor, using the minimum number or parameters necessary to allow dynamic effects to be included in the simulation. The phenomenological lineshape used contained Lorentzian (0.029 mT) and Gaussian (0.017 mT) components and dynamics were considered in the fast motional regime, $\tau_c \approx 5.4$ ns.

EPR parameters of the MNP di-adduct DTBN were obtained by fitting a spectrum acquired from a solution of MNP which had been illuminated overnight with a 465 nm LED (*ca*. 7 mW), in order to promote the formation of DTBN. Hyperfine couplings arising from both the nitroxidic nitrogen and statistical abundance of nearest neighbour ^{13}C nuclei were included and considered to be fully isotropic. An isotropic **g**-tensor was used and dynamic effects were neglected.

Simulation of the MNP-mannitol spin-adduct signal was performed by including only the hyperfine coupling arising from the nitroxidic nitrogen, which was considered to be fully isotropic. An isotropic **g**-tensor was used and dynamic effects were neglected.

Supplementary Figures

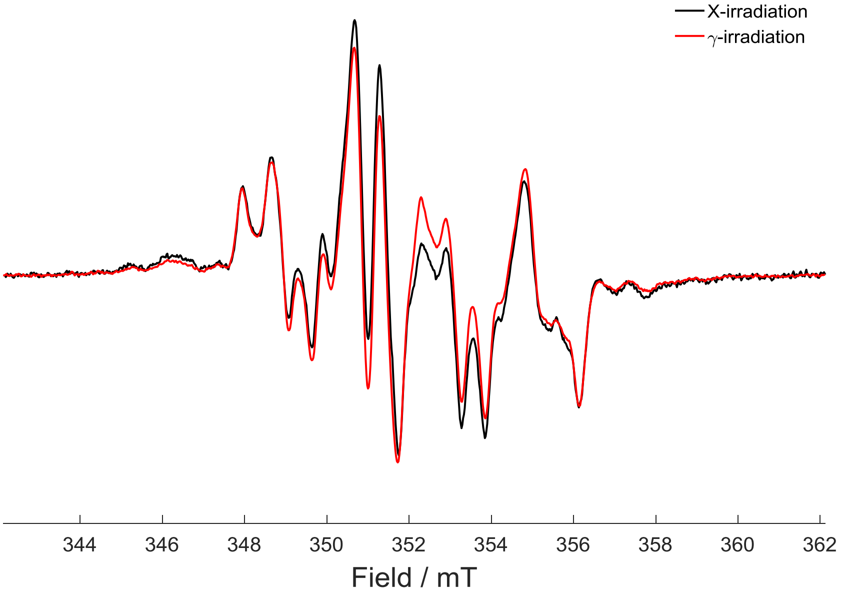

Fig. S1. CW X-band EPR spectra of L-histidine powder irradiated using X-rays (black) and γ-rays (red) with irradiation doses of 10 kGy (X-rays) and 25 kGy (γ-rays). The EPR parameters used to record the spectra were: time constant 20.48 ms; conversion time 20.48 ms; number of points 1024; number of scans 30 (X-rays), 10 (γ-rays); microwave frequency 9.8 GHz; microwave power 3.2 µW; modulation amplitude 0.1 mT.

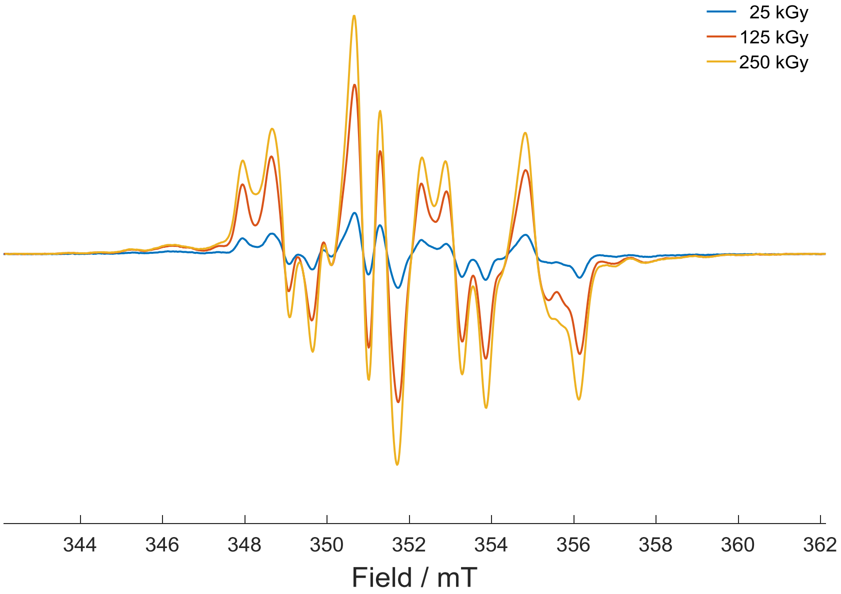

Fig. S2. CW X-band EPR spectra of γ-irradiated L-histidine powder with irradiation doses of 25 kGy (blue), 125 kGy (red) and 250 kGy (yellow). The EPR parameters used to record the spectra were: time constant 20.48 ms; conversion time 20.48 ms; number of points 1024; number of scans 10; microwave frequency 9.8 GHz; microwave power 3.2 µW; modulation amplitude 0.1 mT.

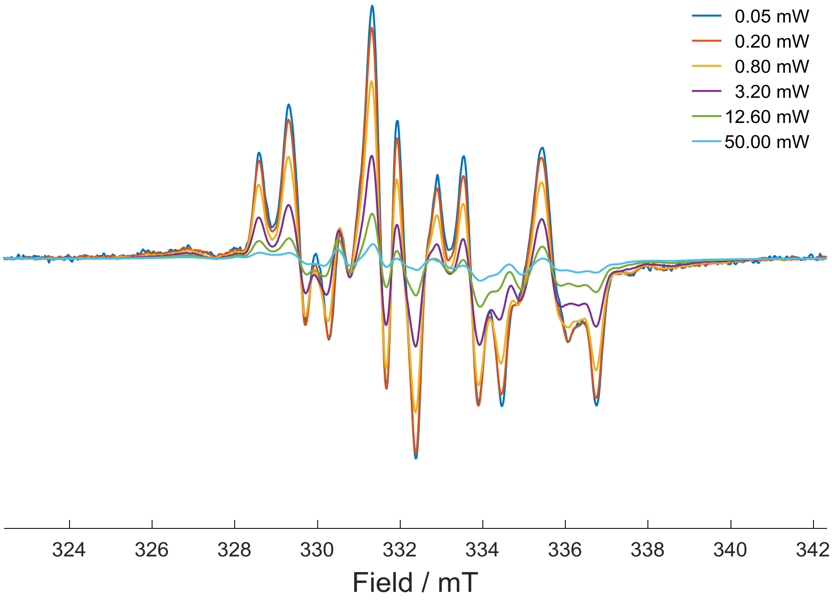

Fig. S3. CW X-band EPR spectra of γ-irradiated L-histidine powder with an irradiation dose of 25 kGy recorded at various microwave powers. Each spectrum was scaled by the square root of the microwave power such that in a non-saturating regime the intensity of each spectrum would be equal. The EPR parameters used to record the spectra were: time constant 81.92 ms; conversion time 40.96 ms; number of points 1024; number of scans 4; microwave frequency 9.3 GHz; modulation amplitude 0.1 mT.

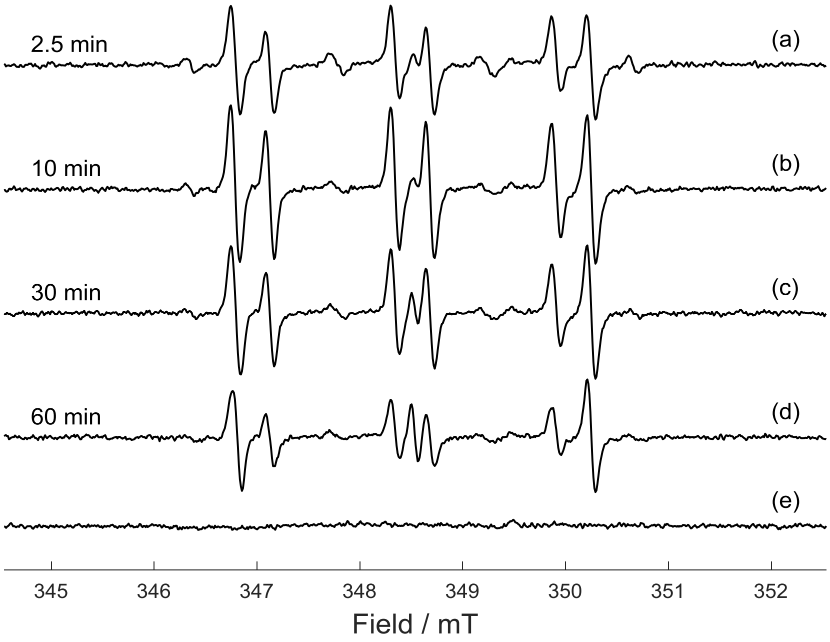

Fig. S4. CW X-band EPR spectra of the MNP spin-adducts showing the regeneration of the deamination radical when the γ-irradiated L-histidine powder (dose = 250 kGy) is dissolved in water with subsequent addition of the MNP spin-trap. The spectra were recorded following MNP (final conc. 40 mM) addition to the solution a) 2.5 mins, b) 10 mins, c) 30 mins and d) 60 mins after dissolution. In all cases the sample solution was transferred using a syringe with a metal needle. The intensity of the spin-adduct signal in d) is *ca*. 50% of the intensity in a). The control X-band EPR spectrum of γ-irradiated L-histidine powder (dose = 250 kGy) dissolved in water containing a phosphate buffer 50 mM at pH 7.2 is shown in e). The EPR parameters used to record the spectra were: time constant 40.96 ms; conversion time 40.96 ms; number of points 1024; number of scans 20; microwave power 20 mW; microwave frequency 9.8 GHz; modulation amplitude 0.1 mT.

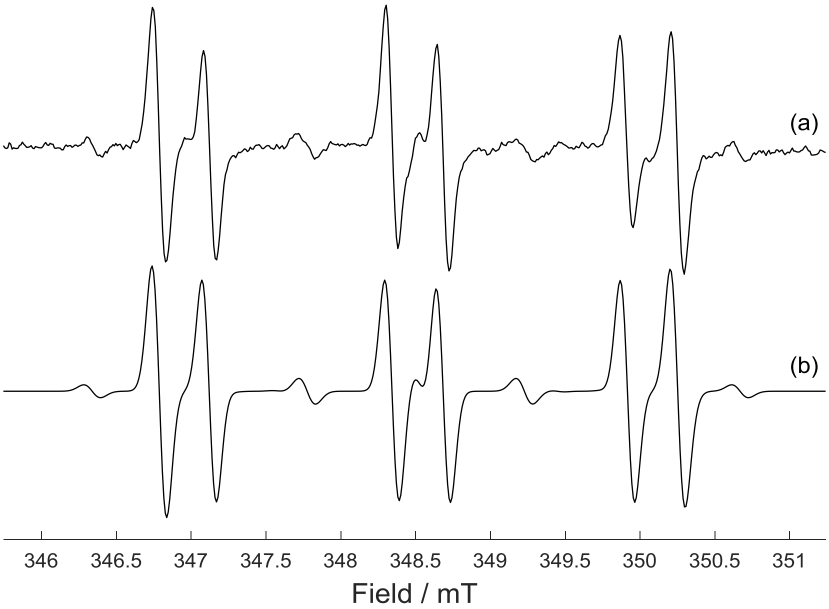

Fig. S5. a) CW X-band EPR spectra of the MNP spin-adducts obtained by dissolution of γ-irradiated L-histidine powder (dose = 250 kGy) in water with MNP (final conc. 40 mM) added 2.5 mins after dissolution and transferred by using a syringe with a metal needle. b) Simulation (performed using EasySpin (Stoll and Schweiger, 2006)) containing contributions from both the deamination radical and the hydroxyl radical spin-adducts, obtained using the parameters reported in Table S1. The EPR parameters used to record the spectra were: time constant 40.96 ms; conversion time 40.96 ms; number of points 1024; number of scans 20; microwave power 20 mW; microwave frequency 9.8 GHz; modulation amplitude 0.1 mT.

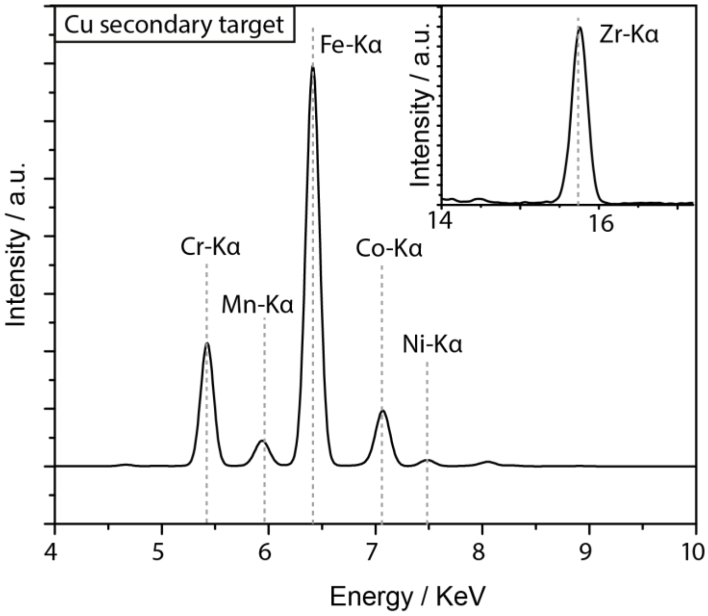

Fig. S6. Background subtracted XRF spectra for the metal needles. Six key metals were identified: Fe-Kα (6.405 KeV); Cr-Kα (5.415 KeV); Co-Kα (6.931 KeV); Ni-Kα (7.480 KeV); Zr-Kα (15.775 KeV) and Mn-Kα (5.900 KeV). Qualitatively, the data suggests that there is significantly higher Fe in the needles than the other metals. The presence of these metals is consistent with the manufacturer information describing the needles as stainless, chromium-nickel steel.

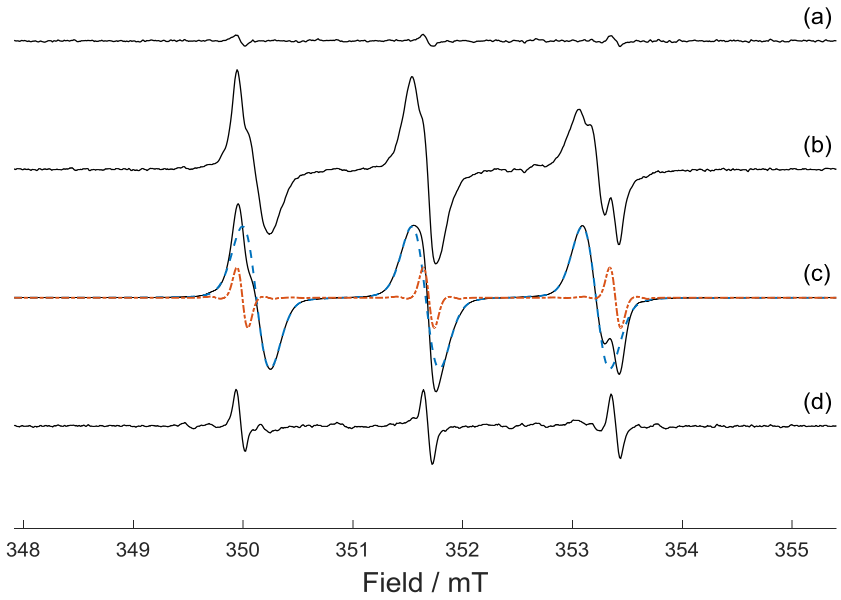

Fig. S7. CW X-band EPR spectra of the MNP spin-adducts obtained by dissolution of the γ-irradiated D-mannitol powder. Irradiation of the powder induces the formation of persistent radical species as previously reported (Karakirova and Yordanov, 2015), which have not yet been identified. When the powder is then dissolved in a MNP solution a broad 3-line spectrum is detected, but the radicals do not appear to regenerate upon delayed addition of spin trap after dissolution in contrast to L-his. a) EPR spectra of the non-irradiated D-mannitol powder dissolved in a (40 mM) MNP solution; b) γ-irradiated powder (dose = 250 kGy) dissolved in MNP (40 mM); c) simulation of the spin adduct signal (using EasySpin (Stoll and Schweiger, 2006)) with contributions from the mannitol-MNP spin-adduct (dashed blue) and DTBN (dashed-dotted red), and were obtained using the parameters reported in Table S2; d) irradiated D-mannitol powder dissolved in water with MNP (final conc. 40 mM) added 3 minutes after dissolution and transferred by using a syringe with a metal needle. The EPR parameters used to record the spectra were: time constant 40.96 ms; conversion time 40.96 ms; number of points 1024; number of scans 60; microwave power 20 mW; microwave frequency 9.8 GHz; modulation amplitude 0.1 mT.

Supplementary Tables

Table S1. EPR hyperfine coupling (mT) and *g*-values for the trapped deaminated L-histidine radical (MNP-his), hydroxyl radical (MNP-OH) and the MNP di-adduct di-*tert*-butyl nitroxide (DTBN) extracted from Fig. S5.

Radical		$a^{\mathrm{N}}_{\mathrm{NO}}$	a^{H}_{α}	$a^{^{13}\mathrm{C}}$	**g-value**
MNP-his	This study	1.57(1)	0.34(1)	-	2.0055(2)
	(Rustgi et al., 1977)	1.55	0.39	-	-[a]
MNP-OH	This study	1.45(1)	1.44(1)	-	2.0054(2)
	(Lai, C-S and Piette, L. H., 1997)	1.44	1.44	-	-[a]
DTBN	This study	1.75(1)	-	0.48(1)	2.0056(2)
	(Rustgi et al., 1977)	1.72	-	-[a]	-[a]

[a] not determined

Table S2. EPR hyperfine coupling (mT) and *g*-value for the trapped D-mannitol radical and the MNP di-adduct di-*tert*-butyl nitroxide (DTBN) extracted from Fig. S7.

Radical		$a^{\mathrm{N}}_{\mathrm{NO}}$	$a^{^{13}\mathrm{C}}$	**g-value**
MNP-Mannitol	This study	1.56(1)	-	2.0054(2)
DTBN	This study	1.71(1)	0.48(1)	2.0053(2)
	(Rustgi et al., 1977)	1.72	-[a]	-[a]

[a] not determined

References

Brown, T.A., 1991. Molecular Biology LabFax. BIOS Scientific Publishers Ltd., Oxford, UK.

Karakirova, Y., Yordanov, N., 2015. Mannitol as a radiation sensitive material for electron paramagnetic resonance dosimetry. Bulg. Chem. Commun. 47, 144–148.

Lai, C-S, Piette, L. H., 1997. Further evidence for OH radical production in Fenton’s reagent. Tetrahedron Lett. 20, 775–778. doi: 10.1016/S0040-4039(01)86036-6

Rustgi, S., Joshi, A., Riesz, P., Friedberg, F., 1977. E.S.R. of spin-trapped radicals in aqueous solutions of amino acids. Reactions of the hydrated electron. Int. J. Radiat. Biol. 32, 533–552. doi: 10.1080/09553007714551321

Stoll, S., Schweiger, A., 2006. EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. J. Magn. Reson. 178, 42–55. doi: 10.1016/j.jmr.2005.08.013