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1 **An Electron Paramagnetic Resonance (EPR) spectroscopy study on**  
2 **the  $\gamma$ -irradiation sterilization of the pharmaceutical excipient**  
3 **L-histidine: regeneration of the radicals in solution**

4  
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16

17 **ABSTRACT**

18 The effects of  $\gamma$ -radiation sterilization on the parenteral excipient L-histidine were analysed by  
19 means of EPR spectroscopy. The irradiation process was found to induce the formation of a  
20 deamination radical which was persistent in the solid state. The nature and reactivity of the radicals  
21 following dissolution in water was evaluated using spin-trapping EPR experiments. The deamination  
22 radical was found to regenerate in solution in the presence of trace metals, potentially leading to  
23 radical induced degradation reactions occurring up to an hour after the dissolution process.  
24 Understanding this process is significant for the improved design of parental pharmaceutical  
25 formulations in which unwanted radical reactions after  $\gamma$ -radiation sterilization could lead to  
26 degradation of active ingredients.

27

28 **Keywords**

29 Electron Paramagnetic Resonance (EPR)

30 Spin-trapping

31 Sterilization

32 Irradiation

33 Dissolution

34 Fenton reaction

35

36 **Chemical compounds studied in this article**

37 L-histidine (PubChem CID: 6274); 2-Methyl-2-nitrosopropane (PubChem CID: 23272);

38

39 **1. Introduction**

40 Radiation sterilization of pharmaceuticals has been studied for over 50 years as a means to allow  
41 sterilization of heat sensitive materials (Gopal, 1978). When applicable, this terminal-sterilization  
42 process is preferable to other methods such as gas sterilization, which is difficult to control, or  
43 aseptic processing, which is a complex and costly procedure (Food and Drug Administration, 2011;  
44 Hasanain et al. 2014). Radiation sterilization is usually carried out with a  $\gamma$ -source primarily due to  
45 the high penetrating power, speed, reliability and facile control of the process (Abuhamoğlu and  
46 Özer, 2010). This irradiation process may produce free radicals (Symons, 1995); these extremely  
47 reactive species frequently promote a number of different chemical reactions, which are difficult to  
48 predict beforehand (Schulman and Achey, 2007). It is therefore necessary to assess the stability of  
49 each irradiated sample and thereby characterize the degradants formed as a result of the  
50 sterilization process (Ambrož et al., 2000; Gibella et al., 2000; Hasanain et al. 2014; Jacobs, 1995,  
51 1985).

52 Excipients are substances other than the pharmacologically active drugs or prodrugs which are  
53 included in the manufacturing process or are contained in a finished pharmaceutical product dosage

54 form (Pikal and Costantino, 2004). These excipients improve the properties of the drug, either by  
55 enhancing the therapeutic effect of the Active Pharmaceutical Ingredients (APIs) or by facilitating the  
56 manufacturing process (García-Arieta, 2014), and are typically the major components in a  
57 pharmaceutical product. Not only could direct degradation of the APIs diminish the action of the  
58 product, but degradation of excipients can also affect the efficacy of the drug either by altering its  
59 chemico-physical properties or by reacting with the APIs. It is therefore crucial to assess the stability  
60 of such components after they undergo industrial processes which could affect their stability.

61 In this work we focus on the amino acid L-histidine (hereafter labelled L-his), an excipient typically  
62 used in parenteral formulations as a buffering agent and a stabilizer for subcutaneous, intramuscular  
63 and peritoneal injections (Kaisheva et al., 2003; Kamerzell et al., 2011). The effects of  $\gamma$ - and  
64 X-irradiation on L-his has been studied by means of Electron Paramagnetic Resonance (EPR)  
65 spectroscopy, which detects specifically paramagnetic species, such as free radicals, with the  
66 unaltered L-his or non-radical degradation products remaining EPR silent (Mangion et al., 2016). The  
67 identity of the main radical species generated by irradiation was confirmed by EPR analysis of both  
68 the L-his powder and the single crystal. The irradiation products of numerous amino acids have been  
69 investigated previously by EPR in the solid state (Aydin, 2010; Dicle et al., 2015; Karabulut and  
70 Yildirim, 2015), but here the reactivities of the radicals following dissolution were also evaluated by  
71 means of spin-trapping EPR experiments (Davies, 2016). While studies involving the spin trapping of  
72 radicals formed in the solid state have been previously reported for several organic compounds,  
73 including amino acids (Kuwabara et al., 1981; Lagercrantz and Forschult, 1968; Makino and Riesz,  
74 1982; Minegishi et al., 1980; Talbi et al., 2004) we are not aware of previous reports of the  
75 regeneration and trapping of amino acid radicals in solution upon addition of the trapping agent  
76 many minutes after dissolution of an irradiated powder.

77

## 78 **2. Material and methods**

79 *2.1.  $\gamma$ -irradiation of powder*

80 L-his free base was purchased from Sigma Aldrich and irradiated in the supplied powder form.

81 Samples were sealed in glass vials and  $\gamma$ -irradiated at room temperature (r.t.) at the Dalton

82 Cumbrian Facility (UK) using a dose rate of approximately 2.3 kGy/h to achieve total doses of either

83 25, 125 or 250 kGy. The samples were exposed to gamma rays emitted from high activity sealed

84 cobalt-60 sources loaded into a model 812 irradiator, supplied by Foss Therapy Services, Inc,

85 California, USA. Absorbed dose rates were determined using a model 2060C radiation detection

86 instrument equipped with ion chamber type 20X60-0.18, supplied by Radcal Corporation, California,

87 USA. The model 2060C instrument was calibrated annually to traceable national or international

88 standards. In addition, routine cross-checks of dose rates were performed using Fricke dosimetry, a

89 widely used chemical method, with an acceptable tolerance of within +/- 5% of the Radcal measured

90 values.

91 *2.2. Single crystal growth and X-irradiation*

92 Single crystals of L-his were grown from a saturated aqueous solution by slow evaporation at r.t.

93 Their structure was determined by single crystal X-ray diffraction on a Rigaku Oxford Diffraction

94 Gemini R instrument and was found to be orthorhombic with the space group  $P2_12_12_1$  ( $a =$

95 5.1480(3) Å,  $b = 7.2330(4)$  Å,  $c = 18.8122(11)$  Å), in agreement with previously published structures

96 (Lehmann et al., 1972; Westhof et al., 1974). X-irradiation of the sample, delivering a total dose of

97 4 kGy, was performed on a Bruker D5005 X-ray powder diffractometer at a dose rate of 0.72 kGy/h.

98 The diffractometer dose rate was calibrated by irradiation of alanine dosimetry pellets (Bruker) and

99 subsequent EPR analysis using a Bruker e-scan Alanine Dosimeter, which has a specified accuracy of

100 better than 1%.

101 *2.3. Spin trapping*

102 Stock solutions of 2-methyl-2-nitrosopropane (hereafter abbreviated to MNP) at 0.8 M or 1.6 M

103 concentrations were prepared using acetonitrile as a solvent for subsequent 1:20 dilution into the

104 aqueous sample solution. In order to investigate the effects of trace metal contamination on the

105 generation of radicals in solution, spin-trapping experiments were performed by dissolving  
106  $\gamma$ -irradiated L-his powder ( $\approx 0.27$  M) either i) in an aqueous solution of MNP; ii) in water, followed by  
107 subsequent addition of MNP (after 3 minutes unless otherwise stated); and iii) in an aqueous  
108 solution of ethylenediaminetetraacetic acid (abbreviated to EDTA) at 1 mM concentration, followed  
109 by the subsequent addition (after 3 mins) of MNP. Samples were inserted into quartz EPR capillaries  
110 (Wilmad-LabGlass 712-SQ-100M) using either a glass Pasteur pipette or a syringe bearing a sterile  
111 stainless, chromium-nickel steel needle (Braun Sterican 4665643; 21 G, 120 mm). EPR spectra were  
112 recorded as described below, with acquisition of the spectra commencing *ca.* 6 mins after the  
113 addition of the spin trapping agent MNP. The free radical 4-hydroxyl-2,2,6,6-tetramethyl-piperidine-  
114 1-oxyl (abbreviated TEMPOL) was used to quantify the spin adduct concentrations, as previously  
115 reported (Barr et al., 2001). Milli-Q water ( $18.2$  M $\Omega$ ·cm) buffered at pH 7.2 with a 50 mM phosphate  
116 buffer was utilised in all of the spin-trapping experiments.

#### 117 *2.4. EPR experiments and simulations*

118 All EPR experiments were performed on a Bruker EMX spectrometer operating at X-band with a  
119 cylindrical cavity (ER 4122 SHQE). Unless otherwise stated experimental parameters were as follows:  
120 modulation amplitude 0.1 mT; conversion time and time constant 40.96 ms; number of scans 20. For  
121 solid state samples the microwave power was 50.7  $\mu$ W at 9.7 GHz and in spin-trapping experiments  
122 (solution-state) the microwave power was 20 mW at 9.8 GHz. EPR simulations were performed with  
123 the Matlab package EasySpin (Stoll and Schweiger, 2006).

#### 124 *2.5. XRF analysis*

125 X-ray fluorescence (XRF) analysis was performed on a Rigaku NEX CG Energy Dispersive(ED)-XRF  
126 instrument, using a Cu secondary target.

127 Additional experimental and simulation details are described in the supplementary material.

128

### 129 **3. Results and discussion**

130 The  $\gamma$ -irradiation of L-his powder induces the formation of a persistent radical species as revealed by  
131 X-band continuous wave (CW) EPR spectroscopy (Fig. 1a). The same radical species is observed  
132 irrespective of the radiation dose or the source of ionising radiation ( $\gamma$ - vs X-ray) (see Fig. S1 and S2  
133 in the supplementary material). The EPR spectrum of the L-his single crystal exposed to X-ray  
134 radiation is shown in Fig. 1b. The main feature of the single crystal spectrum is an eight line pattern  
135 which can be readily attributed to couplings with an  $\alpha$ -proton and two inequivalent  $\beta$ -protons of the  
136 radical. This observation is consistent with the main radical species being the product of  
137 deamination (Scheme 1), as previously reported (Westhof et al., 1974). Weaker additional lines can  
138 also be observed in the spectrum (Fig.1b) suggesting the presence of a second, as yet unidentified,  
139 radical species. Whilst these features are similar in appearance to satellite lines, which are known to  
140 arise from the fraction of radicals containing  $^{13}\text{C}$  ( $I = 1/2$ ), their intensity is inconsistent with the  
141 natural abundance of this isotope. The microwave power saturation behaviour of the powder  
142 sample (Fig. S3) is also indicative of the presence of one or more additional radical species.

143  
144 Dissolution of the irradiated L-his powder was undertaken in order to study the reactivity of the  
145 radical species in solution, and thereby mirror the treatment of excipients in parenteral formulations  
146 reconstituted before injection. Following dissolution of the irradiated powder in water, no EPR signal  
147 was detected (Fig. S4e), as expected for a short-lived carbon centred radical (Ambrož et al., 2000;  
148 Iravani, 2017). However, when the irradiated powder was dissolved in a spin-trap solution of MNP, a  
149 persistent spin-adduct signal was detected with a concentration of *ca.* 0.25  $\mu\text{M}$  (Fig. 2). The  
150 observed 18-line EPR spectrum can be assigned to the coupling of the unpaired electron with the  
151 nitroxidic nitrogen of the spin-trapping agent, along with one  $\alpha$ -proton and two almost equivalent  $\beta$ -  
152 protons of the trapped radical species. The hyperfine couplings extracted by simulation of the  
153 spectra are in good agreement with previous reports in which the deaminated L-his radical was  
154 either formed from  $\gamma$ -irradiation of histidine in the solid (Minegishi et al., 1980) or solution state  
155 (Rustgi et al., 1977) followed by spin trapping in solution with MNP (Table 1). This 18-line signal was

156 not obtained by dissolving the non-irradiated L-his powder in a solution containing the spin-trap  
 157 MNP (Fig. 2a), indicating that the trapped adduct species was indeed formed as a result of the  
 158 irradiation process. The low-intensity three line background signal evident in Fig. 2a was assigned to  
 159 the formation of an MNP di-adduct, di-*tert*-butyl nitroxide (DTBN), which commonly occurs in low  
 160 concentrations with this particular spin-trapping agent (Rustgi et al., 1977).

161

162 **Table 1.** Hyperfine parameters (mT) and *g*-values for the MNP-deaminated L-histidine radical spin-adduct.

		$a_{\text{NO}}^{\text{N}}$	$a_{\alpha}^{\text{H}}$	$a_{\beta 1}^{\text{H}}$	$a_{\beta 2}^{\text{H}}$	$g_{\perp}$	$g_{\parallel}$
This study	$a_{\perp}$	1.442(5)	0.400(5)	0.057(5)	0.048(5)	2.0057(2)	2.0052(2)
	$a_{\parallel}$	1.771(5)	0.333(5)	0.079(5)	0.046(5)		
Previous work (Minegishi et al., 1980)	$a_{\text{iso}}$	1.54	0.41	0.06	0.06	- <sup>a</sup>	- <sup>a</sup>
Previous work (Rustgi et al., 1977)	$a_{\text{iso}}$	1.545	0.392	0.05	0.05	- <sup>a</sup>	- <sup>a</sup>

163 <sup>a</sup> not determined

164 If the irradiated powder is firstly dissolved in water and the spin trap MNP added subsequently (i.e.,  
 165 only after the dissolution of the powder), then the spin-adduct signal of the trapped radical is still  
 166 detectable, at a concentration of *ca.* 0.1  $\mu\text{M}$  (Fig. 3a); due to the lower signal intensity, the  
 167 modulation depth was increased and as a result the  $\beta$ -proton coupling is not resolved. A second,  
 168 though less intense, four line signal with a 1:2:2:1 pattern can also be observed in the spectrum. The  
 169 MNP-histidine spin-adduct has been detected following addition of MNP one hour after dissolution  
 170 of the irradiated powder in water (Fig. S4). Such a long persistence time of the radical in solution is  
 171 inconsistent with the expected reactivity of carbon centred radicals, and is in contrast with our  
 172 inability to directly detect the radical in the absence of a spin-trap which implies a short radical  
 173 lifetime. It is therefore proposed that, rather than invoking long radical lifetimes in solution to  
 174 account for the observed spin adduct signals, the deaminated histidine radical must be regenerated  
 175 in solution after addition of the spin-trap. If for example iron is present in the solution, together with

176 a strong oxidant, Fenton-type reactions may take place leading to the formation of reactive oxygen  
177 species (ROS) (Neyens and Baeyens, 2003). Such species could facilitate the regeneration of the  
178 deaminated histidine radical which is readily and subsequently trapped by MNP at some prolonged  
179 time-interval following dissolution of L-his.

180 To test this hypothesis, irradiated L-his powder was dissolved in an aqueous solution containing the  
181 chelating agent EDTA (1 mM), and MNP was subsequently added to this solution 3 mins after  
182 dissolution of L-his. As shown in Fig. 3b, the EPR signal from the L-his radical-adduct could not be  
183 detected when the chelating agent was present in the solution. In our initial experiments, a syringe  
184 fitted with a sterile metal needle was used to transfer the solution to the quartz capillary for EPR  
185 measurement. However, when a glass pipette was used instead to transfer the sample solution into  
186 the EPR quartz capillary tube, no spin-adducts were detected even in the absence of EDTA (Fig. 3c).  
187 An X-ray fluorescence analysis of the metal needles showed the presence of large amounts of iron,  
188 together with chromium, manganese, nickel and trace levels of other metals (Fig. S6). It therefore  
189 appears that the deaminated radical trapped after dissolution in water and subsequent addition of  
190 MNP is the result of a Fenton-type reaction catalysed by traces of the metals contained in the  
191 syringe needle, which takes place as soon as the needle comes in contact with the sample solution.  
192 Furthermore, such behaviour appears to be characteristic of L-histidine, as other excipients such as  
193 D-mannitol did not show any radical regeneration properties (Fig. S7). This result is of great  
194 significance for the use of irradiation sterilization of excipients in parenteral formulations, for which  
195 the reconstitution process or drug delivery might involve use of similar needles providing sufficient  
196 trace metals for radical regeneration.

197  
198 According to these considerations, the four line EPR spectrum of the previously unidentified radical  
199 species can be attributed to the formation of the MNP-OH spin-adduct (Fig. S5), further supporting  
200 the assertion that Fenton-type reactions are operative. Additionally, the concentration of the MNP-  
201 histidine radical-adduct formed from direct dissolution of the irradiated powder in the spin trap

202 solution was found to be four times higher (*ca.* 1  $\mu\text{M}$ ) when the glass pipette was used in place of  
203 the syringe with metal needle (*ca.* 0.25  $\mu\text{M}$ ). The proposed Fenton-type reactions could also explain  
204 why the concentration of the MNP-His spin-adduct was found to be significantly lower when using  
205 the syringe and needle for sample transfer, as the reactions of the ROS produced might compete  
206 with direct formation of the spin-adduct from the L-his deaminated in the solid state.

207

208 As mentioned earlier, the presence of strong oxidants is required for the Fenton chemistry to occur.  
209 Such oxidants can easily form as a result of the irradiation process. In fact, the primary effect of  
210 exposing L-his to ionising radiations is the ejection of an electron from the molecule itself (Symons,  
211 1995) (Scheme 2). Thus, in addition to the formation of a histidine radical, in the presence of air, the  
212 ejected electron can also combine with molecular oxygen leading to the formation of a superoxide  
213 radical. This reactive oxygen species can in turn lead to the generation of other ROS such as  
214 hydrogen peroxide and histidine hydroperoxides. Irradiation in solution is known to produce amino  
215 acid hydroperoxides from which spin adducts can be trapped on addition of  $\text{Fe}^{2+}$  (Davies et al., 1995;  
216 Gebicki, S., Gebicki, J.M., 1993), but to our knowledge generation of these species by irradiation in  
217 the solid state and survival into solution has not previously been observed. All these species can be  
218 responsible for initiating the observed Fenton chemistry in solution and the consequent production  
219 of additional ROS. The well-known scavenging properties of L-his towards ROS (Foote and Clennan,  
220 1995; Pazos et al., 2006; Wade and Tucker, 1998; Zs.-Nagy and Floyd, 1984) suggest that these  
221 reactive species are quenched by histidine in solution, hence further generating deamination  
222 radicals which are readily trapped by MNP.

#### 223 **4. Conclusions**

224 The effects of irradiation sterilization on the parenteral excipient L-his has been analysed by CW EPR  
225 spectroscopy and spin-trapping. Whilst the identity of the irradiation induced deamination radical  
226 formed has been confirmed previously through both direct analysis of the irradiated solid and spin-  
227 trapping experiments, in this work we have further explored the fate of the irradiation products in

228 solution. As expected upon dissolution of the irradiated powder in a physiological solution, the  
229 radical species were found to have a short lifetime; however, spin-trapping experiments show not  
230 only the formation of C-centred radical adducts, but also the regeneration of radical species long  
231 after the initial dissolution of the irradiated material. Fenton-type chemistry involving strong  
232 oxidants generated during the irradiation process, and catalysed by trace metals from a standard  
233 sterile syringe needle, was implicated in this process.

234 Knowing the behaviour of the reactive degradation products in solution is essential when dealing  
235 with excipients intended for parenteral formulations. Avoiding the regeneration of the radicals in  
236 solution is necessary in order to eliminate the potential for radical-induced degradation of other  
237 drug components, such as APIs in particular, in a complete pharmaceutical formulation.

238 Unintentional injection of free radical containing solutions into patients could also have direct  
239 toxicological implications. Whilst a thorough analysis of each irradiation sterilized product remains  
240 necessary, assessing the effects of  $\gamma$ -irradiation on single drug ingredients is an essential first step  
241 towards the analysis of multi-component systems. We have shown that not only the degradants  
242 formed directly by the irradiation procedure, but also the subsequent products of potentially  
243 complex solution mechanisms, must be taken into account.

244

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251

252

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349 **Figure Captions**

350 **Fig. 1.** a) X-band CW EPR spectrum of L-histidine (L-his) powder after  $\gamma$ -irradiation at r.t. with a total dose of  
351 25 kGy. b) X-band CW EPR spectrum of L-his single crystal with the magnetic field aligned parallel to the *c*-axis  
352 after X-irradiation at r.t. (black) and EasySpin (Stoll and Schweiger, 2006) simulation (dashed red). The EPR  
353 parameters used to record the powder & single crystal spectra respectively were: a) time constant 81.92 ms;  
354 number of points 1024; number of scans 4 and b) time constant 40.96 ms; number of points 2048; number of  
355 scans 20, with other parameters as in section 2.4.

356

357 **Fig. 2.** X-band CW EPR spectra of a) non-irradiated L-his powder dissolved in a spin-trap solution of MNP  
358 (80 mM), b) 250 kGy  $\gamma$ -irradiated L-his powder dissolved in a spin-trap solution of MNP (80 mM), c) simulated  
359 EPR spectrum (obtained using EasySpin (Stoll and Schweiger, 2006)) for a combination of the MNP-his adduct  
360 and DTBN, and d) simulation of DTBN only. The EPR parameters used to record the spin-adduct spectra were  
361 as detailed in section 2.4 with the exception of the lower modulation amplitude of 0.01 mT; the number of  
362 points was 4096.

363

364 **Fig. 3.** X-band CW EPR spectra of L-his powder dissolved in water with a) MNP (final conc. 20 mM) added  
365 3 minutes after dissolution and transferred using a syringe with metal needle; b) aqueous EDTA (1 mM)  
366 followed by processing as described in a); c) same as a) but transferred by using a glass Pasteur pipette. In a)  
367 the low-intensity four line EPR spectrum with a 1:2:2:1 pattern, indicated by ▼, was attributed to the spin-  
368 adduct MNP-OH (see Fig. S5 and Table S1). The EPR parameters used to record the spin-adduct spectra were  
369 as detailed in section 2.4, with number of points 1024.

370

371 **Scheme 1.** Radiolytic deamination of L-his.

372

373 **Scheme 2.** Irradiation of L-his powder with formation of strong oxidants (labelled in blue) involved in the  
374 regeneration of L-his radicals in solution. The ROS produced from the Fenton reaction (in red) are scavenged

375 by L-his, with the consequent formation of L-his radicals readily trapped by MNP to form species detected by  
376 EPR (magenta).