JOURNAL OF MASS SPECTROMETRY
*J. Mass Spectrom.* 2004; **39**: 890–902
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jms.656

JMS

# Liquid chromatographic/electrospray ionization mass spectrometric identification of the oxidation end-products of metformin in aqueous solutions

**F. Collin,[1]* H. Khoury,[1] D. Bonnefont-Rousselot,[2] P. Thérond,[2] A. Legrand,[2] D. Jore[1] and M. Gardès-Albert[1]**

[1] Laboratoire de Chimie Physique, CNRS UMR 8601, Université Paris 5, 75270 Paris, France
[2] Laboratoire de Biochimie Métabolique et Clinique, EA 3617, Université Paris 5, 75270 Paris, France

Received 9 March 2004; Accepted 19 April 2004

**Metformin is an antihyperglycemic drug that exhibits some antioxidant properties. HO•-induced oxidation of metformin was studied in aqueous solution, in both aerated and deaerated conditions. Gamma radiolysis of water was used to generate HO• free radicals, capable of initiating one-electron oxidation of metformin. Oxidation end-products were identified by direct infusion mass spectrometry (MS) and high-performance liquid chromatography/mass spectrometry (HPLC/MS$^n$): for every product, structure elucidation was based on its mass (simple mass spectra confirmed by HPLC/MS). In addition, fragmentation spectra (MS$^2$, MS$^3$ and MS$^4$) and the determination of deuterium–hydrogen exchange sites provided valuable information allowing the complete identification of some of the end-products. At low radiation dose, four products were identified as primary ones, since they result from the direct attack of HO• radicals on metformin. These primary oxidation end-products were identified respectively as hydroperoxide of metformin, covalent dimer of metformin, methylbiguanide and 2-amino-4-imino-5-methyl-1,3,5-triazine. At high radiation dose, seven other products were identified as secondary ones, resulting from the HO•-induced oxidation of the primary end-products. A reaction scheme was postulated for the interpretation of the results.** 

**KEYWORDS:** metformin; oxidative stress; HO free radicals; liquid chromatography/electrospray ionization; mass spectrometry

## INTRODUCTION

Metformin, *N*,*N*-dimethylbiguanide (Fig. 1), is an oral antihyperglycemic agent widely used for the management of type 2 diabetes.[1,2] Previous *in vivo* and *in vitro* studies suggested that this molecule, which is an aminoguanidine-like compound, has other beneficial effects that could be involved in the prevention of diabetic complications. It has been shown that metformin could contribute to the inhibition of the formation of advanced glycation end-products (AGEs),[3,4] to lower systemic methylglyoxal concentrations[5] and to react with $\alpha$-dicarbonyl compounds.[6] In addition, it has been hypothesized that metformin could directly scavenge reactive oxygen species or indirectly act by modulating the intracellular production of superoxide anions,[7] exhibiting antioxidant properties.

Reactive oxygen species (ROS) play an important role in the formation of AGE[8] and, conversely, AGE are involved in the enhancement of cellular oxidative stress.[9] It has been demonstrated that elevated glucose levels induce oxidative stress in diabetes, i.e. an imbalance between the production of oxidant species, particularly radical species, and the antioxidant defences.[10,11] This might partly explain the elevated risk factors towards cardiovascular complications in diabetic patients.[12,13]

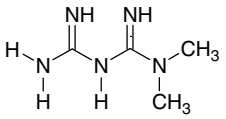


**Figure 1.** Structure of metformin (*N*,*N*-dimethylbiguanide).

Gamma radiolysis is a powerful tool for the modeling of oxidative stress because it allows ROS to be generated in solution, with known radiolytic yields for every species[14] (the radiolytic yield is the number of free radicals produced per unit of energy absorbed, i.e. per joule). This method can be used to initiate the one-electron oxidation (via HO• free radicals) of bio-organic substrates (see, for example, Refs 15 and 16). Among the ROS generated by water radiolysis (i.e. HO•, $O_2^{\bullet-}$ and $H_2O_2$), the hydroxyl radical is the most reactive species. Indeed, it possesses a high oxidation potential of $E'^{\circ}(HO^{\bullet}/H_2O) = 2.3$ V at pH 7.0[14] and reacts on numerous biomolecules

*Correspondence to: F. Collin, Laboratoire de Chimie Physique, CNRS UMR 8601, Université Paris 5, 45 rue des Saints Pères, 75270 Paris cedex 06, France. E-mail: fabrice.collin@univ-paris5.fr
Contract/grant sponsor: Merck Laboratories.

with high rate constants (usually $10^9$–$10^{10}$ l mol$^{-1}$ s$^{-1}$ (Ref. 17)).

Since metformin has been shown to increase the antioxidant status in patients treated for type 2 diabetes, it could act as an antioxidant, that is a reducing agent, by reacting with ROS and leading to metformin-oxidized products. Hence this work was aimed at studying how metformin could be oxidized *in vitro* by HO$^\bullet$ free radicals produced by water radiolysis and at identifying the oxidation products by using high-performance liquid chromatography coupled with mass spectrometry (HPLC/MS$^n$).

## EXPERIMENTAL

### Aqueous solutions

Metformin hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). All aqueous solutions of metformin ($M$ = 129 g mol$^{-1}$) were prepared at a concentration of 200 μmol l$^{-1}$ (pH 6.8) with ultra-pure water (resistivity 18 MΩ, Maxima Ultra Pure Water, ELGA). Methylbiguanide ($M$ = 115 g mol$^{-1}$) was synthesized by Merck-Lipha (Lyon, France) and aqueous solutions (50 μmol l$^{-1}$) were prepared as described previously for metformin.

### Generation of HO$^\bullet$ free radicals by water gamma radiolysis

Gamma irradiations were performed with an IBL 637 irradiator (CIS Biointernational, Gif-Sur-Yvette, France), using a cesium 137 $\gamma$-ray source whose activity was ~222 TBq (6000 Ci). The main advantage of gamma radiolysis is that it allows the modulation of the total amount of reactive oxygen species produced by increasing or decreasing the radiation dose (expressed in Gy; 1 Gy = 1 J kg$^{-1}$), i.e. by selecting the time during which the sample is exposed to the $^{137}$Cs gamma ray source: the longer the exposure, the higher is the radiation dose. It can be noted that for dilute solutions, no direct interaction of $\gamma$-radiation with the molecule under focus occurs and the radiolytic effect is due only to the radical species produced by water radiolysis (i.e. HO$^\bullet$, $e^-_{aq}$ and H$^\bullet$).[14] The dosimetry was determined by Fricke and Morse's method[18] and the dose rate was found to be 9.76 Gy min$^{-1}$ in our experiments.

In the presence of oxygen, i.e. for aerated solutions, water radiolysis leads to the generation of both hydroxyl (HO$^\bullet$) and superoxide ($O_2^{\bullet-}$) free radicals ($O_2^{\bullet-}$ coming from the scavenging of $e^-_{aq}$ and H$^\bullet$ by $O_2$), with formation yields of $2.8 \times 10^{-7}$ and $3.4 \times 10^{-7}$ mol J$^{-1}$, respectively.[14] Bonnefont-Rousselot *et al*. have shown that metformin is not able to scavenge superoxide free radicals *in vitro*.[7] As a consequence, hydroxyl radicals are the only radical species initiating the oxidation process. In the absence of oxygen, i.e. for deaerated solutions saturated with $N_2O$ gas, hydroxyl radicals HO$^\bullet$ are generated with a formation yield of $5.6 \times 10^{-7}$ mol J$^{-1}$, that is, twice the generation yield obtained under aerated conditions, because of the transformation of $e^-_{aq}$ free radicals into hydroxyl radicals.[14] In this work, both aerated and deaerated solutions were irradiated and analyzed in order to compare the oxidation end-products created.

Depending on the radiation dose, the oxidation is called primary or secondary: at a radiation dose below 50 Gy, oxidation products are primary, i.e. result from a direct HO$^\bullet$-induced oxidation of metformin (MTF) in solution. At high radiation dose (e.g. at 300 Gy), other products can be detected because they are derived from the disappearance of the primary end-products, which are attacked by HO$^\bullet$ free radicals. Hence the oxidation of the primary oxidation end-products gives rise to secondary end-products. By analyzing solutions of MTF irradiated at increasing radiation doses, identified oxidation end-products can be classified as primary or secondary.

For irradiation under aerated conditions, 5 ml of solution were introduced into a test-tube in order to be directly irradiated. To remove oxygen in solution completely, and thus to work under deaerated conditions, 5 ml of MTF solution were introduced into a special test-tube, closed at the top and equipped with two thin glass tubes allowing bubbling of the solution with any gas. $N_2O$ gas was used and the solution was bubbled for 1 h at a flow-rate of ~1 ml min$^{-1}$. The test-tube was then hermetically closed until analysis, in order to avoid any contact of the solution with the oxygen present in the air before or after irradiation.

Volumes of 5 ml of a non-irradiated solution were systematically taken as a control for each experiment. Prior to each set of experiments, glassware was carefully washed with TFD4 soap (Franklab, France), rinsed with ultra-pure water (resistivity 18 MΩ, Maxima Ultra Pure Water, ELGA) and finally heated at 400 °C for 4 h to avoid any pollution by organic compounds.

### Analyses

Mass spectrometry was performed on an ion-trap mass spectrometer (LCQ Advantage, Thermofinnigan, Les Ulis, France) equipped with an electrospray ionization (ESI) source. Irradiated aqueous solutions of metformin were diluted by 10:1 in methanol prior to being infused continuously into the ESI source with an SGE 250 μl syringe at a flow-rate of 12.5 μl min$^{-1}$. Experiments performed at lower pHs by adding formic acid solution (1% of the total volume) did not provide any gain in peak intensity. The capillary temperature was held at 250 °C and the relative sheath and auxiliary gas flow-rates were set at 20 and 5, respectively (sheath gas, 0–100 units corresponds to 0–1.5 l min$^{-1}$; auxiliary gas, 0–60 units corresponds to 0–18 l min$^{-1}$). Other parameters, such as lens or capillary voltages, were tuned systematically to obtain the best signal intensities for each ion of interest. All experiments were performed in the positive-ion mode, and each spectrum was typically an average of 15 acquired scans. For MS/MS experiments, a typical isolation width of 1 Da was used.

In addition, some experiments were conducted by coupling a high-performance liquid chromatograph (Surveyor, Thermoquest, Les Ulis, France) to the mass spectrometer. Volumes of 50 μl of the sample were injected in the column, which was a 250 × 4.6 mm i.d. Atlantis dC18 (5 μm) reversed-phase column (Waters, St. Quentin en Yvelines, France) maintained at 40 °C, and eluted with 100% ammonium acetate buffer (10 mmol l$^{-1}$), pumped at a flow-rate of 500 μl min$^{-1}$. Prior to the mass spectrometer, the eluate was detected with a photodiode-array UV–visible detector

(Thermoquest Surveyor, PDA detector), working in both the scan mode (between 200 and 400 nm) and single-wavelength mode at 232 nm, previously identified as a typical wavelength for metformin.[19]

In order to complete these experiments, samples of oxidized MTF were evaporated to dryness, then rediluted in the labelled medium $D_2O$–MeOD (1 : 2, v/v); deuterium exchanges were observed for both MTF and oxidation products. Deuterium oxide and deuterated methanol have minimum isotopic purities of 99.96 and 99.5% D, respectively, and were purchased from Aldrich Chemical (Milwaukee, WI, USA).

An experiment was also carried out in order to characterize the presence of hydroperoxide in solution. The method is based on HPLC analysis with both spectrometric and spectrofluorimetric detection as developed by Thérond *et al*.[20] The operating conditions were as following: isocratic running with an eluent consisting of MeOH and 10 mmol $l^{-1}$ ammonium acetate (96 : 4, v/v), flow-rate 1.2 ml $min^{-1}$ and injection volume 200 µl. Two columns were used: a 25 cm reversed-phase $C_{18}$ followed by a 15 cm reversed-phase $C_8$ (both of 4.6 mm i.d.), maintained at 40 °C. Hydroperoxides were specifically quantified by chemical luminescence, after reaction with a microperoxidase.

## RESULTS AND DISCUSSION

### ESI-MS study of non-oxidized metformin

To obtain the mass spectra of non-oxidized MTF, under ESI in the positive mode (ESI+), its fragmentation pathway was determined. Collision-induced dissociation (CID) spectra were acquired in both $H_2O$–MeOH (Fig. 2(a)) and $D_2O$–MeOD (Fig. 2(b)) media. The parent ion of MTF was detected at $m/z$ 130.2, as protonated MTF. MTF has two $pK_a$ values at 2.8 and 11.5,[21] and therefore occurs as a monoprotonated ion at pH 7, which facilitates its ionization into the ESI source. The fragmentation of $m/z$ 130.2 shows four ions detected at $m/z$ 113.1, 88.3, 85.1 and 60.1 (Fig. 2(a)), along with their equivalent labelled ions at $m/z$ 116.0, 92.3, 90.0 and 66.1 (Fig. 2(b)). These ions could come from the fragmentation of MTF by the breaking of every carbon–nitrogen single bond (except those of the terminal *N*-methyl groups). An ion at $m/z$ 71, previously found by Heinig and Bucheli to be a specific fragment of MTF and used for its quantitation in plasma samples,[22] was detected, probably owing to different experimental conditions.

CID spectra of MTF were also acquired at increasing collision energies, from 15 to 27% (units as given by the manufacturer; 0–100% relative collision energy corresponds to 0 to −100 V multiple offset voltage for positive ions), and the abundances of each detected fragment were plotted as a function of the collision energy (data not shown). When the collision energy increases, the abundance of $m/z$ 130.2 (protonated MTF) decreases and the fragments increase. Based on these experimental data, a fragmentation scheme is proposed in Fig. 3, which includes detected ions for MTF fragmentation in both $H_2O$–MeOH and $D_2O$–MeOD media (ions in parentheses).

The most abundant ion is produced at low collision energy and detected at $m/z$ 60.1 (data not shown). Another fragment starts to appear at low collision energy and is detected at $m/z$ 85.1. The two other fragments appear at higher relative collision energies, above 20% for $m/z$ 113.1 and 25% for $m/z$ 88.3. These ions result from the fragmentation of a carbon–nitrogen bond located in the α-position of one of the imino groups of MTF (Fig. 3, pathways a and b). The ions at $m/z$ 60.1 and 85.1 are shifted up in mass by 6 and 5 units respectively, when the CID spectrum is acquired in $D_2O$–MeOD medium, indicating the number of H–D exchange sites for each ion and thus confirming the structures postulated for these two ions (H–D exchanges only occur on acidic hydrogens, i.e. those of amino or imino groups).

The two other detected fragments, at $m/z$ 88.3 and 113.1, result from the same fragmentation pattern applied to the second imino group (Fig. 3, pathways c and d). The structures proposed for these two ions are confirmed by the shift in

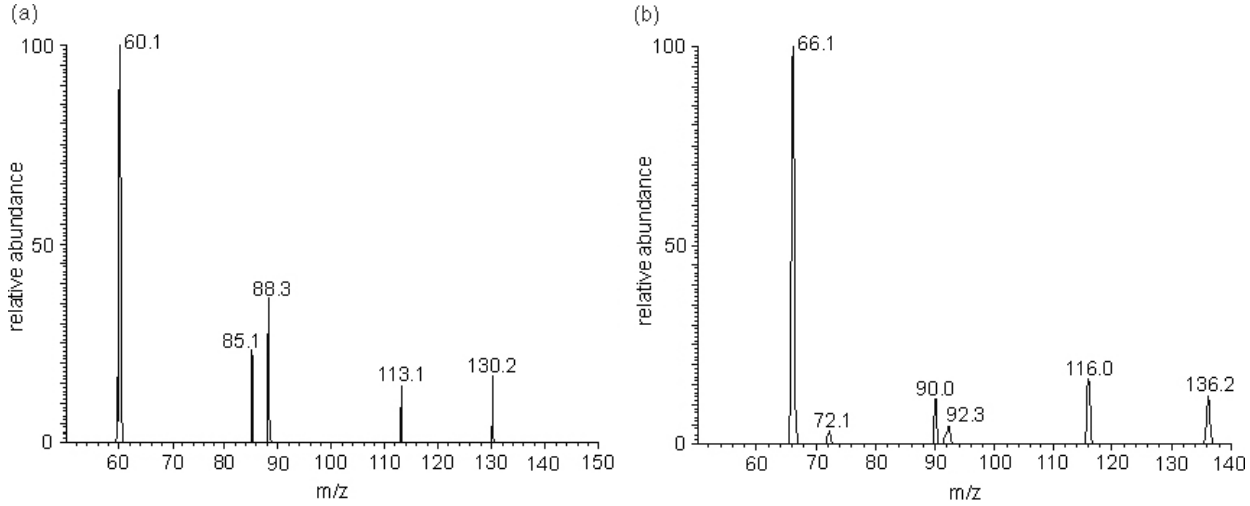



**Figure 2.** (a) CID spectrum of MTF (200 µmol $l^{-1}$) in $H_2O$–MeOH (1 : 10) at 33% collision energy and (b) CID spectrum of MTF (200 µmol $l^{-1}$) in $D_2O$–MeOD (1 : 1) at 28% collision energy. Collision energies are as given by the manufacturer. Ordinate, relative abundance; abscissa, mass/charge ratio (*m/z*).

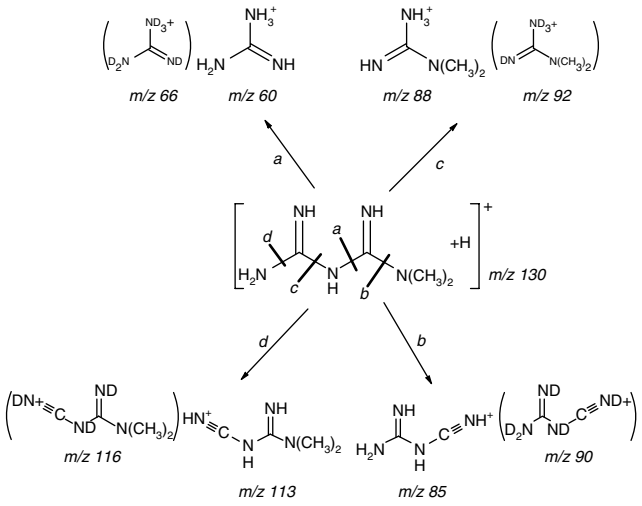



**Figure 3.** Proposed scheme for the fragmentation of the protonated MTF at *m/z* 130 (ions in parentheses are labelled equivalent fragments detected in $D_2O$–MeOD).

mass of 3 and 4 units for $m/z$ 113.1 and $m/z$ 88.3, respectively, when the CID spectrum is acquired in $D_2O$–MeOD medium.

## MS and HPLC/MS study of oxidized metformin

Hydroxyl radicals can initiate three types of oxidizing reactions: abstraction of an H-atom, abstraction of an electron (charge transfer) or addition to double bonds.[23] MTF has many reaction sites towards the oxidation of $HO^{\bullet}$ radical: two carbon–nitrogen double bonds, five electronic doublets and six H-atoms on two methyl groups. Hence the potential structures of oxidation products are difficult to predict. As a consequence, the first approach in this work was to study the MS of an aerated oxidized (300 Gy) aqueous solution of MTF (200 μmol $l^{-1}$) by infusing directly a dilute sample ($H_2O$–MeOD, 1 : 10, v/v) into the ESI source and acquiring a full mass spectrum from 50 to 180 Da (Fig. 4(a)). Six ions are present in this spectrum: the $[M + H]^+$ protonated MTF at $m/z$ 130.1, along with the isotopic contribution of $^{13}C$ at $m/z$ 131.1, and four others at $m/z$ 116.1, 126.1, 144.1 and 162.0. No peaks were detected above 180 Da.

Experiments were also carried out on deaerated oxidized (400 Gy) aqueous solutions of MTF (200 μmol $l^{-1}$) and the mass spectrum between 50 and 400 Da allowed the detection of many ions (Fig. 4(b)). Among them, some are of interest *a priori*: the ion at $m/z$ 384.1, along with its equivalent doubly protonated ion at $m/z$ 192.5, whose mass is close to the mass of three molecules of MTF ($3 \times 129 = 387$); the ion detected at $m/z$ 257.1, along with its equivalent doubly protonated ion at $m/z$ 129.1, whose mass is close to the mass of two molecules of MTF ($2 \times 129 = 258$); the ion at $m/z$ 116.1 that has been identified under aerated conditions; and the ion at $m/z$ 130.1, which is the protonated MTF. Above 400 Da, no peaks were detected.

These results were taken as a base in the identification of the oxidation products by HPLC/MS. In addition, all masses from 50 to 400 Da were sought step by step (1 Da). Figure 5 shows trace chromatograms obtained for an aerated (Fig. 5(a) and (b)) and a deaerated (Fig. 5(c) and (d)) solution of MTF (200 μmol $l^{-1}$), oxidized at 50 and 300 Gy, for every mass of detected ions.

For a solution of MTF oxidized at 300 Gy under aerated conditions (Fig. 5(b)), previous identifications are confirmed ($m/z$ 162.1, 126.1 and 116.1), except for $m/z$ 144.1. New ions are also detected at $m/z$ 148.1, 112.1 and 102.1. It appears that two products have the same mass ($m/z$ 126.1) but have significantly different retention times (5.4 and 8.6 min), indicating a possible different molecular structure. The same results are observed for $m/z$ 112.1, detected at two retention times (7.1 and 13.5 min). When MTF is oxidized at 50 Gy (low radiation dose), the chromatogram (Fig. 5(a)) shows peaks corresponding only to the ions at $m/z$ 130.1, 162.1, 126.1 and 116.1: they correspond to the primary oxidation products of MTF (i.e. resulting from a direct attack of $HO^{\bullet}$ radicals on MTF), since they are detected at low radiation dose (50 Gy). For the other products (not detected below 50 Gy, but present at 300 Gy), they are secondary ones because they are produced by the $HO^{\bullet}$-induced oxidation of the primary

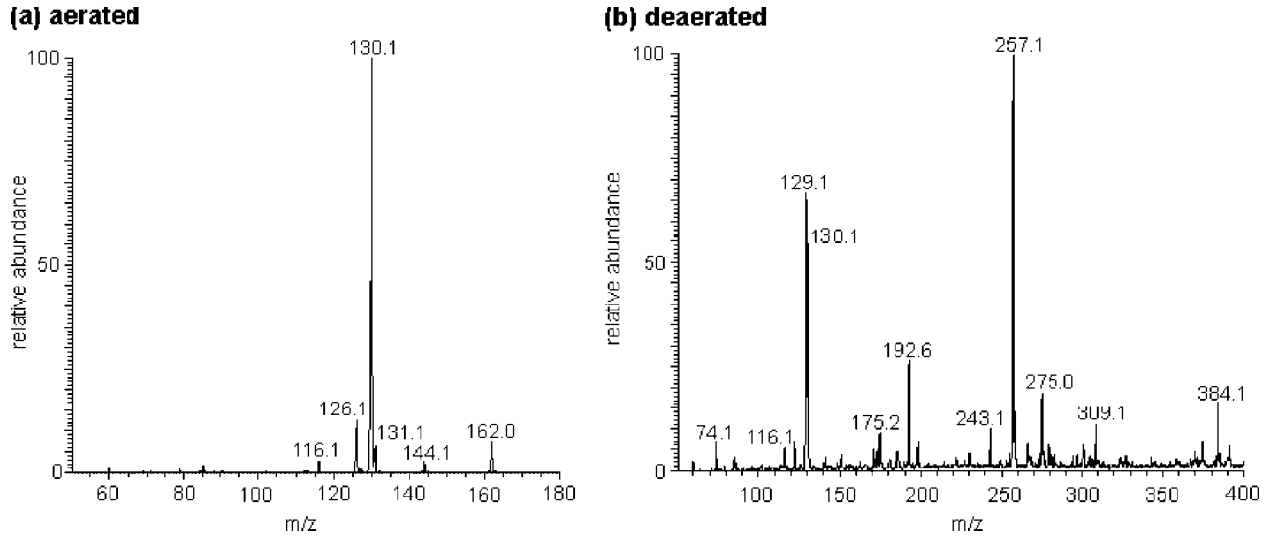



**Figure 4.** Full mass spectra of an aqueous solution of MTF (200 μmol $l^{-1}$), oxidized (a) at 300 Gy under aerated conditions and (b) at 400 Gy under deaerated conditions. Abscissa, mass/charge ratio (*m/z*); ordinate, relative abundance.

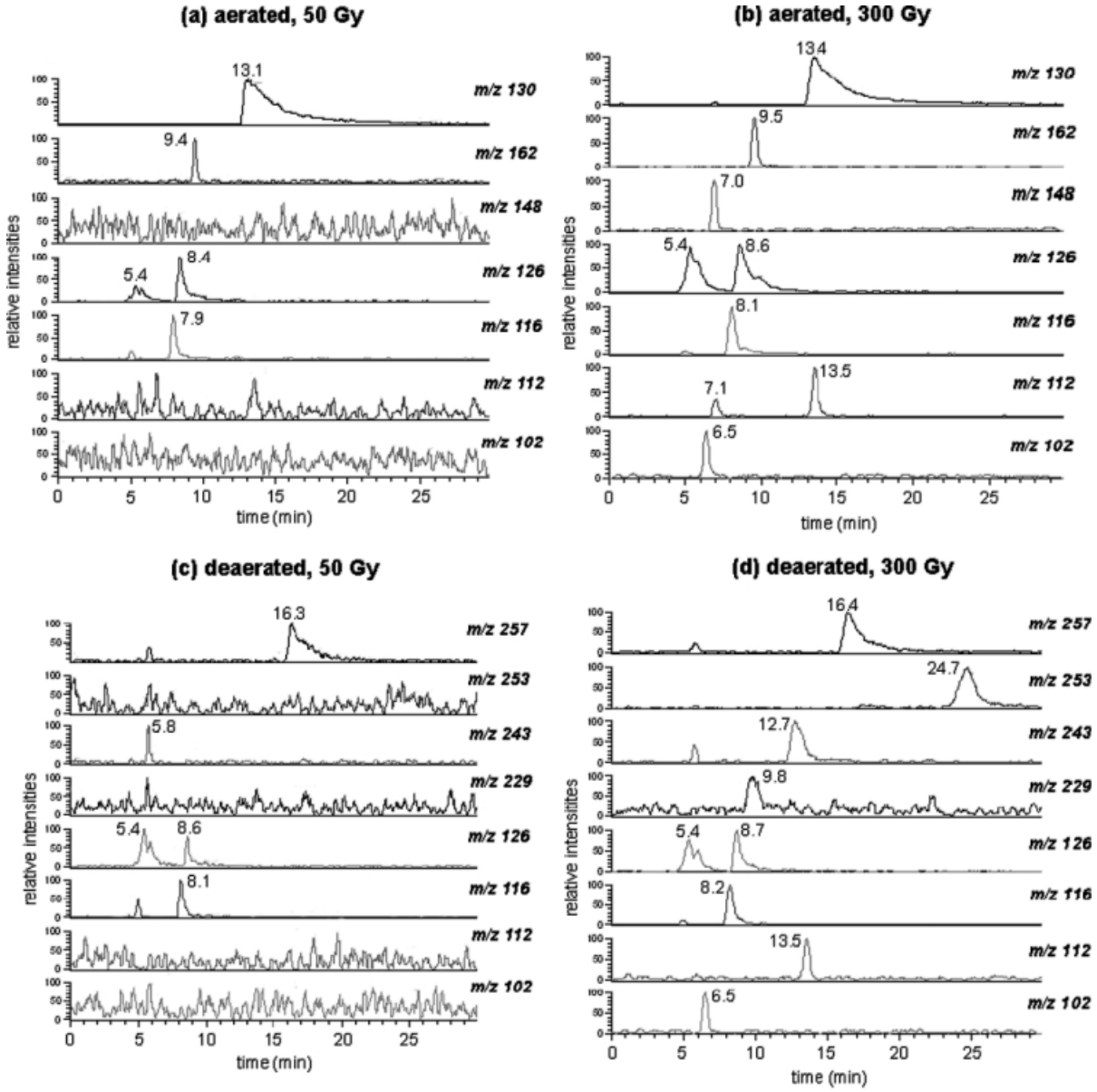



**Figure 5.** Aqueous solution of MTF (200 μmol $l^{-1}$), oxidized under aerated conditions at (a) 50 and (b) 300, and under deaerated conditions at (c) 50 and (d) 300 Gy. Trace chromatograms for each detected ion. —Abscissa, retention time; ordinate, relative intensity.

oxidation end-products of MTF when they are sufficiently accumulated.

Under deaerated conditions, for a solution of MTF oxidized at 300 Gy (Fig. 5(d)), some of the ions previously detected by full MS infusion are confirmed ($m/z$ 257.1, 243.0 and 116.1) and new ions are found at $m/z$ 253.1, 229.1, 126.1, 112.1 and 102.1. By comparing the results obtained at high (Fig. 5(d)) and low radiation doses (Fig. 5(c)), as done previously, three products are found to be primary ($m/z$ 257.1, 126.1 and 116.1) and five secondary ($m/z$ 253.3, 243.0, 229.1, 112.1 and 102.1).

Table 1 summarizes all the masses of the ions detected for a solution of MTF oxidized at 300 Gy, along with their retention times and a possible identification for each one. The first ion at $m/z$ 257.1 could result from a covalent dimerization of MTF (H-abstraction on two molecules of MTF, leading to two MTF radicals, and formation of a covalent bond between these two radicals). The second ion at $m/z$ 162.1 corresponds to a formal addition of molecular oxygen to MTF ($162 = 130 + 32$). As oxygen is known to react rapidly with a carbon-centered free radical leading to peroxyl free radicals and finally to hydroperoxides ($k \approx 10^8$–$10^9$ l $mol^{-1}$ $s^{-1}$ (Ref. 24)), this ion could be assumed to be a protonated hydroperoxide of MTF. The ion detected at $m/z$ 126.1 corresponds to a loss of 4 Da in relation to MTF. There are not many possibilities for such a very small loss, and, in a first approach, it could be the result of two losses of molecular dihydrogen. The last primary product, detected at $m/z$ 116.1, could come from a loss of a formal $CH_2$ group from MTF.

As previously described, the oxidation of MTF seems to lead to four primary oxidation end-products, generated by four different reactions: a covalent dimerization, a peroxidation, a demethylation and a double dehydrogenation.

**Table 1.** Oxidation products of MTF identified by HPLC/MS, in both aerated and deaerated conditions: products are sorted according to whether they result from a primary (low radiation dose) or a secondary oxidation (high radiation dose), and based on a comparison of their masses with a reference parent mass, a possible identification is proposed

| Parent ion (Da) | (A)erated or (D)eaerated | $t_r$ (min) | (P)rimary or (S)econdary | Difference in mass/MTF[a] | Possible identification |
|---|---|---|---|---|---|
| 257.1 | D | 16,4 | P | $[2 \times (MTF - 1) + 1]$ | $[MTF - MTF + H]^+$ covalent dimer of MTF |
| 162.0 | A | 9,5 | P | $[MTF + 1] + 32$ | $[MTFOOH + H]^+$ hydroperoxide of MTF |
| 126.1 | Both | 5,4 and 8,6 | P | $[MTF + 1] - 4$ | $[MTF - 2H_2 + H]^+$ |
| 116.1 | Both | 8,1 | P | $[MTF + 1] - 14$ | $[MTF - CH_2 + H]^+$ methylbiguanide (MBG) |
| 384.1 | D | ??? | S | $[3 \times (MTF - 1) - 1 + 1]$ | $[MTF - MTF - MTF + H]^+$ covalent trimer of MTF |
| 253.1 | D | 24,7 | S | $257 - 4$ | $[MTF - MTF - 2H_2 + H]^+$ |
| 243.1 | D | 12,7 | S | $257 - 14$ | $[MTF - MTF - CH_2 + H]^+$ |
| 229.1 | D | 9,8 | S | $[2 \times (115 - 1) + 1]$ | $[MBG - MBG + H]^+$ covalent dimer of MBG |
| 148.1 | A | 7,0 | S | $116 + 32$ | $[MBGOOH + H]^+$ hydroperoxide of MBG |
| 112.1 | Both | 7,1 and 13,5 | S | $116 - 4$ | $[MBG - 2H_2 + H]^+$ |
| 102.1 | Both | 6,5 | S | $116 - 14$ | $[MBG - CH_2 + H]^+$ biguanide (BGN) |

[a] MTF: 129 g mol$^{-1}$; protonated molecule $[M + H]^+$ detected at $m/z$ 130.2. MBG: 115 g mol$^{-1}$; protonated molecule $[M + H]^+$ detected at $m/z$ 116.1.

The formation of the secondary oxidation products that have been detected in this study (Table 1, bottom) can be explained by a similar reaction scheme, applied to two primary oxidation products ($m/z$ 116.1 and 257.1). Indeed, ions at $m/z$ 229.1, 148.1, 112.1 and 102.1 could be generated by covalent dimerization, peroxidation, double dehydrogenation and demethylation of methylbiguanide (MBG, $m/z$ 116.1), respectively. Likewise, ions detected at $m/z$ 253.3 and 243.0 could come from covalent dimers of MTF ($m/z$ 257.1) via a double dehydrogenation and a demethylation, respectively. These results give a first approach to the behavior of metformin when oxidized by $HO^\bullet$ free radicals.

## Identification of *m/z* 257.1

Figure 6 shows the mass spectra of an aqueous solution of MTF oxidized at 300 Gy under deaerated conditions (aqueous solution saturated with $N_2O$). The full mass spectrum (Fig. 6(a)) exhibits two major peaks at $m/z$ 257.1 and 129.2. If the first one could be assumed to correspond to the monoprotonated diMTF, the second one could be the doubly protonated ion $((257 + 2)/2 = 129)$. The other ions present in this spectrum ($m/z$ 116.1, 126.1, 243.0 and 253.3) have been previously identified as potential oxidation products of MTF under deaerated conditions (see Fig. 5). Full mass spectrum of oxidized MTF acquired in the labeled medium, $D_2O$–MeOD (50 : 50, v/v), shows a single peak detected at $m/z$ 268.1 (Fig. 6(a), inset), indicating that diMTF has exchanged 11 times its acidic hydrogen atoms with deuterium, meaning that diMTF contains 11 exchange sites.

There are only three possibilities for diMTF to be generated from two molecules of MTF by abstracting two hydrogen atoms and creating a covalent bond: the

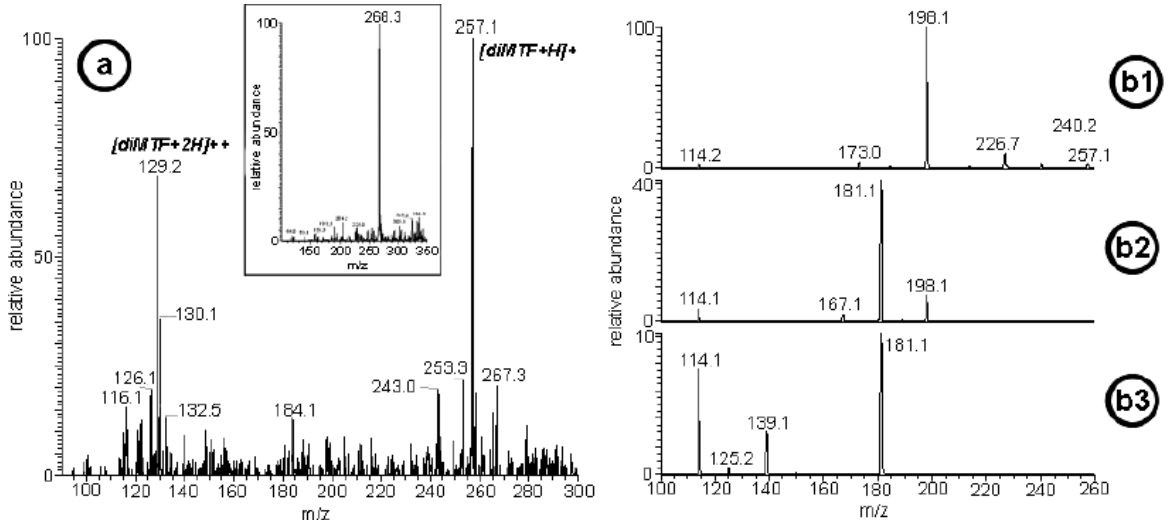



**Figure 6.** Mass spectra of an aqueous solution of MTF (200 μmol l$^{-1}$), oxidized at 300 Gy, under deaerated conditions ($N_2O$ saturated): (a) full mass spectrum in $H_2O$–MeOH and (inset) $D_2O$–MeOD, and (b) CID spectra of (b1) $m/z$ 257 ($MS^2$ at 25% collision energy), (b2) $m/z$ 198.1 ($MS^3$ at 30% collision energy) and (b3) $m/z$ 181.1 ($MS^4$ at 30% collision energy). Abscissa, mass/charge ratio ($m/z$); ordinate, relative abundance. diMTF = covalent dimer of metformin.

first is to create a carbon–carbon bond, the second a nitrogen–nitrogen bond and the third a carbon–nitrogen bond. Since deuterium only exchanges with acid hydrogen, i.e. hydrogens of the amino and imino functional groups of MTF, this result (Fig. 6(a) and inset) demonstrates that the formation of diMTF involves the creation of a carbon–carbon single bond. Indeed, MTF possesses five exchange sites, diMTF has 10 potential exchange sites (plus one for the protonation) and is detected at $m/z$ 268.1 ($268.1 = 257.1 + 10 + 1$). Hence the molecular structure shown in Fig. 7 is proposed for diMTF.

CID spectra of $m/z$ 257.1 are presented in Fig. 6(b) and confirm this proposal. First, on fragmenting the ion of $m/z$ 257.1 ($MS^2$, Fig. 6(b1)), two losses of 17 and 42 Da are observed, corresponding to the transitions 257.1 → 240.2 and 240.2 → 198.1 respectively. They come from the fragmentation of the non-methylated part of MTF, as previously proposed in Fig. 3 (pathways c and d). Fragmentation of $m/z$ 198.1 ($MS^3$, Fig. 6(b2)) and $m/z$ 181.1 ($MS^4$, Fig. 6(b3)) leads to the same losses of 17 Da (198.1 → 181.1) and 42 Da (181.1 → 139.1). Hence the fragmentation pathway proposed for MTF can be applied twice to the ion of $m/z$ 257.1, confirming that this ion contains two molecules of MTF. However, the loss of 45 Da, corresponding to the neutral fragment $(CH_3)_2NH$ and taking place for MTF (Fig. 3, pathway b), is not observed on fragmenting $m/z$ 257.1. This shows that diMTF cannot be fragmented to lose the same neutral fragment $(CH_3)_2NH$ as MTF because the dimethylamino group is involved in the formation of diMTF, since a carbon–carbon bond has been created.

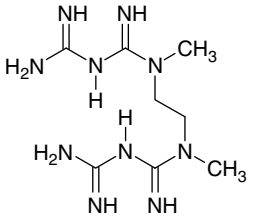



**Figure 7.** Proposed structure of the covalent dimer of metformin.

## Identification of *m/z* 162.1

MS of the HPLC peak detected at a retention time $t_r = 9.54$ min is presented in Fig. 8. The base peak of this spectrum is detected at $m/z$ 162.1 and could correspond to the ion $[M + H]^+$ of the protonated molecule, previously proposed (in Table 1), i.e. a hydroperoxide of MTF (MTFOOH). The peak at $m/z$ 163.0 ($[M + H + 1]^+$) is due to the natural contribution of $^{13}C$ (1%) and $^{15}N$ (0.4%) and accounts for about 6% of the abundance of the parent ion: MTFOOH contains four carbon atoms and five nitrogen atoms ($4 \times 1\% + 5 \times 0.4\% = 6\%$).

This spectrum shows that $m/z$ 162.1 is easily fragmented because three other peaks are simultaneously detected. The two first are at $m/z$ 144.1 and 126.1. The transitions 162.1 → 144.1 and 144.1 → 126.1 involve the same neutral loss (18 Da), which could correspond to the mass of water. Hence this double loss of water implies the presence of two atoms of oxygen for the ion of $m/z$ 162.1. The last peak of this spectrum (at $m/z$ 60.0) has been previously detected for the fragmentation of authentic MTF (Fig. 3, pathway a) and has been shown to be present at low collision energies. The other fragments involved in the fragmentation of MTF (see Fig. 2(a)) are not detected for MTFOOH because they need relatively high collision energies to be generated and the experiment described in Fig. 8 present a full mass spectrum where only in-source fragmentation could occur.

The molecular structure shown in Fig. 9 is proposed for the oxidation product detected at $m/z$ 162.1.

To verify this assumption, an aqueous solution of MTF (200 μmol $l^{-1}$), irradiated at 300 Gy in aerated medium, was diluted twofold with $D_2O$–MeOD (50 : 50, v/v) and introduced directly into the mass spectrometer (infusion). The full mass spectrum thus obtained (Fig. 8(b)) shows a statistical distribution of H–D exchanges on MTFOOH. It was not

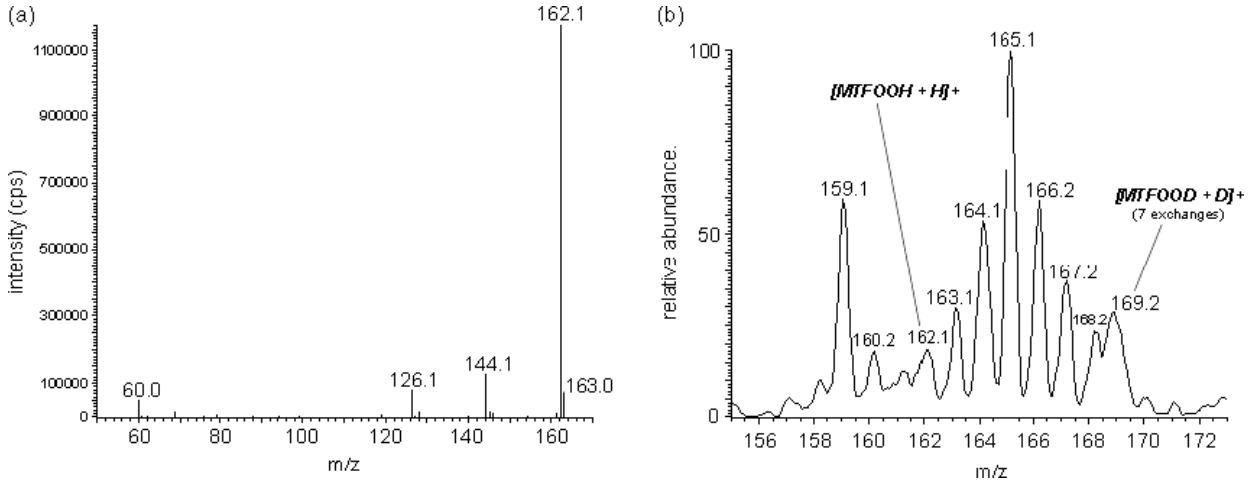



**Figure 8.** Mass spectra of an aqueous solution of MTF (200 μmol $l^{-1}$), oxidized at 300 Gy, under aerated conditions. (a) Full mass spectrum of the HPLC peak detected at $t_r = 9.5$ min. Abscissa, mass/charge ratio (*m/z*); ordinate, absolute intensity (cps). (b) Full mass spectrum of the solution diluted in $D_2O$–MeOD (1 : 1) and analyzed with direct infusion. Abscissa, mass/charge ratio (*m/z*); ordinate, relative abundance.

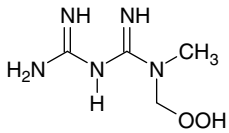



**Figure 9.** Structure of hydroperoxometformin (MTFOOH).

possible to obtain a single peak corresponding to the highest number of exchanges because the solution still contains $H_2O$: to remove it, and thus avoid possible back-exchanges between deuterium and hydrogen, an evaporation should have been performed by heating the solution. However, this procedure could initiate the decomposition of hydroperoxides with temperature. The protonated MTFOOH is detected at $m/z$ 162.1 and the statistical distribution of exchanges seems to end at $m/z$ 169.2, showing seven possible exchange sites on this molecule. In relation to MTF, which has five hydrogen atoms susceptible to be exchanged plus one for the protonation, a new exchange site is detected in MTFOOH: it could correspond to the hydrogen atom of the hydroperoxide functional group. This result allows the previous proposed structure for MTFOOH to be confirmed.

It should be noted that no ion at $m/z$ 162.1 was detected for solutions of MTF irradiated under deaerated conditions (Fig. 5). Indeed, the generation of hydroperoxides by water radiolysis involves the reaction of a carbon-centered free radical with molecular oxygen ($O_2$) as an intermediate step.[24]

To finalize this identification, an aqueous solution of MTF (200 μmol $l^{-1}$) was analyzed specifically for hydroperoxides by HPLC/chemiluminescence, as described previously (see Experimental section). The chromatogram obtained for the previous solution irradiated at 200 and 400 Gy (data not shown) contains a peak whose area depends on the radiation dose (area of the peak: 53 075 at 400 Gy and 22 467 at 200 Gy), thus showing that the oxidized solution of MTF contains a hydroperoxide.

## Identification of *m/z* 116.1

Based on the mass of the ion detected at $m/z$ 116.1, a possible identification was proposed in Table 1: it could be the protonated demethylmetformin, called methylbiguanide (MBG). This product was analyzed by MS. Because its molecular structure is very close to that of MTF, its fragmentation should follow the same pathway as MTF (see Fig. 3). Indeed, by subtracting 14 from the mass of the different fragments of MTF (those containing a dimethylamino group), the fragmentation spectrum of MBG could be predicted: it should present peaks at $m/z$ 60, 74, 85 and 90. This is exactly what is observed when the CID spectrum of an aqueous solution of synthetic MBG (non-oxidized) is acquired, as shown in Fig.10(a). This result confirms that MBG exhibits the same mass spectrometric behaviour as MTF. The structure in Fig. 11 is proposed.

A similar behaviour is also observed for MS performed in $D_2O$–MeOD. Figure 10(b) shows a CID spectrum of the ion detected at $m/z$ 123.1 for an aqueous solution of MTF (200 μmol $l^{-1}$) irradiated at 300 Gy under aerated conditions, then evaporated to dryness and rediluted in $D_2O$–MeOD (50 : 50, v/v). MBG shows six exchange sites plus one for the protonation and is detected at $m/z$ 123.1 (123 = 116 + 6 + 1). The proposed structures of the ions detected, based on the fragmentation pathway of MTF (Fig. 3), are all in agreement with the shifts in mass due to H–D exchanges. For example, the ion at $m/z$ 99.1 (Fig. 10(a)) can exchange four times to give an ion at $m/z$ 103, detected when working in $D_2O$–MeOD (Fig. 10(b)). The peak detected at $m/z$ 116.1 for a solution of oxidized MTF can exchange seven times (one more time than MTF) and its fragmentation leads to fragment ions that are all compatible with ions detected when fragmenting synthetic MBG (non-oxidized). These results demonstrate that methylbiguanide is one of the oxidation products of MTF.

It can be noted that a peak at $m/z$ 116.1 is also detected for solutions of MTF irradiated under deaerated conditions

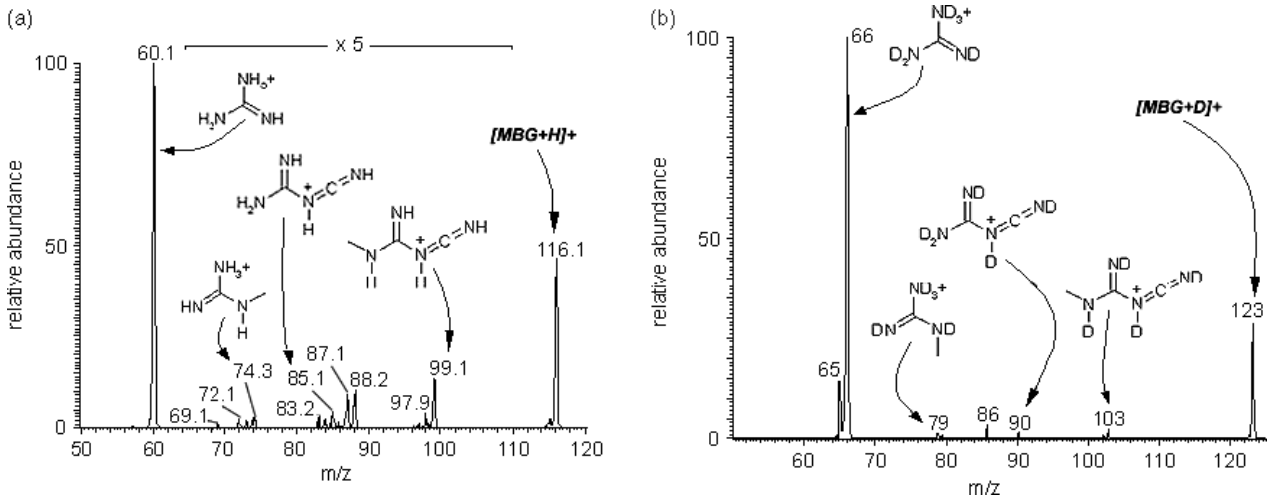



**Figure 10.** CID spectrum of the ion detected at *m/z* 116.1 for an aqueous solution of synthetic methylbiguanide (MBG) at 50 μmol $l^{-1}$, non-oxidized, diluted in $H_2O$–MeOH (1 : 10) and analyzed with direct infusion (collision energy = 23%). Abscissa, mass/charge ratio (*m/z*); ordinate, relative Abundance. (b) CID spectrum of the ion detected at *m/z* 123 for an aqueous solution of MTF (200 μmol $l^{-1}$), oxidized at 300 Gy, under aerated conditions, diluted in $D_2O$–MeOD (1 : 1) and analyzed with direct infusion (collision energy = 26%).

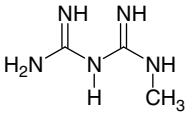


**Figure 11.** Structure of methylbiguanide (MBG).

(see Fig. 5). As a consequence, the generation of MBG seems not to be dependent on the presence of molecular oxygen in solution; this point will be considered further in the discussion of a possible reaction mechanism.

## Identification of *m/z* 126.1

The presence of ions detected at $m/z$ 126.1, corresponding to a mass of 125.1 Da for the oxidation products, implies a chemical transformation of MTF leading to a loss of 4 Da. The simplest assumption that can be made is that such a low loss should correspond to two molecular dihydrogens ($H_2$) (double dehydrogenation). According to the molecular structure of MTF, there is only one mechanism that would allow a double dehydrogenation: cyclization of MTF followed by the formation of a double bond (oxidation). Assuming that the reactive part of MTF towards $HO^\bullet$ radicals is the dimethylamino group (previously shown to be involved in the formation of MBG, diMTF and MTFOOH), a hypothetical mechanism could be considered, leading to an oxidation product of mass 125.1 Da (Fig. 12).

The first step is the action of $HO^\bullet$ radical, abstracting a hydrogen atom from a terminal methyl group, leading to a carbon-centered free radical. This radical is then susceptible to react intramolecularly with an imine group by creating a nitrogen–carbon single bond, giving a six-membered ring radical: *endo* cyclization seems to occur, even though it is usually unfavorable for other systems.[25] No reaction of this carbon-centered free radical with molecular oxygen is involved because the end-product ($m/z$ 126.1) is also detected under deaerated conditions (see chromatogram in Fig. 5). A biradical reaction followed by a two-electron oxidation by $H_2O_2$ (one of the molecular species generated during

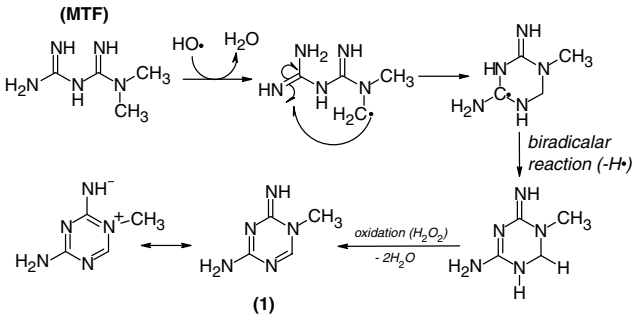



**Figure 12.** Proposed mechanism of formation of the ion detected at *m/z* 126.1 during $HO^\bullet$ radical-induced oxidation of metformin in solution.

water radiolysis) leads finally to an oxidation end-product that has a molecular structure close to an aromatic 1,3,5-triazine (compound **1** along with its mesomeric form for which the aromatic feature appears more clearly). Once again, molecular oxygen cannot be involved in this last step because of the occurrence of $m/z$ 126.1 under deaerated conditions.

The aromatic feature of this product is confirmed by the UV spectrum of the HPLC peak detected at $t_r = 8.6$ min (on the chromatogram presented in Fig. 5). The differential spectrum, i.e. for which the UV spectrum of MTF is taken as a reference, exhibits two main bands with maximum absorptions at around 208 and 255 nm (data not shown). This last wavelength is characteristic of the aromatic compounds.

The structure of $m/z$ 126.1, parent ion of 4-amino-2-imino-1-methyl-1,2-dihydro-1,3,5-triazine (4,2,1-AIMT (**1**)), was confirmed by the CID spectra in the direct infusion mode (Fig. 13). The fragmentation of the ion of $m/z$ 126.0 is characterized by three main losses of 27, 42 and 56 Da, which could correspond to each part of the molecule previously suggested, as indicated in Fig. 13(a). This kind of fragmentation was proposed previously by Baglio *et al*. for triazine herbicides.[26] In addition, for a solution of MTF diluted in $D_2O$–MeOD, the ion at $m/z$ 126.0 seems to

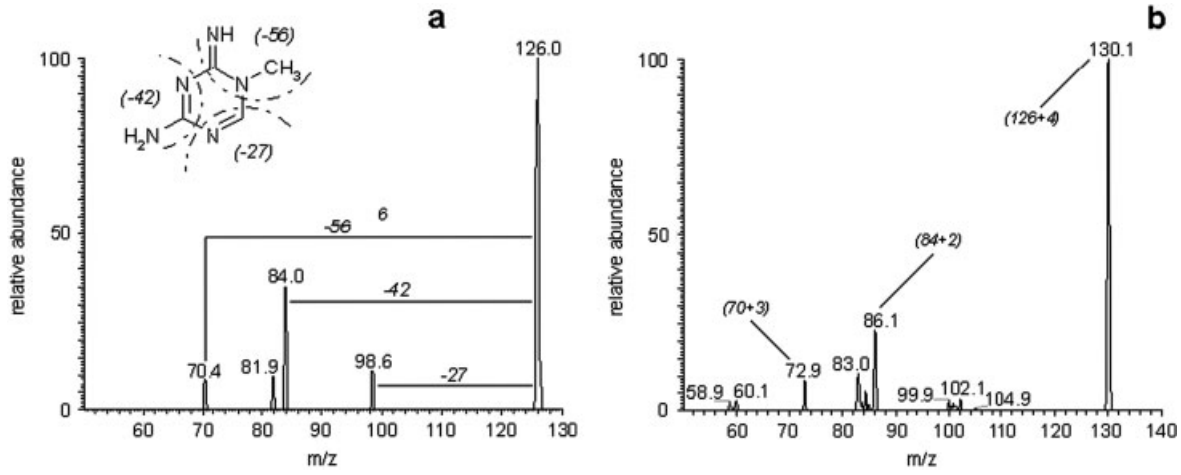



**Figure 13.** CID spectra of an aqueous solution of MTF (200 μmol $l^{-1}$) oxidized at 300 Gy, under aerated conditions, in direct infusion mode. Abscissa, mass/charge ratio (*m/z*); ordinate, relative abundance (30 accumulated scans). (a) Fragmentation of the ion detected at *m/z* 126.0 (collision energy = 29%), for the solution diluted in $H_2O$–MeOH (1 : 10). (b) Fragmentation of the ion detected at *m/z* 130.1 (collision energy = 26%), for the solution diluted in $D_2O$–MeOD (1 : 1).

exchange four times to give an ion at $m/z$ 130.1 (Fig. 13(b)), in good agreement with the molecular structure of 4,2,1-AIMT (three exchange sites plus one for protonation). Its fragmentation leads to ions with masses compatible with those of the fragments detected in $H_2O$–MeOH (Fig. 13(a)): fragments at $m/z$ 70.4 and 84.0 can exchange three times and twice, respectively, to give ions detected at $m/z$ 72.9 and 86.1. These results demonstrate that 4,2,1-AIMT is one of the primary oxidation end-products of MTF in aqueous solution.

As shown in Fig. 5, another HPLC peak is detected at $m/z$ 126.1 ($t_r = 5.4$ min), which probably corresponds to a second oxidation product that has the same mass as 4,2,1-AIMT. A simple way to find a second molecular structure consists in assuming the migration of a methyl group from 4,2,1-AIMT (**1**), as represented in Fig. 14.

This rearrangement leads to an aromatic molecule, called 2-amino-4-methylamino-1,3,5-triazine (2,4-AMT (**2**)). The molecular structure of this second compound is compatible with the CID spectra presented in Fig. 13. In particular, the three main losses of 27, 42 and 56 Da observed in the CID spectrum acquired in the non-labeled medium ($H_2O$–MeOH) could correspond to each part of 2,4-AMT. Moreover, the number of H–D exchange sites observed on the CID spectrum acquired in $D_2O$–MeOD (Fig. 13(b)) are also compatible with the molecular structure of **2**. These results confirm the assumption that 2,4-AMT would be a possible oxidation end-product of MTF in aqueous solution.

Ions at $m/z$ 126.1 were difficult to observe as they could arise from aromatic compounds (triazines), usually very difficult to fragment. By accumulating a lot of scans when working in MS infusion, a correct CID spectrum was finally obtained. However, this CID spectrum corresponds to a mixture of the two peaks detected at $m/z$ 126.1 by HPLC/MS. Indeed, the accumulation time, equal to the peak width, was not sufficient to obtain valuable MS/MS information (intensities are too low) when we tried to obtain the CID spectra for the individual HPLC peaks.

Finally, other experiments using nuclear magnetic resonance (NMR) and high-resolution Fourier transform ion cyclotron resonance (FT-ICR) methods in the search for additional data for structure elucidation were carried out in order to provide elements of confirmation for the proposed structures of the products detected at $m/z$ 126.1. However, no valuable information was obtained since dilute aqueous solution (radiolysis conditions) is not a good medium for NMR work and the mass of metformin (129 g mol$^{-1}$) is too low to work correctly with FT-ICR.

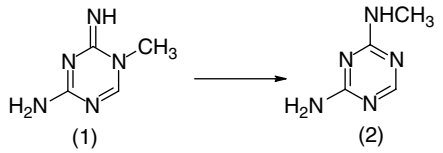



**Figure 14.** Proposed mechanism involved in the formation of the second ion detected at *m/z* 126.1 during HO$^\bullet$ radical-induced oxidation of metformin in solution.

## Identification of secondary oxidation products

As previously explained (see Experimental section), secondary oxidation products are generated at high radiation doses (above 50 Gy) and are the result of the attack of HO$^\bullet$ radicals on some of the primary oxidation end-products. Based on MS identifications, a general reaction mechanism has been postulated, that includes four different reactions (covalent dimerization, peroxidation, double dehydrogenation, demethylation). Since the molecular structures of primary oxidation end-products and the reactions involved in their formation have been characterized, molecular structures were deduced for each secondary oxidation end-product and are presented in Table 2.

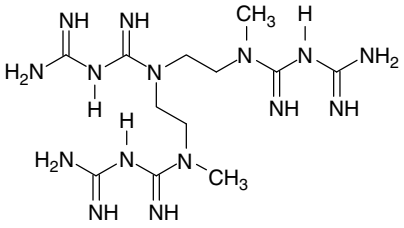
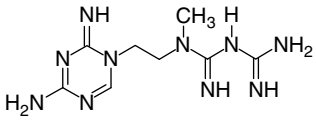
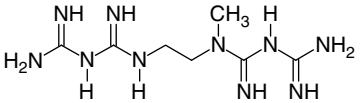
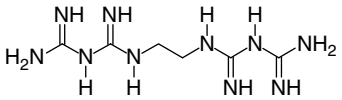
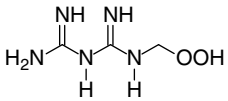
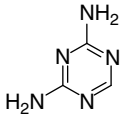
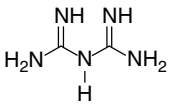
The heaviest secondary oxidation product is detected at $m/z$ 384.1 and could correspond to a covalent trimer of MTF (called triMTF). Full mass and CID spectra confirm this assumption. A doubly protonated ion is detected at $m/z$ 192.6, indicating that this ion contains at least two potential sites for protonation. The triply charged ion that could be detected at $m/z$ 128.7 is not clearly identified because of a lack of resolution of the mass spectrometer: it should be observed together with the peak at $m/z$ 129.1, the doubly protonated diMTF. The full mass spectrum for a solution of oxidized MTF (300 Gy, deaerated conditions) diluted in $D_2O$–MeOD (1 : 1) shows a peak at $m/z$ 400.3, due to 16 H–D exchanges on the ion of $m/z$ 384.1, which confirms the formation of two carbon–carbon single bonds (triMTF contains three molecules of MTF that can exchange $3 \times 5 = 15$ times plus one for protonation). Finally, the CID spectrum of $m/z$ 384.1 exhibits three losses of 17 and 42 Da, as previously observed for diMTF (see Fig. 6(b)). These observations confirm that a covalent trimer of MTF is one the secondary oxidation end-products.

The next two products, detected at $m/z$ 253.1 and 243.1, could come from the double dehydrogenation and the demethylation of diMTF, respectively, and are called 4-amino-2-imino-1-[2-(1-methylbiguanid-1-yl)ethyl]-1,2-dihydro-1,3,5-triazine (4,2,1-AIBT) and *N*-demethyldimetformin (DMdiMTF). Actually, DMdiMTF could also be generated by a radical–radical reaction between an MTF radical and an MBG radical (both carbon-centered). Full mass and CID data provide elements of confirmation for these proposals. For 4,2,1-AIBT, no peak at $m/z$ 127 is detected, meaning that the double protonation of its parent ion is not easy, probably because of the cyclic part of this molecule (in relation to MTF). Concerning DMdiMTF, the doubly protonated molecule is detected at $m/z$ 122.1, and the CID spectrum of this last ion exhibits fragment ions at $m/z$ 113.6, 85.1 and 60.1, which are typical MTF fragments as determined previously (see Fig. 3).

The last reaction that could have been applied to diMTF, to find another product, could be peroxidation. However, all of these products (diMTF, triMTF, 4,2,1-AIBT and DMdiMTF) are generated only under deaerated conditions; peroxidation was made impossible because of the absence of molecular oxygen.

The other secondary oxidation products could come from reactions on MBG: covalent dimerization, peroxidation, double dehydrogenation and demethylation that could

**Table 2.** Proposed structures for the secondary oxidation end-products of MTF, along with the reaction type involved in their formation and any experimental results allowing confirming the proposals

| Parent ion (Da) | Postulated structure (name) | Reaction involved | Analytical results[a] |
|---|---|---|---|
| 384 |  (*triMTF*) | Covalent dimerization of diMTF and MTF | $m/z$ 192.7 (++) detected 16 exchange sites CID of $m/z$ 384.1 = losses of 17 and 42 Da (as for diMTF) |
| 253 |  *4-Amino-2-imino-1-[2-(1-methylbiguanid-1-yl) ethyl]-1, 2-dihydro-1, 3, 5-triazine (4, 2, 1-AIBT)* | Dehydrogenation of diMTF | $m/z$ 127 (++) not detected |
| 243 |  *N-Demethyldimetformin (DMdiMTF)* | Demethylation of diMTF | $m/z$ 122.1 (++) detected CID of $m/z$ 122.1 = 113.6, 85.1, 60.1 |
| 229 |  *(diMBG)* | Covalent dimerization of MBG | Signal too weak[b] |
| 148 |  *Hydroperoxomethylbiguanide (MBGOOH)* | Peroxidation of MBG | CID of $m/z$ 148 = double loss of water (as for MTFOOH) |
| 112 |  *2, 4-Diamino-1, 3, 5-triazine (2, 4-DAT)* | Dehydrogenation of MBG | Signal too weak[b] |
| 102 |  *Biguanide (BGN)* | Demethylation of MBG | Signal too weak[b] |

[a] Data not shown but available on request.

[b] The signal was too weak to perform any MS/MS experiment.

generate a covalent dimer of MBG (diMBG at $m/z$ 229.1), hydroperoxomethylbiguanide (MBGOOH at $m/z$ 148.1), 2,4-diamino-1,3,5-triazine (2,4-DAT at $m/z$ 112.1) and biguanide (BGN at $m/z$ 102.1), respectively. In the case of MBGOOH, the full mass spectrum of the HPLC peak detected at $t_r = 7.0$ min shows three main peaks at $m/z$ 148.1, 130.1 and 112.1, indicating a possible double loss of water (148.1 → 130.1 and 130.1 → 112.1) occurring easily without any CID of the ion. A similar result was observed for MTFOOH, showing the presence of two oxygen atoms in the molecule (see Identification of $m/z$ 162.1). For the other detected ions (at $m/z$ 229.1, 122.1 and 102.1, Table 2), the intensity of the signal was not high enough to perform MS/MS experiments and full mass spectra of the corresponding HPLC peaks do not provide any information (except the mass of the protonated molecule) that confirms the postulated structures.

## CONCLUSION

MTF was oxidised by $HO^{\bullet}$ free radicals, the most reactive species involved in the oxidative stress phenomena *in vivo*. Towards this entity, MTF acts as an antioxidant, i.e. a

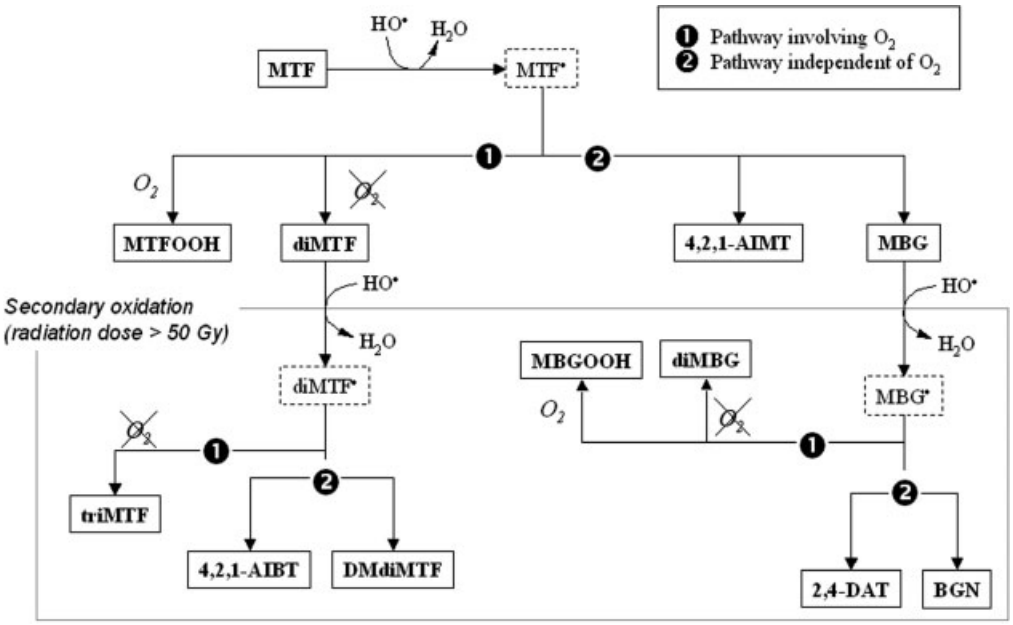



**Figure 15.** Proposed scheme of the general reaction mechanism for the oxidation of MTF by HO radicals. MTF = metformin; MTFOOH = hydroperoxometformin; diMTF = covalent dimer of metformin; 4,2,1-AIMT = 4-amino-2-imino-1-methyl-1,2-dihydro-1,3,5-triazine; MBG = methylbiguanide; triMTF = covalent trimer of metformin; 4,2,1-AIBT = 4-amino-2-imino-1-[2-(1-methylbiguanid-1-yl)ethyl]-1,2-dihydro-1,3,5-triazine; DMdiMTF = *N*-demethyldimetformin; MBGOOH = hydroperoxo-methylbiguanide; diMBG = covalent dimer of methylbiguanide; 2,4-DAT = 2,4-diamino-1,3,5-triazine; BGN = biguanide.

reducing agent that can be oxidized, leading to various products. The primary and secondary oxidation end-products were characterised by HPLC/$MS^n$.

As discussed previously, a hypothetical scheme of the reaction mechanism could be deduced from the identification of the primary and secondary oxidation end-products of MTF, and is presented in Fig. 15. The first step is the attack of hydroxyl radicals on MTF, leading to an MTF radical. Two pathways are then postulated, since differences were observed in the identifications under aerated and deaerated conditions. The first pathway (1) is $O_2$-dependent because it leads to MTFOOH under aerated conditions and diMTF under deaerated conditions. The second pathway (2) does not depend on the presence of $O_2$ and leads to 4,2,1-AIMT and MBG. These two latter oxidation products were detected under both aerated and deaerated conditions. To summarize, the four compounds MTFOOH, diMTF, MBG and 4,2,1-AIMT characterize the primary $HO^{\bullet}$-induced oxidation of MTF observed in aqueous solution at low radiation doses. The secondary oxidation of MTF at high radiation dose corresponds to the attack of $HO^{\bullet}$ radicals on the primary oxidation products diMTF and MBG. Pathways (1) and (2) applied to diMTF give triMTF, 4,2,1-AIBT and DMdiMTF, whereas when applied to MBG they lead to 2,4-DAT, BGN, MBGOOH and diMBG (depending on the experimental conditions, i.e. aerated or deaerated). Such a reaction scheme (Fig. 15) clearly shows the similar reactivities of MTF, diMTF and MBG towards $HO^{\bullet}$ radical-induced oxidation. However, kinetic studies should be carried out in order to confirm each elementary step of this mechanism.

These experimental results could open the way to the development of new drugs, that could potentially have the same properties as MTF in terms of increasing the antioxidant status.

## Acknowledgements

The authors gratefully thank Y. Hoppilliard and J. Chamot-Rooke (Laboratoire des Mécanismes Réactionnels, CNRS UMR 7651, Ecole Polytechnique, Palaiseau, France) for their advices on mass spectrometry, and R. Azerad (CNRS UMR 8601, Université René Descartes, Paris, France) for the use of the HPLC/MS facilities. We are indebted to Jamila Mekhloufi for the HPLC/chemiluminescence experiments. We acknowledge N. Wiernsperger and Merck Laboratories (Lyon, France) for financial support and for providing methylbiguanide.

## REFERENCES

1. Wiernsperger NF, Bailey CJ. The antihyperglycaemic effet of metformin. Therapeutic and cellular mechanism. *Drugs* 1999; **58**: 31.
2. Bailey CJ, Turner RC. Drug therapy: metformin. *N. Engl. J. Med.* 1996; **334**: 574.
3. Tanaka Y, Iwamoto H, Onuma T. Inhibitory effect of metformin on formation of advanced glycation end products. *Curr. Ther. Res.* 1997; **58**: 693.
4. Tanaka Y, Uchino H, Shimizu T, Yoshii H, Njwa M, Ohmura C, Mitsuhashi N, Onuma T, Kawamori R. Effect of metformin on advanced glycation end product formation and peripheral nerve function in streptozocin-induced diabetic rats. *Eur. J. Pharmacol.* 1999; **376**: 17.
5. Beisswenger PJ, Howell SK, Touchette AD, Lal S, Szwergold BS. Metformin reduces systemic methylglyoxal levels in type 2 diabetes. *Diabetes* 1999; **48**: 198.
6. Ruggiero-Lopez D, Lecomte M, Moinet G, Patereau G, Lagarde M, Wiernsperger N. Reaction of metformin with dicarbonyl compounds. Possible implication in the inhibition of advanced glycation end product formation. *Biochem. Pharmacol.* 1999; **58**: 1765.
7. Bonnefont-Rousselot D, Raji B, Walrand S, Gardès-Albert M, Jore D, Legrand A, Peynet J, Vasson MP. An intracellular

modulation of free radical production could contribute to the beneficial effects of metformin towards oxidative stress. *Metabolism* 2003; **52**: 586.
8. Giardino I, Edelstein D, Brownlee M. BCL-2 expression or antioxidant prevent hyperglycemia-induced formation of intracellular advanced glycation endproducts in bovine endothelial cells. *J. Clin. Invest.* 1996; **97**: 1422.
9. Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, Pinsky D, Stern D. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptor/binding proteins. *J. Biol. Chem.* 1994; **269**: 9889.
10. Betteridge DJ. What is oxidative stress? *Metabolism* 2002; **49**: 3.
11. Ceriello A. Oxidative stress and glycemic regulation. *Metabolism* 2000; **49**: 27.
12. Chu NV, Kong APS, Kim DP, Armstrong D, Baxi S, Deutch R, Caulfield M, Mudaliar SR, Reitz R, Henry RR, Reaven PD. Differential effects of metformin and troglitazone on cardiovascular risk factors in patients with type 2 diabetes. *Diabetes Care* 2002; **25**: 542.
13. Rosen P, Nawroth PP, King G, Moller W, Tritchler HJ, Packer L. The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a congress series sponsored by UNESCO–MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab. Res. Rev.* 2001; **17**: 189.
14. Spinks JWT, Woods RJ. *Water and Inorganic Aqueous Systems. Introduction to the radiation chemistry* (3rd edn). Wiley: New York, 1990; 243.
15. Bonnefont-Rousselot D, Gardès-Albert M, Delattre J, Ferradini C. Oxidation of low-density lipoprotein by $HO^{\bullet}$ and $O_2^{\bullet -}/HO^{\bullet}$ free radicals produced by gamma radiolysis. *Radiat. Res.* 1993; **134**: 271.
16. Hauville C, Remita S, Thérond P, Jore D, Gardès-Albert M. Radiation induced peroxidation of polyunsaturated fatty acids: recent results on formation of hydroperoxides. *Can. J. Physiol. Pharmacol.* 2001; **79**: 176.
17. Buxton GV, Greenstock CL, Helman WP, Ross AB. Critical review of rate constants for reaction of hydrated electrons, hydrogen atoms and hydroxyl radicals in aqueous solutions. *J. Phys. Chem. Ref. Data.* 1988; **17**: 513.
18. Fricke H, Morse S. The chemical action of Röntgen rays on dilute ferrosulfate solutions as a measure of dose. *Am. J. Roentgenol. Radium Ther.* 1927; **18**: 430.
19. Beckmann R. The fate of biguanides in man. *Ann. N.Y. Acad. Sci.* 1968; **148**: 820.
20. Thérond P, Couturier M, Demelier JF, Lemonier F. Simultaneous determination of the main molecular species of soybean phosphatidylcholine or phosphatidylethanolamine and their corresponding hydroperoxides obtained by lipogenase treatment. *Lipids* 1993; **28**: 245.
21. Elpern B. Chemistry of the biguanides. *Ann. N.Y. Acad. Sci.* 1968; **148**: 577.
22. Heinig K, Bucheli F. Fast liquid chromatographic–tandem mass spectrometric (LC–MS–MS) determination of metformin in plasma samples. *J. Pharm. Biomed. Anal.* 2004; **34**: 1005.
23. Steeken S. Addition–elimination paths in electron-transfer reactions between radicals and molecules. *J. Chem. Soc., Faraday. Trans. 1* 1987; **83**: 113.
24. Ross AB, Neta P. *Rate Constants for Reactions of Aliphatic Carbon-centered Radicals in Aqueous Solution*. National Standard Reference Data System. US Department of Commerce, National Bureau of Standards: Washington, DC, 1982.
25. Fossey J, Lefort D, Sorba J. *Free Radicals in Organic Chemistry*. Wiley: New York, 1995; 151.
26. Baglio D, Kotzias D, Larsen BR. Atmospheric pressure ionisation multiple mass spectrometric analysis of pesticides. *J. Chromatogr. A* 1999; **854**: 207.