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Methodological Considerations and Factors Affecting 8-Hydroxy-2′-deoxyguanosine Analysis

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Oxidative stress is related to a number of diseases due to the formation of reactive oxygen species (ROS). There are also several substances found in the occupational environment or as life style related situations that generates ROS. A stable biomarker for oxidative stress on DNA is 8-hydroxy-2′-deoxyguanosine (8-OH-dG).

A potential problem in the work-up and analysis of 8-OH-dG is oxidation of dG with false high levels as a result of analysis. This paper summarizes and discusses some of the critical moments in terms of auto-oxidation. The removal of transition metals, low temperatures, absence of isotopes (or 2′-deoxyguanosine) and incubation times are all important factors. Removal of oxygen is complicated while the problem is reduced if a nitroxide (TEMPO) is added during work-up. Certain reducing agents and enzymes could be critical if added during work-up.

The application of the ^{32}P-HPLC method to analyze 8-OH-dG is discussed. The ^{32}P-HPLC method is suitable for 8-OH-dG analysis and avoids several factors that oxidizes dG by removal of dG before addition of isotopes. Factors of crucial importance (columns, eluents, gradients and detection of ^{32}P) for the analysis of 8-OH-dG are commented upon and certain recommendations are made to make it possible to apply the ^{32}P-HPLC methodology for this type of analysis.

Keywords: 8-hydroxy-2′-deoxyguanosine

Abbreviations: 8-OH-dG = 8-hydroxy-2′-deoxyguanosine,
dG = 2′-deoxyguanosine,
EC = electrochemical detection,
^{32}P-HPLC = high-performance liquid chromatography with on-line detection of ^{32}P-postlabeled substances,
^{32}P-TLC = thin layer chromatography with autoradiography for ^{32}P-postlabeled substances,
GC-MS = gas chromatography-mass spectrometry,
SOD = superoxide dismutase,
TEMPO = 2,2,6,6-tetramethylpiperidine-*N*-oxyl,
PBN = α-phenyl-*N*-*tert*-butylnitrone,
POBN = α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone,
DMPO = 5,5-dimethyl-1-pyrroline-*N*-oxide

INTRODUCTION

Organisms living under aerobic conditions are continuously exposed to oxygen related radicals and other reactive oxygen species (ROS). Examples of ROS are superoxide, hydrogen peroxide and hydroxyl radicals. These radicals are normally eliminated by the metabolism of the cell but a small fraction react with DNA causing oxidative stress of DNA. One example is the formation of oxidized 2′-deoxyguanosine (dG), 8-hydroxy-2′-deoxyguanosine (8-OH-dG, see Figure 1). This type of reaction is estimated to cause 90,000

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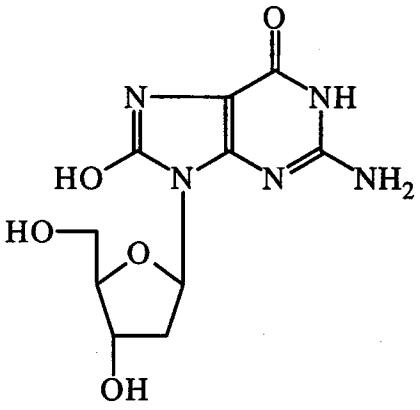

FIGURE 1 The DNA addition product and biomarker for oxidative stress, 8-OH-dG.

DNA-lesions per cell and day.[1] Studies on the DNA repair of 8-OH-dG lesions in prokaryotic cells[2,3] and mammalian cells[4,5] indicate that this is a common form of DNA lesion in oxygen dependent organisms. Inactivation of the DNA repair enzyme DNA glycosylase (found in *E. coli* as well as mammalian cells) which repairs 8-OH-dG leads to a 10-fold increase in the spontaneous mutation frequency.[6]

Oxidative DNA lesions are considered to be an important event related to the development of cancer and aging.[6–9] There is a large number of diseases that are related to formation of ROS and thereby a risk to form 8-OH-dG. In some cases the oxidative stress is the disease while in other cases the oxidative stress is a side effect or one among other critical events. Table I compiles a large variety of diseases related to formation of ROS. Most of them have also been shown to induce 8-OH-dG.

Chronic hepatitis gives significant increases of 8-OH-dG in liver DNA,[10] and smokers excrete significant elevated levels of 8-OH-dG in urine.[11] Ionizing radiation from radiotherapy results in a dose dependent formation of 8-OH-dG in blood cell DNA.[12] Diabetes patients have been found to have 8-OH-dG levels of 96–223 fmol/mg DNA in white blood cells which is statistically significantly higher than the 15–73 fmol/mg DNA found in healthy individuals.[13] Lipid peroxidation, another biomarker for oxidative stress, is also increased in diabetes patients.[14,15] It is further suggested that 8-OH-dG is related to heart disease found in diabetes patients.[13]

TABLE I Examples of situations and diseases that are related to the formation of reactive oxygen species

Disease	Reference	
Diabetes	[70,71]	/§/
Aging	[72,73]	/§/
Cardiovascular disease	[74,75]	
Infections	[74,76]	/§/
Inflammation	[74,77]	
Heliobacter pylori	[76]	/§/
Rheumatism	[78]	
Asthma	[79]	
Fanconi's anemia	[80]	/§/
Cancer	[10,81–83]	/§/
Chron's disease	[84]	/§/
Atherosclerosis	[85,86]	
Ischemia	[87,88]	/§/
Lipid peroxidation	[89]	
Cystic fibrosis	[90]	/§/
Immunological disease	[91]	
Alzheimer's disease	[92]	/§/
Parkinson's disease	[93,94]	
Cataract	[94]	
Septic shock	[95]	
Hepatitis	[10]	/§/
Cirrhosis	[10]	/§/

/§/ = Increased levels of 8-OH-dG in humans confirmed.

Occupational or environmental (including life-style factors) exposures involve a number of factors/substances shown to induce oxidative stress in different assays. In most cases formation of 8-OH-dG is shown to occur. These data are summarized in Table II. Examples are smoking,[11] alcohol[16,17] and UV-irradiation.[18–20] In a recent report, hamsters were chronically administered estradiol which resulted in a high incidence of kidney tumors. 8-OH-dG was not elevated in liver but up to 400% of the background level in kidney.[21] Other life style factors are related to the protection of ROS, e.g. consumption of fruit and vegetables and thereby vitamins and anti-oxidants. Glutathione prevents oxidative stress (measured as 8-OH-dG) in rat kidney after administration of potassium bromate.[22]

TABLE II Examples of endogenous and exogenous exposure related to oxidative stress

Exposure	Reference	
Ethanol	[16,17]	/§/
Asbestos	[96]	/§/
Iron	[16,25,97]	
Copper	[16,25]	/§/
Chrome	[98]	
Vanadium	[99]	/§/
Cadmium	[100]	
Aflatoxin	[101]	
Halogenated acetic acids	[102]	/§/
Smoking	[11,103]	/§/
Pentachlorophenol	[104]	/§/
2-Nitropropane	[32]	
2,7-Dinitrofluorene	[88]	/§/
Ozone	[105]	
Phorbol esters	[106]	/§/
Nitrosamines	[107]	
Nitrogen dioxide	[105]	/§/
Benzene	[108,109]	
UV irradiation	[18–20]	
Gamma irradiation	[67,110]	

/§/ = Increased levels of 8-OH-dG in humans confirmed.

Tumor promotion can be related to a decreased stability of DNA owing to oxidative lesions. The formation of 8-OH-dG makes the otherwise stable aromatic five atom ring of guanine unstable. Destabilization leads to an increased risk of other chemical reactions, for instance hydrolysis and loss of bases which further can cause strand breaks. There was a dose dependent formation of 8-OH-dG in nuclear DNA when tumor promoters were administered to human cell cultures.[23] This could be one explanation to the reduction of cancer risk found in many cancer studies after intake of antioxidants.

In general ROS are extremely short lived. As a consequence, it is very complicated to measure ROS and therefore it is common to use indirect measurements, for instance decrease of antioxidant levels in organisms. The advantage of analysis of 8-OH-dG in cell nucleus DNA is that this is a stable reaction product found in the nucleus after the reactants have passed a number of protective systems.

In order to determine the background level of 8-OH-dG/dG in cells, and to be able to see differences in samples having low but different 8-OH-dG levels, it is of the utmost importance to reduce the work-up formation of 8-OH-dG. Unless work-up and analysis of 8-OH-dG have been performed in a controlled manner, oxidation of dG during these steps can add the major part of 8-OH-dG detected. Commercially available DNA and dG also contain a substantial amount of 8-OH-dG which is of importance when using standards and reference samples.

The aim of this paper is to comment on and summarize the different parts of work-up and analysis that are critical in terms of artifactual formation of 8-OH-dG and to suggest alternative approaches to enable precise measurements of 8-OH-dG.

RESULTS AND DISCUSSION

1. Removal of Transition Metals

Several of the 32 transition metals found in the *d* block in the periodic table can attain many different oxidation numbers, thereby acting as radicals, and switch between these by gaining or losing one or several electrons.[24] For example, manganese is most stable as Mn^{2+} in aqueous solution, but can also exist as Mn(III), Mn(IV) and Mn(VII).[24] Several transition metals have been shown to generate oxygen radicals and a probable mechanism for 8-OH-dG formation during work-up is shown in Figure 2, where Fe^{2+} may be substituted by other reduced transition metals, but with different reactivities in reactions 1–4 due to their different redox potentials. Nanomolar levels of Fe^{2+} were found to catalyze 8-OH-dG formation when incubated with dG.[25] Use of water and chemicals low in transition metals is recommended, as well as use of plastic bottles wherever possible. Avoidance of using enzymes that contain transition metals is also recommended. For example, acid phosphatase contains iron at its catalytic site, whereas alkaline phosphatase does not.[26] Acid phosphatase induced

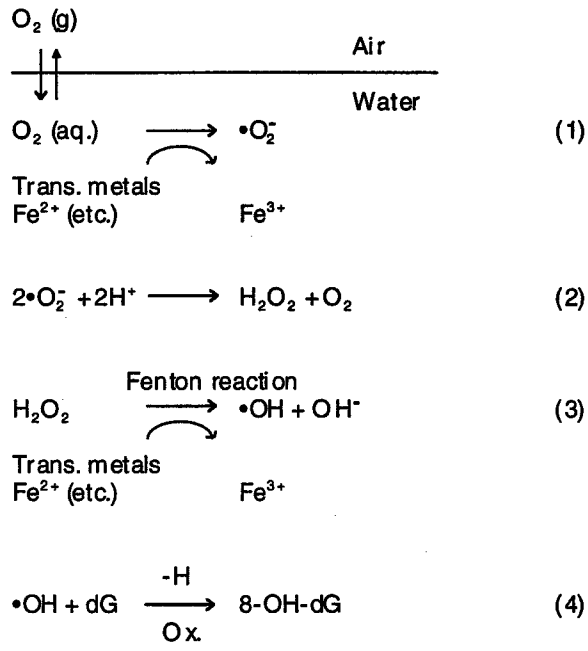

FIGURE 2 Probable causes of 8-OH-dG formation during sample work-up procedure.

8-OH-dG formation when incubated with dG.[25] Wash of enzymes by dialysis is recommended. Repeated wash of nuclei after cellular homogenization, to remove transition metals and reducing agents, is recommended prior to warm enzymatic incubations. Chelex treatment of solutions to remove transition metals could work but will be difficult for small volumes of enzymes and it is probably not realistic to remove all transition metals. It is not necessarily the case that a complex-bound metal is inactivated, still bound it could redox cycle and thereby have the potential to act as a catalyst in oxidation processes.

2. Reduction of Temperature

8-OH-dG formation followed a third order equation when incubating dG in an ultra pure solution at 0–140°C. An incubation temperature of 37°C was used when cleaving proteins and DNA. Centrifugation was performed at 0°C and almost all liquid handling was performed on ice.[25] Formation of 8-OH-dG from dG, catalyzed by 1 μM Fe^{2+}, could be blocked by incubation on ice,[25] indicating heat dependency for reactions (1)–(4) in Figure 2. Another reaction to consider is the cleavage of the relatively weak O–O bond in H_2O_2 by homolytic fission,[24] a mechanism which requires further investigations as to at what temperatures it will occur.

$$H_2O_2(+\text{energy}) \rightarrow 2\ \bullet OH \qquad (5)$$

DNA and dG should be stored as cold as possible. The GC-MS method requires elevated temperature (140–150°C) to hydrolyze DNA, followed by derivatization of the bases. One problem with GC-MS is the high temperature used. Another is that cleavage of the N-glycosidic bond in dG requires less energy than in 8-OH-dG which will generate false low 8-OH-dG levels if not a 100% hydrolysis is accomplished.[25,27] Jenner *et al.* reported that decreasing the temperature to 23°C during derivatization lowered the levels of 8-hydroxyguanine.[28] Enzymatic hydrolysis of DNA followed by LC-MS analysis does not require elevated temperatures.[29]

3. Avoidance of High Energetic Radiation

Ionizing radiation can be absorbed directly by DNA, leading to ionization of the bases, or react with the surrounding water molecules giving e^-_{aq}, •OH and •H.[30] It was shown that ^{32}P-ATP substantially mediated the formation of 8-OH-dG when incubated with dG at room temperature.[31] ^{32}P-postlabeling assays such as ^{32}P-HPLC and ^{32}P-TLC therefore require the separation of dG before labeling of 8-OH-dG. A small dose of natural background ionizing radiation is always present which can possibly lead to build up of $\bullet O_2^-$ and H_2O_2 in the solutions. Ultraviolet radiation has been shown to form singlet oxygen ($^1O_2$) which selectively attacks the guanine base in DNA, forming 8-OH-dG.[20] Normal laboratory light had no effect on 8-OH-dG formation.[25] Also, many steps such as centrifugation and hydrolysis of proteins and DNA are usually performed in the dark.

4. Reduction of Incubation Time

Shortening the time between tissue collection and 8-OH-dG analysis will reduce the oxidation during work-up, especially in the warm enzymatic steps. However, the work-up cannot be shortened too much, since enzymes etc. must be allowed to cleave DNA and proteins properly.

5. Removal of Oxygen

Removal of oxygen from buffers will reduce artificial 8-OH-dG formation during work-up as few new superoxide radicals can be formed. However, the H_2O_2 already present in the buffers will not be removed. Also, even in an anaerobic environment H_2O_2 could possibly be built up from background irradiation of the buffers due to splitting of water molecules. Use of anaerobic boxes is complicated, expensive, time consuming and will not completely remove all oxygen. Due to the high cost of commercial anaerobic boxes we used a self-made anaerobic polycarbonate box filled with nitrogen. However, removal of O_2 from the box was a tedious procedure and the O_2-concentration was never below 0.2%. Solutions were sparged with argon to remove oxygen, but this proved complicated when working with small volumes. As oxygen can pass when samples were taken out of the box, a centrifuge and a vortex were placed inside the box. This procedure was abandoned since it was complicated and time consuming, and required a lot of expensive gas. Also, heat generated especially from the centrifuge elevated the temperature in the box. If an anaerobic box is considered for use during work-up, we recommend a refrigerated box. Use of automatic DNA isolation systems under inert gas have been described,[32] but do not remove O_2 during DNA hydrolysis.

6. Increasing the dG Concentration

Increased 8-OH-dG/dG levels when working with small amounts of DNA has been shown by several groups.[33–35] If there is a constant production of •OH in the solutions during work-up, resulting in a fixed 8-OH-dG work-up generated number of molecules per volume, higher 8-OH-dG/dG ratios will be detected when using low amounts of dG (or DNA). Thus, the use of high dG concentrations will "dilute" the work-up generated 8-OH-dG resulting in lower 8-OH-dG ratios. Beckman and Ames recommend the hydrolysis of $>20\,\mu g$ DNA,[34] whereas Helbock *et al.* recommend the hydrolysis of $>100\,\mu g$ DNA[35] to reduce the effect of the work-up 8-OH-dG formation.

7. Oxidation of Transition Metals

Nitroxides such as TEMPO (Figure 3) acting as mild oxidants can quickly oxidize Fe^{2+} and Cu^{+} to Fe^{3+} and Cu^{2+} respectively.[36] $100\,\mu M$ of TEMPO completely inhibited a $1\,\mu M$ Fe^{2+} catalyzed formation of 8-OH-dG from dG.[25] Nitroxides do not react with O_2 or H_2O_2.[36] Further studies are needed to investigate the oxidizing effect of nitroxides on other transition metals.

8. Avoidance of Transition Metal Reducing Agents

The antioxidant function of ascorbate (vitamin C) *in vivo* has been described as:

$$AscH^- + \bullet X \rightarrow Asc^- + XH \qquad (6)$$

where •X is a free radical such as •OH, RO• (aliphatic alkoxyl radical) and ROO• (alkyl peroxyl radical).[37] Transition metals are available in solution *in vitro* and can be reduced by ascorbate.[38] Reduced transition metals can

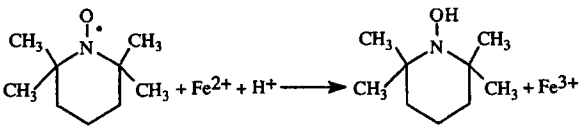

FIGURE 3 Oxidation of Fe^{2+} to Fe^{3+} by TEMPO. TEMPO is reduced to the hydroxylamine form.

donate an electron to O_2:

$$AscH^- + Fe^{3+} \rightarrow \bullet Asc^- + Fe^{2+} \qquad (7)$$

with the subsequent generation of •OH as outlined in Figure 2. Theoretically, very low concentrations of transition metal ions are needed since they can redox cycle. We showed[25] that ascorbate is a very potent mediator of 8-OH-dG formation when incubated together with dG. The ability to reduce transition metals is dependent on the redox potential of the particular transition metal and the redox potential of the reducing agent. Reducing agents such as glutathione (GSH),[39,40] dithiothreitol (DTT),[41] cysteine[40,41] and dihydrolipoic acid[40] have been shown to mediate reduction of Fe^{3+} to Fe^{2+} *in vitro*, with subsequent generation of 8-OH-dG. Jenner *et al.*, however, reported reduced 8-OH-dG formation when using ethanethiol during anaerobic derivatization for GC-MS analysis.[28] The mechanisms for this need to be further investigated. Reduction of transition metals by the nucleotides themselves is not likely to occur, and there are, to our knowledge, no reports available discussing this event.

9. Removal of H_2O_2 and $\bullet O_2^-$

Catalase can split H_2O_2 into H_2O and O_2, but can also oxidize several substances with H_2O_2.[42] When catalase was incubated with dG, the dG molecule was efficiently cleaved and guanine (G) was identified by HPLC/UV.[25] Catalase was hence not suitable for H_2O_2 removal. Catalase mimics could work, however, but binuclear manganese complexes have been reported to oxidize organic substrates as well.[43] Neither $\bullet O_2^-$ nor H_2O_2 are known to oxidize DNA.[44] Scavengers of $\bullet O_2^-$ could function to reduce the build-up of H_2O_2, however, the spontaneous dismutation of $\bullet O_2^-$ into H_2O_2 and O_2 will compete with the $\bullet O_2^-$ scavenger. Removal of $\bullet O_2^-$ by SOD or SOD mimics by $\bullet O_2^-$ dismutation produces H_2O_2 and O_2, which will enhance •OH formation. In addition, catalase, SOD, catalase mimics and SOD mimics often contain transition metals which could increase •OH production.

10. Chelation of Transition Metals

Transition metal chelators with high affinity for a specific transition metal, such as desferrioxamine for Fe^{3+} (affinity constant 10^{31}), can scavenge •OH from the Fenton reaction, but can also change the transition metals' redox potential so that it cannot redox cycle.[45] However, since most metal chelators are specific for one metal, other metals may be only loosely bound and allowed to redox cycle. Loosely bound transition metals may have a redox potential such that redox cycling is enhanced compared to the free transition metal.[46]

11. Addition of a Scavenger Molecule for •OH

Several substances can be considered as scavengers for •OH, that is react with •OH before the radical can react with e.g. dG. However, •OH reacts with many known •OH scavengers (PBN, POBN and DMPO) at similar rates as with dG.[47] Thus, high concentrations have to be used, possibly affecting cleavage of proteins and DNA by enzymes. A •OH scavenger with a considerable higher reaction rate than dG for •OH would be of interest for the work-up procedure for 8-OH-dG analysis. We showed that phenol and pink phenol did not catalyze 8-OH-dG formation,[25] on the contrary, phenol with its aromatic structure may act as a •OH scavenger.

12. ^{32}P-postlabeling

The major requirement on methods for DNA damage analysis is a high sensitivity. This is because DNA damage is generally present at very low levels, and samples are often available only in small amounts. To detect 8-OH-dG in the lymphocyte DNA from a 5 ml blood sample an

absolute sensitivity of 5–50 pg or 15–150 fmol is necessary. One method offering a very high sensitivity is ^{32}P-postlabeling,[48–51] meaning the selective radioactive labeling of DNA adducts from samples with ^{32}P-phosphate groups. Today, ^{32}P-postlabeling of DNA adducts is generally performed after extraction of DNA, purification from proteins and RNA, usually enzymatically by proteinase K and ribonucleases (RNases) A and T_1. DNA is then enzymatically digested to mononucleotide 3′-phosphates by micrococcal nuclease and spleen phosphodiesterase. The presence of the 3′-phosphate group is necessary for the labeling of the compounds. The adducted nucleotides are labeled by transfer of a radioactive [^{32}P]phosphate group from adenosine 5′-[gamma-^{32}P]triphosphate (^{32}P-ATP) by T4 polynucleotide kinase.

13. Nuclease P_1 Treatment and Butanol Extraction

Since the adducted nucleotides only constitute a very small fraction of the total DNA digest, it is desired that the number of non-adducted nucleotides is reduced, to reduce interference and amount of ^{32}P-ATP required. Such enrichment of the adducts can be achieved by nuclease P_1, i.e. enzymatic treatment selectively cleaving off 3′-phosphate from non-adducted nucleotides or by extraction of the adducted nucleotides with butanol.[50]

14. Chromatography

The original separation method with ^{32}P-postlabeling is thin-layer chromatography (^{32}P-TLC).[48,49] ^{32}P-TLC has been used extensively to analyze DNA adducts formed both *in vitro*, in laboratory animals and in humans.[52] The ^{32}P-TLC method offers a rather good separation and requires little advanced or expensive equipment. Many samples can also be run in parallel with this method. However, when complex mixtures are analyzed, e.g. DNA adducts from humans, the separation is rarely enough to resolve all compounds into individual spots. The reproducibility of this method is also limited. Significant variations in the positions of the resulting spots are sometimes seen even between analyses of the same sample performed in parallel with TLC plates from the same batch.

Already a few years after the introduction of the ^{32}P-postlabeling method, attempts were made to combine the very high sensitivity offered by the postlabeling method with the high resolution and reproducibility of HPLC.[53,54] These and more recent attempts proved HPLC to be of value for separation of ^{32}P-postlabeled DNA adducts. Although proving valuable, many of these methods suffer drawbacks in that they require extra sample work-up procedures, e.g. a TLC step, have their sensitivity reduced owing to high radioactive backgrounds, or require radioactivity measurements on collected fractions of the eluate.

^{32}P-HPLC was developed in our laboratory to enable fast and reproducible analyses with a high separation efficiency in complex mixtures of DNA adducts.[55–63] The following are commentaries on the application of ^{32}P-HPLC to the analysis of 8-OH-dG.

15. HPLC Columns

An important factor in ^{32}P-HPLC for DNA adducts is the choice of column. The column must both be able to give a high resolution of very similar compounds and also to survive rather harsh chromatographic conditions. During method development several different types of columns were tested.[55] The one that was found to have the best performance was a high-performance reverse-phase column with a C18 coat on 5 µm silica particles with 100 Å pore size called Delta Pak.[55] These types of columns also proved to survive intense analyses with harsh conditions for several years. To improve resolution further, two HPLC columns can be connected in series (data not yet published).

16. HPLC Eluents

In order to separate compounds having a vast range of polarity in a single ^{32}P-HPLC analysis, a gradient system of acetonitrile in ammonium formate buffer was developed.[55,56] The use of acetonitrile gave somewhat sharper peaks than methanol did. Ammonium formate was chosen because of the positive experiences from using it in ^{32}P-TLC analysis of DNA adducts. Even at high concentrations ammonium formate allowed for a high percentage of acetonitrile in the gradient without any problems from salt precipitation observed with phosphate buffers that were also tested. It is worth noticing that at high levels of acetonitrile in high concentration ammonium formate buffer, a liquid two-phase system appears at rest. However, during the turbulent flow through the HPLC system this will still act as a one-phase system.

17. HPLC Detection

In order to get a convenient detection of radioactivity in the samples, an on-line radioactivity detector was used.[55] A disadvantage with this kind of flow-through detector is that the radioactivity only can be measured during a short period of time, usually a few seconds, while measurements during indefinite lengths of time can be performed on HPLC fractions or radioactive spots on ^{32}P-TLC. HPLC fractions can be measured for many minutes or even hours and ^{32}P-TLC spots are routinely measured by autoradiography for up to several days, while the on-line detection was carried out in 12 s cycles. Since the radioactive signal from a sample is proportional to detection time, one could expect an advantage for HPLC fractions in detection limit by a factor of approximately 100 and for ^{32}P-TLC by a factor of up to 10,000. However, this was not the case. The detection limit with radioactivity is usually defined as a certain factor, often approximately 3, above the background noise. The background noise is proportional to the square root of the background and the background is proportional to the detection time. This means that the difference in detection limit with equal background is approximately the square root of the difference in detection time, for HPLC fractions a factor of 10 and for ^{32}P-TLC a factor 100. Also, the better chromatographic efficiency on ^{32}P-HPLC compared to ^{32}P-TLC usually means a signal to surrounding background ratio that is a factor 10–100 higher in ^{32}P-HPLC with on-line detection. Since the background noise is proportional to the square root of the background, the detection limit advantage for ^{32}P-TLC was approximately one order of magnitude or a factor 10–30. Also, this is based on the assumption that the same amount of DNA is used. The better resolution and lower background in ^{32}P-HPLC allows the use of far more DNA than in ^{32}P-TLC analyses.

18. HPLC Gradient

A gradient starting point at a certain percentage of acetonitrile allowed for a high resolution of lipophilic DNA adducts but also caused a high background from tailing polar postlabeled compounds.[55] By choosing a gradient starting point without any acetonitrile and a slow increase of acetonitrile content this problem was much reduced.[56] A 70 min linear gradient from 0 to 35% acetonitrile in 2 M ammonium formate at pH 4.5 resulted in good separation both between normal nucleotides, other polar compounds and lipophilic DNA adducts while at the same time the background radioactivity was kept at a low level.[56]

The basic resolution was not always enough when complex mixtures of DNA adducts were analyzed, e.g. formed by exposure to a multitude of DNA adduct forming agents or to agents capable of forming a multitude of DNA adducts. This problem could be solved by inserting isocratic plateaus into the otherwise linear gradient. This expanded and enhanced the resolution of certain areas of the chromatograms. There was a direct linear relation between at what

acetonitrile level the isocratic plateau was inserted and around which retention time using the standard gradient the resolution was maximized. This method was used to resolve complex mixtures of DNA adducts including up to eight different stereoisomers of the same DNA adduct.[57]

19. ^{32}P-HPLC Applications

^{32}P-HPLC has been used to build retention time libraries for DNA adducts from a multitude of substances, e.g. polycyclic aromatic hydrocarbons, nitrated compounds and aromatic amines.[58] Due to the good reproducibility of ^{32}P-HPLC these libraries were used to tentatively include or exclude possible DNA adduct sources in analysis of DNA adducts formed in humans exposed to complex environmental mixtures of DNA adduct forming agents.[59] DNA adducts were also analyzed in several different organs of laboratory animals exposed to the same group of substances[55,56,60–62] (data not yet published). DNA adducts have also been analyzed by ^{32}P-HPLC in 18 different organs from humans.[59] (Figure 4.)

20. ^{32}P-postlabeling Adapted to 8-OH-dG

^{32}P-postlabeling is proposed to be suitable for analyses of 8-OH-dG since it was originally developed to analyze DNA adducts at a level of 1 adduct per 10^9–10^{10} normal nucleotides in μg amounts of DNA, corresponding to fg on nmol amounts of DNA adducts.[50,51] The first attempts to use ^{32}P-postlabeling for 8-OH-dG analyses were made already back in 1981.[48,51]

^{32}P-HPLC developed for DNA adducts from e.g. polycyclic aromatic hydrocarbons proved to be able to separate both normal nucleotides and small adducts like 7-methyl-2′-deoxyguanosine.[56] Therefore this method could also be expected to be able to separate a similar small modification like 8-OH-dG. However, to overcome problems with interference from normal

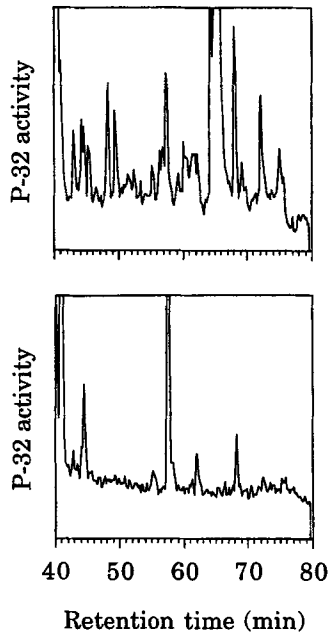

FIGURE 4 ^{32}P-HPLC chromatograms of lipophilic DNA adducts from two human individuals without any known specific exposure. The samples are from liver tissue and illustrate the span of normally occurring DNA adducts. The chromatograms are cropped to focus on the retention time window (46–74 min) where lipophilic and bulky DNA adducts occur.

nucleotides, some modifications were required. 8-OH-dG and normal nucleotides showed to be so polar that they eluted before the chromatogram was effected by the acetonitrile gradient. Therefore the system was simplified by discarding the gradient. The polarity of the compounds was decreased by use of Nuclease P_1 after postlabeling to remove the unlabeled 3′-phosphate group. This increased the retention time in reverse-phase HPLC and enhanced the separation. To further increase retention times for the polar compounds and thereby further enhancing resolution, the buffer strength was reduced from 2 M ammonium formate to between 5 and 50 mM. To optimize the resolution between 8-OH-dG and the normal nucleotides further, the pH was adjusted from 4.5 to 3.0 (Figure 5).[63]

^{32}P-postlabeling with TLC separation has also been used for analyses of 8-OH-dG.[64–66] This method gave a reasonably good separation but

resulted in 8-OH-dG values 20–50 times higher than those usually expected. This is possibly owing to problems with oxidation of dG during sample handling and analysis, partly owing to the presence ^{32}P-ATP.[31]

21. Pre-separation

There is always a risk of oxidation of 2′-deoxyguanosine (dG) to 8-OH-dG during sample handling and analysis. When using ^{32}P the radiation may cause this kind of oxidation.[31] Therefore, it is important to reduce or eliminate this risk, either by using conditions where oxidation will not occur or by eliminating dG from the sample. This can be achieved by degrading dG, e.g. using trifluoroacetic acid,[64] or by separating it from 8-OH-dG in a pre-separation step, e.g. through chromatography. When DNA is enzymatically digested for ^{32}P-postlabeling it is hydrolyzed to 3′-phosphate nucleotides. These have chromatographic properties very similar to the 5′-phosphate nucleotides produced after ^{32}P-postlabeling and removal of the 3′-phosphate group by Nuclease P_1. Therefore, a pre-separation can be achieved with a chromatographic system very similar to the one used for ^{32}P-HPLC on 8-OH-dG. To facilitate the handling of the sample after pre-separation, i.e. drying and postlabeling, it is desirable to use an HPLC eluent with low salt content. A 5 mM ammonium formate buffer at pH 2.5 resulted in a good separation of normal nucleotides and 8-OH-dG.[63] Other HPLC methods to separate 8-OH-dG from normal nucleotides have also been reported.[67,68] However, these methods appear to have problems in separating 8-OH-dG from some commonly found contaminants, e.g. from RNA, in the hydrolyzed samples.

Another method to perform the pre-separation of 8-OH-dG and normal nucleotides is capillary electrophoresis.[68] This method appears to be well suited for the enrichment of 8-OH-dG in small samples but suffers the drawback of not being able to give a complete separation of dG and 8-OH-dG.

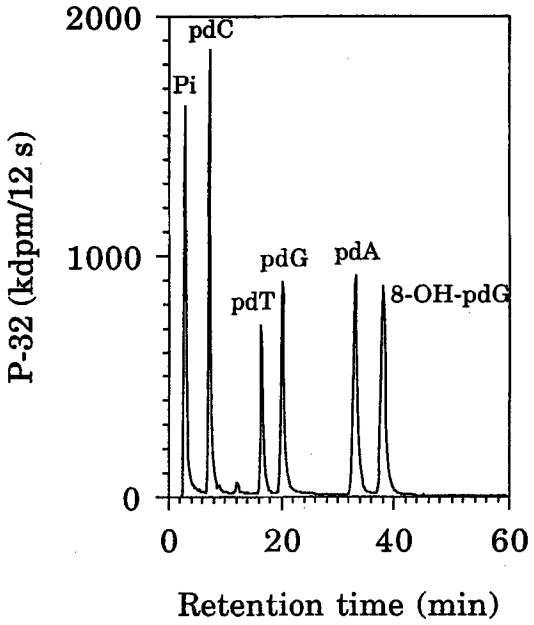

FIGURE 5 ^{32}P-HPLC chromatogram of equal amounts of standards of normal nucleotide and 8-OH-dG 5′-monophosphates. When pre-separation is performed virtually all 2′-deoxyadenosine (pdA) and 2′-deoxyguanosine (pdG) 5′-monophosphates are eliminated while the retention time differences between 8-OH-dG on one hand and orthophosphate (Pi), 2′-deoxycytidine (pdC) and thymidine (pdC) 5′-monophosphates on the other hand are sufficient to prevent interference.

22. Data Processing

Data from a radioactive flow-through detector are characteristically different from the data obtained from flow-through light absorbance or fluorescence detectors. Especially at low intensities of radioactivity the background noise, being equal to the square root of the number of radioactive decays counted, causes a very jagged chromatogram compared to the usually smooth chromatograms from other types of detectors. This makes most chromatogram integrators and computer software less suitable to handle radioactivity chromatograms. However, the strict mathematical relation between signal and noise permits a well designed computer software to detect and validate peaks in a radioactivity chromatogram with a very high sensitivity and

certainty. This fact was used to develop a software that allows the detection and validation of peaks approximately one magnitude below what can be detected visually or by ordinary chromatographic software on a smoothed radioactivity chromatogram.[55] The electrochemical detector in HPLC-EC is usually very sensitive to pressure fluctuations. This can cause a chromatogram containing periodic variations owing to HPLC pump strokes. To remove this kind of confounding factor the chromatogram can be subjected to Fourier transform analyses where any periodic variations in the chromatogram can easily be removed.[69]

23. Conclusion

8-OH-dG is a stable biomarker for oxidative damage to DNA. The possible problem in the analysis of this DNA adduct is that the adduct could be formed during work-up and analysis, in contrast to other DNA adducts like polycyclic aromatic hydrocarbons. Therefore certain precautions are necessary. By using the precautions discussed in this paper a 5–10- fold (and up to a 100-fold in extreme cases) decrease of 8-OH-dG in control tissues can be achieved. The key factor is either to remove dG or inhibit formation of oxidants and their reaction with dG. Further investigations are necessary and on going with the aim to further reduce auto-oxidation, especially when small amounts of DNA with a low level of modification are analyzed.

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References

[1] C.G. Fraga, M.K. Shingenaga, J.W. Park, P. Degan and B.N. Ames (1990) Oxidative damage to DNA during aging: 8-hydroxy-2′-deoxyguanosine in rat organ DNA and urine. *Proceedings of the National Academy of Sciences in the United States of America*, **87**, 4690–4537.

[2] T. Tchou, H. Kasai, S. Shibutatni, M.H. Chung, J. Laval, A.P. Grollman and S. Nishimura (1991) 8-Oxoguanine (8-hydroxy-guanine) DNA glycosilase and its substrate specificity. *Proceedings of the National Academy of Sciences in the United States of America*, **88**, 4690–4694.

[3] J. Tchou, M.L. Michaels, J.H. Miller and A.P. Grollman (1993) Function of the zincn finger in *Escherichia coli* Fpg protein. *Journal of Biological Chemistry*, **268**, 26738–26744.

[4] Y.S. Lee, H.S. Lee, M.K. Park, E.S. Hwang, E.M. Park, H. Kasai and M.H. Chung (1993) Identification of 8-hydroxyguanine glycosylase activity in mammalian tissues using 8-hydroxyguanine specific monoclonal antibody. *Biochemical & Biophysical Research Communications*, **196**, 1545–1551.

[5] K. Sakumi, M. Furuichi, T. Tsuzuki, T. Kakuma, S. Kawabata, H. Maki and M. Sekiguchi (1993) Cloning and expression of cDNA synthesis. *Journal of Biological Chemistry*, **268**, 23524–23530.

[6] T. Lindahl (1993) Instability and decay of the primary structure of DNA. *Nature*, **362**, 709–715.

[7] R.A. Floyd (1990) The role of 8-hydroxyguanine in carcinogenesis. *Carcinogenesis*, **11**, 1447–1450.

[8] M. Hayakawa, K. Torii, S. Sugiyama, M. Tanaka and T. Ozawa (1991) Age associated accumulation of 8-hydroxyguanosine in mitochondrial DNA of human diaphragm. *Biochemical & Biophysical Research Communications*, **179**, 1023–1029.

[9] D.B. Clayson, R. Mhta and F. Iverson (1994) International commission for protection against environmental mutagens and carcinogens. Oxidative DNA damage – the effects of certain genotoxic and operationally non-genotoxic carcinogens. *Mutation Research*, **317**, 25–42.

[10] R. Shimoda, M. Nagashima, M. Sakamoto, N. Yamaguchi, S. Hirohashi, J. Yokota and H. Kasai (1994) Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. *Cancer Research*, **54**, 3171–3172.

[11] S. Loft, A. Astrup, B. Buemann and H.E. Poulsen (1994) Oxidative damage correlates with oxygen consumption in humans. *FASEB Journal*, **8**, 534–537.

[12] A.C. Povey, V.L. Wilson, A. Weston, V.T. Doan, M.L. Wood, J.M. Essigmann and P.G. Shields (1993) Detection of oxidative damage by ^{32}P-postlabelling: 8-hydroxydeoxyguanosine as a marker of exposure. In: *Postlabelling Methods for Detection of DNA Adducts* (Eds. Phillips, Castegnaro and Bartsch), IARC, Lyon, France, pp. 105–114.

[13] P. Dandona, K. Thusu, B. Snyder, J. Makowski, D. Armstrong and T. Nicotera (1996) Oxidative damage to DNA in diabetes mellitus. *The Lancet*, **347**, 444–445.

[14] M. Kitahara, H.J. Eyre, R.E. Lynch, M.L. Rallison and H.R. Hill (1980) Metabolic activity of diabetics monocytes. *Diabetes*, **29**, 251–256.

[15] D. Armstrong and F. Al-Awadi (1991) Lipid peroxidation and retinopathy in steptozotocin induced diabetes. *Free Radical Biology & Medicine*, **11**, 433–436.

[16] R.S. Britton and B.R. Bacon (1994) Role of free radicals in liver diseases and hepatic fibrosis. *Hepato-Gastroenterology*, **41**, 342–348.

[17] A. Bjorneboe and G.E. Bjorneboe (1993) Antioxidant status and alcohol related diseases. *Alkohol & Alkoholism*, **28**, 111–116.

[18] M.S. Stewart, G.S. Cameron and B.C. Pence (1996) Antioxidant nutrients protect against UVB-induced

oxidative damage to DNA of mouse keratinocytes in culture. *Journal of Investigative Dermatology*, **106**, 1086–1089.
[19] Y. Hattori, C. Nishigori, T. Tanaka, K. Uchida, O. Nikaido, T. Osawa, H. Hiai, S. Imamura and S. Toyokuni (1997) 8-hydroxy-2′-deoxyguanosine is increased in epidermal cells of hairless mice after chronic ultraviolet B exposure. *Journal of Investigative Dermatology*, **107**, 733–737.
[20] H. Wei, Q. Cai, R. Rahn and X. Zhang (1997) Singlet oxygen involvement in (254 nm) radiation-induced formation of 8-hydroxy-deoxyguanosine in DNA. *Free Radical Biology & Medicine*, **23**, 148–154.
[21] H. Xueliang and J. Liehr (1994) 8-hydroxylation of guanine bases in kidney and liver DNA of hamsters treated with estradiol: role of free radicals in estrogen-induced carcinogenesis. *Cancer Research*, **54**, 5515–5517.
[22] K. Sai, T. Umemura, A. Takagi, R. Hasegawa and Y. Kurokawa (1992) The protective role of glutathione, cysteine and vitamin C against oxidative DNA damage induced in rat kidney by potassium bromate. *Japanese Journal of Cancer Research*, **83**, 45–51.
[23] T. Takeuchi, M. Nakajima and K. Morimoto (1994) Establishment of a human system that generates O_2 and induces 8-hydroxyguanosins, typical of oxidative DNA damage, by a tumor promotor. *Cancer Research*, **54**, 5837–5840.
[24] B. Halliwell and J.M.C. Gutteridge (1989) *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford.
[25] T. Hofer and L. Möller (1998) Reduction of oxidation during the preparation of DNA and analysis of 8-hydroxy-2′-deoxyguanosine. *Chemical Research in Toxicology* (in press).
[26] J.B. Vincent, M.W. Crowder and B.A. Averill (1992) Hydrolysis of phosphate monoesters: a biological problem with multiple chemical solutions. *TIBS*, **17**, 105–110.
[27] A.V. Chernikov, A.M. Usacheva and V.I. Bruskov (1996) Depurination of 8-oxo-7,8-dihydroguanine (8-hydroxyguanine) nucleosides. *Biochemistry (Moscow)*, **61**, 35–38.
[28] A. Jenner, T.G. England, O.I. Aruoma and B. Halliwell (1998) Measurement of oxidative DNA damage by gas chromatography-mass spectrometry: ethanethiol prevents artifactual generation of oxidized DNA bases. *Biochemical Journal*, **331**, 365–369.
[29] J. Serrano, C.M. Palmeira, K.B. Wallace and D.W. Kuehl (1996) Determination of 8-hydroxydeoxyguanosine in biological tissue by liquid chromatography/electrospray ionization-mass spectrometry/mass spectrometry. *Rapid Communications in Mass Spectrometry*, **10**, 1789–1791.
[30] A.P. Breen and J.A. Murphy (1995) Reactions of oxyl radicals with DNA. *Free Radical Biology & Medicine*, **18**, 1033–1077.
[31] L. Möller and T. Hofer (1997) [^{32}P]ATP mediates formation of 8-hydroxy-2′-deoxyguanosine from 2′-deoxyguanosine, a possible problem in the ^{32}P-postlabeling assay. *Carcinogenesis*, **18**, 2415–2419.
[32] A. Tagaki, K. Sai, T. Umemura, R. Hasegawa and Y. Kurokawa (1995) Inhibitory effects of vitamin E and ellagic acid on 8-hydroxy-deoxyguanosine formation in liver nuclear DNA of rats treated with 2-nitropropane. *Cancer Letters*, **91**, 139–144.
[33] D. Nakae, Y. Mizumoto, E. Kobayashi, O. Noguchi and Y. Konishi (1995) Improved genomic/nuclear DNA extraction for 8-hydroxydeoxyguanosine analysis of small amounts of rat liver tissue. *Cancer Letters*, **97**, 233–239.
[34] K.B. Beckman and B.N. Ames (1996) Detection and quantification of oxidative adducts of mitochondrial DNA. *Methods in Enzymology*, **264**, 442–453.
[35] H.J. Helbock, K.B. Beckman, M.K. Shigenaga, P.B. Walter, A.A. Woodall, H.C. Yeo and B.N. Ames (1998) DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. *Proceedings of the National Academy of Sciences in the United States of America*, **95**, 288–293.
[36] A. Samuni and M.C. Krishna (1997) Antioxidant properties of nitroxides and nitroxide SOD mimics. In: *Handbook of Synthetic Antioxidants* (Eds. L. Packer and E. Cedenas), M. Dekker, New York, pp. 351–373.
[37] G.R. Buettner and B.A. Jurkiewicz (1995) Ascorbate radical: a valuable marker of oxidative stress. In: *Analysis of Free Radicals in Biological Systems* (Eds. A.E. Favier, J. Cadet, B. Kalyanaraman, M. Fontecave and J.-L. Pierre), Birkhäuser Verlag, Basel, pp. 145–164.
[38] B. Halliwell (1996) Vitamin C: antioxidant or pro-oxidant *in vitro*? *Free Radical Research*, **25**, 439–454.
[39] J.-W. Park and R.A. Floyd (1997) Glutathione/Fe^{3+}/O_2-mediated DNA strand breaks and 8-hydroxydeoxyguanosine formation. *Biochimica et Biophysica Acta*, **1336**, 263–268.
[40] J.-W. Park and R.A. Floyd (1994) Generation of strand breaks and formation of 8-hydroxy-2′deoxyguanosine in DNA by a thiol/Fe^{3+}/O_2-catalyzed oxidation system. *Archives of Biochemistry and Biophysics*, **312**, 285–291.
[41] N. Spear and S.D. Aust (1998) The effects of different buffers on the oxidation of DNA by thiols and ferric iron. *Journal of Biochemistry and Molecular Toxicology*, **12**, 125–132.
[42] O.M. Lardinois, M.M. Mestdagh and P.G. Rouxhet (1996) Reversible inhibition and irreversible inactivation of catalase in presence of hydrogen peroxide. *Biochimica and Biophysica Acta*, **1295**, 222–238.
[43] D.H.R. Barton, S.-Y. Choi, B. Hu and J.A. Smith (1998) Evidence for a higher oxidation state of manganese in the reaction of dinuclear manganese complexes with oxidants. Comparison with iron based gif chemistry. *Tetrahedron*, **54**, 3367–3378.
[44] O.I. Aruoma, B. Halliwell and M. Dizdaroglu (1989) Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase. *The Journal of Biological Chemistry*, **264**, 13024–13028.
[45] J.-B. Galey (1997) Potential use of iron chelators against oxidative damage. In: *Antioxidants in Disease Mechanisms and Therapy* (Ed. H. Sies), Academic Press, London and New York, pp. 167–203.
[46] M.J. Burkitt, L. Milne, P. Nicotera and S. Orrenius (1996) 1,10-Phenanthroline stimulates internucleosomal DNA fragmentation in isolated rat-liver nuclei by promoting the redox activity of endogenous copper ions. *Biochemical Journal*, **313**, 163–169.
[47] H.K. Young, R.A. Floyd, M.L. Maidt and J.R. Dynlacht (1996) Evaluation of nitrone spin-trapping agents as radioprotectors. *Radiation Research*, **146**, 227–231.
[48] K. Randerath, M.V. Reddy and R.C. Gupta (1981) ^{32}P-Labelling test for DNA damage. *Proceedings of the National Academy of Sciences of the United States of America*, **78**, 6126–6129.
[49] R.C. Gupta, M.V. Reddy and K. Randerath (1982) ^{32}P-Postlabelling analysis of nonradioactive aromatic carcinogen-DNA adducts. *Carcinogenesis*, **3**, 1081–1092.

[50] R.C. Gupta (1985) Enhanced sensitivity of ^{32}P-postlabelling analysis of aromatic carcinogen:DNA adducts. *Cancer Research*, **45**, 5656–5662.

[51] M.V. Reddy and K. Randerath (1986) Nuclease P_1-mediated enhancement of sensitivity of ^{32}P-postlabelling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543–1551.

[52] A.C. Beach and R.C. Gupta (1992) Human biomonitoring and the ^{32}P-postlabeling assay. *Carcinogenesis*, **13**, 1053–1074.

[53] G.N. Levy and W.W. Weber (1988) High-performance liquid chromatographic analysis of ^{32}P-postlabeled DNA-aromatic carcinogen adducts. *Analytical Biochemistry*, **174**, 381–392.

[54] E.H. Weyand, J.E. Rice and E.J. LaVoie (1987) ^{32}P-postlabeling analysis of DNA adducts from non-alternant PAH using thin-layer and high performance liquid chromatography. *Cancer Letters*, **37**, 257–266.

[55] L. Möller and M. Zeisig (1993) DNA adduct formation after oral administration of 2-nitrofluorene and *N*-acetyl-2-aminofluorene, analyzed by ^{32}P-TLC and ^{32}P-HPLC. *Carcinogenesis*, **14**, 53–59.

[56] L. Möller, M. Zeisig and P. Vodicka (1993) Optimization of an HPLC method for analyses of ^{32}P-postlabeled DNA adducts. *Carcinogenesis*, **14**, 1343–1348.

[57] M. Zeisig and L. Möller (1997) ^{32}P-Postlabeling high-performance liquid chromatographic improvements to characterize DNA adduct stereoisomers from benzo[]-pyrene and benzo[*c*]phenanthrene, and to separate DNA adducts from 7,12-dimethylbenz[]anthracene. *Journal of Chromatography B Biomedical Applications*, **691**, 341–350.

[58] M. Zeisig and L. Möller (1995) Accelerated paper: ^{32}P-HPLC suitable for characterization of DNA adducts formed *in vitro* by polycyclic aromatic hydrocarbons and derivatives. *Carcinogenesis*, **16**, 1–9.

[59] L. Möller, E. Grzybowska, M. Zeisig, B. Cimander, K. Hemminki and M. Chorazy (1995) Seasonal variations of DNA adduct pattern in human lymphocytes analyzed by ^{32}P-HPLC. *Carcinogenesis*, **17**, 61–66.

[60] L. Möller, X.-S. Cui, U.-B. Torndal and L.C. Ericsson (1993) Preneoplastic lesions and DNA adduct formation of the airborne genotoxic agents 2-nitrofluorene and 2,7-dinitrofluorene. *Carcinogenesis*, **14**, 2627–2632.

[61] L. Möller, M. Zeisig, T. Midtvedt and J.Å. Gustafsson (1994) Intestinal microflora enhances formation of DNA adducts following administration of 2-NF and 2-AAF. *Carcinogenesis*, **15**, 857–861.

[62] P. Wohlin, M. Zeisig, J.-Å. Gustafsson and L. Möller (1996) ^{32}P HPLC analysis of DNA adducts formed *in vitro* and *in vivo* by 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine and 2-amino-3,4,8-trimethyl-3*H*-imidazo [4,5-*f*]quinoxaline, utilizing an improved adduct enrichment procedure. *Chemical Research in Toxicology*, **9**, 1050–1056.

[63] M. Zeisig, J. Cadet and L. Möller (1998) ^{32}P-Postlabeling high-performance liquid chromatography (^{32}P-HPLC) adapted for analysis of 8-hydroxy-2′-deoxyguanosine (submitted).

[64] J.T. Lutgerink, E. de Graaf, B. Hoebee, H.F.C. Stavenuitez, J.G. Westra and E. Kriek (1992) Detection of 8-hydroxyguanine in small amounts of DNA by ^{32}P postlabeling. *Analytical Biochemistry*, **201**, 127–133.

[65] U.-S. Devanaboyma and R.C. Gupta (1996) Sensitive detection of 8-hydroxy-2′-deoxyguanosine in DNA by ^{32}P-postlabeling assay and the basal levels in rat tissues. *Carcinogenesis*, **17**, 917–924.

[66] D. Schuler, M. Otteneder, P. Sagelsdorff, E. Eder, R.C. Gupta and K. Lutz (1997) Comparative analysis of 8-oxo-2′-deoxyguanosine in DNA by ^{32}P- and ^{33}P-post-labeling and electrochemical detection. *Carcinogenesis*, **18**, 2367–2371.

[67] V.L. Wilson, B.G. Taffe, P.G. Shields, A.C. Povey and C.C. Harris (1993) Detection and quantification of 8-hydroxydeoxyguanosine adducts in peripheral blood of people exposed to ionizing radiation. *Environmental Health Perspectives*, **99**, 261–263.

[68] K. Podmore, P.B. Farmer, K.E. Herbert, G.D.D. Jones and E.A. Martin (1997) ^{32}P-Postlabelling approaches for the detection of 8-oxo-2′-deoxyguanosine-3′-monophosphate in DNA. *Mutation Research*, **178**, 139–149.

[69] S. Adachi, M. Zeisig and L. Möller (1995) Improvements in the analytical method for 8-hydroxydeoxyguanosine in nuclear DNA. *Carcinogenesis*, **16**, 253–258.

[70] B. Tesfamariam (1994) Free radicals in diabetic endothelial cell dysfunction. *Free Radical Biology and Medicine*, **16**, 383–391.

[71] M. Hayakawa and F. Kuzuya (1990) Free radicals and diabetes mellitus. *Nippon Ronen Igakkai Zasshi*, **27**, 149–154.

[72] S.P. Wolff, Z.Y. Jiang and J.V. Hunt (1991) Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radical Biology and Medicine*, **10**, 339–352.

[73] P. Mecocci, U. MacGarvey, A.E. Kaufman, D. Koonz, J.M. Shoffner, D.C. Wallace and M.F. Beal (1993) Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain. *Annals of Neurology*, **34**, 609–616.

[74] E.J. Crary and M.F. McCarty (1984) Potential clinical applications for high-dose nutritional antioxidants. *Medical Hypotheses*, **13**, 77–98.

[75] K.S. Kilgore and B.R. Lucchesi (1993) Reperfusion injury after myocardial infarction: the role of free radicals and the inflammatory process. *Clinical Biochemistry*, **26**, 359–370.

[76] S.-H. Baik, H.-S. Youn, M.-H. Chung, W.-K. Lee, M.-J. Cho, G.-H. Ko, C.-K. Park, H. Kasai and K.-H. Rhee (1996) Increased oxidative DNA damage in *Heliobacter pylori*-infected human gastric mucosa. *Cancer Research*, **56**, 1279–1282.

[77] V.R. Winrow, P.G. Winyard, C.J. Morris and D.R. Blake (1993) Free radicals in inflammation: second messengers and mediators of tissue destruction. *British Medical Bulletin*, **49**, 506–522.

[78] A.M. Peretz, J.D. Neve and J.P. Famaey (1991) Selenium in rheumatic diseases. *Seminars in Arthritis & Rheumatism*, **20**, 305–316.

[79] G.E. Hatch (1995) Asthma, inhaled oxidants and dietary antioxidants. *American Journal of Clinical Nutrition*, **61** (3 Suppl), 625–630.

[80] P. Degan, S. Bonassi, M. De Caterina, L.G. Korkina, L. Pinto, F. Scopacasa, A. Zatterale, R. Calzone and G. Pagano (1995) *In vivo* accumulation of 8-hydroxy-2′-deoxyguanosine in DNA correlates with release of reactive oxygen species in Fanconi's anaemia families. *Carcinogenesis*, **16**, 735–742.

[81] H. Ohshima and H. Bartsch (1994) Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutation Research*, **305**, 253–264.

[82] D.C. Malins and R. Haimanot (1991) Major alterations in the nucleotide structure of DNA in cancer of the female breast. *Cancer Research*, **51**, 5430–5432.
[83] J. Musarrat, J. Arezina-Wilson and A.A. Wani (1997) Prognostic and aetiological relevance of 8-hydroxyguanosine in human breast carcinogenesis. *European Journal of Cancer*, **32A**, 1209–1214.
[84] L. Lih-Brody, S.R. Powell, K.P. Collier, G.M. Reddy, R. Cerchia, E. Kahn, G.S. Weissman, S. Katz, R.A. Floyd, M.J. McKinley, S.E. Fisher and G.E. Mullin (1996) Increased oxidative stress and decreased antioxidant defense in mucosa of inflammatory bowel disease. *Digestive Diseases and Sciences*, **41**, 2078–2086.
[85] R. Dargel (1992) Lipid peroxidation – a common pathogenic mechanism? *Experimental & Toxicologic Pathology*, **44**, 169–181.
[86] D.D. Bankson, M. Kestin and N. Rifai (1993) Role of free radicals in cancer and atherosclerosis. *Clinics in Laboratory Medicine*, **13**, 463–480.
[87] P.K. Liu, C.Y. Hsu, M. Dizdaroglu, R.A. Floyd, Y.W. Kow, A. Karakaya, L.E. Rabow and J.-K. Cui (1996) Damage, repair and mutagenesis in nuclear genes after mouse forebrain ischemia-reperfusion. *The Journal of Neuroscience*, **16**, 6795–6806.
[88] L. Möller *et al.* (1900) Karolinska Institutet, unpublished results.
[89] M. Nakano (1992) Free radicals and their biological significance. *Human Cell*, **5**, 334–340.
[90] R.K. Brown, A. McBurney, J. Lunce and F.J. Kelly (1995) Oxidative damage to DNA in patients with cystic fibrosis. *Free Radical Biology & Medicine*, **18**, 801–806.
[91] A. Bendich (1993) Physiological role of antioxidants in the immune system. *Journal of Dairy Science*, **76**, 2789–2794.
[92] P. Mecocci, U. MacGarvey and M.F. Beal (1994) Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Annals of Neurology*, **36**, 747–751.
[93] M.B. Youdim and L. Lavie (1994) Selective MAO-A and B inhibitors, radical scavengers and nitric oxide synthase inhibitors in Parkinson's disease. *Life Sciences*, **55**, 2077–2082.
[94] J. Ward (1994) Free radicals, antioxidants and preventive geriatrics. *Australian Family Physician*, **23**, 1297–1301.
[95] J. Pincemail (1995) Free radicals and antioxidants in human diseases. In: *Analysis of Free Radicals in Biological Systems* (Eds. A.E. Favier, J. Cadet, B. Kalyanaraman, M. Fontecave and J.-L. Pierre).
[96] S. Adachi, K. Kawamura, S. Yoshida and K. Takemoto (1992) Oxidative damage on DNA induced by asbestos and man-made fibers *in vitro*. *International Archives of Occupational and Environal Health*, **63**, 553–557.
[97] S.M. Plummer and S.P. Faux (1994) Induction of 8-hydroxy-deoxyguanosine in isolated DNA and HeLa cells exposed to fecapentaene-12: evidence for the involvement of prostaglandin H synthase and iron. *Carcinogenesis*, **15**, 449–453.
[98] X. Shi, X. Sun, P.M. Gannett and N.S. Dalal (1992) Deferoxamine inhibition of Cr(V)-mediated radical generation and deoxyguanine hydroxylation: ESR and HPLC evidence. *Archives of Biochemistry and Biophysics*, **293**, 281–286.
[99] X. Shi, H. Jiang, Y. Mao, J. Ye and U. Saffiotti (1996) Vanadium(IV)-mediated free radical generation and related 2′-deoxyguanosine hydroxylation and DNA damage. *Toxicology*, **106**, 27–38.
[100] M.V. Mikhailova, N.A. Littlefield, B.S. Hass, L.A. Poirier and M.W. Chou (1997) Cadmium-induced 8-hydroxydeoxyguanosine formation, DNA strand breaks and antioxidant enzyme activities in lymphoblastoid cells. *Cancer Letters*, **115**, 141–148.
[101] H.M. Shen, C.N. Ong, B.L. Lee and C.Y. Shi (1995) Aflatoxin B_1-induced 8-hydroxydeoxyguanosine formation in rat hepatic DNA. *Carcinogenesis*, **16**, 419–422.
[102] E.W. Austin, J.M. Parrish, D.H. Kinder and R.J. Bull (1996) Lipid peroxidation and formation of 8-hydroxydeoxyguanosine from acute doses of halogenated acetic acids. *Fundamental and Applied Toxicology*, **31**, 77–82.
[103] S. Loft, K. Vistisen, M. Ewertz, A. Tjønneland, K. Overvad and H.E. Poulsen (1992) Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis*, **13**, 2241–2247.
[104] K. Sai-Kato, T. Umemura, A. Takagi, R. Hasegawa, A. Tanimura and Y. Kurokawa (1995) Pentachlorophenol-induced oxidative DNA damage in mouse liver and protective effect of antioxidants. *Food & Chemical Toxicology*, **33**, 877–882.
[105] M.G. Mustafa (1990) Biochemical basis of ozone toxicity. *Free Radical Biology & Medicine*, **9**, 245–265.
[106] R.S. Bhimani, W. Troll, D. Grunberger and K. Frenkel (1993) Inhibition of oxidative stress in HeLa cells by chemopreventive agents. *Cancer Research*, **53**, 4528–4533.
[107] M.A. Sipowicz, S. Amin, D. Desai, K.S. Kasprzak and L.M. Anderson (1997) Oxidative DNA damage in tissues of pregnant female mice and fetuses caused by the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Cancer Letters*, **117**, 87–91.
[108] S. Lagorio, C. Tagesson, F. Forastiere, I. Iavarone, O. Axelson and A. Carere (1994) Exposure to benzene and urinary concentrations of 8-hydroxydeoxyguanosine, a biological marker of oxidative damage to DNA. *Occupational and Environmental Medicine*, **51**, 739–743.
[109] T.-Y. Liu, C.-L. Chen and C.-W. Chi (1996) Oxidative damage to DNA induced by areca nut extract. *Mutation Research*, **367**, 25–31.
[110] R. Olinski, T.H. Zastawny, M. Foksinski, W. Windorbska, P. Jaruga and M. Dizdaroglu (1996) DNA base damage in lymphocytes of cancer patients undergoing radiation therapy. *Cancer Letters*, **106**, 207–215.