Chapter 13

HPLC-ECD, HPLC-MS/MS (Urinary Biomarkers)

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1. **Introduction** 234
2. **Principles for HPLC and GC Separation** 234
3. **Comparison of Different Methods** 244
4. **Interpretation of Tissue Levels and Urinary Excretion** 244
5. **What is Known from Human Studies of Biomarkers of Oxidative DNA Damage?** 247
6. **Proper Design of Human Intervention Studies** 248
7. **Future Perspectives** 250
8. **Conclusions** 251

References 251

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1. Introduction

Mammalian life is based on oxygen and uses oxygen reduction for energy production and synthetic processes. By 4-electron reactions oxygen is reduced to water and the energy released is stored for controlled use. However, one electron reduction occurs in minor amounts giving rise to various reactive oxygen species (ROS).[1,2] The reactive oxygen species potentially oxidizes important macromolecules and structures in the body. Although posing serious threats of deleterious effects on vital functions, it is now increasingly realized that ROS play important roles as part of defence, signaling and transcription mechanisms.[3–5]

A multiplicity of different oxidative DNA modifications has been described.[6,7] Still, there is much more scarce data on their occurrence in the *in vivo* situation, and whether they have biological significance or relates to diseases. The exception is the 8-hydroxylation of guanine (8-oxodG), this lesion has been investigated both *in vitro* and *in vivo*, including human studies.

Oxidation processes are prone to occur in the earth's environment, including in test tubes, refrigerators, freezers, laboratories etc. due the ubiquitous oxygen. This poses a major challenge to anybody studying these processes since artefacts can arise from oxidation during sample handling. Particularly, most methods rely on storage or prolonged preparation of samples before the initial analysis. In addition to storage, most procedures are carried out at conditions that clearly makes spontaneous oxidation possible. Often it will be found that immense differences are reported between different laboratories. Consequently published data always should be scrutinized bearing this aspects in mind.

There is no doubt that data considered to reflect the *in vivo* situation with regard to oxidative stress to some extend are contradictory and difficult to interpret, and that this may be because the necessary precautions against oxidation were not taken. This review focus on the methodologies that presently are available, mainly with regard to estimating oxidative DNA products excreted into urine, but also in the context of tissue levels. The methodology is very similar and information from tissue levels and urinary excretion provide information on the oxidative stress to DNA.

2. Principles for HPLC and GC Separation

Two powerful techniques are available for separation of substances in samples in order to enable detection. HPLC, high performance (or pressure) liquid chromatography, is particularly suited for small water-soluble molecules and proteins. Most used for analysis of DNA fragments is the reverse phase HPLC. Gas chromatography (GC) is well suited for volatile compounds, however, with the use of various derivatization procedures it is possible to separate e.g. DNA bases with GC.

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In essence chromatography corresponds to a series of organic extractions, e.g. of water and chloroform. However, the theoretical series of extractions corresponds to maybe 5000 or more. Considering this is done within minutes on a HPLC or GC column, it is clear how powerful this separation technique is. The HPLC technique requires few preparative steps of the sample which saves time and work and can also avoid potential problems related to the clean-up procedures. The GC technique requires derivatization and extraction procedures but provides very high and sharp peaks that are particularly suitable for mass spectrometry.

To some degree, theoretical considerations can be a great aid in setting up the analysis. For HPLC computer programs like DryLab® exists and can be of some help. To our experience there are many problems that cannot be foreseen from theoretical considerations, and development/implementation rests to a large degree on trial and error, experience and a little inspiration. Special consideration should be given to factors that reduce noise. Among these, heavy pulse damping of the HPLC system, use of high quality water and solvent and selection of the right column is of particular importance.

Other separation techniques have large potentials, e.g. capillary electrophoresis, but have not been much used for analysis of DNA oxidation.

For HPLC with tandem mass spectrometric detection (HPLC-MS/MS), the HPLC separation is not as demanding as for HPLC-ECD. We have found that a single column is sufficient,[8] however, we must emphasize that unknown substances similar in mass to 8-oxodG needs to be separated from 8-oxodG. For high sensitivity in mass spectrometry the peak height in HPLC is very important. The amount detected is proportional both to the peak height and to the area under the curve.

By derivatization it is possible to use the GC separation procedure coupled with mass spectrometry to measure oxidized DNA products. However, this method has with few exceptions not been used for urinary measurements on DNA, but has been the method used for estimation of 8-oxodG, actually the base after hydrolysis, and other DNA oxidation products in tissue. For urine measurements a semi-preparative HPLC procedure was applied, followed by hydrolysis, derivatization and GC-MS.[9–11]

The immunological methods do not include a prior HPLC separation procedure.

2.1. Electrochemical Detection (ECD)

This method relies on the oxidation of a compound in an electrical field and detection of the change in current by this process. The particular virtue of the ECD detection is the extreme sensitivity making it possible to detect e.g. 8-oxodG in the nM concentration range, i.e. fmol injection. Since the first publication of the HPLC-ECD method,[12] this has been the preferred method and used in a large

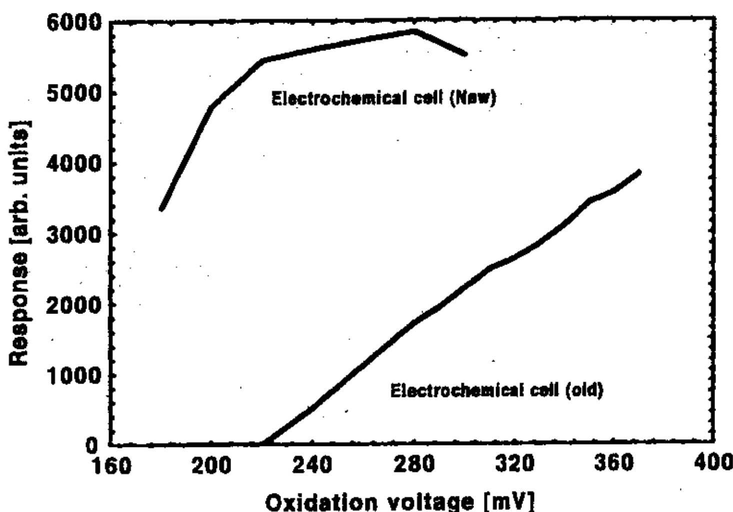

Fig. 1. Dynamic voltamogram of 8-oxodG using a Coulochem Electrochemical detector and a 5011 analytical cell. The graph is constructed from injection of pure 8-oxodG on the system with different voltage setting for oxidation current. The difference between a worn-out and a new cell is depicted.

number of laboratories mostly for detection of 8-oxodG in DNA, but also for detection of 8-oxodG in urine.

The applied electrical potential that can oxidize a compound exhibits several plateaus. The actual plateau for a given compound and brand of apparatus has to be identified for that specific combination. This is done[13] by establishing a dynamic voltamogram by measuring the signal after injection of a known sample and increasing the oxidation voltage. An example is shown in Fig. 1 that depicts a worn out electrochemical cell and a new cell. This figure also illustrates that it is necessary to check the voltamogram with regular intervals and to adjust the oxidation voltage.

There are many different brands of ECD detectors and electrodes/cells. In our laboratory we have found that the ESA Coulochem is working excellent for our purposes and provides excellent sensitivity. Similar experience with other detectors can be found.

We have found that for HPLC-ECD analysis of urine, separation is critical due to electrochemically active peaks eluting close to that of 8-oxodG. Ways to detect a false peak is given in details elsewhere.[14]

The quantification also requires special attention since in HPLC it is not possible to use a true internal standard, i.e. an internal standard that behaves exactly as the substance you want to measure. An internal standard, 2,6-Diamino-8-oxopurine, has been suggested,[15] but is probably only useful in controlling

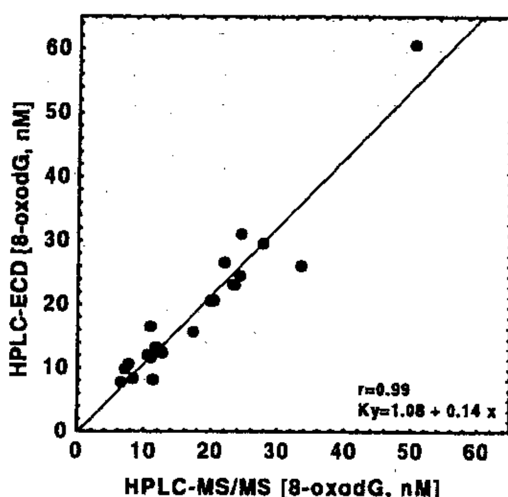

Fig. 2. Correlation between 8-oxodG in 21 different urine samples measured by HPLC-ECD and HPLC-MS/MS. The correlation, $r = 0.99$, the negligible intercept (0.14 nM) and the close to unity slope (1.08) provide evidence for the excellent agreement of the two methods.

variations in the injection volume, and cannot be used for other purposes that poses more severe problems like artificial oxidation, degradation of 8-oxodG on the column etc. Presently there is no experience with the use of this internal standard for urine measurement. We use external standard addition in different concentrations and evaluate the response ratios,[14] and this methodology appears to function satisfactory from comparison with HPLC-MS/MS analysis as seen in Fig. 2.

2.2. Mass Spectrometric Detection (MS)

The detection of a substance from determination of its mass is often stated as a specific methodology, yet many substances have the same mass. With some separation of the substances with GC or HPLC coupled with fragmentation and selection of a suitable fragment, a very high degree of specificity may be achieved. The advancement of the technical side of mass spectrometry and the use of elaborated computer software to control and handle the data output from mass spectrometers have made it possible to use this methodology more widely than previously. For years the dominating methodology has been gas chromatography coupled with mass spectrometry for the detection of oxidized DNA products. However, the development of ionization methods for HPLC, e.g. electrospray has

made possible the use of HPLC coupled with mass spectroscopy. For quantification purposes, triple quadrupole mass spectrometers are probably the most useful apparatus as of today. The progress in other techniques like capillary electrophoresis-MS and mass spectrometry based on time of flight may soon develop to a similar or surpassing degree.

Gas-chromatography-mass spectrometry used for quantification of oxidative DNA products has been criticized for errors due to artificial oxidation, however, provided that sufficient precautions are taken, this can be avoided and results similar to those from HPLC-ECD can be provided regarding 8-oxodG in DNA.[16] Presumably this is also valid for other oxidative DNA products, but needs to be validated. In case of 8-oxodA the validity has been questioned[17] regarding 8-oxodA in an experiment with vitamin C and vitamin E intervention[18] and using HPLC-MS/MS it seems likely that the high reported 8-oxodA values relates to artifactual oxidation.[8] Many of the problems regarding artificial oxidation relates to the very high content of non-oxidized dG in DNA hydrolysates, about 1 000 000 times higher. This means that oxidation of only a very minute fraction of dG gives serious artefacts. For urine measurements the levels of oxidized and non-oxidized nucleosides are similar and would *a priori* not present a problem of the same magnitude.

The basis for using mass spectrometry is ionization of the substances. With ion spray this is done from an HPLC outlet, where the mobile phase is evaporated at high temperature and the substances ionized by a high voltage before the ionized molecules enter the vacuum of the mass spectrometer itself. Ionspray is a soft ionization technique that means that the ions do not show extensive fragmentation. The ionization process used in gas chromatography is more energetic resulting in fragmentation of the substances. GC-MS analysis is normally used with a single quadrupole for detection whereas triple quadrupole instruments are used with HPLC separation.

In the triple quadrupole mass spectrometer (Fig. 3), a first quadrupole, Q1, is used to select one or several wanted mass(es). They are passed on to a second quadrupole, Q2, that functions as a collision cell, where the ions collide with a collision gas. The quadrupole arrangement serves to focus the ions. The collisions can be controlled and in case of oxidized nucleosides specific breakage of the pseudomolecularions N-glycosidic bond can be achieved. The product ions are then selected in the third quadrupole, Q3, followed by final detection. This is usually called Multiple Reaction Monitoring (MRM) and is the basis for the particular usefulness of the triple quadrupole when high specificity and sensitivity is needed. Figure 3 depicts this principal build of the triple quadrupole tandem mass spectrometer. Details of how to operate an HPLC-MS/MS system depend on the brand and the software version. Reference is made to the brands manual and for other details to original papers.[8, 14, 19, 20] It should be noted that in HPLC mass spectrometry non-volatile salts should generally be

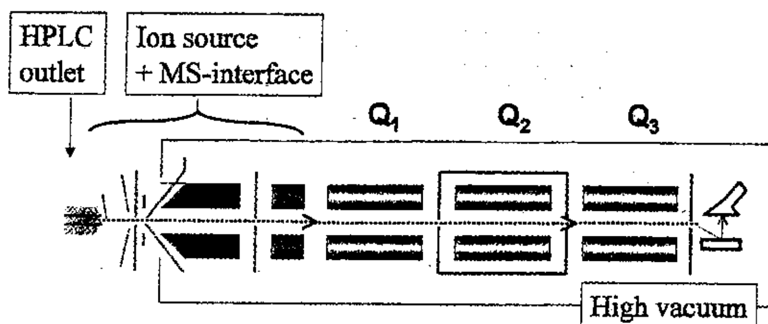

Fig. 3. The principle build of a HPLC-MS/MS instrument. The HPLCC outlet is to a high temperature ion source and MS-interface that provides evaporation and ionization of the substances in the eluent. The ions then enter the vacuum in which the quadrupole select the masses defined, before and after fragmentation.

avoided, mainly because salt suppresses the ionization and also deposits in the apparatus with loss of sensitivity and demand for repeated cleaning procedures.

With careful consideration to these factors, using individual tuning files the HPLC-MS/MS analysis matches the sensitivity of HPLC-ECD, i.e. below 1 nM 8-oxodG in urine with an injection of 20 μL. This opens the possibility for running a parallel column system that with software-controlled valves can give a high sample throughput. The actual practical limit of such a system is still unknown.

One particular virtue of HPLC-MS/MS, as well as GC-MS, is the use of stable isotope labeled internal standards. By labeling e.g. 8-oxodG with stable carbon and/or nitrogen isotopes in non-functional positions, a substance that chemically, chromatographically and in other physical-chemical aspects behaves exactly like the unlabeled compound is provided. In the mass spectrometer the two substances can easily be detected at the same time (for practical purposes) and used for quantification.

Since mass spectrometry does not rely on UV absorption or electrochemical properties, and since mass detection may provide high sensitivity for different compounds the potential for measuring different nucleosides, bases or other DNA products is present. Presently this potential has only been used to a limited degree, but development is ongoing in several laboratories.

As mentioned above GC-MS has been used to measure urinary DNA oxidation products, however, various clean up or up-concentration methods are necessary. The choice for urinary measurement is therefore either HPLC-EC, which is limited mainly to 8-oxodG measurement or to HPLC-MS/MS where multiple products can be measured. Both of these methods can be set up with very little preparation of urine, just a simple centrifugation and dissolving of possible sediments.

The versatility and sensitivity of LC-MS/MS is great. For example, we have developed a method by which it is possible to analyze the promutagenic exocyclic

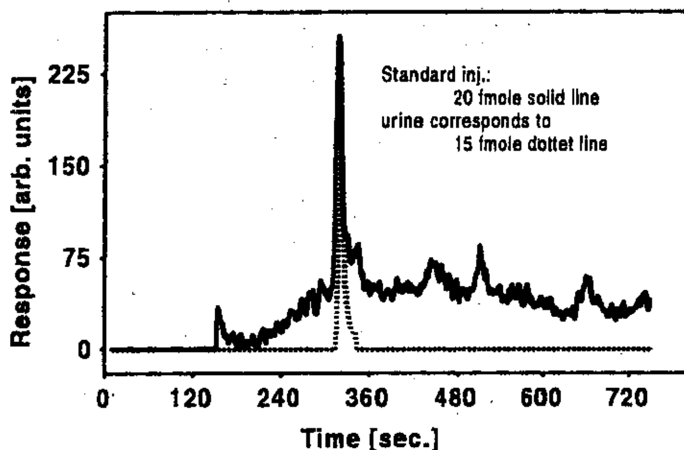

Fig. 4. HPLC-MS/MS analysis of urine from a human volunteer. A Sciex API 3000 mass spectrometer with heated nebulizer (APCI) was used for monitoring m/z 276.2 (MH+) → 160.0 (BH_2+). Urine was separated by column switching between a Zobax Eclipse C_{18} 4.6 * 50 mm, 3.5 μ column and a wacosil C_{18} 4.0 × 50 mm, 3 μ column. Eluents were 5 nM ammonium acetate pH 5.0 and 5 nM ammoniumacetate pH 5.0, 7% acetonitrile. The dotted line is a trace of injection of 20 fmole etheno-deoxyadenosine, the solid line injection of 100 μL urine.

DNA adduct 1,N^6-ethenodeoxyadenosine (εdA), Fig. 4. The analysis was done using atmospheric pressure chemical ionization (APCI) in the positive ion mode with multiple reaction monitoring (MRM) on a Sciex API 3000 triple quadrupole mass spectrometer. Quantification was performed on the characteristic transition m/z 276.3(MH^+) → 160.3(BH_2^+). Promising results have been obtained with εdA standard with a limit of detection below 1 fmole. Figure 4 shows a chromatogram of urine from a healthy volunteer indicating a concentration of εdA of about 150 fmole/ml.[21]

2.3. Immunological Methods

The use of a specific antibody could be the basis for a fast and effective methodology to measure 8-oxodG. However, it has proven difficult to produce an antibody with sufficient specificity for analysis in urine. Several publications have appeared.[22–25] However, although some characterization of the antibody and epitope is given, it appears not to be tested against the many different DNA and RNA products in urine.[26] Furthermore, testing against the present method of choice HPLC-ECD, GC-MS or HPLC-MSMS has only been stated without data, and at present time the data have not been made available in the literature.[22] One particular problem with the immunologically based assays may relate to the high

Table 1. Studies on Urinary Excretion of Oxidative DNA Products

Disease/Condition/ Intervention	Level	Unit	Number of Individuals	Finding	Method	Lesion	ID
Adriamyacin	80.8 ± 8 → 98.7 ± 6.9	nmol/24 h	20	increased	HPLC+GC/MS	5-OH-me-Uracil	(Ref. 10)
Controls	121 ± 56	pmol/ml	10	concentration only	HPLC-GC/MS	5-OH-Me-Uracil	(Ref. 11)
Adriamycin treatment	74.4 ± 9.58 → 96.3 ± 8.7	nmol/24 h	14	corresponds to normal	HPLC-GC/MS	5-OH-Me-Uracil	(Ref. 46)
Controls	58 ± 23	pmol/ml	10	concentration only	HPLC-GC/MS	5-OH-Uracil	(Ref. 11)
Controls	7 ± 4	pmol /ml	10	concentration only	HPLC-GC/MS	8-oxo-Ade	(Ref. 11)
Controls versus cancer	1.19 ± 0.488 → 2.42 ± 2.28	µmol/mol creatinine	10 + 30	increased in cancer patients	multi-D-HPLC-ECD	8oxodG	(Ref. 47)
Hemochromatosis	1.39 ± 0.40	µmol/mol creatinine	12	corresponds to controls	GC/MS	8-oxodG	(Ref. 48)
Lung cancer	272 ± 13/ 19.4 ± 8.5		14/52	increased in small cell carcinoma	ELISA	8-oxodG	(Ref. 22)
Control	3 ± 1	ng/ml	1	—	solid phase extraction LC-MS	8-oxodG	(Ref. 49)
Smoking	1.02 ± 0.35 – 3.37 ± 2.84	µmol/mol creatinene	34 controls 10 smokers 24 non-smokers 10 malignant disease	increased in smokers and malignancy	multi-D-HPLC-ECD	8-oxodG	(Ref. 50)
All out' rowing	2.5	nmol/µmol creatinene	10	no change	multi-D-HPLC-ECD	8-oxodG	(Ref. 51)
Repeated exercise	266 ± 76 – 336 ± 107	pmol /kg/day	10	increased	multi-D-HPLC-ECD	8-oxodG	(Ref. 52)
Single exercise bout	24.7 ± 1.5	nmol/day	28	no change	multi-D-HPLC-ECD	8-oxodG	(Ref. 53)

Table 1 (*Continued*)

Disease/Condition/ Intervention	Level	Unit	Number of Individuals	Finding	Method	Lesion	ID
Long distance running	0.12 – 6.45	µmol/mol creatinine	32	no change	multi-D-HPLC-ECD	8-oxodG	(Ref. 54)
Adriamyacin	34.4 ± 5.1 → 35.5 ± 4.6	nmol /24 h	20	no change	HPLC+HPLC-ECD	8-oxodG	(Ref. 10)
Cystic fibrosis	1.51 ± 0.38 versus 2.78 ± 1.21	nmol/mmol creatinine	23	increased in cystic fibrosis	correlation with plasma vit E HPLC-EC Pre-purification	8-oxodG	(Ref. 55)
Workplace exposure	0.5 – 3.0	µmol/mol creatinine	41 + 30	increased	multi-D-HPLC-ECD	8-oxodG	(Ref. 56)
Normal persons	2.7 ±1.88	µmol/mol creatinine	60	in accordance with literature	multi-D-HPLC-ECD	8-oxodG	(Ref. 57)
Correlation to plasma antioxidants	10 – 105	nmol /24 h	225	no correlation	multi-D-HPLC-ECD	8-oxodG	(Ref. 28)
Vegetable and fruit	49.6 ± 23 → 21.4 ± 19.2		28	not significant	ELISA	8-oxodG	(Ref. 25)
Brussel's sprouts	236 – 1469	pmol/kg/ 24 h	37/52	Reduction	multi-D-HPLC-ECD	8-oxodG	(Ref. 58)
Controls	30 ± 15	pmol /ml	10	concentration only	HPLC-GC/MS	8-oxodG	(Ref. 11)
Comparison of published values ELISA versus HPLC-ECD	2.2 ± 0.9 → 24.3	ng/mg creatinine		3.7–10.1 higher values by ELISA	ELISA and HPLC-ECD	8-oxodG	(Ref. 59)
Exercise ß-carotene intervention	1.5 ± 0.2	nmol/mmol creatinine	14	no change	multi-D-HPLC-ECD	8-oxodG	(Ref. 60)
Children	3.8	µmol/mol creatinine	28 + 14	increase with age, no correlation with urinary malondialdehyde	multi-D-HPLC-ECD	8-oxodG	(Ref. 61)

Table 1 (*Continued*)

Disease/Condition/ Intervention	Level	Unit	Number of Individuals	Finding	Method	Lesion	ID
HIV-infection and AZT treatment ± antioxidants	110 ± 79 → 355 ± 100	pmol/kg/d	8	low in HIV, increased from ATZ, reduced by antioxidants	pre-purification + HPLC-ECD	8-oxodG	(Ref. 62)
Altitude exposure	23.0 ± 11.6 → 30.6 ± 13.9	µmol/L	58	increased with high altitude	ELISA	8-oxodG	(Ref. 63)
Method development	19 – 39	pmol/ml	not given	method development	clean up + HPLC + GC/MS	8-oxodG	(Ref. 64)
Background radiation	19.5 ± 1.2 → 25.3 ± 1.6	nmol/L urine	63	tendency to increase	multi-Dimensional	8-oxodG	(Ref. 65)
Benzyl-8-oxoguanine exposure	1.000 – 9.250	ng/ml urine	1		HPLC-ECD	8-oxoGua	(Ref. 66)
Controls	583 ± 376	pmol /ml	10	concentration only	HPLC-GC/MS	8-oxo-Gua	(Ref. 11)
Allopurinol/children/cancer			2	N2-Me-8oxodGua/ 8-oxodGua ratio increased in infants and cancer patients (22x) only U2-Me-8-oxodGua		N2 methyl-8-oxoguanine	(Ref. 67)

number of DNA/RNA products excreted into urine. In case of RNA products high concentrations of very similar chemical substances are excreted in to urine.[26] A similar myriad of DNA products undoubtedly is also excreted. Together this may make it very difficult to produce a specific antibody. A commercially available kit tested out against the three dimensional HPLC-ECD shoved clear non-specificity.[27] Until clear demonstration of close correlation to the verified HPLC-ECD method the use of immunologically based methods for quantification of 8-oxodG in urine cannot be recommended. Since there is a very close correlation between HPLC-ECD and HPLC-MS/MS (Fig. 2) measurements, presently these methods may be regarded as the golden standard.

3. Comparison of Different Methods

Presently, the only lesion measured in urine in a fair number of studies is 8-oxodG. There is a very close correlation between urinary 8-oxodG measurements with the three dimensional HPLC-ECD method and the HPLC-MS/MS method (Fig. 2). Also, recovery of added 8-oxodG is 100% and storage over many years yields identical results.[13, 27, 28] As stated above there is poor relationship with an immunologically based assay.[27]

With regard to other base modifications the data are so scarce that it is not really possible to establish correlation between methodologies. The various publications can be found in Table 1.

Generally, it can be stated that the huge discrepancy that has been reported for the use of HPLC-ECD and GC-MS to measure tissue levels does not appear to be much of a problem in urinary measurements. This is in agreement with the much lower levels of non-oxidized bases/nucleosides in urine compared with DNA.

4. Interpretation of Tissue Levels and Urinary Excretion

In the following the argumentation is done for 8-oxodG, however, except for specific values, the argumentation is valid for all oxidative DNA products. Specific conditions may exist for the individual products.

The actual unperturbed DNA steady state level (of 8-oxodG) has been the subject of intense debate. While the ESCODD initiative.[29–31] and the extensive work to reduce oxidative artefacts from the GC-MS DNA procedure[16, 32, 33] are steps in the right direction there still is no definite consensus about the "true levels". Very high levels have been reported from GC-MS measurements[33] but also levels close to those measured by HPLC-ECD. Results from the same lab using the same methodology may differ with a factor of 10–100 over the years. The authors do not want to point at single laboratories, since the variation in

levels can be pointed out for virtually all laboratories. The plethora of data on 8-oxodG in DNA is not systematically reviewed here, several reviews can be found in the literature list and in other chapters of this book. A large and comprehensive review has been published.[34]

In case of mitochondrial DNA the variation seems to be even greater. A review of the literature showed that the reported level of 8-oxodG in mitochondria spans more than 60 000-fold[35] from 0.035 to 2200 oxidative adducts per 100 000 unaltered bases. Eliminating the most extreme value the level still spans from 0.8 to 2200 oxidative adducts per 100 000 bases, or almost 3.000 fold. The mitochondria genome is 16 KB and the number of mitochondria in cells from various tissues vary from 220 (peritoneal macrophages) to 1720 (lung macrophages) per cell, skeletal muscle cells having less than 400 genomes per cell.[36] An average number of mtDNA copies in mitochondria of the body could well be about 200–400 per cell, giving a total number of base pairs of 16 000 times 200 or 3×10^6 base pairs per cell compared to the 1×10^9 base pairs in the nuclear genome. Although several authors have stated that the mtDNA is much more damaged than the genomic DNA, maybe with a factor of 10 times, these calculation still indicate that only a small portion of the 8-oxodG excreted in urine originates from mitochondria. Furthermore, the mitochondrial genome only contains 16 000 KB corresponding to about 4000 dG. Assuming correctness of data (unpublished) from our own laboratory, which indicate a level of about 1 8-oxodG per 10 000 dG in mtDNA, only an average of one in about 2–3 mitochondria will carry an 8-oxodG. Taking into account that the oxidative DNA damage has been overestimated[37] and that mitochondrial damage persists longer than nuclear damage[38] there is no reason to argue that the urinary 8-oxodG has a major component from mitochondria. Rather it can be concluded that the majority of the excreted 8-oxodG relates to nuclear DNA. Further support for this is our study on 2-nitropropane induced 8-oxodG in various organs where the nuclear DNA increase of 8-oxodG is reflected in urinary excretion.[39] Also it could be argued that mtDNA is less damaged than nuclear DNA, i.e. evaluated per genome. Probably the best evaluation is done as damage for a specific gene. Techniques for this are only emerging.

The urinary excretion of 8-oxodG in pigs following i/v injection follows simple kinetics with a half-life of a about 2.5 h, a clearance of about 4 mL min^{-1} kg BW^{-1} and a volume of distribution close to 1 L kg^{-1} BW,[40] and moreover the urinary excretion rate corresponded to the infusion rate. After liver transplantation we observed an increased urinary excretion of 8-oxodG and in a caval clamp experiment the excretion was temporarily reduced. These experiments indicate that steady state between formation and urinary excretion is obtained rapidly.

The reported values of urinary excretion of 8-oxodG in the literature are in agreement. The reported 8-oxodG urinary excretion rates measured with HPLC-ECD or GC-MS[13] vary from about 100 to 600 pmol kg BW^{-1} 24 h^{-1}, excluding the

measurements with immunologically based estimations that vary between 1600–4800 pmol kg BW^{-1} 24 h^{-1} most presumably for the reasons about lack of specificity given above. Classic pharmacokinetic consideration gives a theoretical steady state plasma concentration equal to production (dosing rate) divided by clearance, i.e. between 0.017 and 0.100 nmol/L. The conventional HPLC-ECD and HPLC-MS/MS methods have sensitivity close to that level. Using up-concentrations and a HPLC-ECD system with a non-commercially available carbon column Bogdanov *et al.*[41] reported plasma values of 0.014–0.070 nmol/L (4–21 pg/ml), i.e. in close agreement with the theoretical values.

Collectively, these data indicate that the 8-oxodG in the urine mainly originates from genomic DNA. However, on a more detailed level the contribution of 8-oxodG from the nucleotide pool cell turnover, cell death, and from inflammatory cells is unknown. Presently, neither direct nor indirect data from the *in vivo* situation are available.

Accepting that the contribution of nuclear DNA reflects the oxidation of nuclear DNA, the urinary excretion is a reflection of the average total oxidative stress to DNA of all body cells. In most experimental situations *in vivo* it is reasonable to argue that a given person is in a steady state, i.e. a constant 8-oxodG level in DNA and a constant repair. Mass conservation will be applicable and consequently the amount of excreted 8-oxodG will equal newly formed 8-oxodG. The urinary measurement is therefore equal to the rate of oxidative stress to DNA. This is depicted in Fig. 5. If an experimental or other form of change happens (say smoking

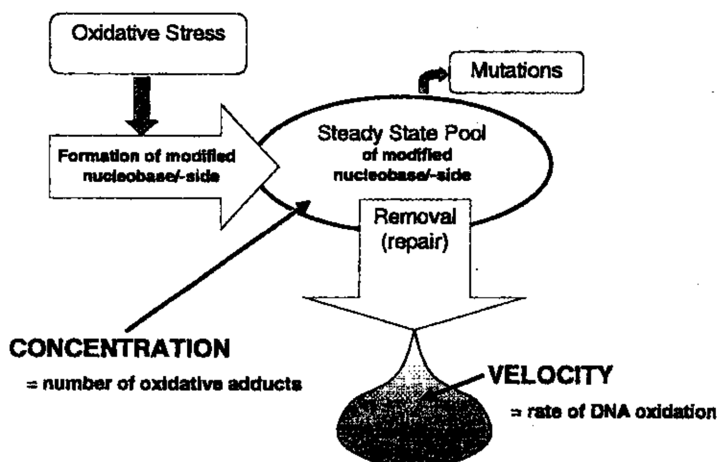

Fig. 5. Model for oxidative stress inducing modification of DNA. A sample taken from tissue will provide information about the concentration of modifications in DNA. Urine measurement is a measure of the amount of oxidative DNA products over a time period and this is a measure of the rate of DNA oxidation, when the system is in steady state. In the latter situation the measurement is independent of repair.

cessation, antioxidant intervention) a new steady state will soon be reached and a change in the rate of oxidation of DNA can be identified. It is important to stress that this measure is independent of DNA repair, a point often not recognized.

The concentration of say 8-oxodG in DNA reflects a balance between newly formed 8-oxodG's and removal. An increased level can consequently reflect either an increased formation (increased oxidative stress) or a decrease in repair or any combination. It is important to note that this cannot be determined from measurement of the level. A similar argumentation can be made for decreased levels.

It can further be argued that comparing two persons with different oxidative stress to DNA, i.e. different urinary excretion rates, the one with the higher stress will statistically have a higher chance for a mutation in DNA. Increased levels cannot necessarily be interpreted in the same way, unless it can be established whether it originates from increased stress or decreased repair.

It should be noted that for urinary excretion studies the preferred design is to collect 24 h urine. In some special designs it can be argued that the use of spot urine samples and correction for urinary creatinine concentration may be a valid measure. A prerequisite for the spot urine — creatinine correction design is a solid argumentation that creatinine excretion is unchanged by the experimental condition or that it is not different between groups. A theoretical example is comparison of lean men versus fat females. Their cell number is comparable but muscle mass very different. Creatinine excretion is mainly dependent on muscle mass, and there can easily be a difference in creatinine excretion of says 3 fold between the two groups. If they have the same oxidative stress to their DNA, females would appear to have 3 times higher values, simply because the male excretion is divided by a three times higher creatinine concentration. The same argumentation can be applied to comparison of catabolic patients versus normal controls, and old versus young adults. Preferentially 24 h urine, overnight urine(s) or at least 8 h urine on a defined period of the day should be collected and the 8-oxodG excretion given as amount per time unit and kg BW, preferentially lean body weight.

5. What is Known from Human Studies of Biomarkers of Oxidative DNA Damage?

The most studied oxidative modification of DNA relates to direct oxidation of DNA, the 8-hydroxylation of guanine being the one most extensively studied, particularly regarding urinary excretion of the repair product 8-oxodG. The excretion of the base, 8-oxoGua, is much less studied, although it is excreted in larger amounts, about 5–10 timer larger than 8-oxodG.[42] There is general agreement that the modifications like 8-oxodG are the result of reactions between DNA and

reactive oxygen species. However, other oxidative processes e.g. lipid peroxidation gives rise to reactive intermediates that in turn can modify DNA. Lipid peroxidation leads to formation of malondialdehyde, crotonaldehyde and acrolein that in turn lead to propano- and etheno-DNA adducts, called exocyclic adducts. These adducts are found in lower quantities than e.g. 8-oxodG and require ultra sensitive methods. The urinary excretion of 1,N^6-ethenodeoxyadenosine (εdA) ranges from about 0.1 to 4 fmol/micromol creatinine in human urine.[43] Human studies on the exocyclic adducts and their excretion into urine so far are limited indeed. A comprehensive overview is given in a recent IARC publication.[44]

With regard to 8-oxodG a large number of human studies have been published. They are listed in Table 1. Several pieces of basic information are known (for references see Table 1). The variation among normal people is about 7-fold with a clear sex difference. Smokers have a 50% increased 8-oxodG, which is reduced 4 weeks after smoking cessation, There is a close relationship between individual oxygen uptake (energy expenditure) and 8-oxodG excretion. Extreme longstanding exercise increases the excretion, moderate exercise or extreme bouts do not change the excretion. The majority of antioxidant interventions do not show any effect on the excretion of 8-oxodG, i.e. β-carotene, vitamin E and vitamin C. Yet there is some controversy about the change in lymphocyte levels in antioxidant intervention trials. Occupational exposure to diesel exhaust/air pollution and styrene increase the urinary excretion. Dietary restriction does not influence the excretion to any detectable extent, whereas Mediterranean diet and Brussel's Sprouts seem to reduce excretion.

For a number of diseases there are reports on increased urinary excretion and higher lymphocyte levels, and both radiotherapy and radiomimetic treatment increases excretion. So far there does not seem to be reports on decreased excretion in any disease.

The general picture that emerges is that a variety of environmental and dietary factors can explain some of the variation. However, these influences generally seem to be in the order of about 50% at maximum. The 7-fold variation in the population thus leaves the major determining factors to be found. Probably, if oxidative stress is maintained at a high level throughout life, changes in the order of 10–20% may be highly relevant, particularly on a group basis. Such a difference may reflect an increased risk of cancer or premature ageing.

6. Proper Design of Human Intervention Studies

Several elements are mandatory for a trial, particularly a trial on humans, to adhere to modern scientific demands. The basic elements are an *a priori* defined primary hypothesis and definition of primary and secondary endpoint and in some cases also tertiary endpoints. Once this is defined the design of the trial, the statistical analysis and the control group can be defined.

Only the two major design types will be dealt with here, crossover and parallel groups designs, also called paired and unpaired designs. A large number of studies use comparison of people before and after, say e.g. antioxidant intervention. Such a design is considered based on historical controls and should not be performed. Rather, the persons should be randomized to two different treatments, placebo and active treatment, the randomization gives the random order of the treatments. By such a design effects e.g. due to season is randomly allocated to the groups. The advantage of the paired design is that each person serves as his own control, and the number of subjects in the trial is reduced compared with the unpaired trial. Among the disadvantages are that every time a person drops out the first measurement he/she cannot be included in the analysis. Furthermore, if the variation within individuals is comparable with that between individuals, extra power is not obtained.

The parallel group is a more simple design. A group of people is randomized to two treatments, e.g. active treatment and placebo or two different active treatments, and the primary variable is then compared between the groups.

More complicated designs can be used but is not mentioned here. Most important is to stress the proper use of randomization and controls.

In the planning of a trial it is necessary to calculate the number of persons needed to be able to detect a predefined difference. In many countries, e.g. in Denmark, ethical approval is not given if a proper statistical power analysis is not given.

The power analysis is a calculation of the number of people to enter the trial, provided there is knowledge about the defined Type I error risk (significance

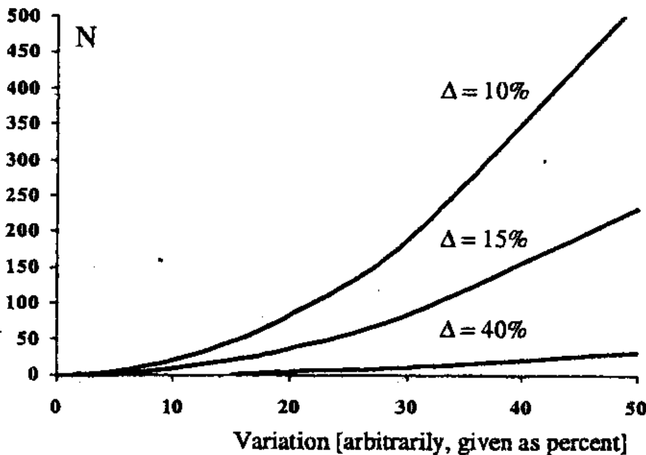

Fig. 6. Relation between the number of persons (*N*), the difference to detect (Δ) and variation on the measurement used. The graphs are calculated for significance $\alpha = 0.05$ and power $\beta = 0.90$.

level), the Type II error risk (power), the defined difference the trial is supposed to detect (delta Δ) and the variation of the measurement in the trial. A simple mathematical relationship between these factors exists. For details readers should look in statistical textbooks. In Fig. 6, the number of persons to enter a parallel group trial can be read if the variation of the measurement is known. The graphs indicate differences of 10, 15 and 40% between the groups, Types I and II errors are set at 5 and 10%, respectively.

It should be noted that the inter-individual variation e.g. in 24 h 8-oxodG excretion is often 30%. If it is desired to detect a difference of say 10% about 200 persons is required. There are very few studies with that number of persons. This should borne in mind when trials are evaluated. Very often a negative trial can represent a Type II error, not finding a true difference.

7. Future Perspectives

The formation of DNA adducts from endogenous processes and from exogenous factors has emerged as an important factor in the pathogenesis of cancer and ageing. The development of accurate, reliable methods to determine DNA oxidation is essential for understanding the processes. Presently, there has been a fast growing knowledge about the 8-oxodG lesion, and particularly there has been improvement in the knowledge about how to avoid artifacts during the process of quantifying the damage. However, there is only limited knowledge about other lesions than 8-oxodG, particularly *in vivo* in humans. Measurement of single lesions may be misleading and just because one lesion is the most dominating it is not necessarily the most import. Free radicals generate many products at the same time.[45]

Furthermore other free radical induced processes, e.g. lipid peroxidation, produce reactive intermediates that may be important. Examples of such other lesions are for example malondialdehyde induced DNA damage and exocyclic DNA adducts.[44]

Development of methodologies to detects these DNA modifications are in progress. Furthermore, molecular biology methods, e.g. variants of the PCR methods, and newer mass spectrometry methods like time of flight will in the future make it possible to detail the various DNA modifications not only by reliable methods for quantification but also for position in specific genes. Increasingly, we will see animal studies using genetically modified animals, studies that will clarify specific mechanisms, including studies with DNA array techniques to quantify mRNA to give deeper insight into the cellular biology of oxidative stress.

Furthermore, the future will improve the technologies for measurement on smaller samples and for measurement of large number of samples with reasonable use of time and money. This will enable large scale epidemiological and

intervention trials with reliable estimates of the precise role of these modification in the pathogenesis of disease and ageing.

8. Conclusions

- Oxidative lesions are implicated in cancer and ageing. Presently the role is based on circumstantial evidence and information in humans *in vivo* is limited.
- Urinary excretion of oxidative DNA products reflects total average DNA oxidation in the body and in the steady state situation it is independent of repair. The urinary excretion reflects mainly the oxidative stress to nuclear DNA.
- DNA levels in tissue of oxidatively modified nucleobases reflect a balance between oxidative stress and repair.
- A timed urine collection, at least 8–16 h preferentially 24 h urine, is recommended and the result should be given as excreted modified nucleobase per time unit and per mass unit body weight (preferentially lean body mass).
- Spot urine samples should only be used if it can be verified that creatinine excretion is unchanged.
- Methods of analysis preferred are HPLC-ECD or HPLC-MS/MS. The latter method has greater potential for measuring multiple oxidation products. Immunologically based methods needs to be verified.
- GC-MS methods can be used provided sufficient measures to counteract artificial oxidation are taken.
- The exact role of DNA oxidation in the pathogenesis of cancer and ageing needs to be established.

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