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Estimation of Oxidative and Lipids Peroxidation DNA Adduct in Urine and DNA. Methodological Aspects and Application in Molecular Epidemiology

Henrik E. Poulsen
Department of Clinical Pharmacology Q7642, Rigshospitalet, University Hospital Copenhagen Blegdamsvej, Copenhagen N, DK-2100, Denmark
E-mail: henrikep@rh.dk

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 oxidises important macromolecules and structures in the body. 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 make spontaneous oxidation possible. Often it will be found that immense differences are reported between different laboratories. Consequently published data always should be scrutinised bearing this aspects in mind.

2. Analysis of oxidised DNA and excreted repair products

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. Detection with electrochemical detectors are preferred. 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 [3].

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 [4], but is probably only useful in controlling variations in the injection volume, and cannot

be used for other purposes that pose 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 [3], and this methodology appears to function satisfactory.

Also HPLC with tandem mass spectrometric detection (HPLC-MS/MS) provides a suitable method of analysis. We have found that a single column is sufficient [3,5], however, we must emphasise 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 oxidised 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 DNA.

For urine measurements a semi-preparative HPLC procedure was applied, followed by hydrolysis, derivatization and GC-MS [6,7].

Gas-chromatography-mass spectrometry used for quantification of oxidative DNA products has been criticised 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 [8]. 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 [9] in an experiment with vitamin C and vitamin E intervention [10] and using HPLC-MS/MS it seems likely that the high reported 8-oxodA values relates to artefactual oxidation [5]. Many of the problems regarding artificial oxidation relates to the very high content of non-oxidised 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 oxidised and non-oxidised nucleosides are similar and would a priori not present a problem of the same magnitude.

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 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 [11-14]. However, although some characterisation of the antibody and epitope is given, it appears not to be tested against the many different DNA and RNA products in urine [15]. Furthermore, testing against the present method of choice HPLC-ECD, GC-MS or HPLC-MS/MS has only been stated without data, and at present time the data have not been made available in the literature [12]. One particular problem with the immunologically based assays may relate to the high 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 [15]. 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 [16]. 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 measurements, presently these methods may be regarded as the golden standard.

The urinary excretion of 8-oxodG in pigs following i/v injection follows simple kinetics with a half-life of a about 2.5 hours, a clearance of about 4 mL min^{-1} kg^{-1} BW^{-1} and a volume of distribution close to 1 L kg^{-1} BW [17], 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 [18] 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 pharmaco-kinetic 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. [19] 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. If an experimental or other form of change happens (say smoking cessation, anti-oxidant 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 recognised.

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 can not 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 say 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 hours urine, overnight urine(s) or at least 8 hours 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.

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 [20]. 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 $1,N^6$-ethenode-oxyadenosine (εdA) ranges from about 0.1 to 4 fmol/micromol creatinine in human urine [21]. Human studies on the exo-cyclic adducts and their excretion into urine so far are limited indeed. A comprehensive overview is given in a recent IARC publication [22].

3. 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 artefacts 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 [23].

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 [22].

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 se 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.

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