CHARLES UNIVERSITY IN PRAGUE

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Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

ANALYSIS OF OXIDATIVE AND FREE RADICAL INDUCED DNA DAMAGE

Diploma thesis

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Hradec Králové, 2013 Nela Váňová

I declare that this thesis is my original author work. Literature and other sources used in the thesis are stated in the list of reference and cited properly. This thesis was not used to acquire any other degree.

Hradec Králové 13.5. 2013

Nela Váňová

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ABSTRACT

Analysis of oxidative and free radical induced DNA damage

Diploma thesis

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Free radicals and reactive oxygen species (ROS) are highly reactive molecules capable of modifications of biomolecules, including DNA. 5',8-cyclopurine-2'deoxynucleosides represent a group of DNA lesions characterized by concomitant damage to both sugar and base moieties of the same purine nucleoside that are together with 8-oxo-2'-deoxypurines among the major lesions formed by attack of free radicals (e.g. hydroxyl radical).

Quantification of oxidative and free radical induced DNA lesions as biomarkers of oxidative stress has a high importance in study of their role in human health and disease. For quantification of these DNA lesions in gamma irradiated samples, high performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) will be utilized. Before injection into the LC/MS/MS, irradiated samples, treated by enzymatic digestion in order to gain free nucleosides, have to be desalted and DNA lesions have to be separated from undamaged nucleosides.

A new HPLC/UV method was developed for separation of (5'R)-5',8-cyclo-2'-deoxyadenosine; (5'R)-5',8-cyclo-2'-deoxyguanosine; (5'S)-5',8-cyclo-2'-deoxyadenosine; (5'S)-5',8-cyclo-2'-deoxyguanosine; 8-oxo-2'-deoxyguanosine and 8-oxo-2'-deoxyadenosine from 2'-deoxyadenosine; 2'-deoxycytidine; 2'-deoxyguanosine and 2'-deoxythymidine in aqueous solutions (without salts). Analytes were separated on Phenomenex LUNA C18 (2) [150 x 4,6 mm, 5μm] analytical column protected by Phenomenex LUNA C18 (2) [30 x 4,6 mm, 5μm] pre-column using gradient elution with acetonitrile (ACN) and 2 mM ammonium formate buffer as mobile phase. Flow rate of mobile phase was 1 mL/min. Detection vas achieved using UV-detector with detection at 260 nm. Separated DNA lesions were collected by Gilson FC 203B Fraction Collector in given time ranges.

Recovery from HPLC was 100 ± 2%; recovery from the subsequent lyophilization step was in the range from 77% to 103%, however further and more precise data will come from LC/MS/MS analysis

This method will be further updated for samples containing salts in the same concentrations as in real samples.

ABSTRAKT

Analýza oxidativního poškození DNA volnými radikály

Diplomová práce

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Volné radikály a reaktivní formy kyslíku (ROS) jsou vysoce reaktivní molekuly schopné modifikovat biomolekuly včetně DNA. 5',8-cyklo-2'-deoxynukleosidy představují skupinu DNA lézí vyznačující se současným poškozením sacharidové složky a nukleové báze stejného purinového nukleosidu a společně s 8-oxo-2'-deoxypuriny patří do hlavní skupiny lézí vznikajících atakem volných radikálů (včetně hydroxylového radikálu).

Kvantifikace oxidativně a volnými radikály způsobených DNA lézí jako biomarkerů oxidačního stresu hraje významnou roli ve studiu jejich vlivu na lidské zdraví a nemoci. Pro kvantifikaci těchto DNA lézí ve vzorcích ozařovaných gamma paprsky bude použito spojení tandemové spojení hmotnostní spektrometrie a vysoce účinné kapalinové chromatografie (LC/MS/MS). Před nástřikem do LC/MS/MS, ozářené vzorky podrobené enzymatickému štěpení za účelem získání volných nukleosidů musí být odsoleny a DNA léze musí být separovány od nepoškozených nukleosidů.

Pro separaci (5'R)-5',8-cyklo-2'-deoxyadenosinu; (5'R)-5',8-cyklo-2'-deoxyguanosinu; (5'S)-5',8-cyklo-2'-deoxyadenosinu; (5'S)-5',8-cyklo-2'-deoxyguanosinu; 8-oxo-2'-deoxyguanosinu a 8-oxo-2'-deoxyadenosinu od 2'-deoxyadenosinu; 2'-deoxycytidinu; 2'-deoxyguanosinu a 2'-deoxythymidinu ve vodných roztocích (bez solí) a pro sbírání separovaných DNA lézí byla vyvinuta HPLC/UV metoda. Anality byly separovány pomocí Phenomenex LUNA C18 (2) [150x4,6 mm; 5µm] analytické kolony chráněné Phenomenex LUNA C18 (2) [30 x4,6 mm; 5µm] předkolonou s použitím gradientové eluce, acetonitrilu (ACN) a 2 mM mravenčanu sodného jako mobilní fáze. Průtok mobilní fáze byl 1 mL/min. Pro detekci byl použit UV-detektor s detekcí při 260 nm. Separované

DNA léze byly sbírány pomocí Gilson FC 203B frakčního kolektoru v daných časových intervalech.

Výtěžnost HPLC byla 100 ± 2%; výtěžnost z následné lyofilizace byla v intervalu od 77% do 103%, nicméně další a přesnější data budou získána z LC/MS/MS analýzy. Tato metoda bude dále aktualizována pro vzorky obsahující soli ve stejné koncentraci jako v reálných vzorcích.

LIST OF ABREVIATIONS

(5'R)-cdA	(5'R)-5',8-cyclo-2'-deoxyadenosine
(5'R)-cdG	(5'R)-5',8-cyclo-2'-deoxyguanosine
(5'S)-cdA	(5'S)-5',8-cyclo-2'-deoxyadenosine
(5'S)-cdG	(5'S)-5',8-cyclo-2'-deoxygua-nosine
2'-dA	2'-deoxyadenosine
2'-dC	2'-deoxycytidine
2'-dG	2'-deoxyguanosine
2'-dT	2'-deoxythimidine
8-oxo-2'-dA	8-oxo-2'-deoxyadenosine
8-oxo-2'-dG	8-oxo-2'-deoxyguanosine
ACN	acetonitrile
C_v	coefficient of variation
dN	2'-deoxynucleosides
$HO^{\cdot}$	hydroxyl radical
IR	ionizing radiation
IS	internal standard
I_v	injection volume
L	DNA lesions
LOD	limit of detection
LOQ	limit of quantification
METC	mitochondrial electron transport chain
MP	mobile phase
MS	mass spectrometry
N	plate number
$O_2^{\cdot -}$	superoxide
ROS	reactive oxygen species
SOD	superoxide dismutase
SP	solid phase
SPE	solid phase extraction
UV	ultraviolet light

1. INTRODUCTION

Free radicals and reactive oxygen species (ROS) are highly reactive molecules which can be induced by both endogenous and exogenous sources. In certain levels, ROS are important for maintaining cellular homeostasis essential for normal cellular functions. However, excess generation of ROS can disrupt this homeostasis. Living organisms develop number of protective mechanisms to balance increased generation of ROS.

In the case of DNA, ROS and the most harmful hydroxyl radical especially can cause chemical modifications at several sites of macromolecule leading to single base or sugar damage, cyclic base-sugar lesions or DNA-protein cross-links endangering the stability and integrity of DNA. Living cells are equipped with several repair mechanisms dealing with these alterations in DNA structure.

Determination and quantification of these DNA lesions as biomarkers of oxidative stress has a high importance which puts demands on development of reliable and sensitive methodologies in order to provide better understanding of their role in human health and diseases.

Chromatographic techniques coupled with mass spectrometry (MS) are currently the mainly employed techniques in analysis of DNA lesions for their high accuracy and sensitivity and ability to provide structural evidence for an analyte. Furthermore, the application of isotope dilution MS (IDMS) with the use of stable isotope-labelled analogues of analytes as internal standards ascertains accurate quantification.

2. FREE RADICALS AND REACTIVE OXYGEN SPECIES

Free radicals are defined as atoms or molecules, with one or more unpaired electrons in the outer shell. The unpaired electron, existing in atomic or molecular orbital by itself, is responsible of the instability and reactivity of free radicals.

The most important class of free radicals generated in living systems during oxidative metabolism is represented by radicals derived from oxygen[1,2]. However, it is worth noting that the term of "reactive oxygen species" (ROS) includes not only radical forms derived from oxygen, but non-radical oxygen derivates as defined in Table 1.[3]

Radicals		**Non-radicals**	
Superoxide	$O_2^{\cdot -}$	Hydrogen peroxide	H_2O_2
Hydroperoxyl	$HO_2^{\cdot}$	Peroxynitrite[a]	$ONOO^-$
Hydroxyl	$OH^{\cdot}$	Peroxynirous acid[a]	ONOOH
Peroxyl	$RO_2^{\cdot}$	Nitrosoperoxycarbonate[a]	$ONOOCO_2^-$
Alkoxyl	$RO^{\cdot}$	Hypochlorus acid	HOCl
Carbonate	$CO_3^{\cdot -}$	Hypobromus acid	HOBr
Carbon dioxide	$CO_2^{\cdot -}$	Ozone	O_3
Singlet oxygen	$O_2{}^1\Sigma g^+$	Singlet oxygen	$O_2{}^1\Delta g$

Table 1. Reactive oxygen species[3]

[a]Also called reactive nitrogen species

The chemistry of ROS is followed for its role in the modification of natural molecules during cellular processes and signaling. Frequent cellular targets of chemical modification by ROS include DNA, lipids and proteins. The order of preference for these oxidative modifications depends upon number of factors, such as location of ROS production, relative ability for the biomolecule to be oxidised and availability of metal ions inside the cell. When oxidative damage of biomolecules occurs there are many molecular and enzymatic systems to repair the damage. However, an excess of radical damage can occur in consequence of inflammatory and infective conditions, or a diminution of defences can also take place as consequence of nutritional and metabolic defeats. Under a variety of conditions, the damages can impair the good cellular

functioning, thus leading to cell injury or even to cell death. The oxidative stress is also correlated to several aspects of the ageing process.

Therefore, it is very important to study the types and extents of free radical damages in order to clarify the molecular mechanisms and provide better understanding of cellular processes involved in health and diseases, also for discovering new protective strategies[4].

2.1. Reactive oxygen species formation

In living systems, ROS can be formed due to effect of both endogenous and exogenous factors. Endogenous ROS formation is a consequence of metabolic and other biochemical reactions[5]. The most important source of endogenous ROS is considered to be the mitochondrial respiration. It is important to underline that the ROS formation normally occurs in the processes of cell response after a stimulus; therefore these species have essential roles in physiological processes such as cell signalling and regulation[6]. On the other hand, exogenous ROS formation is in most cases the result of ionising radiation, ultraviolet light or chemical mutagens. To avoid the destructive effect of ROS cells have developed several protective mechanisms helping to decrease the ROS level[7].

2.1.1. Endogenous formation of reactive oxygen species

The main site of intracellular ROS production is the mitochondrial electron transport chain (METC) which takes part in cellular respiration. METC is located in the inner mitochondrial membrane and is composed of four multimeric integral membrane protein complexes designated as Complex I (NADH-ubiquinone oxidoreductase), Complex II (succinate dehydrogenase), Complex III (ubiquinol-cytochrome c oxidoreductase) and Complex IV (cytochrome c oxidase). In addition to these four protein complexes, coenzyme Q (ubiquinone) and cytochrome c (heme protein) are other compounds participating in the METC. METC course involves the successive transfer of electrons between its particular components. Complex I accepts electrons from Nicotinamide Adenine Dinucleotide (NADH), Complex II accepts electrons from succinate and these electrons are transferred via electrochemical gradient through coenzyme Q_{10} to Complex III. From Complex III, electrons are transferred through cytochrome c to Complex IV where molecular oxygen is reduced to water using 4 electrons. Reaction is shown in scheme 1.

$$4H^+ + 4e^- + O_2 \rightarrow 2H_2O$$

Scheme 1. Molecular oxygen reduction to water[8]

During respiration is most of the oxygen (O_2) reduced to water, but an estimate 1-2% of consumed O_2 is not completely reduced to water. This amount of oxygen can be partially reduced to superoxide ($O_2^{\cdot -}$). One electron reduction of O_2 to $O_2^{\cdot -}$ may occur at Complex I and Complex III. $O_2^{\cdot -}$ generated by the complex I is released into the mitochondrial matrix. Complex III generates $O_2^{\cdot -}$ by the autoxidation of ubisemiquinone formed during Q-cycle and thus formed $O_2^{\cdot -}$ is mainly released into the intermembrane space and partially into the mitochondrial matrix. In the mitochondrial intermembrane space and in the matrix, O2$^{\cdot -}$ is ceded to further reactions. $O_2^{\cdot -}$ is converted into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), an enzyme present in the mitochondrial matrix and intermembrane space. Increased steady state concentration of $O_2^{\cdot -}$ may cause reduction of transition metals. Then, H_2O_2 may react with reduced transition metals and produce hydroxyl radical ($HO^{\cdot}$) (Fig. 1). The scheme of H_2O_2 and $HO^{\cdot}$ production is shown in scheme 2.

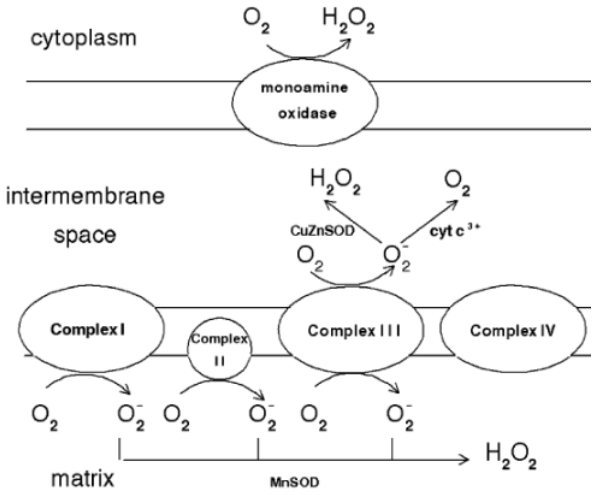

Figure 1. Sites of $O_2^{\cdot -}$ formation in METC

$$O_2^{\bullet -} \xrightarrow{SOD} H_2O_2$$ [a]

$$H_2O_2 + O_2^{\bullet -} \xrightarrow{Fe^{2+}/Fe^{3+}} OH^{\bullet} + OH^- + O_2$$ [b]

Scheme 2. [a]H_2O_2 formation and [b]iron catalyzed Haber-Weiss reaction[8,10]

$O_2^{\cdot -}$ production by METC primarily depends on the concentration of O_2 and on the concentration of reduced electron donors. When the concentration of O_2 and/or the concentration of reduced electron donors is increased, $O_2^{\cdot -}$ production is more extensive[8,9,10].

Besides mitochondria as the main source, there are other cellular sources of $O_2^{\cdot -}$. Xanthine oxidase is an enzyme catalyzing the hydroxylation of purines. In particular, xanthine oxidase catalyses the transformation of hypoxantine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced to $O_2^{\cdot -}$ which is further transformed to H_2O_2. Cytochrome P450 through the metabolism induction has been proposed to be another endogenous source of ROS. ROS are primarily generated by uncoupling of the P450 catalytic cycle. Important sources of H_2O_2 under physiological conditions are microsomes and peroxisomes. In addition, inflammatory activation of neutrophils, eosinophils and macrophages represents a source of ROS. Activated macrophages initiate an increase in O_2 uptake that give a rise to a variety of ROS[6,11,12].

2.1.2. Exogenous formation of reactive oxygen species

In addition to endogenous formation, ROS can be produced on the basis of various exogenous factors. The most common exogenous factors are ionising radiation, ultraviolet light, environmental pollutants and xenobiotics.

2.1.2.1. Ionising radiation

In living cells, ionizing radiation (IR) causes an increase in the ROS levels in two different ways. Immediately after IR exposure, ROS levels are increased due to water radiolysis. Because increased ROS levels were detected even several hours after IR exposure, there a must exist a way that IR stimulates the production of ROS derived from biological sources.

IR initially causes ionization and excitation of H_2O, leading to the formation of various H_2O radiolysis products such as hydrated electron (e_{aq}^-), ionized water (H_2O^+), hydroxyperoxyl radical ($HO_2^{\cdot}$), hydroxyl radical ($HO^{\cdot}$), hydrogen radical ($H^{\cdot}$) and hydrogen peroxide (H_2O_2) in a very short period of time (~10^{-8}s). Except H_2O_2, these formed radicals are unstable and disappear within less than 10^{-3}s.

In proliferating cells, the mitochondrial content, mitochondrial ETC formation and mitochondrial ROS levels are not constant during the cell cycle, but they oscillate in a cell-dependent manner. The highest values can be found in the G2/M phase of cell cycle under the control of the G2/M checkpoint. When irradiated, cells are arrested under the G2/M phase about three times longer than no-irradiated cells .Cells under G2/M phase are expected to have higher mitochondrial membrane potential, mitochondrial respiration and cellular ATP level than non-irradiated cells. It is also possible, that IR-

induced G2/M arrest leads to mitochondrial fission, which causes increased mitochondrial ROS production. In addition, METC function changes may cause variations in mitochondrial ROS production, but further investigation is required to determine its IR-induced mechanism[13].

2.1.2.2. Ultraviolet light

UV-light is composed of two major components; UVA (320-400 nm) and UVB (290-320 nm). UVA inflicts more noticeable oxidative stress through ROS formation than UVB because the exposition to UVB is much lower than to UVA due to the UVB absorption by ozone layer. UV-light also causes depletion of endogenous antioxidant systems[14].

Damage induced by UV radiation can be caused by direct or indirect mechanism. When living system is exposed to UV radiation, UV-light energetic photons are absorbed by a cellular molecule (chromophore, photosensitizer). Direct absorption of UV photons by cellular chromophores (for example DNA bases) can lead to photo-induced reactions. Indirect way includes the photosensitization step. During photosensitization, endogenous or exogenous sensitizers (nucleic acids, aromatic amino acids, NADH, NADPH, heme, quinones, flavins, porphyrins, carotenoids, 7-dehydrocholesterol, eumelanin or urocanic acid) absorb UV light. Absorption of photons energy leads to changes in electron distribution in the sensitizer molecule and creates the excited single state. Excited photosensitizer can cause cellular damage by two major pathways in dependence on the chemical properties of photosensitizer. Type I mechanism is based on direct interaction and one electron transfer between an excited photosensitizer and the target biomolecule. This results in free radical formation with no need of oxygen for the induction of biomolecular damage. Type II mechanism comparing to Type I, involves energy transfer from an excited sensitizer to molecular oxygen and leads to ROS formation. Major product of this reaction is singlet oxygen ($^1O_2$), which is powerful oxidant, but also some $O_2^{\cdot -}$ is formed. $O_2^{\cdot -}$ is then dismutated to H_2O_2 which in presence of metal cations generates $HO^{\cdot}$[15].

2.1.2.3. Xenobiotics

Xenobiotic is term used for all chemical compounds foreign to human body containing drugs, food additives or environmental pollutants. ROS generation induced by xenobiotics include direct metabolization of xenobiotics to primary radical intermediates and activation of endogenous sources of ROS such as METC. Interaction

of xenobiotics with METC occurs in two different pathways. First is based on fact that some xenobiotics block electron transport increasing the reduction level of carriers located upstream of the inhibition site (for example neurotoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine which blocks Complex I). Second is based on ability of some xenobiotics to accept an electron from a respiratory carrier and transfer it to molecular oxygen (for example antineoplastic drug doxorubicin). This stimulates $O_2^{\cdot -}$ formation without inhibiting METC[9]. Other drugs (analgesic drug paracetamol or antineoplastic drug cisplatin) may cause depletion in cell antioxidant systems and raise the harmful effect of generated ROS. Special mechanism of ROS formation belongs to antipsychotic drug chlorpromazine. Chlorpromazine is photoactivated in skin and converted to an excited state with subsequent energy transfer to molecular oxygen and generation of both excited singlet oxygen and superoxide species[16,17].

2.1.3. Cellular defense against reactive oxygen species

In order to protect themselves against the harmful effects of ROS, living organisms have developed a multilevel antioxidant network, which provides the main site of ROS removal. Antioxidants may specifically quench free radicals, chelate redox metals or regenerate other antioxidants within the antioxidant network. There exist two types of antioxidants, enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) which help to decompose ROS to water or less harmful species. Non-enzymatic antioxidants are represented by vitamin C (ascorbic acid), vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin, lipoic acid) and flavonoids. Non-enzymatic antioxidants work as direct scavengers for free radicals and help to regenerate other antioxidants[11].

2.1.4. ROS role in cell signaling and regulation of physiological functions

As mentioned above, ROS are generated at certain levels in living organisms endogenously and continuously. Although the production of ROS is connected notably with their harmful behavior, it has been shown that they have a significant role in cell signaling and cell processes regulation.

ROS may act as messengers thanks to their ability to modify target protein molecules and change the intracellular redox state.

In low levels, ROS may activate the mitogen-activated protein kinase (MAPK) pathway and thus trigger fundamental cell functions such as gene expression, cell proliferation

and differentiation by mitogens, growth factors or cytokines, apoptosis by phosporylation of target proteins. Also, ROS may act as enhancers for some receptors (for example insulin receptor)[18].

2.2. Damage induced by reactive oxygen species

ROS, generated by both endogenous and exogenous pathways, are in certain levels present in living organisms continuously and balanced by the antioxidant network. Therefore, imbalance between pro-oxidants and antioxidant results in macromolecular damage and disruption of physiological redox signaling and cell control mechanisms. Oxidatively modified proteins, lipids and nucleic acids contribute to the development of numerous diseases. To counter oxidative stress, cells have developed several reparative mechanisms to prevent these macromolecular modificatons[19].

2.2.1. DNA damage

In low levels, DNA damage is present even in normally functioning cells. DNA unlike proteins and lipids, once modified, cannot be removed and replaced readily. Thus, DNA damage represents a group of macromolecular modifications of great importance.

ROS and the most harmful $HO^{\cdot}$ especially, give a rise to the chemical modification of several sites of DNA macromolecule leading to single DNA base or sugar lesions, cyclic base-sugar adducts and DNA-protein cross-links[4].

2.2.1.1. DNA base damage.

In human DNA, two pyrimidine bases (thymine and cytosine) and two purine bases (guanine and adenine), are present (Fig. 2).

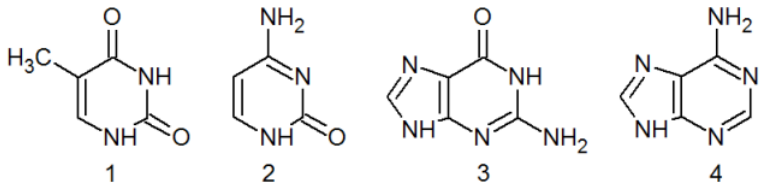

Figure 2. DNA bases; **1** thymine (5-Methylpyrimidine-2,4(1*H*,3*H*)-dione), **2** cytosine (4-aminopyrimidin-2(1*H*)-one), **3** guanine (2-amino-1*H*-purin-6(9*H*)-one), **4** adenine (9*H*-purin-6-amine).

$HO^{\cdot}$ thanks to its electrophilic nature attacks the site of molecule with higher electron density and reacts with DNA base by addition to the double bond of corresponding heterocycle yielding to OH-adduct radicals. Except addition, $HO^{\cdot}$ is capable of hydrogen abstraction from the methyl group of thymine or from each C-H bond of sugar moiety. Addition reaction to double bounds occurs at diffusion-controlled rates with rate constants from 3 to 10 x 10^9 M^{-1} s^{-1}. The rate constant of hydrogen abstraction is

about 2 x 10^9 M^{-1} s^{-1}. In a compare to sugar moiety, majority of $HO^{\cdot}$ attacks the DNA bases (~90%)[4,20,21].

In the case of ionizing radiation, hydrated electron and H atom as radical species are formed from water radiolysis, in addition to $HO^{\cdot}$. Hydrated electron reacts with DNA bases at diffusion controlled rates from 0.9to 1.7 x 10^{10} M^{-1} s^{-1}. The rate constant of H atom reactions amount to (1-5) x 10^8 M^{-1} s^{-1}. Both hydrated electron and H atom add to DNA base generating electron- and H-adduct radicals, respectively. Electron-adduct radicals are easily protonated by H_2O to yield stable H-adduct radicals. This reaction is inhibited in the presence of oxygen, because oxygen scavenges both hydrated electron and H atom[4].

● **Thymine**

Thymine (**1**) is one of the two pyrimidine nucleobases reacting with $HO^{\cdot}$ by two different ways. The first is represented by $HO^{\cdot}$ addition to the double bond between C_5 and C_6 of pyrimidine heterocycle resulting in C_5-OH (**2**) and C_6-OH-adduct radicals of thymine (**3**). In the case of thymine, the addition of $OH^{\cdot}$ occurs at the C_5 (~60%) preferably to the C_6 (~35%). These formed OH-adduct radicals can be reduced or oxidised in dependence of their redox properties, redox environment and on their reaction partners. The important factor affecting the final reaction products and the reaction yields is the presence or absence of oxygen. In the absence oxygen, C_5-OH-aduct radical oxidation is followed by HO^{-} addition (or addition of H_2O and subsequent deprotonation) and leads to thymine glycol (**4**) formation (Fig. 3). When there is no oxygen, C_5-OH and C_6-OH-adduct radicals can be also reduced to 5-hydroxy-6hydro (**5**) and 6-hydroxy-5-hydrothymine (**6**), respectively (Fig. 3).

When oxygen is present, C_5-OH-aduct radical is converted to C_5-OH-6-peroxyl radical (**7**). Peroxyl radical eliminates $O_2^{\cdot -}$, react with water (OH^{-} addition) and thymine glycol is formed. Peroxyl radical can be also reduced by $O_2^{\cdot -}$ to corresponding hydroxyhydroperoxide (**8**), which decomposes to thymine glycol and in a case of C_6-OH-aduct radical to 5-hydroxy-5methyl-hydantoin (**9**) (Fig. 4).

About 5% of $OH^{\cdot}$ reacts with methyl group of thymine by the mechanism of hydrogen abstraction generating the exocyclic allyl radical (**10**). In the absence of oxygen, allyl radical is converted to 5-(hydroxymethyl)uracil (**11**) (Fig. 3). Oxygen reacts with allyl radical forming peroxyl radical and peroxyl radical is reduced to hydroxyhydroperoxide which is then decomposed to 5-(hydroxymethyl)uracil and 5-formuracil (**12**) (Fig.4).

The overall radiation-induced formation yield of allyl radical oxidation products to thymine glycols is much higher in cellular double-stranded DNA that in free nucleosides, probably due to the location of methyl group of thymine in the major groove of DNA[4,21,22,23].

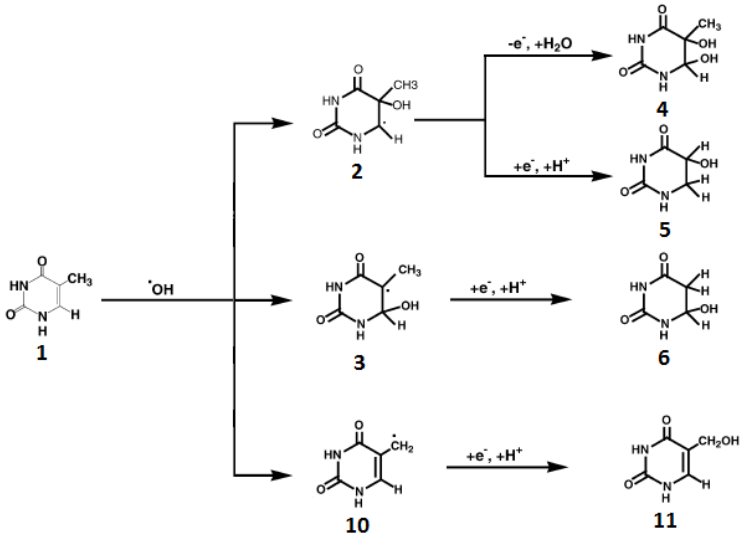

Figure 3. Hydroxyl radical-mediated oxidation products of thymine in the absence of oxygen[4]

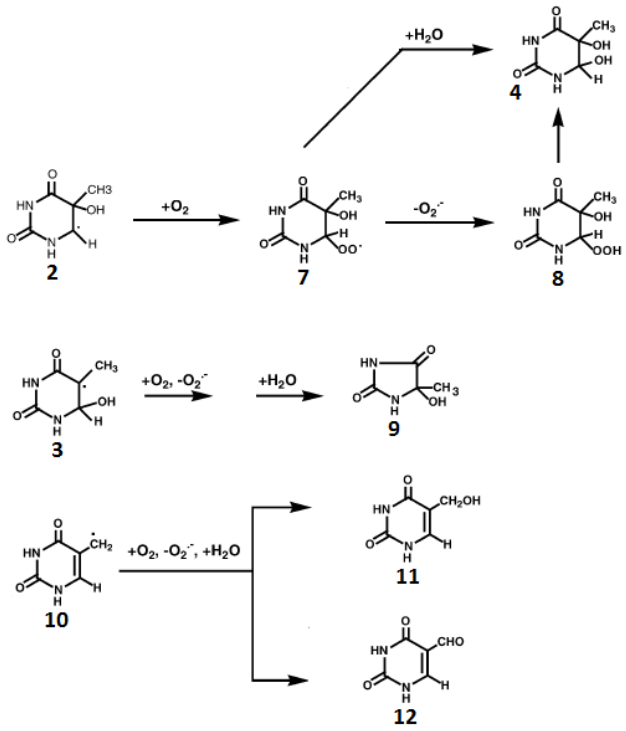

Figure 4. Hydroxyl radical-mediated oxidation products of thymine in the absence of oxygen[4]

● **Cytosine**

Cytosine (**13**) is the second pyrimidine nucleobase. As well as thymine, the addition of $HO^{\cdot}$ occurs at the C_5 (~87%) preferably to the C_6 (~10%) leading to C_5-OH (**14**) and C_6-OH-adduct (**15**) radicals of cytosine. In the absence of oxygen, C_5-OH-adduct radical undergoes oxidation followed by OH^- addition (or addition of H_2O and subsequent deprotonation) and result in cytosine glycol (**16**) formation. In a compare to thymine glycol, cytosine glycol can deaminate and dehydrate. Cytosine glycol deaminates to uracil glycol (**17**) which further dehydrates to 5-hydroxyuracil (**18**). Direct dehydration of cytosine glycol leads to 5-hydroxycytosine (**19**). Simultaneous presence of cytosine glycol, uracil glycol, 5-hydroxyuracil and 5-hydroxycytosine was found in damaged DNA. As well as thymine, cytosine C_5-OH and C_6-OH-adduct radicals can be reduced to 5-hydroxy-6hydro (**20**) and 6-hydroxy-5-hydrocytosine (**21**). Deamination of 5-hydroxy-6-hydrocytosine leads to 5-hydroxy-6-hydrouracil (**22**) (Fig. 5).

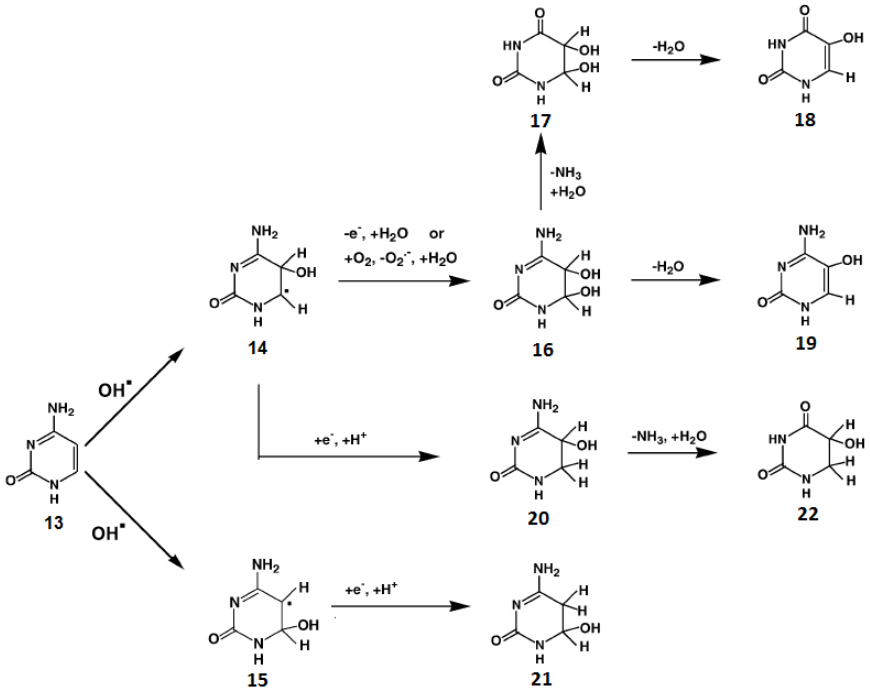

Figure 5. Hydroxyl radical-mediated oxidation products of cytosine in the absence of oxygen[4]

This reaction is inhibited by the presence of oxygen in favor of formation of C_5-OH-6-peroxyl and C_6-OH-5-peroxyl radicals of cytosine and corresponding C_5-OH-6-hydroperoxide (**23**) and C_6-OH-5-hdroperoxide (**24**), respectively. These hydroxyhydroperoxides are relatively unstable. C_5-OH-6-hydroperoxide decompose to

4-amino-5hydroxy-2,6-(1H,5H)pyrimidinedione (**25**) which deaminates to give dialuric acid (**26**). Dialuric acid is oxidised by oxygen to alloxan (**27**) and upon acidic treatment, decarboxylation of alloxan gives a rise to 5-hydroxyhydantoin (**28**). As a minor product in DNA was found to be *tans*-1-carbamoyl-2-oxo-4,5-dihydroxyimidazoline (**29**) formed as a major product of intramolecular cyclization of C_5-OH-6-hydroperoxide. C_6-OH-5-hdroperoxide decompose to 4-amino-5hydroxy-2,5-(1H,6H)pyrimidinedione (**30**) which deaminates to give isodialuric acid (**31**). 4-amino-5hydroxy-2,5-(1H,6H)pyrimidinedione and isodialuric acid can simultaneously exist in DNA as evidenced from the detection of their enol forms, 5,6-dihydroxycytosine (**32**) and 5,6-dihydroxyuracil (**33**) respectively (Fig.6)[4,21].

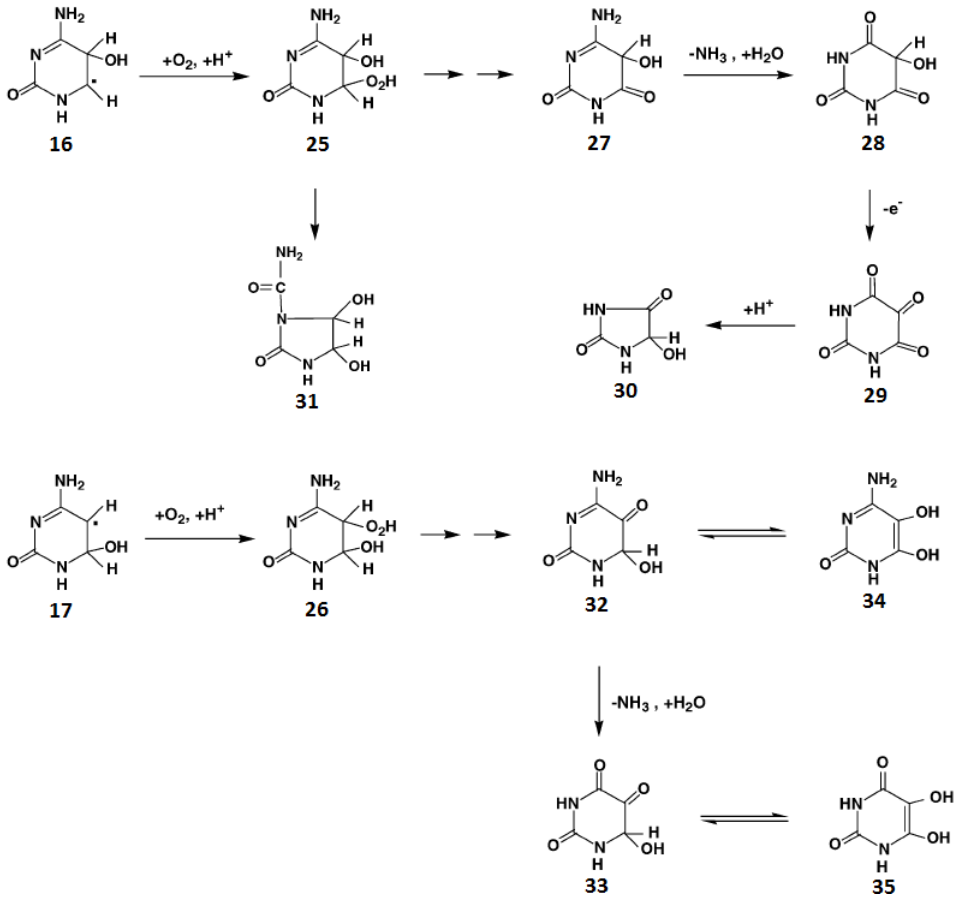

Figure 6. Hydroxyl radical-mediated oxidation products of cytosine in the presence of oxygen[4]

● Guanine

Guanine (**34**) is a derivate of purine and is supposed to be the most susceptible target of oxidative reactions. The addition reaction of $HO^{\cdot}$ to guanine moiety leads to C_4-OH (**35**), C_5-OH (**36**) and C_8-OH-adduct (**37**) radicals formation. Because guanine OH-

adduct radicals might exist in different mesomeric forms, they exhibit a "redox ambivalence" meaning that they can be both oxidizing and reducing. C_4-OH and C_5-OH-adduct radicals dehydrate to produce guanine(-H)$^{\cdot}$ radical (**38**). At neutral pH, dehydration occurs with k = 1.5 x 10^5 s^{-1}. Guanine(-H)$^{\cdot}$ radical is further reduced and protonated to reconstitute the guanine. Reaction of oxygen with guanine(-H)$^{\cdot}$ radical (k = 3 x 10^9 s^{-1}) yielding imidazolone and oxazolone derivates. Elimination of HO^- from the C_4-OH-adduct radical (k = 6 x 10^3 s^{-1}) leads to guanine radical cation (guanine$^{\cdot+}$) (**39**) formation which deprotonates in a pH-dependent manner to give a rise to the guanine(-H)$^{\cdot}$ radical (Fig. 7.). Hydration of guanine$^{\cdot+}$ in double-stranded DNA yielding the C_8-OH-adduct radical subsequently oxidised to 8-hydroxy-guanine (**40**). Nevertheless, monomeric guanine$^{\cdot+}$ reacts with 2'-deoxyribose in DNA by hydrogen abstraction (k ‹ 4 x 10^3 s^{-1}) leading to strand breaks.

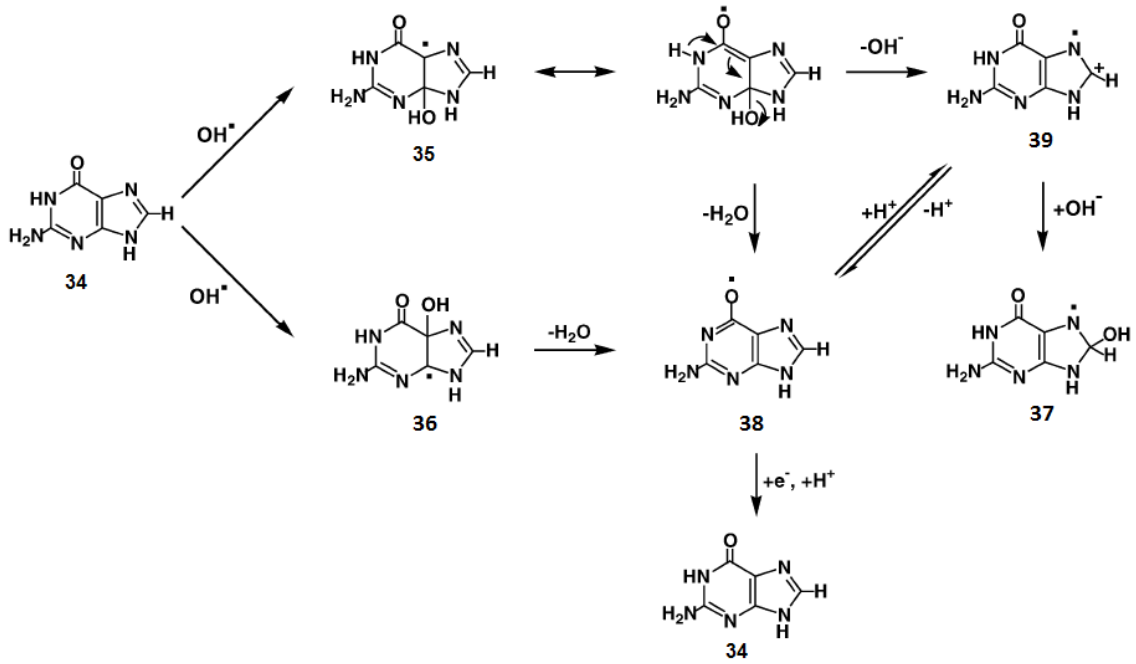

Figure 7. Reactions of C_4-OH and C_5-OH-adduct radicals of guanine[4]

C_8-OH-adduct radical may be except the one-electron oxidation yielding 8-hydroxy-guanine also reduced or transformed by opening its imidazole ring. Reduction of C_8-OH-adduct radical give a rise to 7-hydro-8-hydroxyguanine (**43**) which is converted by ring-opening reaction to 2,6-diamino-4-hydroxy-5-formamidopyrimidine (**44**). Scission of the C_8-N_9 bond (k = 2 x 10^5 s^{-1}) of guanine C_8-OH-adduct radical leads to imidazole ring opening and the ring-opened radical undergoes one-electron reduction to yield 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fig. 8.).

8-hydroxyguanie and formamidopyrimidine is formed both in the presence and absence of oxygen, although the presence of oxygen increases the 8-hydroxyguanie formation[4,21,22].

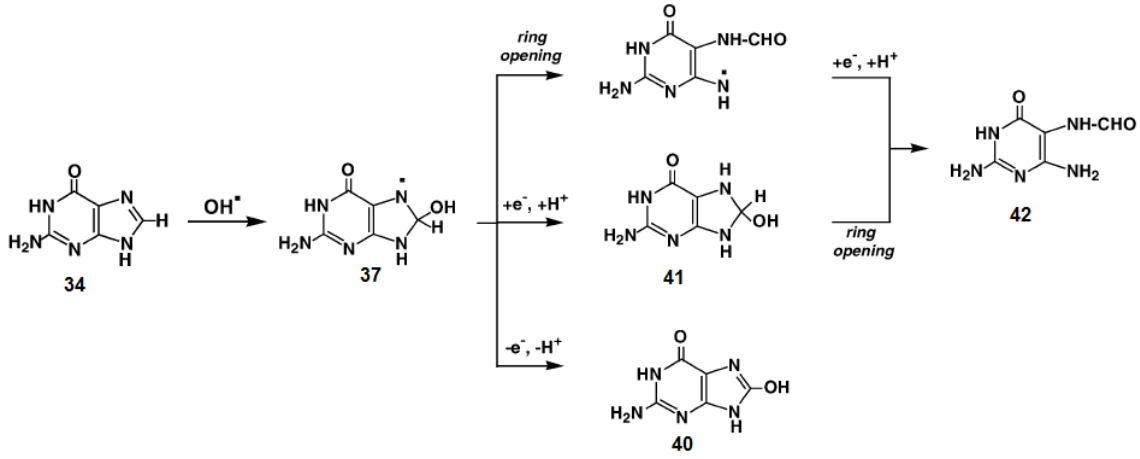

Figure 8. Reactions of C_8-OH-adduct radical of guanine[4]

●Adenine

Adenine as the 6-substitued purine undergoes analogous reactions to guanine. Thus, adenine(-H)˙radical is generated by dehydration of C_4-OH and C_5-OH-adduct radicals of adenine (k = 6 x 10^3 s^{-1}). Adenine(-H)˙ radical undergoes reduction and protonation resulting in adenine reconstitution. Elimination of OH^- from the C_4-OH-adduct radical leads to adenine radical cation (adenine$^{\cdot+}$) formation which deprotonates in a pH-dependent manner to give a rise to the adenine(-H)˙ radical. 8-hydroxyadenine is formed by one-electron oxidation of C_8-OH-adduct radical. C_8-OH-adduct radical can be also reduced to give a rise to 7-hydro-8-hydroxyadenine which can be converted into 4,6-diamino-5-formamidopyrimidine by ring-opening reaction. Unimolecular opening of imidazole ring by scission the C_8-N_9 bond (k = 2 x 10^5 s^{-1}) of adenine C_8-OH-adduct radical leads to the ring-opened radical which is reduced to yield 4,6-diamino-5-formamidopyrimidine. However, HO˙ attack of adenine (**43**) may also lead to C_2-OH-adduct radical (**44**) which undergoes oxidation to yield 2-hydroxyadenine (**45**) (Fig. 9)[4,21,22].

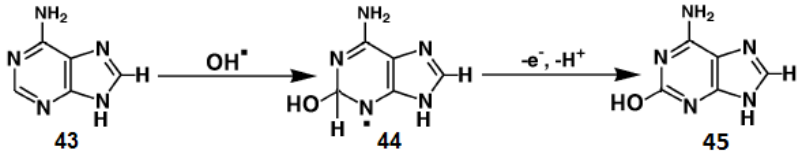

Figure 9. 2-hydroxyadenine formation[4]

2.2.1.2. DNA sugar damage

$HO^{\cdot}$ reacts with DNA sugar moiety, 2'-deoxyribose, by abstracting the hydrogen atom from each of the sugar carbon atoms. Thanks to their location within the minor groove of DNA double helix, C_4' and C_5' hydrogen atoms are supposed to exhibit better accessibility for abstraction by $HO^{\cdot}$ than C_1', C_2' and C_3' hydrogen atoms of 2'-deoxyribose. Hydrogen abstraction leads to carbon centered sugar radical formation resulting in a variety of sugar modifications. Sugar modifications may remain within DNA strand or may be released as free modified sugars. Some of sugar modifications constitute end groups of broken DNA strands.

In the absence of oxygen most of the DNA strand-breaks result from the C_4' radical reactions. C_4' radical is an alkoxyalkyl radical with a phosphate group in the β-position. The phosphate group can be released from either side of the DNA chain resulting in two different radical cations as end groups existing in two mesomeric forms. When C_4' radical cation undergoes reaction with water (HO^{-} addition) followed by unaltered base elimination, 2,3-dideoxypentos-4-ulose as a 3'-end group (**46**) and 2,5-dideoxypentos-4-ulose as a 5'-end group (**47**) of broken DNA chain are formed. If C_4' radical cation undergoes oxidation followed by reaction with water and unaltered base elimination, 2-deoxypentos-4-ulose (**48**) is formed within DNA chain. 2-deoxypentos-4-ulose is also formed in the presence of oxygen, although by different mechanism. Oxygen reacts with C_4' radical to form peroxyl radical as a precursor for 2-deoxypentos-4-ulose which can be released as a free modified sugar (**49**) or remain within DNA chain. On the other hand, C_4' peroxyl radical can undergo fragmentation resulting in glycolic acid residue as a 3'-end group (**50**) of DNA chain. C_2' and C_1' peroxyl radicals undergo fragmentation of sugar ring to produce erythrose (**51**) within DNA chain. In the absence of oxygen, C_1' radical undergoes oxidation followed by reaction with water and unaltered base release to generate 2-deoxyribonic acid lactone (**52**) within the DNA chain. In the presence of oxygen, 2-deoxyribonic acid lactone within the DNA chain is also produced through peroxyl radical as its precursor. C_5' peroxyl radical can be transformed to 2-deoxy-tetra-dialdose as free modified sugar (**53**) as well as 5' end group (**54**) of DNA chain. When C_5' peroxyl radical is formed by the action of antitumor protein, antibiotic or neocarzinostatin in the presence of oxygen, 5'-aldehyde nucleoside as a 5' end group (**55**) of DNA chain is formed without base release. Due to the weakening of glycosidic bond between C_1' of sugar moiety and N atom of altered purine or pyrimidine

base, unaltered sugar (**56**) can be formed within the DNA chain[4,21]. Structure of major products of oxidative damage to the DNA sugar moiety is shown at fig. 10.

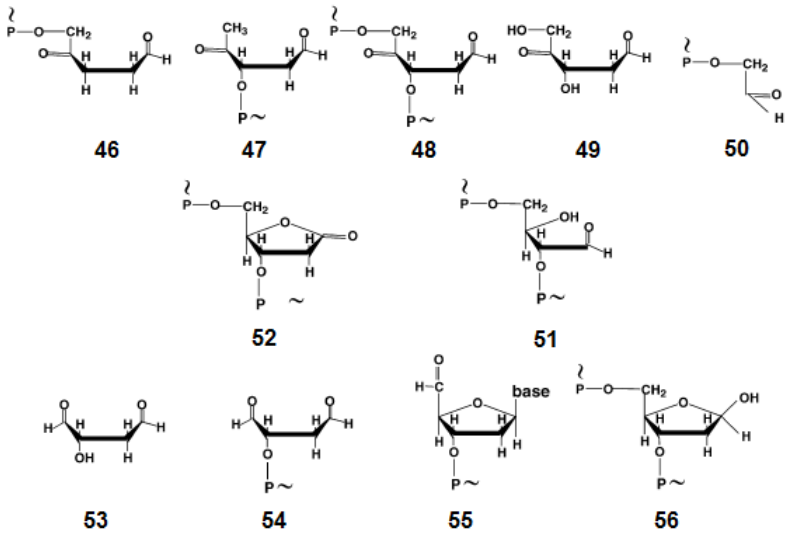

Figure 10. Major products of oxidative damage to the DNA sugar moiety[4]

2.2.1.3. 5',8-Cyclopurine-2'deoxynucleosides

5',8-cyclopurine-2'deoxynucleosides represent DNA lesions also regarded as tandem lesion. 5',8-cyclopurine-2'deoxynucleosides are characterized by concomitant damage to both the sugar and base moieties of the nucleoside, in addition to the glycosidic bond, Structure of 5',8-cyclopurine-2'deoxynucleosides is shown in Fig.11.

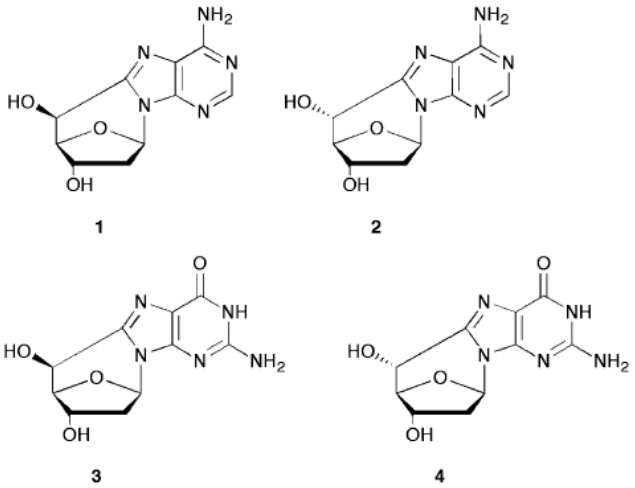

Figure 11. 5',8-cyclopurine-2'deoxynucleosides[20]

(5'S)-5',8-cyclo-2'-deoxyadenosine (**1**), (5'R)-5',8-cyclo-2'-deoxyadenosine (**2**), (5'S)-5',8-cyclo-2'-deoxyguanosine (**3**), (5'R)-5',8-cyclo-2'-deoxyguanosine (**4**)

The mechanism of formation of this lesion includes:

1) Formation of C_5' centered sugar radical as a result of hydrogen abstraction from C_5' carbon of 2'-deoxyribose by an attacking radical (for example $HO^{\cdot}$ radical)
2) Intramolecular attack of C_5' sugar centered radical at the C_8 position of purine base within the same nucleoside leads to N_7 centered radical formation together with the formation of the new bond between the base and the sugar
3) Oxidation of the N7 radical intermediate with formation the final cyclonucleoside.

The reaction mechanism of 5',8-cyclopurine-2'deoxynucleoside formation for 2'-deoxyadenosine is shown in fig.12. In the presence of oxygen, the cyclization reaction is inhibited because oxygen reacts with C_5' radical to give a rise to the corresponding peroxyl radical. The C_5'-C_8 cyclization is stereospecific reaction and the ratio of the R- and S- diastereoisomers is affected by experimental conditions and DNA conformation. In single stranded DNA, R-diastereoisomers are dominant products of cyclization whereas S-diastereoisomers are preferred in double-stranded DNA. In addition, the 5'R/5'S ratio is different for adenosine and guanosine.

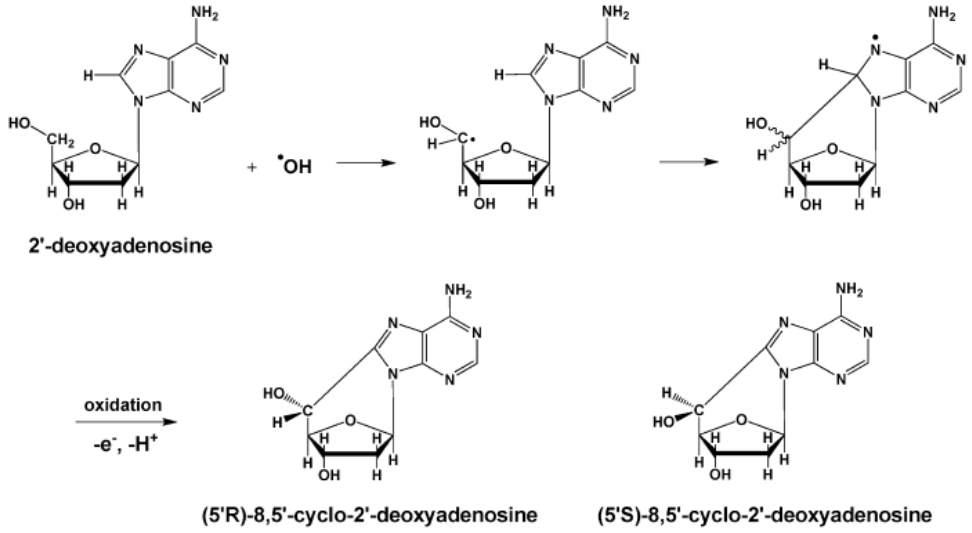

Figure 12. 5',8-cyclo-2'-deoxyadenosine formation[24]

$OH^{\cdot}$ radicals react with base and sugar moieties of guanosine with an overall rate constant of 5.7 x 10^9 M^{-1} s^{-1}. In γ-irradiated N_2O saturated aqueous solutions, the 2'-deoxyguanosine conversion to 5',8-cyclo-2'-deoxyguanosine occurs in the overall yield of 8-10% and in a 5'R/5'S ratio of 8,3:1. When oxygen is present, 2'-deoxyguanosin-5'-yl radical is trapped by oxygen resulting in hydrated-5'-aldehyde.

In the case of adenosine, $OH^{\cdot}$ radicals react with 2'-deoxyadenosine with a rate constant of 4.6 x 10^9 M^{-1} s^{-1}. In γ-irradiated N_2O saturated aqueous solutions, the formation of 5',8-cyclo-2'-deoxyadenosine occurs in the overall yield of ~ 11% and in a 5'R/5'S ratio of 6:1. In the presence of oxygen, the 2'-deoxyadenosine conversion to 5',8-cyclo-2'-deoxyadenosine is inhibited in favor to hydrated-5'-aldehyde formation.

In double stranded DNA, the C_5'-C_8 cyclization leads to the weakening of Watson-Crick hydrogen bonds and double helix distortion near the both R- and S- diastereoisomers as a consequence of changes in backbone torsion angles[20,24].

2.2.1.4. DNA-protein cross-links

DNA-protein cross-link is a covalent bond formed between DNA base radical and aromatic amino acid of protein or amino acid radical[4,21]. An example of DNA-protein cross-link between allyl radical of thymine (**1**) and tyrosine in a protein (**2**) in chromatin is shown at Fig.13.

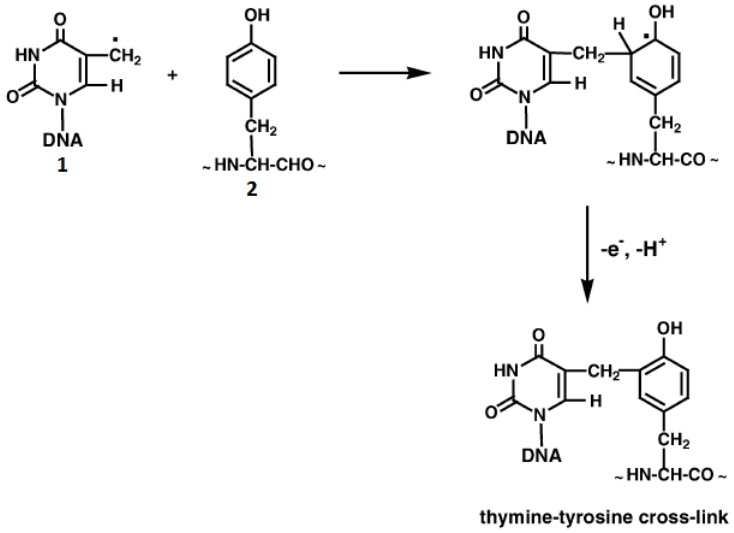

Figure 13. Thymine-tyrosine cross-link formation[4]

2.2.2. DNA damage repair

Alterations in DNA structure are continuously present in living cells as a consequence of both environmental and endogenous factors. It has been estimated that about 1 million individual lesions are generated per cell per day. When variations in essential genes occur, the genome integrity and stability is endangered. To prevent this, cells have developed protective mechanisms able to detect and repair various types of DNA lesions. The control of DNA repair is closely connected to the cell cycle regulation. Cell

cycle checkpoints control the fidelity of DNA repair before DNA undergoes replication and cell division to prevent the accumulation of DNA damage which in turn leads to mutations.

There exist two main mechanisms of DNA repair – direct and excision repair. Direct repair is the simplest response to DNA damage involving the removal and reversion of the lesion in a single step reaction. Excision repair is a multistep repair pathway overlapping with direct repair. Excision repair include base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). BER corrects the majority of base lesions caused by methylation, oxidation, reduction and fragmentation. NER mainly repairs the lesions resulting from UV-induced DNA damage by replacing the whole nucleotide. MMR is involved in repair of misincorporations of normal nucleotides arised during DNA replication and recombination as well in repair of some DNA damages[25-29].

2.2.3. Oxidatively induced DNA damage and ROS in human diseases

The unrepaired or incorrectly repaired DNA lesions lead to mutations or wide-scale genome aberration. Thus threatened genome integrity and stability may affect cell or organism viability. Except DNA lesions, ROS cause damage to cellular proteins, lipids and subcellular organelles which is also related to numerous diseases[4,30].

● Neurodegenerative diseases

Neurodegenerative diseases such as Alzheimer or Parkinson disease are associated with accumulation of DNA lesions in neurons. Elevated production of ROS by neuron mitochondria and consequent DNA damage may trigger neuronal dysfunction and degeneration. In brain, oxidative stress increases with age and the capacity for cell replacement is limited in adulthood. Neurons rely on transcription which is blocked by accumulated DNA damage depriving neurons of vital transcripts and lead to cell dysfunction or apoptosis. Also, defection in repair mechanisms contributes to cumulation of DNA lesions[4,21,30,31].

● Cardiovascular diseases

Cardiovascular diseases connected with ROS-induced oxidative stress involve atherosclerosis, hypertension, ischemic heart disease, cardiac hypertrophy, cardiomyopathies and congestive heart failure.

The peroxynitrile, which is strong oxidant, is formed by oxidation of $NO^{\cdot}$ by $O_2^{\cdot -}$ and is considered to be involved in the process of hypertension. Suppression of $NO^{\cdot}$ which is an important protective molecule in the vasculature is accompanied by endothelial dysfunction and higher risk of development of hypertension and atherosclerosis. The atherosclerotic plaque results as a consequence of lipoprotein and low-density cholesterol (LDL) oxidation in connection with other factors such as hypertension. Additionally, oxidised LDL has been reported to mediate enhanced $O_2^{\cdot -}$ formation causing apoptosis of cells in the umbilical vascular wall[4,30,31,32].

● Diabetes mellitus

Diabetes mellitus (DM) is a chronic disease characterized by absolute (DM type I. or insulin dependent diabetes) or relative (DM type II. or non-insulin dependent diabetes) deficit of insulin. Hyperglycaemia caused by low insulin levels is shown to be one of the major causes of increased oxidative stress which is one of the triggers of hyperglycaemia-induced diabetic complications[4,31].

● Inflammation an autoimmune diseases

During inflammation, important source of ROS are the bactericidal species ($O_2^{\cdot -}$ and H_2O_2) generated from the respiratory burst of invading neutrophils, macrophages and eosinophils damaging surrounding tissues. Tissues damaged by ROS release cytokines promoting infiltration of further inflammatory cells and the primary inflammation spread. Exacerbated inflammation may proceed to chronic inflammation which is hand in hand closely connected to pathogenesis of autoimmune diseases such as rheumatoid arthritis[4,31].

● Cancer

In the process of carcinogenesis, both oxidatively induced redox imbalance and permanent modification of genetic material resulting from oxidative damage of DNA are involved.

It was detected that in pre-malignant and malignant cells nearly 20 purine and pyrimidine lesions have been implicated in cancer development. Formation of DNA lesions results in induction or arrest of transcription, errors in DNA replication and induction of signal transduction pathways which are key players in the process of carcinogenesis. The stages of carcinogenic process seem to be in a dose-dependent relationship with the level of oxidative stress. Tumour promotion starts even in lower

levels of oxidative stress and when the level increases, the process of mutagenesis is more likely to occur. On the other hand, when the level of oxidative stress is too high the cells undergo apoptosis or necrosis in order to prevent tumour progression.

ROS, together with various redox metals are able to generate ROS and non-redox metals ale to bind thiol groups of proteins e.g. antioxidants, have been implicated in the mechanism of carcinogenesis. Alterations of cell signaling mechanisms, such as receptor or tyrosine kinase, levels of specific growth factors, intracellular processes for transduction of membrane signals to the nucleus, portions of the transcription apparatus and genes taking part in cell cycle regulation, can be often traced to abnormal behaviour of neoplastic cells[31,33].

● Aging

Generally, there are two main theories describing the process of aging: damage-accumulation theory and genetic theory. Damage accumulation theory involve "free radical theory" base on the fact that ROS are capable of damage to various cellular components which are accumulated over time.

As important target of ROS is considered to be telomeric DNA which contains multiple TTAGG repeats. Oxidised bases destabilize telomeric DNA and accelerate telomere shortening and below a threshold length, the telomeric proteins dissociate resulting in uncapped telomeres, which in turn trigger cellular growth arrest.

As mentioned above, mitochondria are the major site of endogenous ROS generation. Thereby, mitochondrial DNA (mtDNA) is very susceptible to oxidative damage. Beside nuclear DNA, mtDNA cannot be readily repaired. Therefore, mtDNA damage is accumulated over time and inhibits mitochondria followed by cells death. Thus mtDNA contributes to aging[31,33].

3. HIGH PERFORAMCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) is an instrumental technique based on the separation of a mixture of compounds between the stationary phase in a column and the mobile phase flowing through the system. HPLC is currently one of the most progressive analytical methods used in wide areas of scientific and routine practice with a purpose of identification, quantitation or purification of individual components present in the sample. Compounds are separated thanks to their various affinities to the stationary and to the mobile phase. The major advantages of HPLC involve quantitative and qualitative evaluation of analytes even in small amounts of sample, flexibility due to wide range of analytical columns and mobile phases available, selectivity (in dependence on the detector used), speed of analysis or possibility of process automation[34,35].

3.1. Instrumentation

HPLC system consists of several essential components as shown in Figure 14. High-pressure pump generates sufficient pressure to pump the mobile phase from a reservoir to the column and controls the flow rate. The sample is injected to the system through the injector or by autosampler placed between the pump and the column. From the column, where separation takes place, the mobile phase is further pumped to the detector which monitors changes in analyte concentration during the chromatographic process. The data system monitors the detector output and provides data processing for both graphic and tabular output of data. The HPLC may also comprise a group of individual modular components[34,36].

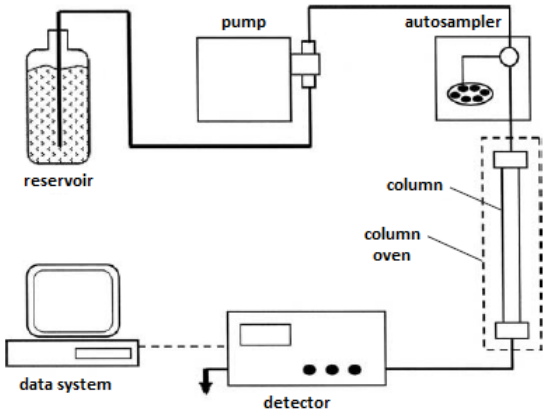

Figure 14. HPLC instrumentation[36]

3.1.1. Mobil phase

Mobile phase (MP) is a liquid phase flowing through the HPLC system. MP is a solvent or a mixture of solvents and some additives such as buffers in which given analytes are soluble. Buffers are used to control the degree of ionization of an analyte. Polarity of used MP depends upon the properties of stationary phase inside the column (see 3.1.5).

The composition of the MP can be constant during the whole analysis or the ratio between individual solvents can vary with time. In isocratic elution, the composition of MP is constant during the entire chromatographic analysis. In gradient elution, the composition of the MP is variable during the chromatographic process typically from low to high eluting strength (polarity) of solvents. In the case of isocratic elution, MP can be hand-mixed or on-line mixed within the HPLC system. For the gradient elution, the on-line mixing is required always. Before entering the HPLC system, MP must be free of particular matter. So if purified solvents (i.e. HPLC-grade) are not available, MP has to be filtered. MP can be filtered by using a vacuum filter apparatus where solvent is poured through a membrane filter[36,37].

3.1.2. Mobile phase degassing

MP degassing is a process used to avoid problems connected with a presence of air bubbles in MP such as pump-delivery problems or spurious peaks in the detector output. Dissolved gas can be removed from the MP by sonication, vacuum filtration or helium sparging. Off-line degassing may not be sufficient in HPLC systems that are highly susceptible to dissolved gas in the mobile phase. In such cases continuous helium sparging or on-line vacuum degassing are better choices.

Helium sparging is the most effective degassing technique using a frit to disperse helium in the MP reservoir.

Vacuum degassing takes place during the solvent filtration by application of a partial vacuum to the MP. Solvent is passed through a piece of polymeric tubing inside a vacuum chamber and the dissolved gas passes through the walls of the tubing while the liquid mobile phase stays inside the tubing. Although this method is not as efficient as helium sparging, the convenience of in-line vacuum degassing and the cost of helium have made this the preferred degassing technique[36].

3.1.3. Pumping systems

The precision and accuracy of the pumping systems and the MP mixing systems have an important role in HPLC analysis. Nowadays, the most popular pumps used are the reciprocating-piston pumps.

The reciprocating-piston pump consists of motor, piston, pump seal and check valves. The piston is driven by the rotation of the motor. Due to the moves of the piston out of and into the pump head, inlet and outlet check valves are alternately opened and closed which allows MP to flow to the column. The pump seal prevents leaking of the MP out of the pump head.

Also, most of the pumps have a purging valve. Purging valve directs the pump output to waste during pump priming, solvent changeover or bubble removal.

The pump generates a high pressure, controls the flow rate and composition of the MP by the solvent delivery to the mixing system. Most of the pumping systems are designed to work at pressures up to 400 bar (6000 psi), but majority of analysis are performed in the 150-200 bar (2000-3000 psi) region[36,37].

3.1.4. Injector and autosampler

The introduction of the sample into the MP stream can be done through manual or automatic injection.

Manual injection is done through the six-port rotary valve by using a conventional syringe. In the loading position, MP flows through the valve to the column and the sample port is connected to the waste port to opposite end of the loop. In this position, the loop is filled by the sample. By rotating switch, the valve is moved to the injection position and the content of the loop is injected into the MP stream and continues to the column.

The advantages of automatic injection lie in improvement of productivity and elimination of personal errors. Samples in the vials, capped with a rubber septum or perforated polypropylene stopper, are kept in the sample tray. Sample tray is usually temperated and protected from light. The process of injection is programmed by computer software and computer also controls the sequence of samples from vials kept in numbered positions of sample tray. Technical principles of sampling are different. The sample can be dispensed using multi-port (usually six-port) valves or several three-port valves. There are three main types of autosamplers:

- the syringe of autosampler is fixed and the sample tray is moving in axial or circular motion
- the sample tray is fixed and the syringe is moving in axial motion
- both syringe and sample tray are fixed and the vials are transported to the syringe by special transport mechanism

To avoid sample cross contamination, the syringe has to be washed from inside and outside after each sequence[36,37,38,39].

3.1.5. HPLC Columns and stationary phases

HPLC chromatographic column is considered to be the heart of the chromatographic system, because separation of individual components of injected sample takes part here. Columns are usually stainless steel or tubes 50-250 mm long with an inner diameter 2.1-4.6 mm equally filled with a suitable stationary phase (SP). The column SP determines retention and selectivity[34,39].

- **Chemically bonded stationary phases based on silica gel**

Chemically bonded SP have found wide application in HPLC thanks to their variability in use in relation with MP used. Silica gel by itself is a polar SP thanks to its free hydroxyl groups present on the surface of the silica gel. These free hydroxyl groups can be chemically bonded with various functional groups with both, polar and non-polar properties (Table 2)[36,39].

Polar functional groups	Non-polar functional groups
Aminopropyl group	Octadecyl group (C18)
Cyanopropyl group	Octyl group (C8)
	Phenyl group
	Phenylalkyl group

Table 2. Examples of polar and non-polar functional groups for silica gel phases[39]

- **Stationary phases based on zirconium oxide**

Solubility of silica gel caused by higher pH of the MP resulted in the development of alternative HPLC sorbents based on metal oxides such as titanium dioxide, aluminum oxide and zirconium oxide especially. Zirconium oxide has no silanol groups on its

surface, but has the adsorptive centres with character of Lewis acids. So the buffers must be added to the MP in order to compensate the strong interactions of these centers with hydroxyl, carboxyl or phosphate groups of substances. Zirconium oxide is stable in the entire pH range at high pressure and temperatures up to 200 ° C [39].

● Monolithic SP

Unlike conventional stationary phases consisting of individual particles, monolithic HPLC stationary phases consist of a single piece of porous material that fills the entire internal space of the column. In a compare to conventional particulate-filled columns, the biggest advantage of monolithic columns are their hydrodynamic properties. Monolithic columns have two types of pores: large pores (macropores) and medium-sized pores (mesopores). Macropores provide fast convective flow of mobile phase through the monolith and significantly accelerate the mass transfer between the MP and SP. Mesopores provide a large enough surface of the monolith, and thus a high separation capacity. The structure of monolithic columns enables the operation under high flow rates without excessive increase of pressure and also without a loss of separation efficiency. Monolithic columns are also suitable for separation of macromolecules. According to the chemical properties and method of preparation, monolithic SP can be divided into inorganic monoliths, macroporous polymer monoliths and compressible monoliths (compressed gels)[39].

3.1.6. Detectors

Sensitivity and selectivity of chromatographic analysis depends upon the detector used. Detector is a device monitoring the changes in MP composition by measurement of chemical or physical properties such as concentration or weight. Ideal detector should be universal for wide range of analytes, reliable, sensitive with low level of noise, linear in a wide range of concentrations, robust and should be able to be used in gradient elution. Unfortunately, no detector able to fulfil all these conditions is now available (Table 3.). The most widely used detectors are ultraviolet/visible light spectrophotometric detectors (UV/VIS), refractive index detectors (RID), fluorescent detectors (FLD) and electrochemical detectors (ECD). Nowadays, combination of HPLC mass spectrometry (MS) is used very frequently[34,36,37].

	UV/VIS	RID	FLD	ECD	MS
Measured value	Absorbance	Refractive index	Fluorescence intensity	Electric current	Mass-to-charge ratio
Response	Selective	Universal	Selective	Selective	Universal
Type	Non-destructive	Non-destructive	Non-destructive	Destructive	Destructive
Sensitivity	ng	μg	pg	pg	pg
Range	10^5	10^4	10^3	10^6	10^5
Dependence of response on flow rate	No	Yes	No	Yes	No
Temperature dependence	Low	High	Low	High	Low
Gradient elution	Yes	No	Yes	No	Yes

Table 3. Properties of HPLC detectors[39,40]

An important indicator of HPLC detectors performance for a particular peak is the signal-to-noise ratio (S/N). Noise is the fluctuation in the baseline that occurs with a periodicity in the same range as chromatographic peaks. Noise is the factor that limits the sensitivity of detector[36].

3.1.6.1. Spectrophotometric detectors

Spectrophotometric detectors are based on the absorption of electromagnetic radiation of given wavelength by particular compounds present in the MP. The most commonly used spectrophotometric detectors operate at UV (100-400 nm) and VIS (400-700 nm) area of the light. Due to their construction, UV/VIS detectors are divided into three types:

- **Variable-wavelength detector**

Detectors with variable wavelength offer a wide selection of UV and VIS wavelengths. However, once the wavelength is set, cannot be changed during the current analysis.

- **Diode-array detector (DAD)**

DAD measures absorption spectra of particular analytes simultaneously in number of wavelengths and thus measured spectra are compared to each others. White light passes through the detector cell and is spread by the diffraction grating across an array of photodiodes. The signal from individual photodiode, that absorbs only the tiny range of wavelengths, is processed to generate a spectrum on an analyte[34,36].

3.1.7 Fraction collector

Fraction collector (FC) is one the HPLC modular systems. FC is a device used to collect the analytes of interest in fixed time ranges. The column effluent is supplied to the FC and in pre-programmed time ranges and FC collects the analytes into the tubes through use of a mechanism that moves the outlet tubing to the desired tube. If the other components of the sample eluted outside of the given time ranges are not desired to be collected, they can be simply directed to the waste. A delay coil of tubing can be mounted between the detector and the FC to allow for peak detection just prior to the capture of a fraction[36].

3.2. Identification in High Performance Liquid Chromatography

To identify an analyte, the retention parameters (t_R or k) of unknown compound and referent material which are determined under the same experimental conditions are compared. Thus, the t_R is the main qualitative characteristic in HPLC[34,36].

3.3. Quantification in High Performance Liquid Chromatography

Quantification involves comparison of the intensity of response from an analyte (peak area or peak height eventually) in the investigated sample with the intensity of response from an analyte in standards measured under identical experimental conditions. There are two main methods used for determination of particular analytes in a mixture: external standard and internal standard (IS) method[36].

●External standardization

External standard method is probably the simplest method of quantification involving two steps. At first, the unknown amount of an analyte in the sample is injected into the chromatographic system. Second, the known amount of standard is injected under identical conditions and the intensity of analytical signal of standard is compared to the intensity of signal obtained during the injection of the sample. As external standard is often used the standard of an analyte under investigation. Amount of particular analytes in the sample is then counted by the ratio of peak area of analyte and peak area of standard. Although the use of external standardization is widely employed, it takes no account of matrix effects, i.e. the effect on the analytical signal caused by interaction of an analyte with the matrix in which is found or ignore the losses of analyte during the sampling, storage and work-up[34,36,37].

● **Internal standardization**

On the other hand, internal standardization involves the injection of an analyte and standard in one step to overcome major sources of inaccuracy and also to improve the precision. An internal standard is a suitable compound added to the sample in a known amount as early in the analytical procedure as possible, ideally at the sampling stage.

Appropriate internal standard should follow next criteria:

- the IS should not be present in the sample to be analyzed
- the IS should be inert to all other compounds present in the sample
- the IS should be chemically and physically similar to analyte to reduce the losses occurring during the sample work-up or storage
- the IS should be eluted in a similar time to the analyte (preferably eluted after analyte) in order to achieve similar precision
- the IS should be added in a similar concentration to analyte so that they may be determined with similar precision

Amount of particular analytes in the sample is then counted as the ratio of peak area of analyte and peak area of internal standard. Because the analyte and the internal standard are injected in the same run, this method is not such a time consuming and is not affected by the error of the double-measurement[34,36,37].

3.4. Chromatographic parameters

The ability to carry out the chromatographic separation depends upon the performance of the chromatographic system which can be defined by theoretical parameters (Fig.15). Although, the performance required for a particular analysis depends upon the separation that is required. This, in turn, depends upon the similarity in the behaviour in the chromatographic system of the analyte(s) of interest to each other and to other compounds present in the mixture[37].

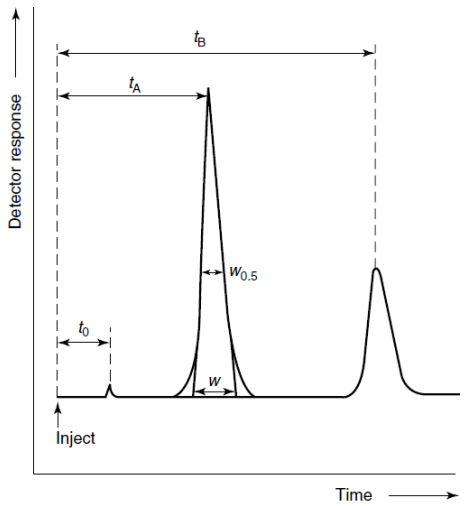

Figure 15. Illustration of some peak parameters[36]

3.4.1. Retention time

Retention time (t_R) is a term describing the time taken by an analyte to elute from a chromatographic column with a particular mobile phase. Retention time is measured from the time of injection to the top of the peak for each analyte of interest. The retention time of the solvent peak is referred to as the column dead time t_0 (or t_m). Because t_R of an analyte is characteristic for a given column, mobile phase and flow rate, capacity factor can be used instead of t_R in some cases[36,37].

3.4.2. Capacity factor

Capacity factor k relates the retention time of an analyte (t_R) to the time taken by solvent or unretained compound (t_0), which passes through the column with no interactions with stationary phase, under identical conditions (Scheme 3.)

$$k = \frac{t_R - t_0}{t_0}$$

Scheme 3. Capacity factor[36]

The *k* values 1-10 are desirable to give an adequate resolution in a reasonable analysis time.

The *k* values can be adjusted by changes in MP composition (polarity) or by optimizing of the temperature[36,37,41].

3.4.3. Selectivity

The selectivity α (or separation factor) is a term describing the separation between two analytes with retention times t_a and t_b (where $t_b > t_a$) using the ratio of their capacity factors k_a and k_b (Scheme 4.). Selectivity can be improved by changing the character of the MP or the SP[36,37,41].

$$\alpha = \frac{k_b}{k_a}$$

Scheme 4. Selectivity[36]

3.4.4. Theoretical Plate number

Plate number (*N*) defines column efficiency (performance). The value depends upon the column length and upon the particle size of SP. The higher the value of *N*, the more efficient is the column and the narrower peaks are in the chromatogram. The equation for plate number calculation is shown at Scheme 5. (t_a - retention time, w_a - peak width at the baseline and $w_{a\ 0,5}$ - peak width in the half-height)[36,37].

$$N = 16\left(\frac{t_a}{w_a}\right)^2 = 5,54\left(\frac{t_a}{w_{a\ 0,5}}\right)$$

Scheme 5. Plate number[36]

To increase the *N*, longer column or column with smaller particle size can be used[41].

The efficiency of column per length unit (*L*) is defined by plate height (*H*) (Scheme 6.)[37].

$$H = \frac{L}{N}$$

Scheme 6. Plate height[36]

3.4.5. Resolution

Resolution (R_s) is used to quantify the level of separation between chromatographic peaks of two analytes with retention times t_a and t_b and peak baseline widths w_a and w_b .

Resolution can be also described as a function of capacity factor, separation factor and plate number. Resolution equations are shown at Scheme 7.[36,37].

$$R_s = \frac{t_b - t_a}{0,5(w_a + w_b)}$$

$$R_s = 0,25\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{1+k}\right)\sqrt{N}$$

Scheme 7. Resolution equations[36]

The higher resolution value, the better separation between particular analytes is. For two peaks of similar size, baseline resolution corresponds to R_s >1.5. A common goal of HPLC method development is the separation of every peak of interest from adjacent peaks with $R_s \geq 2$. When more than two peaks have to be separated, the goal is usually $R_s \geq 2$ for the least well-separated peak-pair. This peak-pair is referred to as the critical peak-pair, and its resolution is referred to as the critical resolution of the separation[36].

4. INTRODUCTION TO EXPERIMENTAL PART

The importance of studying the types and the extent of free radical damage in order to clarify their involvement in human health and disease puts demands on the development of reliable and sensitive methodology for their evaluation and quantitation.

In our laboratory, the main interest is directed to quantitative determination of 5',8-cyclo-2'-deoxypurine DNA lesions as biomarkers of free radical damage in biological samples. In last years, there were numerous attempts to determine the level of 5',8-cyclo-2'-deoxypurines in DNA[20]. The measurement of 5',8-cyclo-2'-deoxypurine in DNA was mainly achieved by using chromatographic techniques coupled with mass spectrometry(MS). The advantage of the techniques that employ MS over others is that mass analysis provides structural evidence for an analyte and that the application of MS with isotope-dilution ascertains accurate quantification of 5',8-cyclo-2'-deoxypurines[24,42,43,44].

At the moment of writing this thesis, DNA samples in our laboratory were represented by DNA hexamers, DNA lesions were generated by ionising radiation and free nucleosides will be obtained by enzymatic digestion of irradiated samples. In some methodologies, enzymatic hydrolysates of irradiated samples were injected directly into the LC/MS/MS[43,44]. In our case, problems occurred due to the presence of salts coming from buffers used during enzymatic digestion because salts can interfere with the performance of MS by overloading the system with charged salt ions or multiple salt-adducts of the analyte.

In several cases, the solid phase extraction (SPE) procedure for 8-oxo-2'-deoxyguanosine determination in human urine was developed[45].

SPE procedure for desalting of our samples containing 5',8-cyclo-2'-deoxypurine DNA lesions and 8-oxo-2'-deoxypurines was also developed in our laboratory. When DNA samples are irradiated by γ-rays, ~ 1 DNA lesion per 10^6 of 2'-deoxynucleosides per 1Gy is generated. Even thought this method was effective for removing of salts and had good recovery of analytes, problems with solubility caused by disproportional concentrations of DNA lesions (pg/mL) and undamaged nucleosides (mg/mL) made this method inapplicable.

Wang and co-workers[47] employed the HPLC/UV method for sample clean-up prior the LC/MS^3 analysis of DNA lesions in the tissues of Long-Evans Cinnamon rats. They

used C18 HPLC column for the enrichment of oxidatively induced DNA lesions from the enzymatic digestion products of DNA., The gradient of methanol and 10 mM ammonium formate buffer was utilised. Separated 5-formyl-2'-deoxyuridine, 5-hydroxymethyl-2'-deoxyuridine, and the 5'R and 5'S diastereomers of 5',8-cyclo-2′-deoxyguanosine and 5',8-cyclo-2′-deoxyadenosine were collected in given time ranges.

We decided to develop a new HPLC/UV method for:

1) Sample desalting
2) Separation of DNA lesions from 2'-deoxynucleosides
3) collection and enrichment of 5',8-cyclo-2'-deoxypurines and 8-oxo-2'-deoxypurines
4) and finally for quantitation of undamaged 2'deoxynucleosides

The liquid chromatography/isotope dilution tandem mass spectrometry methodology, using [$^{15}N_5$] labelled compounds as internal standards will be than applied for quantitation of thus separated DNA lesions. Scheme of DNA lesion analysis is shown at Figure 16.

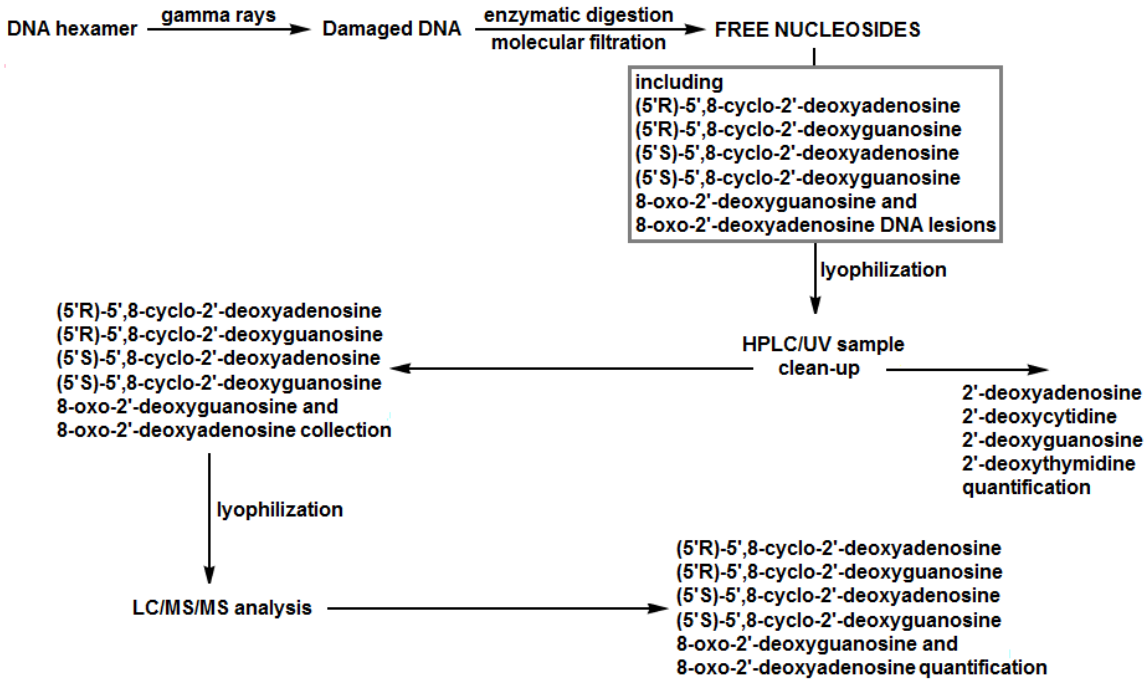

Figure 16. DNA lesions analysis

5. AIMS OF THE THESIS

The main goal of this thesis was to develop and optimize the HPLC/UV method for:

- 2'-deoxyadenosine; 2'-deoxycytidine; 2'-deoxyguanosine; 2'-deoxythymidine; (5'R)-5',8-cyclo-2'-deoxyadenosine; (5'R)-5',8-cyclo-2'-deoxyguanosine; (5'S)-5',8-cyclo-2'-deoxyadenosine; (5'S)-5',8-cyclo-2'-deoxyguanosine; 8-oxo-2'-deoxyguanosine and 8-oxo-2'-deoxyadenosine separation
- (5'R)-5',8-cyclo-2'-deoxyadenosine; (5'R)-5',8-cyclo-2'-deoxyguanosine; (5'S)-5',8-cyclo-2'-deoxyadenosine; (5'S)-5',8-cyclo-2'-deoxyguanosine; 8-oxo-2'-deoxyguanosine and 8-oxo-2'-deoxyadenosine collection for further analysis
- 2'-deoxyadenosine; 2'-deoxycytidine; 2'-deoxyguanosine; 2'-deoxythymidine quantitation

All this will be developed and tested only for aqueous solutions.

The developed method will be updated for samples containing the same amount of salts as in the samples coming from enzymatic digestion. Method will be utilized by the laboratory as pre-analytical step before injection into the LC/MS/MS, where quantitation of DNA lesions will be performed utilizing isotopic IS added to the sample before the HPLC/UV clean-up step.

6. EXPERIMENTAL PART

6.1. List of chemicals

● 2'-deoxynucleosides

2'-deoxyadenosine monohydrate, 2'-deoxyguanosine monohydrate (Berry & Associates, Dexter, USA)

2'-deoxycytidine, 2'-deoxythymidine (Sigma-Aldrich, Steinheim, Germany)

● DNA Lesions

(5'R)-5',8-cyclo-2'-deoxyadenosine, (5'S)-5',8-cyclo-2'-deoxyadenosine (Previously synthesized by application of procedure of Navacchia and co-workers[48])

(5'R)-5',8-cyclo-2'-deoxyguanosine, (5'S)-5',8-cyclo-2'-deoxyguanosine (Previously synthesized by application of procedure of Chatgilialoglu and co-workers[49])

8-oxo-2'-deoxyadenosine, 8-oxo-2'-deoxyguanosine (Berry & Associates, Dexter, USA)

● HPLC mobile phase preparation

Acetonitrile CHROMASOLV® Plus, for HPLC, ≥99.9% (Sigma-Aldrich, Steinheim, Germany)

Ammonium formate for HPLC, ≥99.0% (Fluka, Steinheim, Germany)

Methanol CHROMASOLV®, for HPLC, ≥99.9% (Sigma-Aldrich, Steinheim, Germany)

Milli-Q water (Milli-Q water was prepared from deionized water using Milipore water purification system)

6.2. Chromatographic system and other laboratory equipment

Liquid chromatography system Agilent 1100 Series HPLC System equipped with Agilent G1311A Quat Pump, Agilent G1322A Degasser and G1314A VWD variable wavelength detector (Agilent Technologies Inc., Santa Clara, CA, USA)

Rheodyne 7125 Stainless Steel Injection Valve with standard rotor seal (Rheodyne, Oak Harbor, WA, USA)

Rheodyne Stainless Steel Loop 100 µL (Rheodyne, Oak Harbor, WA, USA)

HPLC Column Phenomenex PhenoSphere ODS (1) 80A 250x4.6 mm; 5µm (Phenomenex, Torrance, CA, USA)

HPLC Column Phenomenex LUNA C18 (2) 150x4.6 mm; 5µm (Phenomenex, Torrance, CA, USA)

HPLC Pre-column Phenomenex LUNA C18 (2) 30x4.6 mm; 5µm (Phenomenex, Torrance, CA, USA)

Rheodyne 7315 Pre-Column Inlet Filter; 0.5µm x 1.5 mm (Rheodyne, Rohnert Park, CA, USA)

Software Agilent ChemStation Rev. A 10.02 (Agilent Technologies Inc., Santa Clara, CA, USA)

Gilson FC 203B Fraction Collector (Middleton, WI, USA)

Hamilton 250 µL syringe (Hamilton Company, Reno, NV, USA)

Millipore water purification system Millipak 40 Gamma Gold 0.22 µm (Millipore, Bedford, MA, USA)

Julabo USR1 Ultrasonicator (Julabo Labortechnik, Seelbach, Germany)

Mettler H20 analytical balance (Mettler Toledo, Greifensee, Switzerland)

Gibertini E154 analytical balance (Thermal Analysis & Surface Solutions, Friedberg, Germany)

Edwards RV8 lyophilizer (Edwards High Vacuum International, Crawley, England)

1.5 mL Safe.-lock Eppendorf tubes (Eppendorf, Hamburg, Germany)

6.3. Stock solutions, working solution and mobile phase preparation

6.3.1. Stock solutions

● **Stock solutions of 2'-deoxynucleosides (dN) for method development and optimization**

2'-deoxyadenosine (2'-dA) stock solution 2.6 mg/mL

5.6 mg of 2'-deoxyadenosine monohydrate (6.69 % H_2O) was dissolved in 2 mL of Milli-Q water.

2'-deoxycytidine (2'-dC) stock solution 1.8 mg/mL

3,6 mg of 2'-deoxycytidine was dissolved in 2 mL of Milli-Q water.

2'-deoxyguanosine (2'-dG) stock solution 2.2 mg/mL

4,6 mg of 2'-deoxyguanosine monohydrate (5,55 % H_2O) was dissolved in 2 mL of Milli-Q water.

2'-deoxythymidine (2'-dT) stock solution 3.2 mg/mL

6.4 mg of 2'-deoxythymidine was dissolved in 2 mL of Milli-Q water

● (dN1) 1 mg/ml stock solution, (dN2) 500 µg/ml stock solution and (dN3) 100 µg/ml stock solution for method development and optimization were prepared by diluting of (2'-dA), (2'-dC), (2'-dG) and (2'-dT) stock solutions by MQ water.

● **L stock solution of 5',8-cyclo-2'-deoxypurines and 8-oxo-2'-deoxypurines for method development and optimization**

The stock solution with concentration 10 µg/mL of each DNA lesion (**L**) (5'R)-5',8-cyclo-2'-deoxyadenosine, (5'S)-5',8-cyclo-2'-deoxyadenosine, (5'R)-5',8-cyclo-2'-deoxyguanosine, (5'S)-5',8-cyclo-2'-deoxyguanosine, 8-oxo-2'-deoxyadenosine and 8-oxo-2'-deoxyguanosine in Milli-Q water was prepared earlier in the lab.

All stock solutions were stored at 4°C in the fridge in Eppendorf tubes for two months.

6.3.2 Working solutions

Working solutions were prepared by diluting the dN1, dN2, dN3 and L stock solutions by Milli-Q water in order to obtain following concentrations (Table 4.):

Concentration of dN and L	**Volume of dN stock solution [µL]**	**Volume of L stock solution [µL]**	**Final volume [µL]**
500 µg/mL of dN; 100 ng/ml of L	500 µL of **dN1**	10 µL	1000
500 µg/mL of dN; 10 ng/ml of L	500 µL of **dN1**	1 µL	1000
100 µg/mL of dN; 100 ng/ml of L	200 µL of **dN2**	10 µL	1000
100 µg/mL of dN; 10 ng/ml of L	200 µL of **dN2**	1 µL	1000
10 µg/mL of dN; 100 ng/ml of L	100 µL of **dN3**	10 µL	1000
10 µg/mL of dN; 1 µg/ml of L	100 µL of **dN3**	100 µL	1000
1 µg/mL of dN; 1 µg/ml of L	10 µL of **dN3**	100 µL	1000
0 µg/mL of dN; 100 ng/ml of L	0	10 µL	1000

Table 4. Working solutions

Working solutions were prepared freshly every week and stored at 4°C in the fridge in Eppendorf tubes.

6.3.3 Mobile phase

Aqueous and organic components of MP were prepared and placed into the mobile phase reservoirs separately and mixed together by the HPLC mixing system in appropriate ratio according to the gradient conditions.

Aqueous MP was 2 mM ammonium formate buffer in Milli-Q water. 1,261 g of ammonium formate was dissolved in 20 mL of Milli-Q water in order to reach 1 M concentration. 2.0 mL of 1 M ammonium formate was used to prepare 1000 mL of the buffer. Before used, the buffer was put for 15 minutes to the sonicator for degassing. 1 M ammonium formate was stocked at 4°C in the fridge for one month.

Organic MP was pure acetonitrile. Before used, acetonitrile was put for 15 minutes to the sonicator for degassing

Mobile phase was freshly prepared every day.

6.4. HPLC/UV parameters

Column: LUNA C18 (2) 150x4,6 mm; 5µm

Pre-column: LUNA C18 (2) 30x4,6 mm; 5µm

Pre-column filter: Rheodyne 7315 Pre-Column Inlet Filter 0.5µm x 1.5 mm

Mobile Phase: 2 mM ammonium formate buffer; Acetonitrile (ACN)

Chromatography separations were carried out under gradient conditions described below (Table 5.).

Time	0	2.2	6.2	11.0	12.0	18.0	26.0	30.0	35.0	37.0	37.1	43.0
% ACN	**0**	**0.3**	**0.8**	**1.5**	**1.9**	**1.9**	**8.0**	**10.0**	**20.0**	**20.0**	**0**	**0**

Table 5. Gradient elution method

Analysis time: 43 minutes

MP flow rate: 1 mL/min

Injected volume: 20 µL

Injector loop: 100 µL Rheodyne Stainless Steel Loop

Room temperature: 25°C

UV detector λ: 260 nm

6.5. Fraction collector parameters

The analytes of interest were collected in the 1.5 mL eppendorf tubes placed in the fraction collector in time ranges defined in Table.6.

Collected analytes	Time range [min]
(5'R)-5',8-cyclo-2'-deoxyguanosine	9.4 – 10.8
(5'R)-5',8-cyclo-2'-deoxyadenosine	15.8 – 17.2
(5'S)-5',8-cyclo-2'-deoxyguanosine	19.5 – 21.0
8-oxo-2'-deoxyguanosine	24.4 – 25.8
(5'S)-5',8-cyclo-2'-deoxyadenosine	26.4 – 27.7
8-oxo-2'-deoxyadenosine	28.9 – 30.3

Table 6. Time ranges for collected

7. RESULTS

7.1. HPLC method development and optimization

7.1.1. Solubility of samples

Based on previous studies of solubility of DNA lesions in acetonitrile/water solutions performed in our laboratory, DNA lesions and 2'-deoxynucleosides are soluble in water and in acetonitrile/water solutions not exceeding the content of acetonitrile 20%.

7.1.2. Column selection

During method development, two columns Phenomenex LUNA C18 (2) (150x4.6 mm; 5μm) and Phenomenex PhenoSphere ODS (1) [80A 250x4.6 mm; 5μm] were tested. Better separation among individual peaks and better symmetry of peaks were obtained with Phenomenex LUNA C18 (2) [50x4.6 mm; 5μm],

7.1.3. Mobile phase selection

Two solvents, acetonitrile and methanol, were tested as the organic components of the MP. Better separation was obtained with acetonitrile.

As aqueous component of the MP was used 2 mM ammonium formate buffer[47].

7.1.4. Detector wavelength

Based on literature, detector wavelength was set up at 260 nm[50].

7.1.5. Injection volume

Three injection volumes were tested; 5 μL, 20 μL and 50 μL. In all three tests, peak broadening was not observed and consistent $w_{0.5}$ values were obtained.

7.1.6. Gradient optimization

At first, linear gradient from 0% to 20% of acetonitrile in 20 minutes was employed and by successive changes (Table 7.) improved into final conditions (Table 12.) in order to reach sufficient separation of peaks for DNA lesions collection.

Time	0	2.2	12.0	16.0	29.0	30.0	30.1	36.0
% ACN	**0**	**1.0**	**2.0**	**2.0**	**13**	**20**	**0**	**0**

Time	0	2.2	12.0	16.0	29.0	31.0	33.0	33.1	38.0
% ACN	**0**	**1.0**	**2.0**	**2.0**	**8.0**	**20.0**	**20.0**	**0**	**0**

Time	0	2.2	6.2	11.0	15.0	24.0	25.0	28.0	28.1	33.0
% ACN	**0**	**0.5**	**1.3**	**2.0**	**2.0**	**8**	**20**	**20.0**	**0**	**0**

Table 7. Example of gradients used during method development

Time	0	2.2	6.2	11.0	12.0	18.0	26.0	30.0	35.0	37.0	37.1	43.0
% ACN	**0**	**0.3**	**0.8**	**1.5**	**1.9**	**1.9**	**8.0**	**10.0**	**20.0**	**20.0**	**0**	**0**

Table 8. Final gradient method

It was determined that the time needed for column re-equilibration has to be at least six minutes.

It was also tested that the initial composition of the mobile phase (level of organic component) does not affect the repeatability of retention times.

Chromatogram obtained under final conditions (1μg/mL of dN and 1μg/mL of L mixture) is shown at Figure 17.

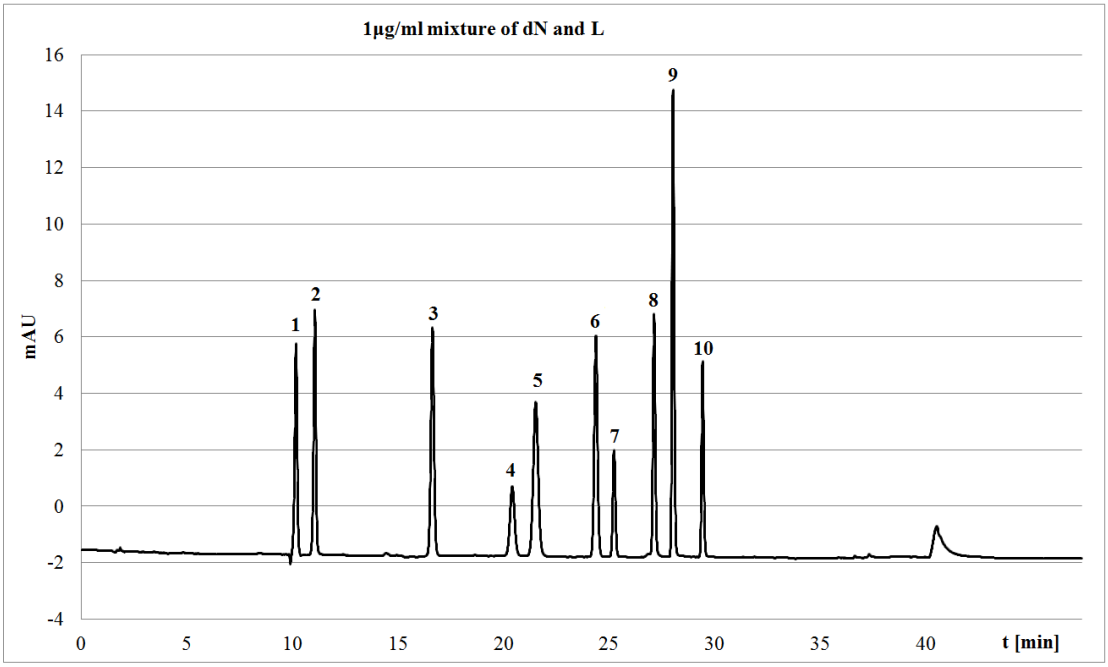

Figure 17. Chromatogram under final conditions.

1. (5'R)-5',8-cyclo-2'-deoxyguanosine (t_R = 10.1 min)

2. 2'-deoxycytidine (t_R = 10.9 min)

3. (5'R)-5',8-cyclo-2'-deoxyadenosine (t_R = 16.5 min)

4. (5'S)-5',8-cyclo-2'-deoxyguanosine (t_R = 20.2 min)

5. 2'-deoxyguanosine (t_R = 21.3 min)

6. 2'-deoxythymine (t_R = 24.1 min)

7. 8-oxo-2'-deoxyguanosine (t_R = 25.3 min)

8. (5'S)-5',8-cyclo-2'-deoxyadenosine (t_R = 26.9 min)

9. 2'-deoxyadenosine (t_R = 27.8 min)

10. 8-oxo-2'-deoxyadenosine (t_R = 29.2 min)

7.1.7. Windows for DNA lesions collection

Time ranges for collection of (5'R)-5',8-cyclo-2'-deoxyadenosine (**(5'R)-cdA**), (5'S)-5',8-cyclo-2'-deoxyadenosine (**(5'S)-cdA**), (5'R)-5',8-cyclo-2'-deoxyguanosine (**(5'R)-cdG**), (5'S)-5',8-cyclo-2'-deoxygua-nosine (**(5'S)-cdG**), 8-oxo-2'-deoxyadenosine (**8-oxo-2'-dA**) and 8-oxo-2'-deoxyguanosine (**8-oxo-2'-dG**) were determined and adjusted based on retention times and peak widths of particular analytes. When adjusting these time ranges, 0.1 minute delay caused by the transport of effluent from the detector to the fraction collector should be taken into account. 0.1 minute delay was determined in dependence of flow rate and the length of the tubing connecting the detector and the fraction collector.

Intra-day values were obtained by four injections of the same homogenous sample (500 μg/mL of dN and 10 ng/mL of L). Inter-day values were obtained by four injections of the same homogenous sample (500 μg/mL of dN and 10 ng/mL of L) in four different days. The measured values are shown in Table 9. and Table 10.

Analyte	**Peak interval [min]**	**Peak interval [min]**	**Peak interval [min]**	**Peak interval [min]**	**Time ranges [min]**
(5'R)-cdG	9.9 – 10.4	9.9 – 10.3	10.0 – 10.4	9.9 – 10.3	**9.4 – 10.8**
2'-dC	10.7 – 11.4	10.6 – 11.3	10.8 – 11.4	10.7 – 11.5	**-**
(5'R)-cdA	16.2 – 16.9	16.3 – 16.9	16.3 -16.8	16.3 – 16.8	**15.8 – 17.2**
(5'S)-cdG	19.9 – 20.7	19.9 – 20.6	19.9 – 20.6	19.8 – 20.4	**19.5 – 21.0**
2'-dG	21.0 – 22.0	20.8 – 21.7	20.8 – 21.8	20.6 – 21.7	**-**
2'-dT	23.8 – 24.6	23.8 – 24.6	23.7 – 24.5	23.7 – 24.7	**-**
8-oxo-2'-dG	24.9 – 25.4	25.1 – 25.6	25.1 – 25.6	25.1 – 25.5	**24.4 – 25.8**
(5'S)-cdA	26.8 – 27.4	26.8 – 27.3	26.8 – 27.2	26.7 – 27.2	**26.4 – 27.7**
2'-dA	27.6 – 28.2	27.6 – 28.2	27.6 – 28.1	27.5 – 28.2	**-**
8-oxo-2'-dA	29.1 – 29.6	29.1 – 29.6	29.1 – 29.5	29.1 – 29.5	**28.9 – 30.3**

Table 9. Intra-day values

Analyte	Peak interval [min]	Peak interval [min]	Peak interval [min]	Peak interval [min]	Time ranges [min]
(5'R)-cdG	9.9 – 10.3	10.1 – 10.4	10.1 – 10.5	10.1 – 10.4	**9.4 – 10.8**
2'-dC	10.6 – 11.3	10.8 – 11.4	10.8 – 11.3	10.9 – 11.4	**-**
(5'R)-cdA	16.3 – 16.9	16.3 – 16.9	16.4 – 17.0	16.3 – 17.0	**15.8 – 17.2**
(5'S)-cdG	19.9 – 20.5	19.9 – 20.6	19.6 – 20.3	19.6 – 20.3	**19.5 – 21.0**
2'-dG	20.8 – 21.7	20.8 – 21.7	21.1 – 21.9	21.0 – 21.9	**-**
2'-dT	23.8 – 24.6	23.8 – 24.5	23.8 – 24.6	23.8 – 24.5	**-**
8-oxo-2'-dG	25.1 – 25.6	25.1 – 25.5	25.2 – 25.6	25.0 – 25.5	**24.4 – 25.8**
(5'S)-cdA	26.8 – 27.3	26.8 – 27.2	26.9 – 27.3	26.8 – 27.3	**26.4 – 27.7**
2'-dA	27.6 – 28.1	27.6 – 28.1	27.7 – 28.3	27.7 – 28.2	**-**
8-oxo-2'-dA	29.1 – 29.6	29.2 – 29.7	29.2 – 29.6	29.1 – 29.7	**28.9 – 30.3**

Table 10. Inter-day values

As is apparent from the previously shown data, in all cases a complete collection of all analytes of interest was achieved in given time ranges**.**

7.2. Method performance

7.2.1. Retention times repeatability

Intra-day and inter-day retention time values for (5'R)-cdA, (5'S)-cdA, (5'R)-cdG, (5'S)-cdG, 8-oxo-2'-dA, 8-oxo-2'-dG, 2'-deoxyadenosine (**2'-dA**), 2'-deoxycytidine (**2'-dC**), 2'-deoxyguanosine (**2'-dG**) and 2'-deoxythimidine (**2'-dT**) are shown in Table 11. Intra-day and Inter-day values were obtained by four injections of the same homogenous sample (500 μg/mL of dN and 10 ng/mL of L.

Analyte	**Retention time [min]**	**Intra-day C_v [%]**	**Retention time [min]**	**Inter-day C_v [%]**
(5'R)-cdG	10.1	**0.833**	10.2	**1.126**
2'-dC	10.9	**0.483**	11.0	**0.414**
(5'R)-cdA	16.5	**0.268**	16.6	**0.417**
(5'S)-cdG	20.1	**0.546**	20.3	**0.296**
2'-dG	21.2	**0.557**	21.4	**0.349**
2'-dT	24.1	**0.393**	24.1	**0.345**
8-oxo-2'-dG	25.2	**0.364**	25.3	**0.650**
(5'S)-cdA	26.9	**0.197**	27.0	**0.230**
2'-dA	27.8	**0.214**	27.8	**0.264**
8-oxo-2'-dA	29.3	**1.386**	29.4	**0.940**

Table 11. Intra-day and inter-day values of retention times

7.2.2. Peak widths and peak widths at half height

Peak width (w) is the measure of the broadness of the peak. Intra-day ad Inter-day values were obtained by four injections of the same homogenous sample (500 μg/mL of dN and 10 ng/mL of L) (Table 12.).

Analyte	w [min]	Intra-day C_v [%]	w [min]	Inter-day C_v [%]
(5'R)-cdG	0.4	**11.8**	0.4	**16.5**
2'-dC	0.7	**11.17**	0.6	**16.7**
(5'R)-cdA	0.6	**16.7**	0.6	**8.0**
(5'S)-cdG	0.7	**11.7**	0.7	**7.4**
2'-dG	1.0	**8.2**	0.9	**5.7**
2'-dT	0.9	**11.8**	0.8	**7.7**
8-oxo-2'-dG	0.5	**10.5**	0.5	**12.8**
(5'S)-cdA	0.5	**16.3**	0.5	**12.8**
2'-dA	0.6	**13.6**	0.5	**9.5**
8-oxo-2'-dA	0.5	**12.8**	0.5	**16.3**

Table 12. Intra-day and inter-day values of peak widths

Peak width at half height ($w_{0.5}$) is the distance from the front slope to the back slope of the peak measured at 50% of the maximum peak height. Values of peak widths at half height were obtained from injection of 1µg/mL of dN and 1µg/mL of L mixture and for dN from injection of 500 µg/mL of dN and 10 ng/mL of L mixture (Table 13.).

Analyte	**$w_{0,5}$ [min] 1µg/mL of dN and L**	**$w_{0,5}$ [min] 500 µg/mL of dN**
(5'R)-cdG	0.11	-
2'-dC	0.11	0.12
(5'R)-cdA	0.17	-
(5'S)-cdG	0.22	-
2'-dG	0.22	0.22
2'-dT	0.12	0.17
8-oxo-2'-dG	0.11	-
(5'S)-cdA	0.11	-
2'-dA	0.11	0.12
8-oxo-2'-dA	0.11	-

Table 13. Peak widths at half height

By injection of 1µg/mL of dN and 1µg/mL of L mixture and by injection of 500 µg/mL of dN and 10 ng/mL of L mixture, similar values of $w_{0.5}$ were obtained.

7.2.3. Symmetry factor

Symmetry factor (A_s) is the parameter used to describe the shape of the peak. The symmetry factors for particular peaks were calculated using following formula (Scheme 8.)

$$A_s = \frac{w_{0,5}}{2d}$$

Scheme 8. Symmetry factor

where $w_{0,5}$ is the peak width at half height and d is the back half-width at 5% of the maximum peak height. Values for peak symmetry calculations were obtained from injection of 1μg/mL of dN and 1μg/mL of L mixture (Table 14.).

Analyte	**A_s**
(5'R)-cdG	1.09
2'-dC	1.18
(5'R)-cdA	1.01
(5'S)-cdG	0.97
2'-dG	0.96
2'-dT	0.97
8-oxo-2'-dG	0.92
(5'S)-cdA	0.91
2'-dA	0.87
8-oxo-2'-dA	0.87

Table 14. Symmetry factors

Ideal values of symmetry factors lie within the interval from 0.8 to 1.5. For all peaks was this condition accomplished.

7.2.4. Plate number

Column efficiency was confirmed by calculating of plate numbers (N) for particular analytes. The plate number for an analyte with retention time t_a and peak width in the half-height $w_{a\,0,5}$ was calculated using following formula (Scheme 5).

Values for plate number calculations were obtained from injection of 1μg/mL of dN and 1μg/mL of L mixture (Table 15.).

Analyte	N
(5'R)-cdG	46705
2'-dC	54397
(5'R)-cdA	52189
(5'S)-cdG	46244
2'-dG	50608
2'-dT	223451
8-oxo-2'-dG	290754
(5'S)-cdA	331306
2'-dA	353846
8-oxo-2'-dA	393061

Table 15. Plate numbers

7.2.5. Resolution

Resolution (R_s) describes the level of separation between particular chromatographic peaks (Scheme 7).

Intra-day and Inter-day values were obtained by four injections of the same homogenous sample (500 μg/mL of dN and 10 ng/mL of L (Table 16.).

	R_s	**Intra-day R_s [%]**	**R_s**	**Inter-day R_s [%]**
(5'R)-cdG / 2'-dC	1.4	**9.0**	1.7	**12.0**
2'-dC / (5'R)-cdA	4.4	**10.7**	4.6	**6.2**
(5'R)-cdA / (5'S)-cdG	5.7	**12.7**	5.7	**6.5**
(5'S)-cdG / 2'-dG	1.3	**6.3**	1.6	**3.7**
2'-dG / 2'-dT	3.2	**8.0**	3.4	**3.0**
2'-dT / 8-oxo-2'-dG	1.7	**3.0**	2.0	**8.2**
8-oxo-2'-dG / (5'S)-cdA	3.5	**8.3**	3.9	**13.5**
(5'S)-cdA / 2'-dA	1.7	**14.4**	1.6	**3.1**
2'-dA / 8-oxo-2'-dA	2.9	**10.5**	3.1	**4.8**

Table 16. Intra-day and inter-day resolution values.

7.2.6. Memory effect

Three different concentrations of sample were injected followed by injection of water in order to evaluate the memory effect of the column. After the injection of water, no memory effect was observed.

7.2.7. Recovery of sample collection

To test the recovery of analytes collected by fraction collector, 5 μM mixture of dN was injected into the chromatographic system (I_v=20 μL). Collected analytes were diluted (1:200) in order to reach such a low concentration of ACN that does not affect the chromatographic process. Thus diluted mixture was re-injected into the chromatographic system and areas obtained in this run were compared to areas obtained in previous run. With this test we achieved recovery of analytes 100 ± 2% so the recovery is not affected by the sample collection.

7.2.8. Recovery of the evaporation step

To test the recovery of analytes after lyophilization, 100 ng/mL mixture of L was injected into the chromatographic system (I_v=20μL) and particular analytes were collected in given time ranges into individual eppendorf tubes. Thus collected analytes were lyophilized overnight. After lyophilization, eppendorf tubes used for sample collection were successively washed with the same 100uL of Milli-Q water by transfer from one tube to another. To avoid losses of analyte due to the adhesion to the walls, tubes were vortexed. In the end of this process we obtained one eppendorf tube containing all six DNA lesions (~20 ng/mL of each) which was re-injected into the chromatographic system (I_v=20μL).

Calibration curves for (5'R)-cdA, (5'S)-cdA, (5'R)-cdG and (5'S)-cdG were constructed by five injections of each 10, 100 and 1000 ng/mL L calibration solutions (Table 17.).

Analyte	Calibration equation	Correlation coefficient (R^2)
(5'R)-cdA	y = 0.0847x + 0.2944	1.0000
(5'S)-cdA	y = 0.0630x – 0.0200	0.9999
(5'R)-cdG	y = 0.0376x + 0.4056	0.9999
(5'S)-cdG	y = 0.0610x – 0.0833	0.9998

Table 17. Calibration curves parameters (5 injection media)

Recovery for individual analytes was determined by the comparison of the peak areas obtained from injection of lyophilized samples with those obtained by interpolating the value of 20 ng/mL mixtures in the calibration curve equations (Table 18.). No satisfactory data for 8-oxo-2'-deoxyadenosine and 8-oxo-2'-deoxyguanosine were

obtained because of their instability, however further and more precise data will come from LC/MS/MS analysis.

Analyte	Recovery [%]
(5'R)-cdA	88
(5'S)-cdA	77
(5'R)-cdG	103
(5'S)-cdG	103

Table 18. Recovery of evaporation step

7.2.9. Limits of detection for DNA lesions and 2'-deoxynuclesides

Limit of detection (LOD) as the lowest concentration of an analyte detectable with a given method used was calculated as the concentration of analyte with the S/N ratio 3/1 (Table 19.).

Values for **LOD** calculations were obtained from injection of 1μg/mL of dN and 1μg/mL of L mixture. The level of noise was determined as 0.01 mAU.

Analyte	Peak high [mAU]	LOD [ng/ml]
(5'R)-cdG	9.9	**3.0**
2'-dC	10.8	**2.7**
(5'R)-cdA	10.4	**2,8**
(5'S)-cdG	3.3	**9,0**
2'-dG	7.4	**4,0**
2'-dT	9.9	**3,0**
8-oxo-2'-dG	5.0	**6,0**
(5'S)-cdA	11.6	**2,6**
2'-dA	22.4	**1,3**
8-oxo-2'-dA	9.1	**3,3**

Table 19. Limits of detection

8. DISCUSSION

Phenomenex LUNA C18 (2) column is ultra-pure silica-based HPLC column with a higher carbon load than Phenomenex PhenoSphere ODS (1). High carbon load ensures more interactions between non-polar analytes and bonded phase and this leads to higher resolution which is required in our samples when non-polar interactions are determinant for selectivity.

Acetonitrile was chosen as the organic component of MP. In a compare to methanol, ACN has lower viscosity. The lower the viscosity of the solvent, the better is the transfer of analytes between SP and MP and faster dynamic equilibrium is obtained. This in turn gives higher separation efficiency and sharper peaks. Also lower viscosity decreases the back pressure.

Considering the solubility of analytes, level of ACN during gradient optimization should not exceed 20%. With final conditions, separation among individual analytes was sufficient for collection of DNA lesions so a column length of 150 mm is enough and there is no need for a 250 mm column.

In reverse phase chromatography, *k* is influenced by the elution strength of MP. With low level of ACN, some analytes are compressed on the top of the column. This analytes do not start to move till the elution strength of MP change the *k* to lower values. This is on the basis of developing elution gradient profile and we can see the change of *k* during the gradient: during chromatographic run, peak broadening within the column is caused by mass transfer between SP and MP, longitudinal and eddy diffusion. In our case, we observed peak broadening (Table 13.) till the elution of 2'-deoxyguanosine [$w_{0.5}$ was: (5'R)-cdG =0.11 min; 2'-dC = 0.11 min; (5'R)-cdA = 0.17 min; (5'S)-cdG 0.22 min; 2'-dG = 0.22 min] From elution of 2'-dT we obtained constant peak widths [$w_{0.5}$ was; 2'-dT = 0.12 min; 8-oxo-2'-dG = 0.11 min; (5'S)-cdA = 0.11 min; 2'-dA = 0.11 min; 8-oxo-2'-dA = 0.11 min]; it seems that these analytes start to move into the analytical column after a delay of about 10 min [2'-dT] , 12 min [8-oxo-2'-dG], 13 min [(5'S)-cdA], 14 min [2'-dA], 16 min [8-oxo-2'-dA].

For the same reason, N values (Table 15.) are similar till 2'-deoxyguanosine (~50000) and then rapidly increase because t_r increases artificially more than peak broadening does.

In real samples, there is significant difference between concentration of DNA lesions and undamaged nucleosides. In samples coming from gamma irradiation is ~ 1 DNA lesion generated per 10^6 2'-deoxynucleosides per 1 Gy. During method development, different concentrations of DNA lesions and 2'-deoxynucleosides were injected to get close to these values. According to LOD (Table 22.) of particular analytes [LOD were; (5'R)-cdA = 2.8 ng/mL, (5'S)-cdA = 2.6 ng/mL, (5'R)-cdG = 3.0 ng/mL, (5'S)-cdG = 9.0 ng/mL, 8-oxo-2'-dG = 6.0 ng/mL, 8-oxo-2'-dA = 3.3 ng/mL], the lowest concentration of DNA lesions to be used in samples during method development was determined as 10 ng/mL.

Overlapping of time ranges for collection of DNA lesions with peak intervals of 2'-deoxynucleosides occurred during chromatographic run (Table 9. and 10.). However, to be sure that those lesions were collected completely, small amounts of collected 2'-deoxynucleosides are acceptable because they do not affect the solubility and LC/MS/MS analysis.

In recovery studies, we are limited by HPLC/UV values of LOD (Table 19.) and LOQ. To ensure complete collection of DNA lesions, recovery of sample collection was tested. Collected and diluted fractions of 2'-deoxynucleosides were re-injected into the HPLC system and the $100 \pm 2\%$ recovery yield that was achieved showed that collection step will not affect the overall recovery.

Recovery of evaporation step was also tested in order to determine the influence of lyophilization on collected DNA lesions (Table 18.). Recovery we obtained was in the range from 77% to 103%. Because we were limited by the sensitivity of UV detector, more precious and satisfactory data will be obtained by LC/MS/MS analysis and if there were any looses of analytes during the evaporation step, they will be determined by comparing of response obtained from analytes to response obtained from internal standards added to samples before injection to HPLC/UV. As internal standards will be used [$^{15}N_5$] labelled compounds because of the same physical and chemical properties and behaviour during sample pre-treatment, chromatographic process and storing.

9. CONLUSION

HPLC/UV method for separation of (5'R)-5',8-cyclo-2'-deoxyadenosine; (5'R)-5',8-cyclo-2'-deoxyguanosine; (5'S)-5',8-cyclo-2'-deoxyadenosine; (5'S)-5',8-cyclo-2'-deoxyguanosine; 8-oxo-2'-deoxyguanosine and 8-oxo-2'-deoxyadenosine from 2'-deoxyadenosine; 2'-deoxycytidine; 2'-deoxyguanosine and 2'-deoxythymidine in aqueous solutions was successfully developed as the preanalytical step before quantification of DNA lesions by LC/MS/MS.

This developed method will be further updated for samples containing salts in order to be applicable to real samples.

When this method will be updated for real samples, the concentration of DNA lesions will be lower than 1 ng/mL so lower than the sensitivity of UV detector and all recovery studies will be repeated with samples containing salts in real concentrations and LC/MS/MS will be utilized. Before undergoing HPLC/UV clean-up step, [$^{15}N_5$] labelled internal standards will be added to samples and in this way, IS will evaluate the overall recovery of every analyte in real samples.

To be able to quantify 2'-deoxynucleosides in real samples, calibration curves for 2'-deoxyadenosine; 2'-deoxycytidine; 2'-deoxyguanosine and 2'-deoxythymidine in samples containing salts in the same concentrations as in real samples will be constructed.

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