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Measurement of level of oxidative DNA damage in liver perfusate after pretreatment with copper solution using HPLC with electrochemical detection

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ABSTRACT

The aim of the present study was to develop a RP-HPLC method for the determination of a background level of 8-oxodG (a biomarker of oxidative stress) in rat liver after addition of copper to perfusate. The reversed phase analytical column Purospher® STAR C18e (150x4.6 mm, I.D., 5 μm, Merck) with Purospher® STAR RP-18e (4x4mm, I.D., 5 μm, Merck) as a precolumn were applied for the analysis. The mobile phase consisted of 8% (v/v) methanol in 50 mmol/L phosphate buffer, pH 5.5. Oxidative damage to nuclear DNA was determined by the simultaneous measuring of 2'- deoxyguanosine (dG) with UV detection folowed by electrochemical detection of 8-oxodG. The validation of the HPLC method according to linearity, accuracy and precision was investigated. A detailed investigation of experiments have been discussed in this paper.

KEYWORDS

DNA damage;
8-oxodG;
HPLC;
Electrochemical detection;
Liver.

INTRODUCTION

In eukaryotic organisms, oxygen is partially reduced to form reactive oxygen species (ROS). Some of these have an unpaired electron, resulting in radicals, including hydroxyl radical (•OH) and superoxide anion ($\bullet O_2^-$). Unscavenged reactive oxygen species (ROS) can cause extensive damage to all the major groups of biochemical macromolecules, including peroxidation of lipids, protein fragmentation and DNA modification[1,2]. The most important oxygen-free radical is the hydroxyl radical, which can cause damage of biomolecules. Hydroxy radicals react with all DNA bases whereas singlet oxygen selectively modifies guanine. It has been difficult to elucidate the exact mechanisms and significance of oxidative damage. One important factor for the difficulty is a lack of precision and accuracy in the measurement of oxidative DNA. The oxidation of guanine in DNA during sample preparation is a serious artefact. The elimination of this problem, standardisation of protocols and reduction of variability and errors in the different assays are essential. The European Standards Commitee on Oxidative DNA Damage (ESCODD) was set up in 1997 with 27 analytical laboratories as members. It has attempted to resolve methodological problems and improve the accuracy and specificity in

measurements of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), an oxidation product of 2-deoxyguanosine (dG), which is the most commonly measured marker of oxidative DNA damage[3-6].

Results of 8-oxodG analysis from nuclear DNA samples such as tissue or cells are often expressed normalized to the unmodified base (8-oxodG/dG) and enzymatic DNA digestion is required to liberate and measure free 8-oxodG. Measurements of this type represent oxidative damage at the specific sampling site at the time of sampling[7]. Alternatively, analysis of 8-oxodG as a repair product in urine[8-10] probably reflects the level of oxidative DNA damage in the body as a whole. Authors Peoples and Karnes[11] present recent analytical developments with respect to sample preparation and instrumental considerations for the analysis of urinary 8-oxodG.

Analytical approaches for biomarkers of oxidative damage have focused on achieving sensitive detection levels and improving sample preparation procedures. Mostly methods were developer for the measurement of DNA lesions. The direct approaches involve chromatographic methods[12], such as high-performance liquid chromatography (HPLC) with different detection techniques: laser-induced fluorescence[13], electrochemical (HPLC-EC)[14-17] or mass spectrometry (HPLC-MS/MS)[18-22]; gas chromatography-mass spectrometry (GC-MS)[2,23,24]. Alternative methods (enzymic approaches) are based on measurement of single strand breaks. They can include enzyme-linked immunosorbent assay (ELISA)[23,25,26] or single cell gel electrophoresis (Comet assay)[17].

Transition metals are able to participate in reactions that can generate free radicals. It is well known that the formation of highly reactive and damaging hydroxyl radicals is posssible only in the presence of transition metals (usually Fe or Cu) and hydroperoxide. Copper and iron participate, with hydrogen peroxide, in Fenton and other reactions that generate potentially deterious reactive oxygen species, which can damage DNA to form modifications implicated in mutagenesis, carcinogenesis, aging, and some degenerative diseases[27-29]. Only unbound metal ions are toxic. Copper and iron are essential trace elements, being integral components of a great number of important enzymes and cellular macromolecules[30,31].

In the present study, the HPLC method with a Purospher® STAR C18e analytical column and the connection of electrochemical and spectrophotometric detectors in series was investigated for the simultaneous determination of 8-oxodG and dG, respectively. The level of oxidative DNA damage expressed as a molar ratio of 8-oxodG to dG was investigated after addition of copper to the liver rat perfusate.

EXPERIMENTAL

Chemicals and materials

Analytical standards-8-oxo-2'-deoxyguanosine and 2'-deoxyguanosine were supplied by Sigma-Aldrich (St.Louis, MO, USA). Anhydrous potassium dihydrogen phosphate, HPLC grade methanol, phosphoric acid were purchased form Merck AG (Darmstadt, Germany). Enzyme and buffer solutions were prepared using deionized water of highest purity (conductivity 18.2 MQ/cm) from a Simplicity water system UV185 (Millipore). Anesthetics, xylazinum hydrochloride (10 mg/kg) and ketaminum hydrochloride (120 mg/kg) were purchased from Spofa and Léčiva (Prague, Czech Republic), respectively. All other chemicals and solvents were of analytical grade and were used without further purification.

Instrumentation and chromatographic conditions

The liquid chromatographic set-up consisted of a HP 1100 system (Hewlett-Packard, Waldbronn, Germany) equipped with a quarternary pump with on-line vacuum degasser, an autosampler, the thermostated column compartment with Peltier cooling elements, a diode-array detector. The concentration of dG was estimated from the UV peak at absorbance 254 nm. Coulometric detector *Coulochem II* (ESA, USA) with a 5020 guard cell and a 5011A high sensitivity analytical cell was applied for the measurement of levels of 8-oxodG. Potentials were set up at 100 mV for guard cell, 150 mV and 400 mV for channel 1 and channel 2, respectively. Data acquisition and analysis was achieved by HP 3D ChemStation (Hewlett-Packard).

The Purospher® STAR C18e column (150x4.6mm, I.D., 5 um, Merck) was applied as an analytical column and it was protected by a Purospher® STAR C18e precolumn (4x4 mm, I.D., 5 um, Merck). The mobile

phase consisted of 50 mmol/L phosphate buffer, pH 5.5 and methanol (92:8, v/v). Flow rate was 0.6 mL/min for an analytical separation. The column temperature was kept at 20°C and injected volume was 50 L.

Buffers and enzymes preparation

Homogenisation buffer (HB): 10 mmol/L Tris-HCl, 0.4 mmol/L NaCl, 5 mmol/L deferoxamine mesylate (DF; Sigma-Aldrich). HB was prepared in deionized water, adjusted to pH 8.0 with dilute HCl and aliquots were freezed at -20°C. DF is light sensitive, so cover containers in aluminium foil to protect from light during storage and use. Just before use, HB was thawed and Triton X-100 (Merck) was added to 0.5 %.

Ribonuclease buffer: 10 mmol/L Tris-HCl, 0.4 mmol/L NaCl in deionized water and adjusted to pH 8.0 with dilute HCl.

Hydrolysis buffers: a) 1 mol/L sodium acetate containing 45 mmol/L zinc chloride in deionized water adjusted to pH 4.8 with acetic acid. b) 1.5 mol/L Tris-HCl buffer pH 8.0 in deionized water. Aliquots of both buffers were stored at -20°C.

Enzymes

Rnase T1 (Sigma-Aldrich) from Aspergillus oryzae was prepared to a concentration 10^3 U/mL, Rnase IIIA (Sigma-Aldrich) from bovine pancreas was prepared to a concentration 1 mg/L. Both of enzymes were prepared in ribonuclease buffer and put put in 80°C water bath for 15 min. After cooling to room temperature, aliquots were stored at -20°C.

Proteinase K (Roche Diagnostic GmbH Mennheim, Germany) from *Tritirachium album* was supplied as solution ready for use and was stored at 4°C; P1 nuclease (Calbiochem) from *Penicillium citrinum* was dissolved at 1100 U/mL in 25 mmol/L sodium acetate containing 1 mmol/L zinc chloride, pH 4.8 was adjusted with acetic acid.; Alkaline phosphatase (Sigma-Aldrich) Grade I from calf intestine was diluted in 100 mmol/L Tris-HCl pH 8.0 to a concentration 750 U/mL. Aliquots of prepared enzymes were stored at -20°C.

Preparation of stock solutions

Preparation of individual stock solutions of both standards 8oxodG and dG with concentrations of 500 nmol/L and 1 mmol/L was done by a dissolution of weighed solid standards in deionized water. Concentration of individual standards was determined by calculation from the known molar absorption coefficients[32]. At 1 mmol/L 8-oxodG and dG have an absorbance of 12.3 AU (245 nm) and 13 AU (254 nm), respectively. These stock standards were stored at -20°C for daily use. Working standards were prepared daily fresh by an appropriate dilution of the stock standards in a solution of 10 mmol/L Tris-HCL, pH 7.3.

METHODS

Isolation and hydrolysis of DNA from liver samples

A recommended procedure for extraction and hydrolysis of DNA was based on the method carried out by members of ESCODD[33].

Animals and liver procurement

Male Wistar rats (250-310 g) obtained from Top Velaz Co. (Prague, Czech Republic) were kept under conventional conditions of animal house, having free access to food and tap water. The study was approved by the local animal welfare committee. The rats were given ketamin (120 mg/kg) xylazin (10 mg/kg) intraperitoneally to induce anesthesia before surgery and the liver was prepared as described by Kukan[34].

Liver perfusion

The liver was perfused for 60 min through the portal vein in a recirculating perfusion system at a pressure of 12 cmH_2O. Krebs-Henseleit buffer, pH 7.4, containing glucose (10mmol/L) and saturated with 95% oxygen and 5% carbon dioxide, was used as the perfusion medium. Copper ($CuSO_4$) was added to the perfusate at the start of perfusion in concentration of 0; 0.01 and 0.03 mmol/L. At the end of perfusion the liver was blotted, weighed and parts of the left lateral lobe were frozen in liquid nitrogen using precooled Wollenberger clamps. The frozen tissue was subsequently stored at -70°C for 8-oxodG and dG assays.

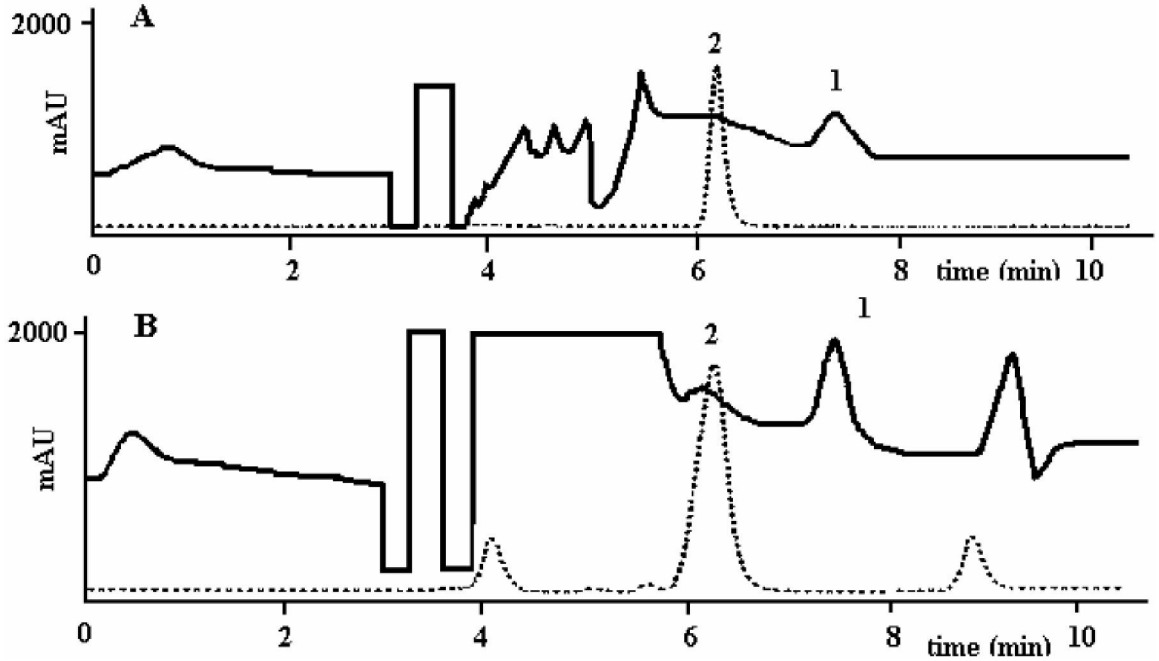
RESULTS AND DISCUSSION

Optimisation of detection conditions

The separately analysis of 8-oxodG and dG standards was important because dG inevitably contained

a low level of 8-oxodG. For the measurement of 8-oxodG, there was a requirement for its sensitive detection and an electrochemical (coulometric) detection has appeared as the most available approach. The chosen optimal potential 400 mV was the same as previously shown by members of ESCODD[3,5,6].

During coulometric detection, the one of the most important facts is to overcome problems with reduce background noise to a minimum (preparing fresh mobile phase buffer daily; purging the mobile phase with helium; filtration of mobile phase through a 0.2 um nylon filter under vacuum etc.). It is known that in case of matrix contribution to the control sample, the additional calibration process must be designed using the method of standard addition to the sample. Figure 1 A,B show chromatograms obtained from the simultaneous determination of 8-oxodG and dG in water (A) as well as in hydrolyzed DNA from liver (B) spiked with the same concentration of 8-oxodG (2 nmol/L) and dG (200 μmol/L) in both matrices.



A - Chromatograms obtained after injection of 50 μL of water sample spiked with standards.
B - Chromatograms obtained after injection of 50 μL of hydrolyzed DNA sample from liver spiked with standards.
Chromatographic conditions: Analytical column: Purospher® STAR C18e (150x4.6 mm I.D., 5 μm, Merk, Darmstadt, Germany). Precolumn: Purospher® STAR C18e e (4x4 mm I.D.,5μm, Merk, Darmstadt, Germany). Mobile phase:50 mmol.L^{-1} phosphate buffer, pH 5.5 with 8% methanol, flow: 0.6 mL.min^{-1}. Detection: ECD (E1 150 mV, E2 400 mV, guard cell 100 mV) for 8-oxodG; DAD 254 nm for dG.

Figure 1 : RP-HPLC determination of 2 nmol.L^{-1} 8-oxodG (1) and 200 μmol.L^{-1} dG (2) by the simultaneous coulochemical (-) and UV (- -) detection.

Validation of HPLC method

In general, a validated analytical method means that it gives reliable and reproducible results and where are definite and vertifiable parameters and capital operating conditions. The main validation parameters are shown in TABLE 1.

Linearity

Calibration curves were prepared in the range of 0.5-5.0 nmol/L and 60-200 mol/L for 8-oxodG and dG, respectively. Individual points of calibration curves were devised with addition of a small volume of analytes with increase of concentration into hydrolysed DNA liver sample. Calibration curves were obtained by least square linear regression analysis of the peak areas, obtained as a function of the concentration of 8-oxodG and dG. The parameters of the calibration curves achieved following values: y=359.2x+132.6 for 8-oxodG and y=720.2+818.2 for dG. Corelation coefficients were >0.99 for both analytes.

Precision and accuracy

For the determination of intra-day precision and

accuracy of the method were evaluated by replicate analyses (n = 4) of the hydrolysed DNA calibration standards. Inter-day parameters were determined by assaying calibration standards at four separate days within 1 week. Coefficients of variation C.V.% (for precision) and relative errors RE % (for accuracy) were expressed as the estimates of standard and absolute deviations calculated for files with the number of samples less than seven. Values of CV and RE lower than 10% for all concentrations were considered acceptable[35,36].

Repeatibility of peak area

Relative errors (RE) (expressed as an estimation of relative standard deviation) were calculated from 20 injections of the analytes in spiked hydrolysed DNA liver samples at two different concentration E1 (0.5 nmol/L and 2 nmol/Lfor 8-oxodG and dG, respectively) and E2 (2 nmol/L for 8-oxodG and 60 µmol/L for dG).

Repeatability of retention time were calculated for 20 injections of the standard and the blank hydrolysed DNA liver samples at concentration level E2.

TABLE 1 : Validation parameters for HPLC determination of 8-oxodG and dG

Parameters	8-oxodG	dG
	0.5-5 nmol.L^{-1}	60-300 µmol.L^{-1}
Intra-assay		
RE%	1.5-4.3	0.8-1.9
CV%	1.2-3.2	0.7-3.5
Inter-assay		
RE%	2.1-6.1	1.0-5.2
CV%	1.9-5.8	1.2-4.9
Repeatability		
RE%		
-of retention time	0.41	0.37
-of peak area		
E1(0.5 nmol.L^{-1} 8-oxodG		
60 µmol.L^{-1} dG)	4.38	1.45
E2(2 nmol.L^{-1} 8-oxodG		
200 µmol.L^{-1} dG)	2.21	1.62
LOD	0.2	12
LOQ	0.9	61

Measurement of DNA damage

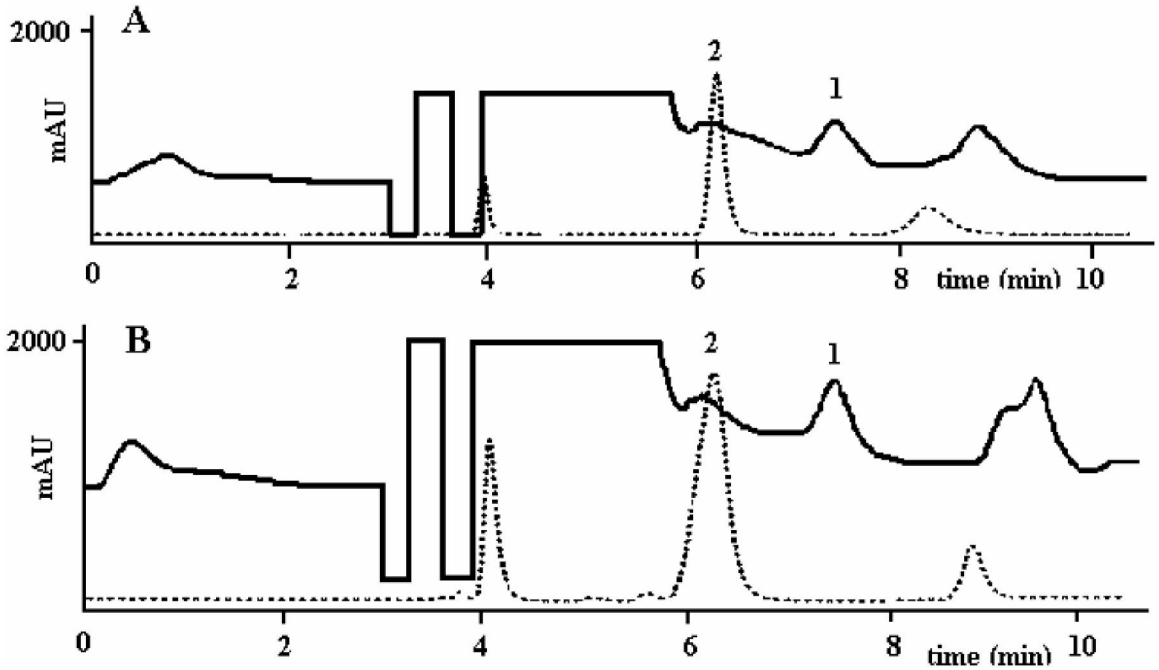
Authors Sagripant and Kraemer[38] suggested that copper ions bind to DNA at sites near guanidine residues. It has been suggested that the formation of a DNA - Cu (I) complex in aerobic aqueous solutions induced in vitro and in vivo copper-mediated DNA damage[39]. The results expressed as the ratio 8-oxodG/10^6 dG in rat liver samples after pretreatment with different concentration of CuSO4 are given in TABLE 2. These results showed a dose-dependent increase (about 50%) of the amount of the oxidized bases (expressed by the ratio) by the copper ion concentration in the perfusate. A representative chromatogram of coulochemical detection of 8-oxodG and UV detection of dG in nuclear DNA obtained from liver sample after pretreatment with 0.03 mmol/L of $CuSO_4$ is shown in Figure 2 B.

TABLE 2 : Measurement of 8-oxodG per 10^6 dG in rat liver DNA samples treated with $CuSO_4$

Concentration of $CuSO_4$ (mmol.L^{-1}) added to the perfusate	8-oxodG (nmol.L^{-1})/ 10^6dG (µmol.L^{-1})
0 (control)	13.8 ± 0.5
0.01	22.5 ± 0.5
0.03	30.8 ± 0.4

Data represent mean±RSD (n=4)

As it can be seen in TABLE 2, the measured ratios 8-oxodG/10^6 dG in the hydrolyzed DNA sample from control liver (without pretreatment with CuSO4) achieved relatively high values (see the chromatogram in Figure 2A). It was possible to assume that the problem observed might be caused by several reasons. Regarding the fact that our study was designed to investigate the effect of copper in the liver perfusate on oxidative DNA damage, the physical liver manipulation during harvest and reperfusion is inevitable. These facts might contribute to the unexpected high levels of oxidative DNA damage in control samples. It corresponds to findings of Schemmer *et al.*[40], who found that in experimental transplantation gentle in situ liver manipulation by touching, retracting and moving liver lobes during harvest, which can not be prevented with standard harvesting techniques, disturbs the hepatic microcirculation. The microcirculatory disturbances cause hypoxia, which leads to activation of Kupffer cells, free radical production and reperfusion injury after cold storage as well[41]. Moreover, the further contribution to the high background level could be related to the time-consuming sample handling during DNA isolation and extraction.



A- Chromatograms obtained after injection of 50 µl of the hydrolyzed DNA sample from control liver.
B- Chromatograms obtained after injection of 50 µL of the hydrolyzed DNA sample from liver after treatment with 0.03 mol.L^{-1} $CuSO_4$.
For chromatographic conditions see caption on Figure 1.

Figure 2 : Representative chromatograms of RP-HPLC determination of 8-oxodG (1) and dG (2) in nuclear DNA by the simultaneous coulochemical (-) and UV (- -) detection.

ABBREVIATIONS

RP-HPLC: reverse-phase high-performance liquid chromatography; EC: electrochemically; GC: gas chromatography; MS: mass spectrometry; DNA: deoxyribonucleic acid; 8-oxodG: 8-oxo-7,8-dihydro-2'-deoxyguanosine; dG: 2'-deoxyguanosine; ROS: reactive oxygen species; ESCODD: European Standards Commitee on Oxidative DNA Damage; HB: homogenization buffer; DF: deferoxamine mesylate; SDS: sodium dodecyl sulphate; UV: ultraviolet; LOD: limit of detection; LOQ: limit of quantification; CV: coefficent of variation; RE: relative error; RSD: relative standard deviation

CONCLUSION

Sumarizing, the presented method was validated according to linearity, precision and accuracy. The HPLC method applying Purospher® STAR C18e analytical column (with coulometric and diode-array detection) proved to be suitable for simultaneously measurements of 8-oxodG and dG in cellular DNA. The treatment of samples before DNA hydrolysis is a critical step in the measurement of 8-oxodG in biological samples and therefore it must be carefully controlled. The procedure with addition of different concentration $CuSO_4$ to the perfused rat livers was tested. Results from HPLC analysis of 8-oxodG and dG pointed to an increase of the amount of oxidized bases by increasing of the copper concentration.

In our study, the problem with the high backgroung levels of oxidized bases in kontrol liver samples was observed. This fact had to account to take into the validation process concerning linearity, LOD, LOQ as well as repeatabilities of peak area and retention time. Limit of detection is 0.2 nmol/L and 12 µmol/L for 8-oxodG and dG, respectively. Limit of quantitation is 0.9 nmol/L for 8-oxodG and 61 ?mol/L for dG.

The high contibution of matrix to the DNA damage (expressed as ratio of 8-oxodG/10^6dG) may be related to artefacts induced during sample preparation (timeconsuming sample treatment before DNA hydrolysis) as well as in situ liver manipulation.

Careful handling, omission of oxidising reagents and/

or addition of antioxidants can reduce this problem, but there is still a need for a further consensus in the measurement techniques and conditions.

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