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Development of an HPLC method with electrochemical detection of femtomoles of 8-oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine in the presence of uric acid

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Abstract

A selective method based on high performance liquid chromatography with electrochemical detection (HPLC-ECD) was developed to enable simultaneous detection of 8-oxo-7,8-dihydroguanine (8-oxoGua) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), products of DNA oxidative damage, in the presence of uric acid (UA), a strong interferent in their electrochemical detection. The method developed consists of HPLC isocratic elution with amperometric detection on a glassy carbon electrode, enabling a detection limit for 8-oxoGua and 8-oxodGuo lower than 1 nM in standard mixtures. Detection of low concentrations up to 25 nM of 8-oxoGua and 8-oxodGuo in the presence of UA in a 10^4-fold higher concentration was achieved after one-step solid phase extraction (SPE). The method was tested with urine samples and it was possible to detect and quantify the presence of 8-oxoGua, and to confirm that UA was eliminated after uricase degradation and SPE. The LOD found in urine samples was about 80 nM, a value higher than in standard mixtures, due to the increase of background current in the urine matrix. The results presented here contribute to the development of a methodological approach to simultaneous determination of 8-oxoGua and 8-oxodGuo in urine samples.

Keywords: DNA damage; Oxidative stress; 8-oxoGua; 8-oxodGuo; 8-oxo-7,8-Dihydroguanine; 8-oxo-7,8-Dihydro-2′-deoxyguanosine; Biomarker; Uric acid; HPLC-ECD; SPE; Urine

1. Introduction

Oxidative DNA damage caused by oxygen-free radicals leads to multiple modifications in DNA, including base-free sites and oxidised bases that are potentially mutagenic [1–7]. The major product of DNA oxidative damage is 8-oxo-7,8-dihydroguanine (8-oxoGua) (Scheme 1a) which is the product of oxidation of guanine, the most easily oxidised base in DNA [8]. This modified base is highly mutagenic [9,10] due to its loss of base pairing specificity [11–13]. Since it was first reported two decades ago [14], 8-oxoGua, namely its deoxynucleoside 8-oxo-7,8-dihydro-2′deoxyguanosine (8-oxodGuo) (Scheme 1b), has been the subject of intensive investigation and became widely accepted as a biomarker of oxidative DNA damage and cellular oxidative stress [15–17]. Elevated levels of 8-oxodGuo were found in the urine and lung tissues of smokers [18,19] as well as in body fluids and DNA from human tissues of patients with disorders such as cancer, atherosclerosis, chronic hepatitis, cystic fibrosis, diabetes, acquired immunodeficiency syndrome, neurodegenerative and age-related diseases [17,20–23].

Two main approaches to assess this oxidative DNA damage exist, one being the measurement of 8-oxoGua or 8-oxodGuo content in DNA isolated from tissues that would represent steady-state levels arising from the balance between oxidative damage and enzymatic repair [16]. However, measurements of 8-oxoGua in cellular DNA resulted in a wide range of values, from about 0.1 to 100 8-oxoGua/10^5 G [6,17,24], attributed to possible artifactual oxidation of unmodified guanine during work-up procedures of DNA isolation [25–30]. This raised concern about

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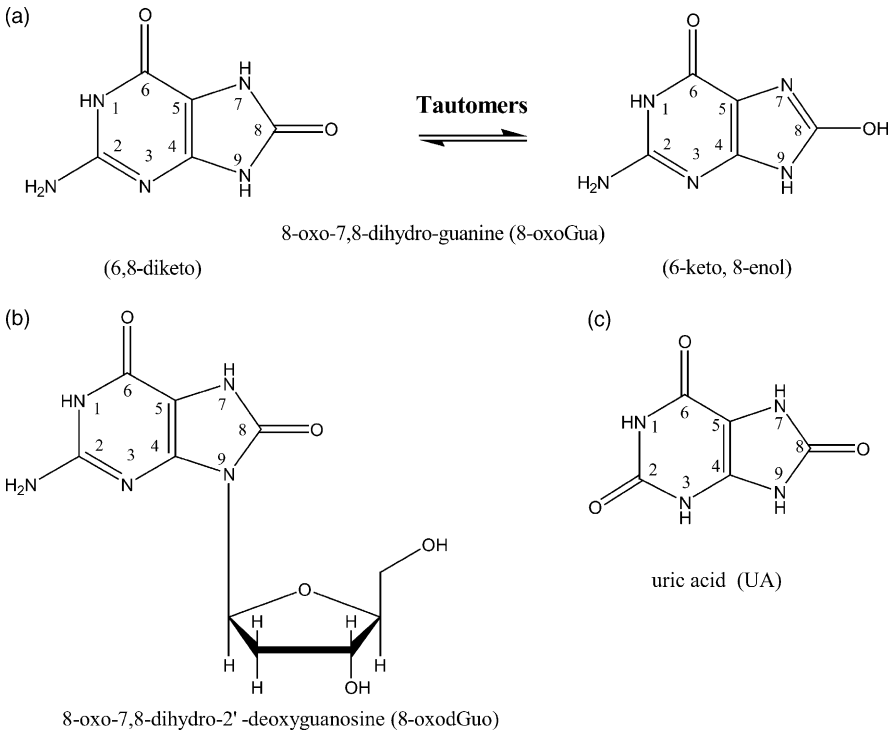

Scheme 1. Chemical structures of: (a) major tautomers of 8-oxo-7,8-dihydroguanine (8-oxoGua) in neutral pH; (b) 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and (c) uric acid (UA).

the validity of the analytical methods employed and led to the investigation of optimal methodologies for 8-oxoGua and 8-oxodGuo measurements in DNA [31–33]. A second approach has been the measurement of urinary levels of 8-oxodGuo that is widely accepted as a biomarker of "whole body" oxidative DNA damage [16,34] since urinary 8-oxodGuo could arise not only from excision repair of oxidised DNA but also, maybe largely, from hydrolysis of oxidised guanine nucleotides in the DNA precursor pool [34,35] and, in a further possibility, from DNA breakdown products upon enzymatic action on dead cells [36]. Initially, urinary 8-oxoGua was rejected as a biomarker of DNA damage because studies on rat urine [20,37] showed that diet could contribute significantly to its urinary levels and be a confusing factor. However, recent studies in humans [22,23] reveal that urinary levels of 8-oxoGua and 8-oxodGuo are not dependent on diet and taking into account that the major DNA repair pathway excises 8-oxoGua and not 8-oxodGuo [38–40], maybe urinary levels of 8-oxoGua could better reflect oxidative DNA damage than 8-oxodGuo. Despite all the controversy, assessment of urinary levels of both 8-oxoGua and 8-oxodGuo may provide a non-invasive approach to evaluate the DNA repair capability in individuals and be used as biomarkers of cellular oxidative stress.

Various methods to detect 8-oxodGuo urinary steady-state levels in humans were found [14,41–47] the majority reporting values below 10 ng 8-oxodGuo mg^{-1} creatinine (excretion rates that correspond to concentrations below 30 nM), with a few publications reporting concentrations still below 100 nM [47–50]. Only a few methods were found to be able to detect human urinary 8-oxoGua [37,41,44,49,51] and even fewer quantified it, reporting levels also below 100 nM [49,51], with the exception of one that presented a concentration around 583 ± 376 nM [44]. In all studies that quantified simultaneously 8-oxoGua and 8-oxodGuo, levels of 8-oxoGua were higher than those obtained for 8-oxodGuo in agreement with the studies indicating that the main DNA repair pathways excise the base and not the nucleoside [39,40].

At present, high performance liquid chromatography with electrochemical detection (HPLC-ECD) is the most commonly used technique to assess urinary 8-oxodGuo, but HPLC with tandem mass spectrometry (HPLC-MS/MS) is increasingly being used, showing high sensitivity and a better specificity. However, HPLC-ECD is easier to use and less demanding of resources. There is a significant lack of HPLC-ECD based methods for 8-oxoGua detection in human urine, despite 8-oxoGua being also electrochemically detectable [52] at a lower potential than 8-oxodGuo [53]. One possible reason for this is that chromatographic separation of 8-oxoGua is problematic due to its low retention on C18 reversed phase columns and to strong interference with another analyte that co-elutes with it, namely uric acid (UA), a purine with a structure very similar to 8-oxoGua (Scheme 1c), also electroactive and excreted in urine at concentrations around 0.5 mM (25–70 mg l^{-1}) [54], 10^4-fold higher than 8-oxoGua and 8-oxodGuo steady-state levels. This introduces serious problems for achieving a high enough separation factor and sensitivity requiring complex,

costly and time-consuming urine clean-up procedures. This paper describes an HPLC-ECD method developed for simultaneous detection of 8-oxoGua and 8-oxodGuo in the presence of excess UA, applying a solid phase extraction (SPE) procedure. The same SPE approach was used for preliminary qualitative analysis of 8-oxoGua and 8-oxodGuo in human urine samples.

2. Experimental

2.1. Chemicals

8-oxo-7,8-Dihydroguanine (8-oxoGua, $C_5H_5N_5O_2$, MW = 167.13), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo, $C_{10}H_{13}N_5O_5$, MW = 283.2), uric acid sodium salt ($C_5H_3N_4O_3Na$, MW = 190.1) and uracil ($C_4H_4N_2O_2$, MW = 112.1) of analytical grade were obtained from Sigma, Steinheim, Germany and used without further purification. Uricase from Bacillus fastidiosus 16.2 U mg^{-1}, EC 1.7.3.3, was obtained from Fluka, Steinheim, Germany.

Sodium dihydrogen phosphate monohydrate, di-sodium hydrogen phosphate dihydrate and ammonium sulphate of analytical grade were obtained from Merck, Darmstadt, Germany. Methanol of HPLC gradient grade was also obtained from Merck. Purified water obtained through a Milli-Q water purification system (resistivity > 18 MΩ cm) from Millipore, Bedford, USA, was used to prepare all solutions. HPLC mobile phase buffers were filtered and degassed under vacuum, through 0.2 μm NL 16 membranes from Schleicher and Schuell, Dassel, Germany.

2.2. Standard solutions preparation

Stock solutions of 8-oxodGuo and UA were prepared by dissolving the powder from Sigma in purified water to final concentration 100 μM. A stock solution of 8-oxoGua was prepared by dissolving the powder from Sigma in ultrapure water to a final concentration of 35 μM. To assure total solubility of 8-oxoGua, 20 μl of 8 M NaOH solution were added to give final pH 9–10, verified with pH-indicator paper. Stocks from each compound were further diluted to 1 μM in pH 6.1 50 mM phosphate buffer and stored at 4 °C for further use. From 1 μM stock solutions, equimolar standard mixtures of 8-oxoGua, 8-oxodGuo and UA were prepared in pH 6.1 50 mM phosphate buffer and used as working standards in the range 2.5–250 nM for electrochemical detection and in the range 100 nM to 10 μM for UV-spectrophotometric detection calibration curves.

2.3. SPE procedure for 8-oxoGua and 8-oxodGuo extraction in the presence of excess UA

2.3.1. Without previous uricase addition for UA degradation

Standard solutions of 0.6 mM UA prepared in pH 6.1 50 mM phosphate buffer were spiked with appropriate volumes of a 1 μM equimolar standard mixture of 8-oxoGua and 8-oxodGuo to give four mixtures with final concentrations of 25, 50, 100 and 250 nM of each analyte. Of each mixture, 3 ml were applied on SPE C18 octadecyl phase cartridges (Chromabond C18 500 mg/3 ml from Macherey-Nagel, Germany) previously conditioned with 3 ml × 3 ml of MeOH, followed by 3 ml of H_2O and 3 ml × 3 ml of pH 6.1 50 mM phosphate buffer at a flow rate of ∼2 ml min^{-1}. The filtrate solutions were then collected for further analysis, the cartridges were washed with 3 ml of pH 6.1 50 mM phosphate buffer and the wash solutions were also collected. Elution from the cartridges was performed with 3 ml of pH 6.1 50 mM phosphate buffer containing 20% MeOH at a flow rate ∼1 ml min^{-1}. Aliquots of 10 μl of the eluate solutions were directly injected into the HPLC system. Another type C18 octadecyl phase SPE cartridges (Chromabond C18ec, where ec means endcapped silanol groups, also from Macherey-Nagel, Germany) was tested for pre-treatment of urine samples. Unlike in the C18 SPE cartridges, in the C18ec cartridges the residual –SiOH groups of immobilised octadecyl chains were reacted with smaller alkylsilane reagents and so lost their capacity to interact with polar groups of some analytes. This difference in capping chemistry leads to the differences in selectivity observed between C18 and C18ec SPE extraction cartridges.

2.3.2. With previous uricase addition for UA degradation

Five milliliters of the standard mixture of 0.6 mM UA were prepared in 0.1 M phosphate buffer (pH 8.2) and spiked with 8-oxoGua and 8-oxodGuo to 100 nM final concentration and 10 μl of a 20 mg ml^{-1} uricase solution were added. The solution was kept at room temperature (∼25 °C) in a stream of pure oxygen for 5 min to reach full enzymatic degradation of uric acid, which was confirmed by spectrophotometric enzymatic assay at 292 nm. After the enzymatic digestion of UA, 3 ml of the mixture were subjected to the same SPE procedure as described in Section 2.3.1.

2.4. Urine collection and clean-up procedure for 8-oxoGua and 8-oxodGuo analysis

The method developed was tested in urine samples from children (age between 3 and 8 years old) with metabolic disorders that may lead to an oxidative stress condition and urines from 65 to 75-year-old persons suffering from cognitive deficit. Urine was collected, divided into 10 ml fractions and stored at −20 °C. For analysis, urine samples were thawed at room temperature and submitted to vortex homogenisation. Salting-out of proteins was performed by adding ∼2 g of ammonium sulphate per 10 ml urine followed by centrifugation at 2200 × *g* for 10 min. The supernatant was removed and the pH of the urine was adjusted with sodium hydroxide to a final value of 7–8, which was checked with pH indicator-paper. From a 20 mg ml^{-1} uricase solution prepared in pH 8.2 0.1 M phosphate buffer, 1.5 μl were added to 1 ml of urine sample and the mixture

kept at room temperature (~25 °C) under a stream of pure oxygen for 5 min. After uric acid degradation, urines were again centrifuged at 2200 × *g* for 5 min. From the supernatant, a fraction of 3 ml was submitted to the SPE procedure described in Section 2.3.1.

2.5. Instrumentation

A Waters 2690 Alliance system with a Photodiode Array Detector 996 (PDA 996) in series with a CONCORDE Electrochemical Detector from Waters Corporation, Milford, USA, was used. The HPLC separation was performed in a reverse phase LC-18-S Supelcosil analytical column with 15 cm × 4.6 mm ID, 5 μm bonded spherical silica, 100 Å pore size, in series with a LC-18-S Supelguard 2 cm × 4 mm ID guard column from Supelco, Bellefonte USA. All chromatograms presented were acquired by a Millenium 32 Chromatography Manager from Waters Corporation. Origin version 6.0 from Microcal Software was used for the presentation of all other plots reported in this work.

2.5.1. Chromatographic conditions

Unless otherwise stated, phosphate buffer 50 mM, pH 6.1 with 6% MeOH and 2 mM KCl, of final pH 6.2 was used as mobile phase. Elution was in isocratic mode at 1 ml min^{-1} flow rate. The guard column and analytical column were kept at $T = 30\,^{\circ}C$ together with the electrochemical flow cell in a Faraday cage with a thermostatic oven.

2.5.2. Electrochemical conditions

The electrochemical cell was a VT-03 flow cell from Antec Leyden, Zoeterwoude, Netherlands, of a confined wall-jet design, in a three-electrode configuration: a glassy carbon working electrode with 2 mm diameter, an in-situ Ag/AgCl reference electrode and a stainless steel auxiliary electrode. The in-situ Ag/AgCl reference electrode, referred to here as ISAAC (in-situ Ag/AgCl) is in direct contact with the mobile phase that contains 2 mM chloride ions (2 mM KCl). There is a difference of +0.19 V between the potential of the Ag/AgCl reference electrode saturated with 3 M KCl and the ISAAC reference electrode in contact with 2 mM KCl. Thus, for an application running at $E = +0.7$ V versus Ag/AgCl saturated with 3 M KCl, the potential setting using ISAAC should be $E = +0.51$ V. All potentials are referred to the ISAAC reference electrode and unless otherwise stated, the cell potential was set at +0.5 V versus ISAAC (+0.69 V versus Ag/AgCl reference electrode).

3. Results and discussion

3.1. Comparison of spectrophotometric and electrochemical detection

Low concentrations up to 2.5 nM of 8-oxoGua and 8-oxodGuo in the presence of UA are selectively and sensitively detected by amperometry but the spectrophotometric/UV signal is badly distinguished from the noise for such low concentrations. Electrochemical signals obtained from injection of a 5 nM standard equimolar mixture of UA, 8-oxoGua and 8-oxodGuocould also be well distinguished from noise whereas, for the same concentration, UV signals for the mixture cannot be detected at all. UV signals with a similar signal to noise ratio were obtained only for mixtures with concentrations above 500 nM, i.e. a 100-fold higher concentration.

Standard mixtures in the concentration range 2.5–250 nM were injected in order to obtain calibration curves for electrochemical signals. The limit of detection (LOD) based on 3.3 × S.D./slope of calibration curve for 8-oxoGua (I_p (nA) = 0.00492 + 0.01178 × 8-oxoGua (nM); $R^2 = 1$; S.D. = 0.00215, $N = 7$, $P < 0.0001$) was 0.6 nM while for 8-oxodGuo (I_p (nA) = 0.00125 + 0.0031 × 8-oxodGuo (nM); $R^2 = 0.99999$; S.D. = 8.50236×10^{-4}, $N = 7$, $P < 0.0001$) was 0.9 nM. For both analytes in standard mixtures, LOD was lower than 1 nM (10 fmol for each analyte injected). Calibration curves for spectrophotometric UV signals at $\lambda = 249$ nm were obtained from injection of standard mixtures in the concentration range 100 nM to 10 μM. The LOD of 179 nM for 8-oxoGua and 193 nM for 8-oxodGuo confirm that electrochemical detection is more sensitive than UV detection by a factor of 300 for 8-oxoGua and 200 for 8-oxodGuo. The HPLC-ECD method presented permits 8-oxoGua and 8-oxodGuo detection in the presence of uric acid, a strong interferent in the electrochemical detection of these substances due to its similar oxidation potential, in less than 12 min.

3.2. Hydrodynamic voltammograms

Hydrodynamic voltammograms for 8-oxoGua, 8-oxodGuo and UA, obtained by running a mixture of the three analytes at a working potential in the range $E = +0.2$ to $E = +0.6$ V versus ISAAC are presented in Fig. 1. The optimum working potential, obtained from the hydrodynamic voltammograms for the value corresponding to maximum current was +0.5 V for all three analytes, and is indicated by an arrow in Fig. 1A. Above this potential and for the flow rate used, the current becomes diffusion limited. Fig. 1A shows clearly that the diffusion-limited current is four times higher for 8-oxoGua than for 8-oxodGuo but the total charge transferred, Fig. 1B, in the oxidation of 8-oxoGua is just two times higher than that of 8-oxodGuo. The difference can be due to the presence of a sugar in the 8-oxodGuo molecule that increases the difficulty to reach the electrode surface. According to these results, the working potential for amperometric detection of 8-oxoGua and 8-oxodGuo was set at +0.5 V versus ISAAC. The half wave potential, $E_{1/2} = +0.3$ V (+0.49 V versus Ag/AgCl reference electrode) obtained in this study is in good agreement with previous work on 8-oxoGua electrochemical oxidation at glassy carbon electrodes by differential pulse voltammetry [52].

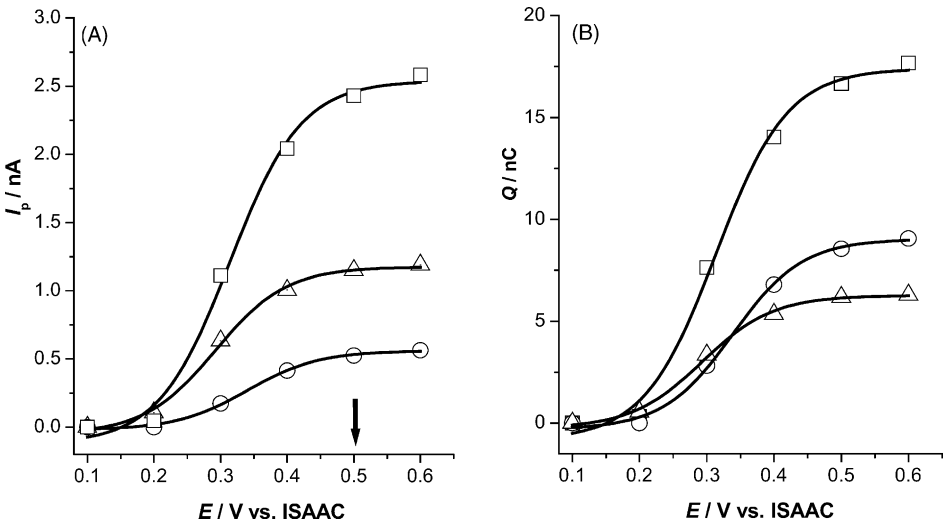

Fig. 1. Hydrodynamic voltammograms of 8-oxoGua (□), 8-oxodGuo (○) and UA (△) obtained by running 10 μl of a 250 nM equimolar mixture of UA, 8-oxoGua, 8-oxodGuo at different working electrode potentials: (A) peak intensity vs. working potential; (B) peak charge vs. working potential. The arrow indicates the optimum working potential. ISAAC (in-situ Ag/AgCl) is −0.19 V vs. Ag/AgCl reference electrode.

3.3. Selection of mobile phase pH to improve separation factor

The effect of the mobile phase pH on the relative retention of UA, 8-oxoGua and 8-oxodGuo was studied by determination of their retention factors as a function of pH in the pH range 4.5–7 as shown in Fig. 2. UA has a behaviour that is typical of an acid with the pH at the inflection point of the sigmoidal curve corresponding to its pK_a in aqueous solution. From this experimental curve, UA pK_a is about 5.5 which is in good agreement with the UA published value of pK_a equal to 5.6 [54]. Above this pH, UA exists predominantly as the urate ion, much more soluble in water and so, its retention on column decreases, as confirmed by the curve in Fig. 2. The relative dependence of 8-oxoGua and 8-oxodGuo retention factor on pH is very similar with no significant variation with pH. Experimental values for 8-oxoGua pK_a are scarce, in part due to its insolubility in water. Nevertheless, from predicted pK_a values of various neutral and charged tautomers of 8-oxoGua in aqueous phase (pK_{a1} = 0.22 (N3), pK_{a2} = 8.69 (N1), pK_{a3} = 11.93 (N9) [13]) it can be concluded that 8-oxoGua as well 8-oxodGuo is neutral below pH 8. This explains well their retention dependence with mobile phase pH, plotted in Fig. 2.

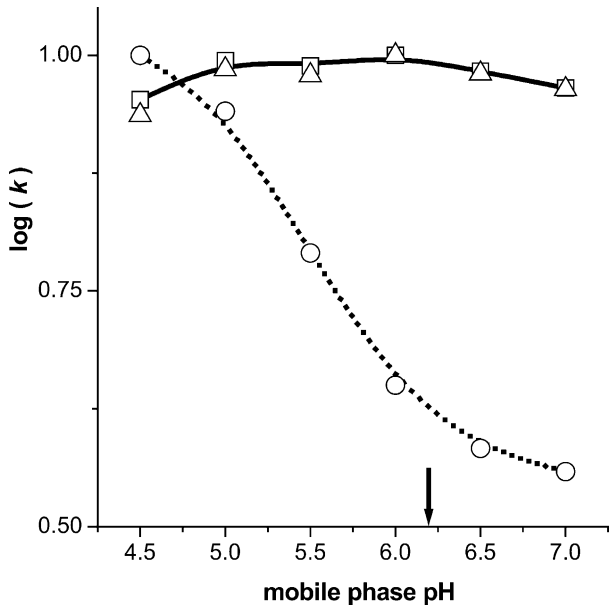

Fig. 2. Retention factor (k) dependence on mobile phase pH for 8-oxoGua (□), 8-oxodGuo (△) and UA (○), obtained from 10 μl injection of a 250 nM equimolar mixture of UA, 8-oxoGua and 8-oxodGuo. Retention factor calculated as: $k = (t_R - t_0)/t_0$, where t_R is the analyte retention time and t_0 is the elution time of an unretained analyte (t_0 = 1.96 min obtained from uracil injection with MeOH/H_2O 66/34 as mobile phase). The arrow indicates the pH chosen for the mobile phase.

From previous studies [52] it is also known that the 8-oxoGua peak current increases with electrolyte pH until pH 10 and thus, a buffer with a pH as high as possible would be ideal for 8-oxoGua detection. Since the operation limit of the analytical column is pH 7.5 and 7.0 did not lead to much signal improvement in the separation factor, pH 6.2 phosphate buffer was chosen as mobile phase.

3.4. Electrochemical detection of 8-oxoGua and 8-oxodGuo in presence of excess UA

As stressed before, in human urine, the concentration of UA is 10^4-fold higher than basal levels of 8-oxoGua and 8-oxodGuo which makes their detection, namely 8-oxoGua, very difficult due to the proximity of UA and 8-oxoGua retention times. With a view to developing a methodology for overcoming this problem in urine samples, solid phase extraction of standard solutions of 8-oxoGua and 8-oxodGuo in the presence of excess UA was carried out to see whether

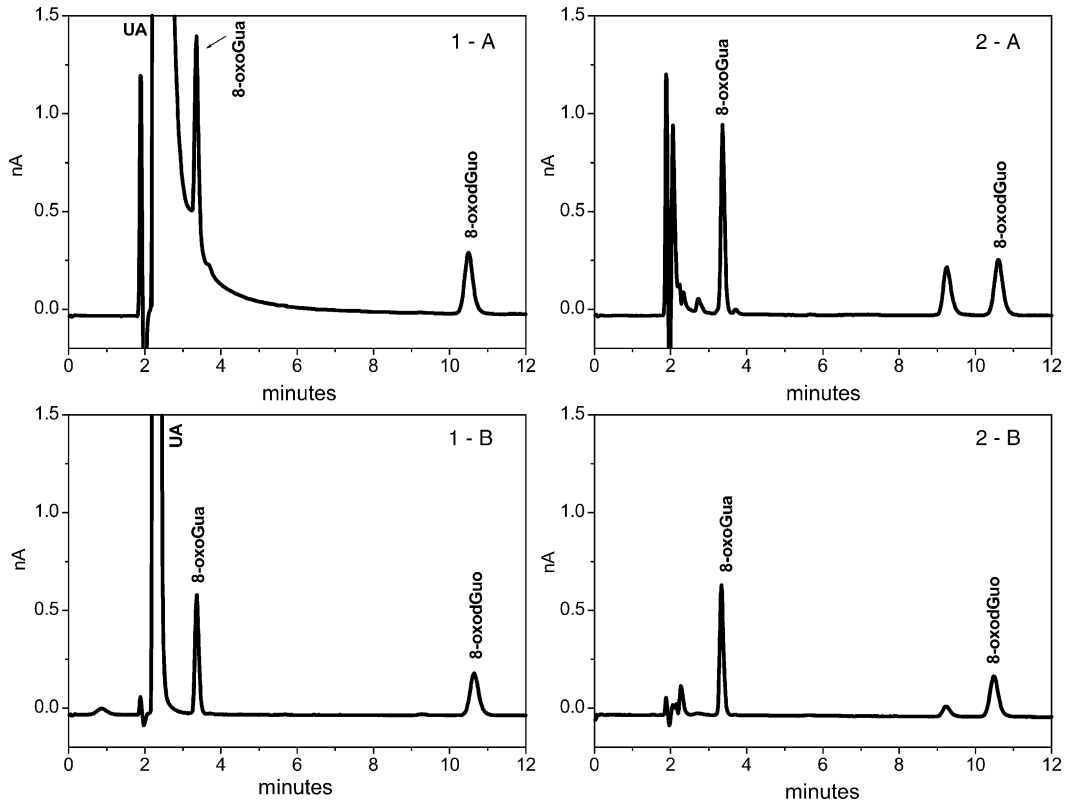

Fig. 3. Chromatograms obtained from 10 μl injection of a standard mixture of UA 0.6 mM spiked with a mixture of 8-oxoGua and 8-oxodGuo to final concentration 100 nM of both: (1) without addition of uricase solution to the mixture; (2) with addition of uricase solution to the mixture for UA digestion; (A) without SPE of the mixture; (B) after SPE treatment of the mixture. Mobile phase: 50 mM phosphate buffer + 6% MeOH + 2 mM KCl, final pH 6.2.

UA could be eliminated by SPE without significant loss of 8-oxoGua and 8-oxodGuo.

In Fig. 3 are presented chromatograms obtained from a standard solution of 0.6 mM UA spiked with a mixture of 8-oxoGua and 8-oxodGuo to give final concentration 100 nM of both. Fig. 3(1-A) presents the chromatogram obtained for the mixture before SPE treatment and Fig. 3(1-B), the same mixture after SPE treatment by the procedure described in Section 2.3.1. It was found (UV data not shown), that 97% of UA is removed by this SPE procedure. The HPLC determination of UA in the filtrate and wash solutions (see Section 2.3.1), showed that UA was partially eliminated in the filtrate (∼33%) and wash (∼64%) solutions (UV data not shown). This indicated that UA is weakly retained (∼3%) in this hydrophobic sorbent, as expected, since at pH 6 UA exists mainly as urate anion that is more soluble in the buffer aqueous phase. The amount of UA (∼3%) that was not removed by SPE still gave a strong electrochemical signal but the resolution between 8-oxoGua and UA peaks, Fig. 3(1-B), is much better than before SPE, Fig. 3(1-A), whereas 8-oxoGua appears as a shoulder following the UA peak. The addition of uricase solution to the mixture before SPE treatment allows the complete removal of UA without degradation of 8-oxoGua and 8-oxodGuo, Fig. 3(2-A). Control experiments were performed with 8-oxoGua and 8-oxodGuo in the presence and absence of uricase solution and no significant difference was found in the electrochemical signals. A peak related to products from UA digestion was detected at a retention time of ∼9.2 min, Fig. 3(2-A), and is almost eliminated after SPE, Fig. 3(2-B). It was concluded that digestion of UA by uricase is very efficient and even causes a slight increase in the 8-oxoGua peak current, Fig. 3(2-B), compared with that without uricase, Fig. 3(1-B)).

For SPE procedure without uricase digestion, quantification of 8-oxoGua and 8-oxodGuo in eluate solutions compared to original peaks before SPE gave minimum recovery rates around 65% for 8-oxoGua and 8-oxodG. Anyway, this calculation gives only apparent recovery rates since 8-oxoGua peak before SPE could not be quantified correctly due to UA interference. After uricase digestion of UA, it was possible to quantify both peaks before and after SPE as presented in Table 1. Recoveries for each mixture gave median recovery rates of 73 ± 3% for 8-oxoGua and 71 ± 3% for 8-oxodGuo (four determinations).

In the wash solutions, an 8-oxoGua peak was also detected which corresponded to a loss of about 10% but no peak was detected for 8-oxodGuo. In the filtrate solutions neither of these analytes was detected. Since no significant amounts of 8-oxoGua and 8-oxodGuo were found in the filtrate and wash solutions it was concluded that the recovery of 8-oxoGua and 8-oxodGuo retained in the adsorbent is not complete,

Table 1
SPE recoveries for 8-oxoGua and 8-oxodGuo in standard mixtures[a]

Mixture 8-oxoGua and 8-oxodGuo (nM)	I_p (8-oxoGua) (nA)			I_p (8-oxodGuo) (nA)		
	Before SPE	After SPE	Recovery (%)	Before SPE	After SPE	Recovery (%)
25	0.24	0.18	75	0.07	0.05	71
50	0.46	0.33	72	0.15	0.1	67
100	0.96	0.66	69	0.28	0.2	71
250	2.4	1.8	75	0.68	0.5	74
			73 ± 3%			71 ± 3%

[a] After excess UA digestion by uricase.

probably due to the low percentage of methanol used in the eluent (pH 6.1 50 mM phosphate buffer + 20% MeOH). Increasing the amount of methanol in the eluent would cause complete elution of 8-oxoGua and 8-oxodGuo but would also cause the elution of other urine interferents. Thus, this SPE procedure is a compromise between recovery of the analytes of interest and retention of undesired interferents. This result should be taken into account when the final goal is the development of a selective and lower detection limit method for 8-oxoGua and 8-oxodGuo in urine with reduced costs in sample pre-treatment.

3.5. Electrochemical detection of 8-oxoGua and 8-oxodGuo in urine samples

The method developed was tested in urine samples from children (3–8 years old) with metabolic disorders, in order to verify its ability to detect 8-oxoGua and 8-oxodGuo.

In Fig. 4A is presented the chromatogram obtained from one of the urine samples directly injected after SPE treatment (full line) and the chromatogram obtained from a 100 nM standard mixture of UA, 8-oxoGua and 8oxodG (dotted line). Comparing the urine chromatogram with the standard mixture it can be concluded that UA was completely removed from urine by uricase since otherwise, a peak from UA in urine would appear at a retention time of 2.3 min. A peak at a retention time of 3.4 min appears in urine sample attributed to the presence of 8-oxoGua. In order to confirm that this peak is due to 8-oxoGua, urine was spiked with a 250 nM 8-oxoGua standard. Fig. 4B shows that the peak at retention time 3.4 min increases (dotted line). Also, this urine was analysed at two different potentials, +0.5 and +0.35 V versus ISAAC and the peak current ratio obtained was 1.6 ± 0.5 (two injections performed for each potential). Peak current ratio from standard 8-oxoGua injection for these two potentials was 1.5, as can be seen from hydrodynamic voltammogram of 8-oxoGua in Fig. 1. These similar values found for peak current ratio at different potentials in the standard solution and in the urine sample give further evidence that the urine peak at 3.4 min can be attributed to 8-oxoGua. The concentration of 8-oxoGua was found to be ~360 nM, calculated from a calibration curve obtained by injection of 8-oxoGua standards on the same day. However, the purity of the peak was not confirmed since no spectroscopic information can be obtained due primarily to the very low concentration of 8-oxoGua, but also because UV spectra shows an intense peak of hypoxanthine at the same retention time as 8-oxoGua, 3.4 min. In order to ensure that hypoxanthine would not constitute an artefact in the electrochemical detection of 8-oxoGua, control experiments were performed and it was confirmed that at the applied working potential, hypoxanthine does not give any electrochemical signal [55] and does not interfere at all in 8-oxoGua signal.

No 8-oxodGuo was detected in this urine sample as no peak at the retention time of 8-oxodGuo (t_R = 10.9 min) was found. Nevertheless, when the urine was spiked with a 250 nM 8-oxodGuo standard, a new clearly-defined peak

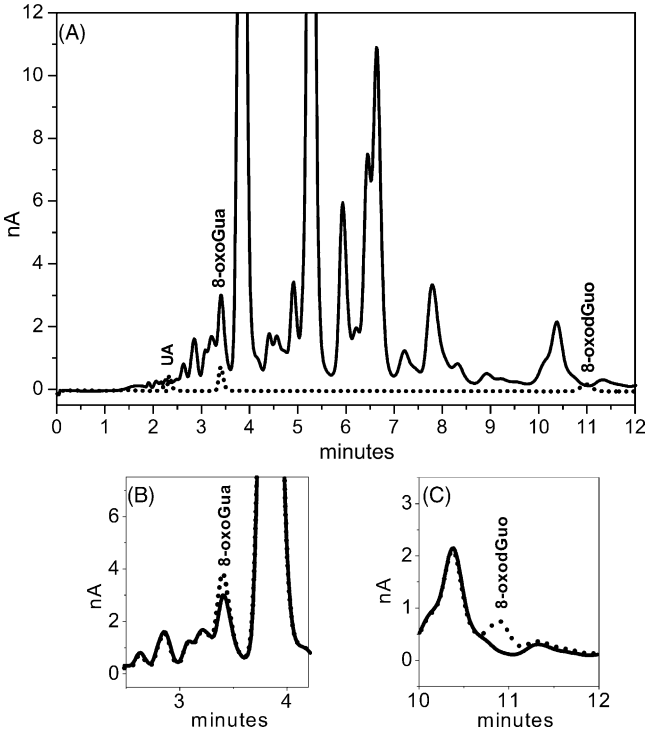

Fig. 4. (A) Chromatograms obtained from (...) 10 μl injection of a 100 nM standard mixture of UA, 8-oxoGua and 8-oxodGuo and (—) 10 μl injection of an urine sample from a child with metabolic disorders after being processed by SPE. Same urine sample (—) before and (...) after being spiked with 250 nM standard solutions of (B) 8-oxoGua and (C) 8-oxodGuo. Mobile phase: 50 mM phosphate buffer + 6% MeOH + 2 mM KCl, final pH 6.2.

appeared at a retention time 10.9 min, Fig. 4C (dotted line) which is evidence that in original urine no 8-oxodGuo was present in a detectable amount, otherwise a peak would appear as in the spiked urine. Undetectable levels of 8-oxodGuo in urine, whilst 8-oxoGua was detected, can be explained taking into account recent studies, applying methodologies able to detect simultaneously 8-oxoGua and 8-oxodGuo in urine, where 8-oxoGua levels were found to be always higher than 8-oxodGuo [22,23,44,49]. This is also in good agreement with studies concerning DNA damage repair mechanisms that show the primary DNA repair pathway excises the base and not the nucleoside [38–40]. On the other hand, looking at the intensity of the peak for 8-oxodGuo in the 100 nM standard mixture, Fig. 4A (dotted line), it can be seen that if 8-oxodGuo were present in this urine at a level below 100 nM (which is quite possible) it would not be detectable since the standard peak is below the baseline. In fact, due to the great complexity of urine samples, the background current increases and the limits of detection in such complicated matrix also increase about 100 times. From calibration curves obtained by spiking urine with 8-oxoGua standards, a LOD of 80 nM for 8-oxoGua (I_p (nA) = 0.010 × 8-oxoGua nM^{-1}; $R^2 = 0.9994$, S.D. = 0.237, $N = 4$) was found.

These results in urine demonstrated that there is no effect of the urine matrix on the retention times of 8-oxoGua and 8-oxodGuo because they are the same in standard mixture and in spiked urine.

In order to show better, the complexity and also inter-individual variability of urine samples, results from a 65-year-old person suffering from Cognitive Deficit, are presented in Fig. 5. This urine was processed by SPE in same way as described in Section 2.3.1 applying two types of SPE cartridges: a C18 octadecyl phase adsorbent (Fig. 5A) and a C18ec octadecyl phase adsorbent, were ec means endcapped silanol groups (Fig. 5B). As can be seen, a slight difference related with the capping chemistry of the SPE cartridges used to process urine leads to a strong difference in the cleaning of the matrix. In fact, even with the same polarity range as C18 cartridges, these C18ec cartridges do not interact with the polar groups of some analytes, which explains the lesser number of compounds retained. With end-capped C18ec cartridges it is possible to eliminate more components from urine which allows a chromatogram with a lower background current (Fig. 5B) when compared to the one obtained for urine processed with C18 adsorbent (Fig. 5A). A peak at same retention time as 8-oxoGua, ∼3.4 min, was detected in urine treated with both SPE cartridges, but in urine cleaned by C18 adsorbent, the complexity is bigger and 8-oxoG peak is not well resolved from neighbour ones. On contrary, in urine treated with C18ec adsorbent, the peak for 8-oxoGua is much better resolved. The identity of the peak was confirmed by a spiking of a 25 nM standard 8-oxoGua (Fig. 5B, dotted line) and quantification by the standards addition method gave a concentration of 7 nM for 8-oxoGua in this urine. However, it should be remembered that C18ec adsorbents remove more components, including more 8-oxoGua, which constitutes a drawback to their use, in comparison with C18 octadecyl phase adsorbent cartridges, if the final goal is the quantification of the analyte of interest.

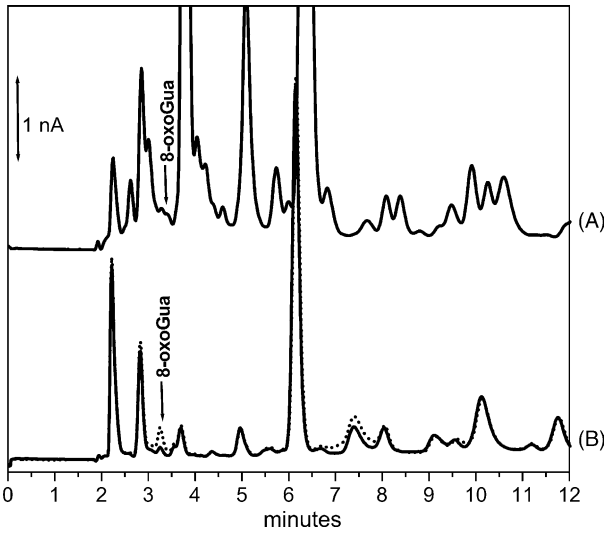

Fig. 5. Chromatograms obtained from 10 μl injection of an urine sample from a 65-year-old person with cognitive deficit after being processed with two different SPE octadecyl phase cartridges: (A) C18 cartridges; (B) C18ec (ec, encapped) cartridges, (▬) before and (...) after being spiked with a 25 nM standard solution of 8-oxoGua. Mobile phase: 50 mM phosphate buffer + 6% MeOH + 2 mM KCl, final pH 6.2.

4. Conclusion

Simultaneous detection of urinary levels of 8-oxoGua and 8-oxodGuo, besides reducing analysis time and being less demanding on resources, may give further insight into the relative significance of the use of 8-oxoGua and 8-oxodGuo as biomarkers of DNA oxidative damage and of oxidative stress. It is widely accepted that 8-oxoGua and 8-oxodGuo are major products of DNA oxidative damage and can be enzymatically repaired being excreted into urine at steady-state levels below 100 nM. There is still a great need for good methodologies for their simultaneous detection in urine that reflects the big difficulty of 8-oxoGua detection in urine samples due to the interference of UA, a strong interferent excreted in urine in 10^4-fold higher concentrations. The present work describes a methodology based on HPLC-ECD for detection of 8-oxoGua in the presence of UA with a good separation factor between 8-oxoGua and UA ($\alpha = 3.7$), enabling a LOD for both analytes below 1 nM in standard mixtures.

The method was applied to urine samples after uricase digestion of excess uric acid and one-step solid phase extraction. It was possible to detect and quantify the presence of 8-oxoGua in urine and to confirm that UA was eliminated after uricase degradation and SPE. The LOD found in urine samples for 8-oxoGua was 80 nM, a value higher than in

standard mixtures, which is due to the increase in the background current. Due to the complexity and inter-individual variability of urine samples, the main application of the present methodology when applied to urine is the simultaneous detection of 8-oxoGua and 8-oxodGuo, reducing as much as possible the sample pre-treatment steps.

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