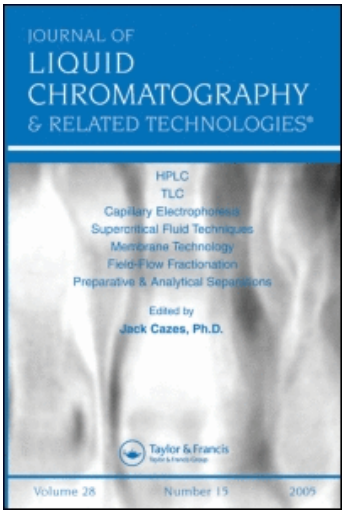

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High Performance Liquid Chromatography-Electrochemical Assay for Monitoring the Formation of 8-Oxo-7,8-dihydroadenine and its Related 2'-Deoxyribonucleoside

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTROCHEMICAL ASSAY FOR MONITORING THE FORMATION OF 8-OXO-7,8-DIHYDROADENINE AND ITS RELATED 2'-DEOXYRIBONUCLEOSIDE

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ABSTRACT

A sensitive method involving the combined use of reversed-phase high-performance liquid chromatography and electrochemical detection has been developed for monitoring the formation of 8-oxo-7,8-dihydro-adenine and 8-oxo-7,8-dihydro-2'-deoxyadenosine. The limit of detection for both compounds is close to 0.2 ng under optimised conditions of detection at an oxidation potential of 850 mV. This assay allows the quantitative measurement of the hydroxylated purine components in aqueous solutions of adenine and 2'-deoxyadenosine exposed to a dose of ionizing radiation as low as 2 Gray (Gy).

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INTRODUCTION

The recent availability of a sensitive high performance liquid chromatographic - electrochemical detection assay for the measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine [1] has given an impetus to investigations on molecular aspects of free-radical processes involved in mutagenesis and carcinogenesis. The formation of this oxidized 2'-deoxyguanosine derivative, as detected by amperometry, has been used for probing cellular radical oxidation processes under various conditions including effects of ionizing radiation [2] and various (pro)oxidizing agents. It is noteworthy that 8-oxo-7,8-dihydro-2'-deoxyguanosine was shown to be generated within DNA of cells exposed to hydrogen peroxide [3-5], tumor promotor tetradecanoylphorbol acetate [6] as well as carcinogens including 4-nitroquinoline-1-oxide [7] and potassium bromate [8]. A similar analytical technique was recently used to monitor the formation of the 8-hydroxylated derivative of 2'-deoxyguanosine within the nucleic acids of rat liver upon treatment with the hepatocarcinogen 2-nitropropane [9] and the peroxisome proliferator ciprofibrate [10]. The high performance liquid chromatography - electrochemical method was also used as a sensitive analytical tool for measuring the release of 8-oxo-7,8-dihydro-2'-deoxyguanosine in human urine as a possible biodosimeter assay [11].

Evidence was recently provided that other hydroxyl radical-mediated nucleobase lesions including 8-hydroxyadenine, 4,6-diamino-5-N-formamidopyrimidine, 5-hydroxyuracil and 5-hydroxymethylcytosine may be also detected by amperometry following HPLC separation [5]. We would like to report in the present paper optimized conditions of both reversed-phase HPLC separation and electrochemical detection of 8-oxo-7,8-dihydroadenine and 8-oxo-7,8-dihydroguanine as well as of their corresponding 2'-deoxyribonucleosides. This method appears to be particularly suitable for detecting the formation of hydroxyl radicals generated in a very low yield in chemical and biochemical systems via the the measurement of the 8-oxo-7,8-dihydroderivative of adenine and 2'-deoxyadenosine.

EXPERIMENTAL

Chemicals

Adenine, guanine (Sigma, St. Louis, MO), 8-oxo-7,8-dihydroguanine (Chemical Dynamics, South Plainfield, NJ) and 2'-deoxyadenosine (Fluka, Buchs, Swizerland) were used without purification. 2'-deoxyguanosine (Genofit, Geneva, Swizerland) was purified to contaminating 8-oxo-7,8-dihydro-2'-deoxyguanosine [12] by reversed-phase HPLC purification on an analytical octadecylsilyl silicagel column Nucleosil using water-methanol (9 : 1) as the eluting system.

8-oxo-7,8-dihydro-2'-deoxyguanosine was prepared by catalytic hydrogenation of 8-(benzyloxy)-2'-deoxyguanosine [13]. A similar procedure involving the chemical synthesis of 8-bromo-2'-deoxyadenosine and 8-(benzyloxy)-2'-deoxyadenosine was used for the preparation of 8-oxo-7,8-dihydro-2'-deoxyadenosine. 8-oxo-7,8-dihydroadenine was quantitatively obtained by mild hydrolsis of the corresponding 2'-deoxyribonucleoside with hydrogen fluoride stabilized in pyridine at 20° for 10 minutes [14].

HPLC-EC Method

The HPLC system consists of a LKB pump Model 2150 (Pharmacia LKB Biotechnology, Uppsala, Sweden) equipped with a Rheodyne Model 7100 loop injector (Berkeley, CA), a LKB Model 2156 solvent conditioner and a LKB Model 2151 variable wavelength monitor (Pharmacia LKB Biotechnology, Uppsala, Sweden). The electrochemical detection was accomplished by amperometry using a Model LC-4B / LC-17A(T) apparatus (Bioanalytical Systems, West Lafayette, IN) set at 0.5 nA. The amperometric detection used two glassy-carbon electrodes in parallel at potentials of + 650 mV and + 850 mV for 8-oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydroadenine components respectively. The octadecylsilylsilica gel column (250 x 4.6 mm I.D.) utilized in the experiments reported here was homepacked

with 10 mm particles of porous silica gel coated with a chemically bonded monolayer of octadecylsilane (Nucleosil, DÜren, FRG). Packing was accomplished by the "non-balanced" slurry technique using 1-butanol as dispersant with a Haskell pump operating (Chromatem, Paris, France) at a delivery pressure of 6000 p.s.i. The three mobile phases which consisted of various mixtures of 50 mM sodium citrate (pH 5.0) and methanol (Table 1) were degassed by filtration through a 0.45 μm HA membrane (Millipore Co, Milford, MA).

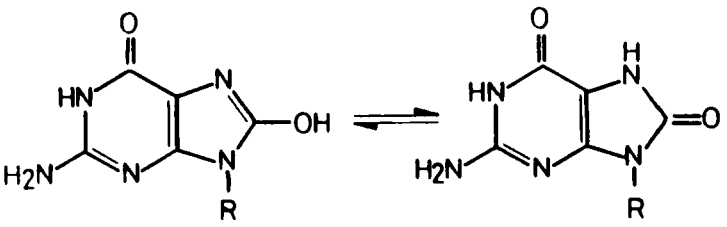
Gamma Radiolysis Experiments

The gamma radiolysis experiments were performed by using three ^{60}Co sources located in a pool. The dose rate which was determined according to Fricke's method, was 80 Gy/min. The flasks which were used for the gamma irradiation experiments were previously filled with deionized water and exposed to high doses of gamma rays (>10^5 Gy) in order to destroy any organic impurity on the surface of the glass.

RESULTS AND DISCUSSION

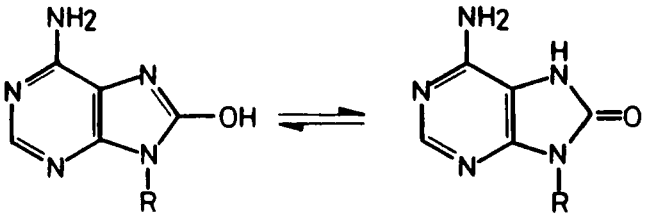
Reversed-Phase HPLC Separation of the 8-Hydroxylated Adenine and Guanine Nucleobases and Nucleosides.

The four 8-hydroxylated DNA nucleobases and nucleosides 1-4, which may be representated as either the enolic tautomer or the more likely ĸetonic derivative in aqueous solution (Fig 1), were completely resolved on an octadecylsilylsilica gel Nucleosil column. This was achieved as illustrated in Fig. 2 in less than 25 minutes by using a binary solvent system which consists of 50 mM sodium citrate buffer (pH 5.0) and methanol [87.5 : 12.5] . The modified bases and nucleosides were found to elute in the



1, R=H,

2, R=2-deoxy-β-D-erythro-pentosyl



3, R=H,

4, R=2-deoxy-β-D-erythro-pentosyl

Figure 1 Chemical structures of the tautomeric forms of the 8-hydroxylated derivatives of adenine and guanine nucleobases and 2'-deoxyribonucleosides (1-4).

following order of mobility : 8-oxo-7,8-dihydroguanine (1) > 8-oxo-7,8-dihydroadenine (3) > 8-oxo-7,8-dihydro-2'-deoxyguanosine (2) > 8-oxo-7,8-dihydro-2'-deoxyadenosine (4) (Table 1). The HPLC behaviour of these hydroxylated purine derivatives is in agreement with the fact that adenine components are usually more retained that the related guanine compounds on reversed-phase HPLC columns [16,17]. In addition the nucleobase is eluted faster than the corresponding 2'-deoxyribonucleoside. This may be rationalyzed in terms of additional hydrophobic interactions between the hydrocarbonaceous moiety of the 2-D-erythropentofuranosyl ring of the nucleoside and the non-polar surface of the octadecylsilylsilica gel ligand [18].

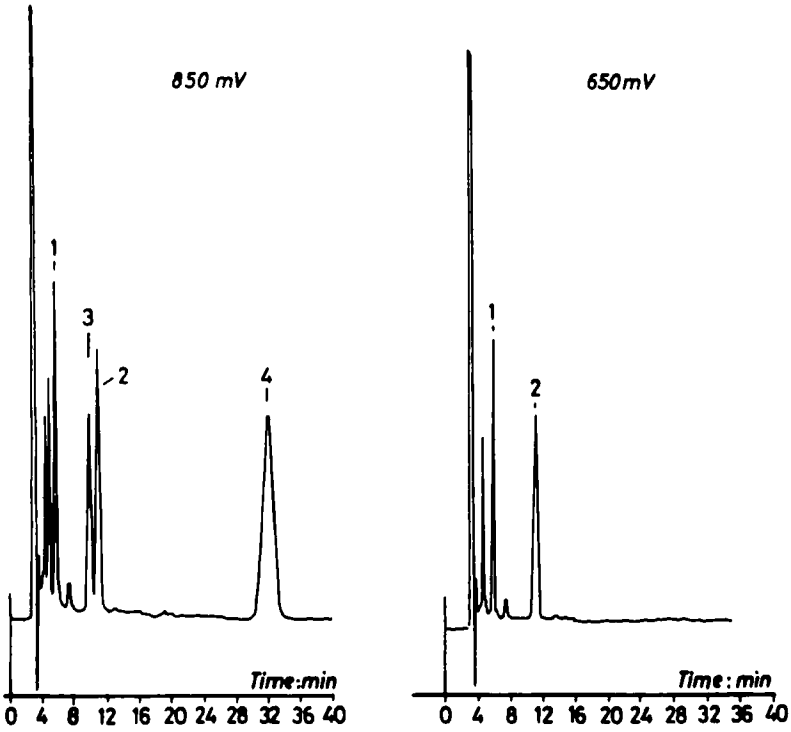


Figure 2 HPLC-EC profile of 8-hydroxylated derivatives of adenine and guanine components column : 250 x 46 mm I.D. C_{18}Nucleosil octadecylsilylsilica gel - Eluent : 50 mM sodium nitrate (pH 5.0) - methanol [87.5:12.5]. Flow-rate : 1 mL/min. EC detection : oxidation potentials (+850 mV (left) and +650 mV (right). Compounds : 8-oxo-7,8-dihydroguanine (1), 8-oxo-7,8-dihydro-2'-deoxyguanosine (2), 8-oxo-7,8-dihydroadenine (3), 8-oxo-7,8-dihydro-2'-deoxyadenosine (4).

TABLE 1

Capacity Factors (k') of the Four 8-Hydroxylated Adenine and guanine derivatives on an Analytical ODS Nucleosil C_{18} Column using Various Mixtures of 50 mM Sodium Citrate (pH 5.0) and Methanol[a,b,c]

Compounds	Capacity factor (k') a	b	c
8-oxo-7,8-dihydroguanine (1)	d	0.6	1.6
8-oxo-7,8-dihydro-2'-deoxyguanosine (2)	d	2.1	5.9
8-oxo-7,8-dihydroadenine (3)	d	1.8	3.8
8-oxo-7,8-dihydro-2'-deoxyadenosine (4)	4.3	7.9	d

[a] 50 mM sodium citrate (pH 5.0)-methanol [80 : 20]

[b] 50 mM sodium citrate (pH 5.0)-methanol [87.5 : 12.5]

[c] 50 mM sodium citrate (pH 5.0)-methanol [90 : 10]

[d] not determined

Amperometric Detection of the 8-Hydroxylated Purine Nucleobases and Nucleosides.

It was previously shown that 8-oxo-7,8-dihydro-2'-deoxyguanosine may be electrochemically detected at an applied potential of 800 mV in the oxidation mode [1,2]. Attempts were made to increase the current response versus voltage provided by the EC detector by modifying the buffer composition which was initially proposed by Kohda et al [6]. Optimized conditions were obtained by eliminating sodium acetate and by slighty increasing the concentration in citric acid content. The hydrodynamic voltammogram of 8-oxo-7,8-dihydro-2'-deoxyguanosine (2) as compared to the Ag/AgCl reference electrode is illustrated in Fig. 3. A similar response curve was observed for 8-oxo-7,8-dihydroguanine

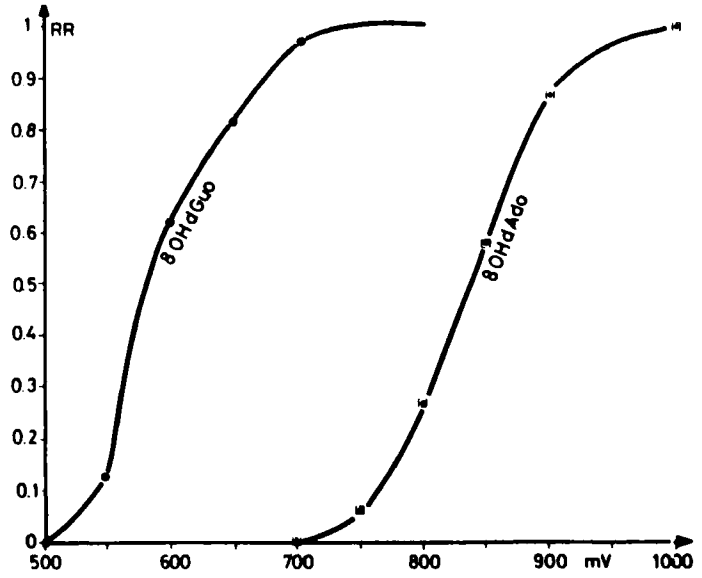


Figure 3 - Hydrodynamic voltammogram of 8-oxo-7,8-dihydro-2'-deoxyguanosine (2) [8OHdGuo] and 8-oxo-7,8-dihydro-2'-deoxyadenosine (4) [8OHdAdo].

(1) with the oxidation potential at half maximum height being 550 mV. The calibration curves for both compounds which were made by applying an oxidation potential of 650 mV were found to be linear over a range of 0.6 pmole to 50 pmoles with a r factor > 0.998 and a coefficient of variation < 3.5% . Under these conditions the detection limits for 1 and 2 at 650 mV were 0.8 and 0.6 pmol respectively.

Higher applied oxidation potential as already noted by Park et al [5] was required for the amperometric detection of 8-oxo-7,8-dihydro-2'-deoxyadenosine (4). The hydrodynamic voltammogram of 4 which exhibits an oxidation potential at half maximum height at 830 mV is presented in Fig. 3. The current response was linear with increasing the amount of 4 over a range of 1 pmole to 50

TABLE 2

Formation of 3 and 4 (in %) in the Gamma Irradiated N_2O Saturated Aqueous Solutions of Adenine and 2'-Deoxyadenosine

dose (Gray)	8-oxo-7,8-dihydroadenine (3)	8-oxo-7,8-dihydro 2'-deoxyadenosine (4)
2.8	0.006	0.016
7.0	0.031	0.043
14.0	0.063	0.084

pmoles with a r factor higher than 0.998 and a coefficient of variation lower than 3%. Similar data were obtained for 8-oxo-7,8-dihydroadenine with its oxidation potential at half-wave close to 820 mV. The EC detection of 3 and 4 at an applied oxidation potential of 850 mV was found to be about two-fold less sensitive than those of 1 and 2 at 650 mV. It is interesting to note that 8-oxo-7,8-dihydroguanine (1) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (2) are selectively detected when an oxidation potential of 600 mV is applied. On the other hand the presence of all four compouds 1-4 can be monitored simultaneously by increasing the oxidation potential to 850 mV (Fig. 2).

Sensitive EC Detection of 3 and 4 in the Gamma Irradiated N_2O Saturated Aqueous Solutions of Adenine and 2'-Deoxyadenosine.

Formation of the 8-oxo-7,8-dihydroadenine derivative was found to be one of the predominant radiation-induced occuring decomposition pathway of the adenine moiety of the related 2'-deoxyribonucleoside [19] and of DNA [20] in N_2O or O_2 saturated aqueous solution. The HPLC-EC assay when operating at

850 mV was found to be suitable to follow the radiation-induced formation of 3 and 4 within the range of very low applied dose (2.8 - 15 Gy). It is interesting to note that the formation of 3 and 4 shows a strict linear dependence with the dose of gamma rays (Table 2).

CONCLUSIONS

The HPLC-EC method which is reported in this paper is a sensitive analytical tool for the quantitative measurement of both 8-oxo-7,8-dihydroadenine and 8-oxo-7,8-dihydroguanine nucleobases as well as the corresponding 2'-deoxyribonucleoside derivatives. This assay may be productively applied to the identification of OH radicals in biochemical and/or chemical systems as the formation of **3** and **4** appears to be specifically induced by this reactive species [21]. On the other hand the formation of **1** and **2** can be used to identify the generation of the oxidizing species involved in Fenton reactions which are likely to be perferryl ions or ferroxo complexes [22,23]. The sensitivity of the HPLC-EC method is sufficient to be used for the search of oxidative purine base damage in cellular DNA when more than 50 μg is available. For smaller quantities of DNA, ^{32}P-postlabeling-HPLC represents a better alternative [24]. However the HPLC-EC assay appears to be very useful in the latter method for calibration purpose.

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