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Micellar electrokinetic capillary chromatography of 8-hydroxydeoxyguanosine and other oxidized derivatives of DNA

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Abstract

8-Hydroxydeoxyguanosine (8-OH-dG) is widely recognized as a marker of DNA oxidation. Until now, 8-OH-dG has been measured by high-performance liquid chromatography or by gas chromatography–mass spectrometry. A method is reported that detects oxidative derivatives of deoxynucleosides by micellar electrokinetic capillary chromatography. Single-stranded DNA was incubated in the presence of 50 m*M* hydrogen peroxide–10 m*M* ascorbic acid and hydrolysed by enzymatic digestion. The order of electrophoretic mobilities of deoxynucleosides was dC > dA > T > dG > 8-OH-dG. 8-OH-dG was determined by introducing a laboratory-prepared internal standard. Two additional major oxidative derivatives were identified by comparing the electropherogram of the oxidized DNA with that of the oxidized standard deoxyguanosine.

1. Introduction

Biological and chemical systems generating oxygen radicals can produce a variety of damage in nucleic acids, such as strand breaks, cross-links and oxidation of sugar and base residues [1]. Such lesions could lead to DNA sequence changes and, consequently, to metabolic dysfunctions. The possibility that the DNA-repair system could be overcome by oxidative damage is the central element of the free-radical theory of ageing [2]. Oxidative modifications of genetic material have also been implicated in carcinogenesis, particularly during promotion and progression [3]. 8-Hydroxy-2′-deoxyguanosine (8-OH-dG) is widely recognized as a marker of DNA oxidation, even though this nucleoside represents only one of the twenty different modifications thought to be formed in DNA by the action of oxy radicals [4]. However, 8-OH-dG is one of the major products of base damage during exposure of DNA to $^{\cdot}OH$-producing systems. For instance, 8-OH-dG represents over 30% of the total base modification products measured in pro-oxidant systems containing iron(III) ion, and more than 50% in copper ion-dependent systems [5]. Until now, 8-OH-dG has been measured by HPLC with

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electrochemical detection [6] or by gas chromatography–mass spectrometry (GC–MS) [7]. The latter technique provides a higher sensitivity of detection and conclusive structure identification of different adducts. However, the equipment required is often too expensive for many laboratories. In this paper we present a method that, for the first time, uses micellar electrokinetic capillary chromatography (MECC) to detect 8-OH-dG and other DNA-oxidative adducts. This relatively simple technique offers significant advantages in the separation of nucleic acid constituents owing to the rapidity of the analysis, the good efficiency and resolution and the extremely small amount of sample to be injected.

2. Experimental

2.1. Reagents

Calf thymus DNA, deoxyguanosine (dG), DNase I, spleen exonuclease, snake venom exonuclease and alkaline phosphatase were obtained from Sigma.

2.2. Preparation of laboratory 8-OH-dG

8-OH-dG was synthesized by oxidation of dG in the presence of hydrogen peroxide according to the method described by Kasai and Nishimura [8]. The medium used in this study should also produce hydroxyl radicals through the Fenton reaction, as traces of iron generally contaminate the reagents. The reaction mixture (100 ml), containing 5 m*M* sodium phosphate (pH 6.8), 1 mg/ml dG, 10 m*M* ascorbic acid and 50 m*M* H_2O_2, was bubbled with O_2 and incubated at 40°C. After 1 h, H_2O_2 was added again and, after O_2 bubbling, the mixture was further incubated for 2 h. After concentration under vacuum to about 10 ml, salts were precipitated with 2 volumes of acetone at 2°C. The solution was then centrifuged at 15 000 *g* and concentrated to 4 ml. 8-OH-dG was purified by HPLC, by injecting 0.6-ml aliquots on to an Ultraspher ODS column (25 × 0.46 cm I.D.). The solvent was 0.2 m*M* ammonium acetate (pH 5.3)–methanol (95:5). The fractions enriched in 8-OH-dG were combined, concentrated to 0.2 ml and further purified by HPLC with a solvent containing only 2% of methanol. NMR and spectral data were similar to those reported [8].

2.2. DNA oxidation and digestion

Calf thymus DNA (50 μg) and dG (1 mg/ml) were separately dissolved in 5 m*M* phosphate buffer (pH 6.8). An aliquot of DNA was boiled for 5 min to separate the two strands. Single-stranded DNA, double-stranded DNA and dG were then incubated in the presence of 50 m*M* H_2O_2–10 m*M* ascorbic acid as previously described for 8-OH-dG synthesis and followed by a tenfold dilution in a buffer containing 10 m*M* $MgCl_2$ and 40 m*M* Tris–HCl (pH 8.5). DNA was subsequently hydrolysed by enzymatic digestion with DNase I (200 units/mg of DNA), spleen exonuclease (0.01 units/mg of DNA), snake venom exonuclease (0.5 units/mg of DNA) and alkaline phosphatase (10 units/mg of DNA) for 2 h at 37°C. The hydrolytic enzymes were removed by ultrafiltration (12 000 *g* for 2 h) using Microcon-3 microconcentrators (Amicon). The filtered solutions (450 μl) were lyophilized and resuspended in 50 μl of distilled water.

2.3. Instrumentation

The nucleosides and their oxidation products were separated by MECC according to the method of Lecoq *et al.* [9]. The analysis was performed with a Beckman P/ACE System 2100. An untreated fused-silica capillary of 57 cm total length × 75 μm I.D. achieved a good compromise between efficiency, resolution and time of analysis. The column temperature was maintained at 25°C. The samples were injected by pressure for 10 s, separated at a constant current of 160 μA (12 kV) and detected at 254 nm. Data were collected at a rate of 20 points per second and processed with Gold software. The running solution contained 20 m*M* sodium phosphate buffer (pH 9.6), 100 m*M* sodium dodecyl sulphate (SDS) and 5% acetonitrile. The addition

of the relatively polar small organic molecule acetonitrile as an organic modifier increased the hydrophobicity of the mobile phase and improved the resolution of all the peaks.

2.4. Capillary treatment

Rinsing between runs was necessary to return the system to the initial conditions and to decrease the fluctuations in retention times. Rinsing was done with 0.1 *M* NaOH and water, forward and reverse, followed by the running buffer without SDS at a slightly higher pH than the running buffer. This sequence avoided sudden changes to the pH inside the capillary. Before separation the capillary was equilibrated with the running buffer. At the end of a set of separations the capillary was treated for 10 min with 0.1 *M* NaOH and 20 min with water, and finally dried with air from an empty vial.

3. Results and discussion

Exposure of the denaturated DNA to the prooxidant mixture produced several adducts (Fig. 1, dotted line) that were identified as additional peaks with respect to the non-oxidized deoxynucleosides (Fig. 1, solid line). The order of electrophoretic mobilities was dC > dA > T > dG > 8-OH-dG. 8-OH-dG was identified by introducing and internal standard prepared in the laboratory (Fig. 2). The lower electrophoretic mobility of 8-OH-dG with respect to the corresponding non-oxidized deoxynucleoside could be due to the increase in both dG mass and negative charge. Nevertheless, the retention time of 8-OH-dG was short enough (mean 9.65 min) to perform a rapid analysis.

The detection of 8-OH-dG was possible only if the sample of DNA was boiled before oxidation. This step leads to the formation of single strands from the native DNA. In contrast, the oxidation of the double-stranded DNA in the presence of H_2O_2–ascorbate did not produce detectable 8-OH-dG (Fig. 3). One possible explanation is that the native structure of DNA could be more protected from the attack of chemical agents.

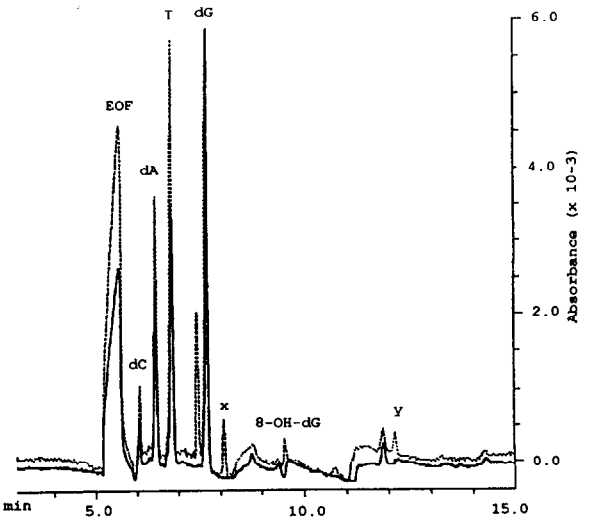

Fig. 1. Comparison between the electropherograms of the digested denatured DNA before (solid line) and after (dotted line) oxidation. EOF = unretained solute; dC = deoxycytosine; dA = deoxyadenosine; T = thymidine; dG = deoxyguanosine; 8-OH-dG = 8-hydroxy-2′-deoxyguanosine. Peaks *x* and *y* also appeared in the electropherogram of the oxidized standard deoxyguanosine.

Free radicals could interact more easily with the bases of a single-stranded DNA than inside a native DNA. Another explanation for why 8-

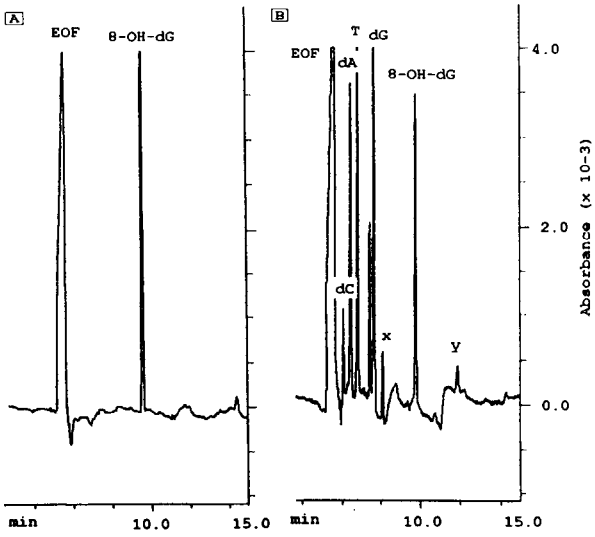

Fig. 2. Electropherogram of (A) standard 8-OH-dG alone and (B) as an internal standard introduced into the sample of the oxidized, single-stranded DNA after enzymatic digestion.

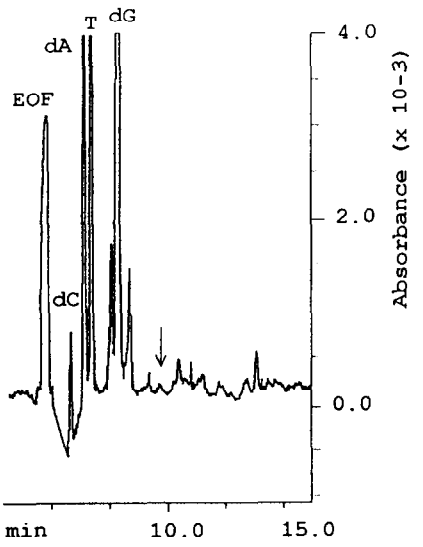

Fig. 3. Deoxynucleosides and oxidative adducts of native DNA. 8-OH-dG was not detectable (arrow).

OH-dG was undetectable in the sample of the oxidized native DNA could be the low sensitivity of the UV spectrophotometer. In fact, the minimum detectable concentration of deoxynucleosides was approximately $5 \cdot 10^{-7}$ M (25 fmol per 50 nl injection volume). Further improvements in the sensitivity of the analysis are expected using a laser-induced fluorescence detector.

The mean values of the 8-OH-dG retention time and the retention time relative to dC are shown in Table 1. The run-to-run value of the coefficient of variation (C.V.) was 0.51% for the retention time and 0.73% for that relative to dC.

Table 1
Run-to-run and day-to-day repeatibilities of the retention times and relative retention times (relative retention time of dC = 1) of 8-OH-dG

Parameter[a]	Retention time (min)	Relative retention time
Run-to-run		
Mean (n = 6)	9.65	1.49
C.V. (%)	0.51	0.73
Day-to-day		
Mean (S = 8)		1.47
C.V. (%)		1.60

The day-to-day C.V. of different series of separations was 1.6%.

The sample of standard deoxyguanosine incubated in the presence of H_2O_2–ascorbate contained its specific by-products of oxidation (Fig. 4, lower panel). In addition to 8-OH-dG, we identified a further two oxidative adducts of

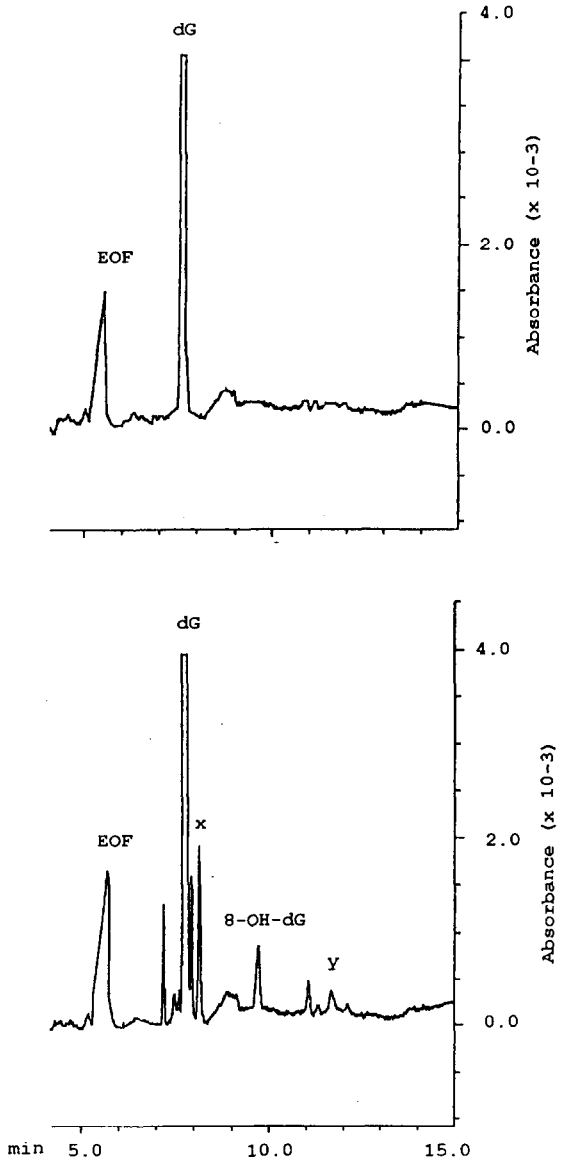

Fig. 4. Electropherogram of the standard deoxyguanosine before (upper panel) and after (lower panel) oxidation.

dG that we called *x* and *y* because their structure is still unknown. These peaks were also present in the electropherogram of the oxidized DNA because they had the same retention times (Fig. 5). Indeed, 8-OH-dG is not the only by-product of dG generated by oxy-radical interactions. $^{\cdot}OH$ can add to guanine also at C-4 and C-5 positions [10]. Moreover, the imidazole ring of 8-OH-guanine adducts can be subsequently opened, followed by the formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine [11]. Thus, 8-OH-dG levels alone could be a misleading quantitative measurement of the oxidative damage to DNA [5]. Studies are in progress to identify the structure of peaks *x* and *y* by mass spectrometry.

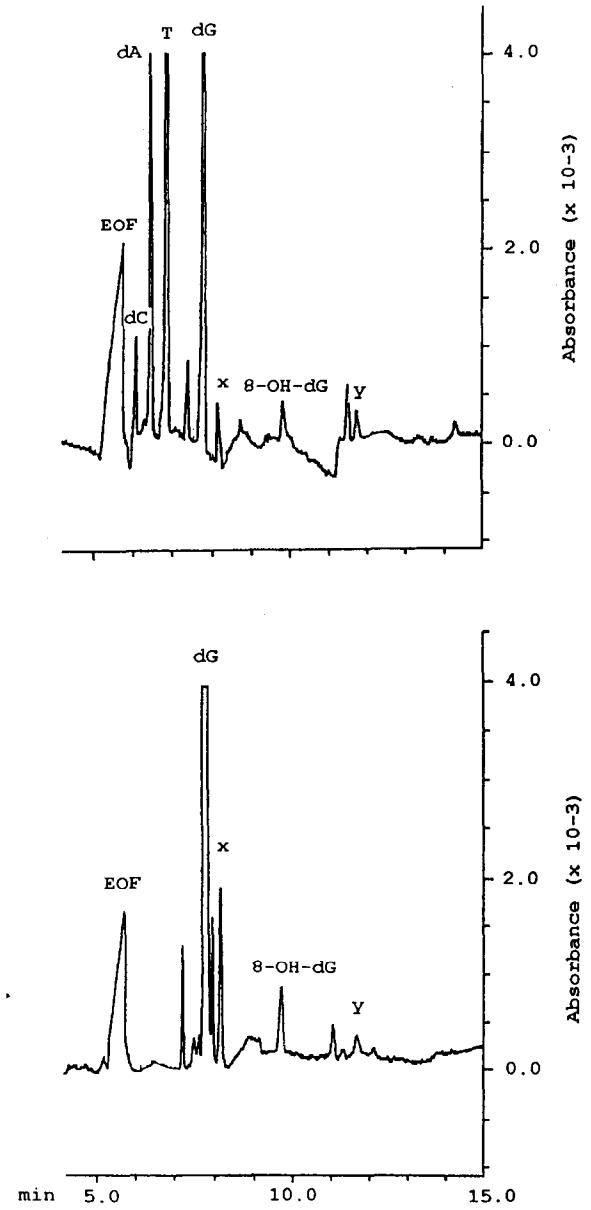

Fig. 5. Comparison between the electropherograms of the digested denatured DNA (upper panel) and the standard deoxyguanosine (lower panel) after oxidation. Peaks *x* and *y* share the same retention times.

In conclusion, the MECC separation of deoxynucleosides and their oxidative adducts offers some advantages such as good efficiency and reproducibility, short run times, very small injection volumes and low costs of analysis. The weak point of this method is the poor sensitivity, which does not allow the extremely low concentrations of oxidized nucleosides generated by endogenous sources of free radicals to be determined. However, this technique can be suitably employed to determine 8-OH-dG and other adducts of DNA produced by *in vitro* pro-oxidant systems.

4. References

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