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Simultaneous determination of O^6-methyl-2′-deoxyguanosine, 8-oxo-7,8-dihydro-2′-deoxyguanosine, and 1,N^6-etheno-2′-deoxyadenosine in DNA using on-line sample preparation by HPLC column switching coupled to ESI-MS/MS

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Abstract

O^6-Methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo) are promutagenic DNA lesions originating from both endogenous and exogenous agents and actions (methylation, hydroxylation, lipid peroxidation products). A highly sensitive quantitative method was developed to measure these DNA adducts simultaneously, using liquid chromatography tandem mass spectrometry with column switching. Deuterated O^6-[$^2H_3$]mdGuo was synthesized and used as internal standard. The limits of quantification for O^6-mdGuo, 8-oxodGuo, and εdAdo were 24, 98, and 48 fmol on column, respectively. The method showed linearity in the range 0.24–125 pmol/ml, 0.98–125 pmol/ml, and 0.49–62.5 pmol/ml for the three adducts, respectively. The inter-day precision in the linear concentration range was between 1.7 and 9.3% for O^6-mdGuo, 10.6 and 28.7% for 8-oxodGuo, and 6.2 and 10.4%, for εdAdo. In DNA isolated from liver of untreated 12-week-old female F344 rats, O^6-mdGuo was above the limit of detection (37 adducts per 10^9 normal nucleosides) but could not be quantified. 8-oxodGuo and εdAdo showed background levels of 500 and 130 adducts per 10^9 normal nucleosides, respectively. DNA analyzed 1 h after treatment of rats with dimethylnitrosamine by oral gavage of 50 μg/kg b.wt. did not affect the levels of 8-oxodGuo and εdAdo but resulted in 200 O^6-mdGuo adducts per 10^9 normal nucleosides. The method developed will be of use to study the biological significance of exogenous DNA adducts as an increment to background DNA damage and the role of modulating factors, such as DNA repair.

Keywords: DNA adducts; Oxidative stress; Background DNA damage; Methylation; Oxidation; Lipid peroxidation; Rat liver; Mass spectrometry

1. Introduction

DNA adduct formation plays an important role in chemical carcinogenesis. Some adducts are also formed as background, from endogenous and unavoidable sources [1]. The biological significance of chemically induced DNA adducts as an increment to background DNA damage is important for a comprehensive risk assessment. Therefore, appropriate analytical techniques are required to provide accurate and reproducible quantification. We chose O^6-mdGuo, 8-oxodGuo, and εdAdo (see Fig. 1 for structures), since these adducts are formed both endogenously and exogenously by DNA-methylation, direct oxidation of DNA, and reaction of DNA with lipid peroxidation products [2].

For the detection of low levels of DNA adducts ^{32}P-postlabeling methods are commonly used due to their high sensitivity. However, these methods provide low specificity, limited reproducibility, and with the enrichment methods available for the selected adducts – e.g., immunoaffinity or nuclease P1 enrichment or butanol extraction – it is difficult to achieve accurate and precise measurements [3,4].

An alternative approach is the use of mass spectrometry. With accelerated mass spectrometry (AMS) the sensitivity can be even higher than with ^{32}P-postlabeling [5]. The disadvantage of AMS

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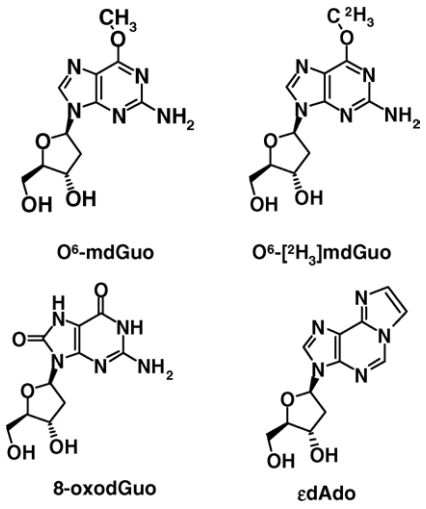

Fig. 1. Chemical structures of the three DNA adducts O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-2′-deoxyguanosine (8-oxodGuo), 1,N^6-etheno-2′-deoxyadenosine (εdAdo) and the isotope-labeled internal standard O^6-[$^2H_3$]mdGuo.

is the need for ^{14}C-radiolabeled chemicals, the lack of structural information, and the problem of biosynthetic incorporation of ^{14}C into DNA. GC–MS methods are available for 8-oxodGuo and εdAdo. These methods generally offer high specificity and allow for accurate and precise quantification by using stable isotope labeled internal standards [6,7]. However, oxidation of 2′-deoxyguanosine (dGuo) to 8-oxodGuo during derivatization and evaporation in the injector system is a problem for quantification [7,8]. 8-oxodGuo has also been analyzed by HPLC-ECD and by the comet assay using the formamidopyrimidine DNA *N*-glycosylase repair enzyme [8]. Both assays cannot be used for the quantification of O^6-mdGuo or εdAdo.

HPLC–MS/MS is another alternative to analyze DNA-adducts either as 2′-deoxyribonucleosides [6,9–11] or as base adducts [12]. Analyzing 2′-deoxyribonucleosides has the advantage of excluding potential contamination of DNA by RNA. For O^6-mdGuo, 8-oxodGuo, and εdAdo, adduct-specific methods have been described [13–15]. The use of tandem mass spectrometers with multiple reaction monitoring (MRM) mode offered high sensitivity paired with high selectivity. Application of most published methods is limited by the sample preparation necessary to quantify DNA adducts in samples isolated from tissue. This step is considered to be critical [6,16] because a complex and time-consuming off-line sample preparation can contribute to loss of analyte and, in the case of 8-oxodGuo, to the formation of artifacts by oxidation of dGuo [4].

On-line sample preparation clean-up techniques coupled with mass spectrometry have proven to be efficient tools in sample preparation from biological matrices [17]. The effectiveness of column switching methods for the analysis of adducts was recently confirmed for DNA in vitro [10,18], nucleosides in urine [19] and for DNA in tissue [20,21]. Therefore, a HPLC–MS/MS method using an on-line column switching technique was developed and validated for the simultaneous analysis of the promutagenic DNA adducts O^6-mdGuo, 8-oxodGuo, and εdAdo.

2. Experimental

2.1. Chemicals

All chemicals for the synthesis of O^6-mdGuo and the internal standard O^6-[$^2H_3$]mdGuo as well as the adduct standards 8-oxodGuo and εdAdo were obtained from Sigma-Aldrich (Taufkirchen, Germany). Alkaline phosphatase from calf intestine was from Calbiochem (Darmstadt, Germany), Nuclease P1 was from MP Biomedicals Inc. (Aurora, Ohio). HPLC-grade methanol and water for liquid chromatography were purchased from Roth (Karlsruhe, Germany).

2.2. Synthesis of O^6-methyl-2′-deoxyguanosine and the labeled internal standard

O^6-mdGuo and its stable isotope-labeled analogue O^6-[$^2H_3$]mdGuo were synthesized based on a published method [22]. First sodium methoxide solution was generated by adding peaces of sodium (310 mg) to 50 ml of cold CH_3OH (for the synthesis of O^6-mdGuo) or C[$^2H_3$]OH (for the synthesis of O^6-[$^2H_3$]mdGuo). Second 2′-deoxyguanosine (dGuo) (94 mg, 0.33 mmol) was dissolved in 1.7 ml of dry pyridine with 0.4 ml (2.8 mmol) trifluoroacetic anhydride under dry nitrogen atmosphere on ice. To this solution 50 ml of the respective methoxide solution was added drop wise. The reaction was stopped after 48 h with pyridine hydrochloride (3 ml of pyridine/1 ml of HCl 30%). Then 0.4 g of $NaHCO_3$ was added. The solution was evaporated to dryness using a rotary evaporator. The residue was dissolved in 3 ml H_2O and injected in fractions of 100 μl into a preparative HPLC column (Nucleosil® 100-7 C18, 250 mm × 10 mm, Machery-Nagel, Düren, Germany) using a gradient of 2–30% acetonitrile in water in 30 min at a flow rate of 2 ml/min. The appropriate fractions were collected, combined, and evaporated to dryness. O^6-[$^2H_3$]mdGuo and O^6-mdGuo were identified by MS/MS and quantified by UV-spectroscopy (extinction coefficients ε(278 nm) = 8.51 cm^2/mmol and ε(274 nm) = 8.13 cm^2/mmol).

2.3. Liquid chromatography

The configuration of the on-line extraction LC–MS/MS system is shown in Fig. 2. The center element of the system was an electrical valve (Valco Valve), which controlled the flow of the solvents from two pumps into two different columns. In the loading position (Fig. 2A) the autosampler (Agilent Series 1100, Waldbronn, Germany) introduced the sample into the system and pump 1 (Agilent Series 1100, Waldbronn, Germany) carried the aqueous mobile phase (5% methanol in 10 mM ammonium acetate, pH 4.3) at 1.5 ml/min to load the sample on the trap column (Oasis® HBL 25 μm 20 mm × 2.1 mm, Waters, Milford, Massachusetts). Sample loading and elimination of matrix components were completed after 1.5 min. Then the valve switched to the elution position (Fig. 2B). Pump 2 (Agilent Series 1100) supplied a gradient (8 min from 10 to 100% methanol) to back flush the trapped analytes from the trap column and to transfer them onto an analytical column

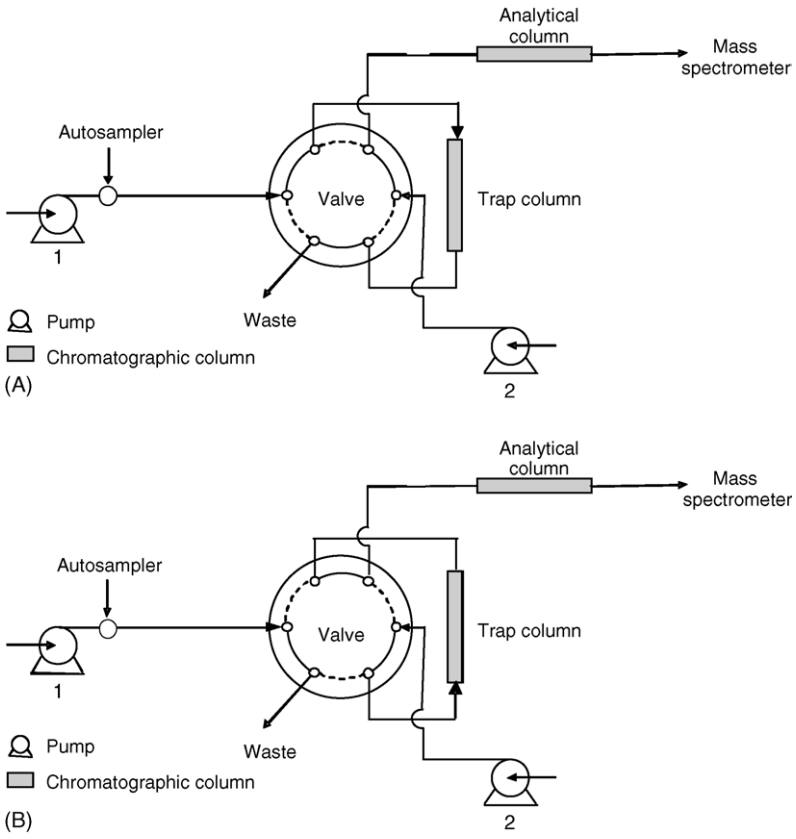

Fig. 2. Schematic configuration and valve positions of the on-line extraction LC–MS/MS system: (A) sample loading position; (B) sample back flush and elution position.

(Reprosil 3.5 μm, 150 mm × 4.6 mm, Dr. Maisch, Ammerbuch, Germany).

2.4. Mass spectrometry

A triple-stage quadrupole mass spectrometer (API 3000, Applied Biosystems, Darmstadt, Germany) equipped with an electrospray ionization source was used. The ionspray voltage was 5500 V, the source temperature 400 °C, nitrogen was used as curtain and collision gas. Positive ions were analyzed by multiple reaction monitoring with a dwell time of 0.100 s for each transition. The compound specific parameters were obtained by infusion of the standards using the quantitative optimization function of the Analyst 1.4.1 software. Details are given in Table 1. Two transitions per adduct were monitored. Peak areas of the most sensitive transitions were used for quantification. Standard curves were generated with O^6-[$^2H_3$]mdGuo as internal standard.

2.5. Method validation

To compensate for matrix effects method validation was conducted in an artificial matrix consisting of the four normal 2′-deoxyribonucleosides (21% each dGuo and dCyd and 29% each dAdo and dThd), similar to a hydrolysate of mammalian DNA. The method was characterized for dynamic range, limit of detection (LOD: defined by a signal-to-noise ratio (S/N) ≥ 3), and limit of quantification (LOQ: S/N ≥ 7.5). Each injection (100 μl) contained 320 nmol of blank matrix (equivalent to 100 μg DNA), the internal standard (1 pmol), and the respective working standards at 11 concentrations in the range from 0.12 pmol/ml to 0.125 nmol/ml. To assess inter-day precision and accuracy measurements were carried out with independently prepared standards on five different days.

2.6. Animals and administration of dimethylnitrosamine

All work conducted with animals was performed in compliance with the policies set forth in the Guide for the Care and Use of Laboratory Animals. Female Fischer F344 rats, 12-week-old (about 200 g) were held on sawdust for 1 week for acclimatization. They were fed ad libitum with standard diet and had free access to tap water. The rats were dosed with DMNA (50 μg/kg b.wt.) in water by oral gavage after 6 h without access to food. After 60 min the rats were killed by CO_2 asphyxiation and livers were removed. The livers were shock frozen in liquid nitrogen and stored at −80 °C until analysis.

2.7. DNA isolation and DNA hydrolysis

DNA was isolated from rat liver using a DNA isolation kit (Nucleobond® AX, Macherey-Nagel, Düren, Germany). Briefly, 250 mg of rat liver was homogenized mechanically (Ultra Turrax). The homogenate was treated with RNase A and proteinase

Table 1
Multiple reaction monitoring parameters for the analysis of O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo) and the deuterated internal standard O^6-[$^2H_3$]mdGuo

Analyte	Precursor ion (Q1)	Product ion (Q3)	Retention time (min)	DP	FP	CE	CXP
O^6-[$^2H_3$]mdGuo (internal std.)	285	169	9.2	26	160	21	10
O^6-mdGuo	282	166[a]	9.2	26	160	21	10
		149				47	12
8-oxodGuo	284	168[a]	8.3	26	130	19	14
		140				45	10
εdAdo	276	160[a]	7.5	31	180	23	14
		106[a]				69	8

DP: declustering potential (V); FP: focusing potential (V); CE: collision energy (V); CXP: collision cell exit potential (V).
[a] Transitions used for quantification.

K. Then the lysate was transferred to a Nucleobond® AX cartridge for adsorption of the DNA. After washing, elution, precipitation with isopropanol, and centrifugation a DNA pellet was obtained. After dissolving the pellet in water to a concentration of approximately 1 μg/μl, the exact concentration and purity were determined by UV spectroscopy by measuring the OD at 260 and 280 nm. Ratios between 1.8 and 2.0 were considered as clean. To digest the DNA to the 2′-deoxyribonucleosides, 2.5 μl of a 1 M NH_4Ac (pH 5.1) buffer containing 45 mM $ZnCl_2$ was added per 100 μl sample. Internal standard (1 pmol) was added and the sample was incubated with Nuclease P1 (0.1 units per μg DNA) for 60 min at 40 °C. Then 10 μl of a 1.5 M NH_4HCO_3 (pH 8) buffer was added prior to incubation with alkaline phosphatase (0.02 units/μg DNA) for 60 min. The sample was centrifuged in a 5000 NMWL cut-off filter tube (Ultrafree, Millipore, Eschborn, Germany) to remove protein. To control whether the digest was complete dGuo was quantified by LC–MS/MS.

3. Results and discussion

3.1. Column switching LC–MS/MS method

Sample clean-up and chromatography of analytes were performed on-line by a column-switching set-up of two HPLC columns and included change in flow direction (Fig. 2). It started with sample loading, trapping of the analytes on the trap column while simultaneously washing matrix components to the waste [17]. Due to similar molecular weights, polarities, and chromatographic properties of the DNA-adducts and the unmodified ("normal") nucleosides, it was necessary to optimize the time for loading/washing the trap column and the concentration of organic solvent in the mobile phase. Using 1.5 min for loading/washing and 5% methanol in the solvent, O^6-mdGuo, 8-oxodGuo, and εdAdo could be quantitatively and reproducibly retained on the trap column. After switching the valve, the compounds were back flushed with a higher organic solvent content onto the analytical column. Preliminary evaluation of the method showed that the normal nucleosides had not been completely washed from the trap column due to their high concentration. However, the reduction was sufficient to allow for adequate chromatographic peaks for all DNA adducts (Fig. 3), as well as reproducible quantification. Since O^6-mdGuo exhibited the lowest background levels in biological samples [23] experimental parameters were optimized for this adduct, while keeping acceptable conditions for the quantification of 8-oxodGuo and εdAdo in the same analytical run.

3.2. Method validation

Method validation was done with an injection volume of 100 μl containing normal nucleosides as matrix, equivalent to a digest of 100 μg of DNA (320 nmol total nucleosides). The matrix was analyzed and contained no measurable levels of DNA adducts. Only upon storage of the matrix solution for more than 2 months in the refrigerator did oxidative reactions occur, which resulted in the formation of low amounts of about 100 fmol 8-oxodGuo.

The LOQs for O^6-mdGuo, 8-oxodGuo, and εdAdo were 24, 98, and 48 fmol on column, respectively. For a sample of 100 μg of DNA this corresponds to 75, 300 and 150 adducts per 10^9 normal nucleosides, respectively. The LOQ for O^6-mdGuo was about four-fold better compared to a previously published LC–MS/MS method [13]. The sensitivity of our method for εdAdo was comparable to other reports [15]. As our method was optimized for O^6-mdGuo, the settings were not the best possible for 8-oxodGuo. Therefore, our LOQ for 8-oxodGuo was about two- to five-fold higher compared to published LC–MS/MS methods [14,24]. However, our LOQ was satisfactory because the lowest value published for 8-oxodGuo in DNA from rat liver is approximately 500 adducts per 10^9 nucleosides [1,25], thus about twice our LOQ.

Table 2 summarizes the assay characteristics for 10 different adduct concentrations monitored on five different days. The matrix-matched calibration curves were linear for O^6-mdGuo, 8-oxodGuo, and εdAdo in the range of 0.24–125 pmol/ml, 0.98–125 pmol/ml, and 0.49–62.5 pmol/ml, respectively. Within the dynamic range linear correlation coefficients r were better than 0.98 for all analytes. The inter-day precision varied in the range of 1.7–9.3%, 10.6–28.7% and 6.2–10.4% for O^6-mdGuo, 8-oxodGuo, and εdAdo, respectively. The inter-day precision of 8-oxodGuo was not as high as for the two other adducts. The formation of 8-oxodGuo from dGuo requires only one oxidation step and the artifact formation of 8-oxodGuo has been discussed

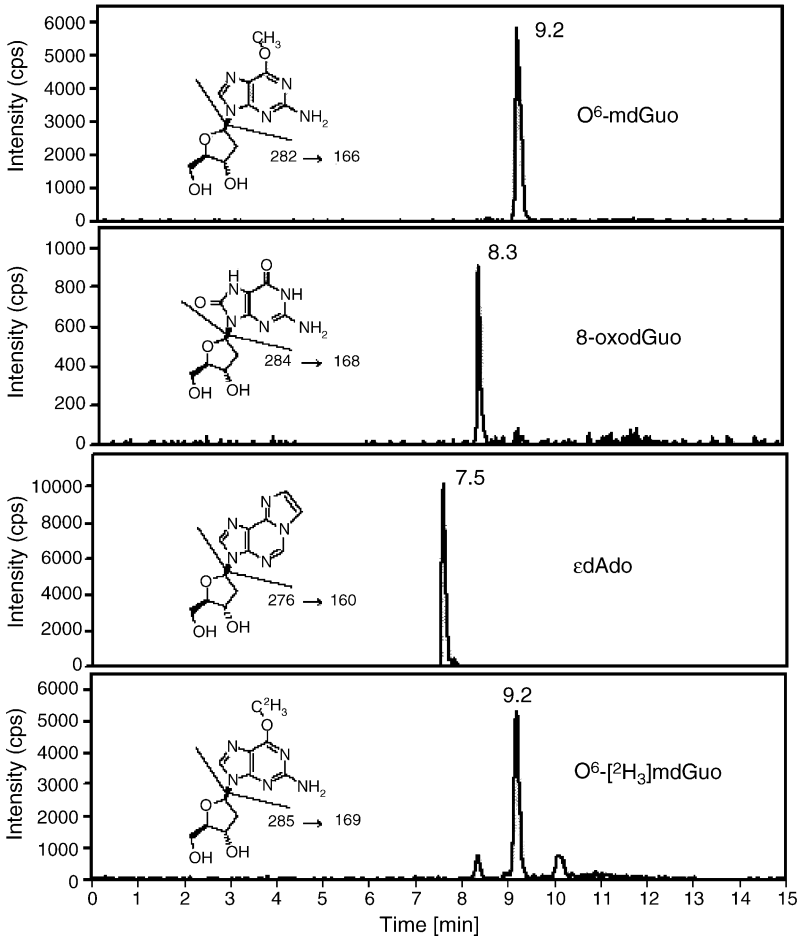

Fig. 3. Multiple reaction monitoring (MRM) chromatograms for the three analytes O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo) (all 8 pmol/ml), and the internal standard.

extensively in the literature [8,25,26]. In general published 8-oxodGuo levels have to be seen critically since the formation of 8-oxodGuo in the presence of large concentrations of dGuo is difficult to control. In comparison to other methods based on LC–MS/MS our method appeared to produce low levels of artifact 8-oxodGuo probably due to reduced sample work-up by on-line sample preparation using the column switching unit.

3.3. Analysis of adducts in rat liver

The validated method was applied to DNA samples from female F344 rats. Analysis of O^6-mdGuo, 8-oxodGuo, and εdAdo in hydrolysate of liver DNA isolated from an untreated rat is shown in Fig. 4 (top panels). O^6-mdGuo was below the LOQ but above the LOD of 37 adducts per 10^9 normal nucleo-

Table 2
Analytical performance for the determination of O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo)

Expected concentration	O^6-mdGuo				8-oxodGuo				εdAdo			
	Mean concentration	S.D.	%CV	Accy	Mean	S.D.	%CV	Accy	Mean	S.D.	%CV	Accy
0.24	0.23	0.02	9.3	94	–[a]	–	–	–	–[a]	–	–	–
0.49	0.47	0.03	6.1	97	–[a]	–	–	–	0.47	0.05	10.4	95
0.98	0.91	0.07	7.4	93	0.91	0.31	28.7	85	1.06	0.07	6.7	108
1.95	1.95	0.09	8.4	100	1.77	0.20	11.5	91	1.95	0.18	9.0	100
3.91	3.96	0.21	5.2	101	3.58	0.56	15.6	92	3.81	0.24	6.2	97
7.81	7.77	0.28	3.6	99	8.53	1.55	18.2	109	7.47	0.54	7.2	96
15.6	14.86	0.43	2.9	95	14.60	1.96	11.8	94	15.6	1.1	7.2	100
31.3	29.85	0.91	3.0	96	34.8	3.7	10.6	112	31.7	2.4	7.7	102
62.5	61.12	1.03	1.7	98	74.3	13.2	17.8	119	65.2	6.3	9.7	105
125	130.8	6.0	4.6	105	137.0	24.2	17.6	110	–[a]	–	–	–

Mean and standard deviation (S.D.; $n = 5$), inter-day precision (coefficient of variation, CV), and accuracy (Accy). Concentrations in pmol/ml.
[a] Outside dynamic range.

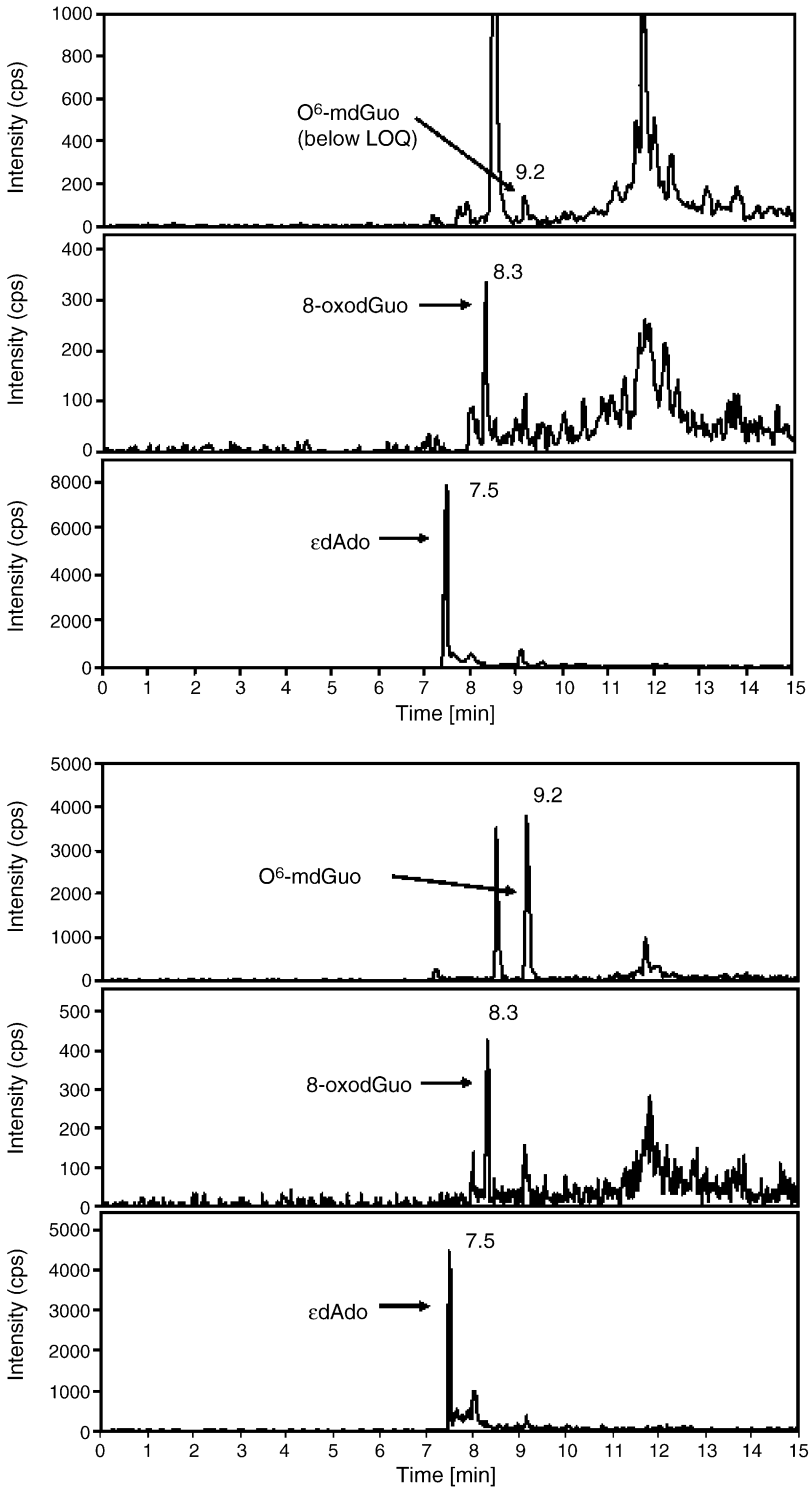

Fig. 4. LC–MS/MS analysis of O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo) in DNA isolated from liver of an untreated rat (top three panels) and of a rat 1 h after treatment with 50 μg/kg b.wt. dimethylnitrosamine (bottom three panels).

sides. Literature values range from 0.3 adducts per 10^9 normal nucleosides determined by immunochemical analysis with a monoclonal antibody [27] and 1300 O^6-mdGuo per 10^9 normal nucleosides determined by a LC–MS/MS method [13]. For 8-oxodGuo we measured a background level of 520 adducts per 10^9 normal nucleosides. A large database on 8-oxodGuo exists and levels determined in liver of untreated rats show big variability [8]. The lowest published values for 8-oxodGuo were on the order of 500 adducts per 10^9 nucleosides [1,25], which corresponds to the level observed here. For εdAdo, we found 130 adducts per 10^9 nucleosides. This value is close to the level of 90 adducts per 10^9 nucleosides measured previously

with LC–MS/MS in livers of untreated female Sprague–Dawley (S.D.) rats [15].

Besides the indicated background DNA lesions O^6-mdGuo, 8-oxodGuo, and εdAdo additional peaks appeared in the DNA adduct profile from rat liver (Fig. 4) when compared to the reference profile (Fig. 3). The only structural information that we can provide is the loss of 116 amu given by the neutral loss of 2′-deoxyribose. We, therefore, assume that these compounds are DNA adducts of other background origin.

In order to verify the applicability of the method for the determination of O^6-mdGuo, two rats were treated by gavage with a single low dose of dimethylnitrosamine (DMNA; 50 μg/kg b.wt.). The levels of 8-oxodGuo and εdAdo, i.e., the two adducts associated with oxidative stress, remained unchanged. For O^6-mdGuo, a clear increase above background was seen, with a mean of 200 adducts per 10^9 normal nucleosides. Fig. 4 (bottom panels) shows the respective chromatograms. One hour after oral gavage of female S.D. rats with 50 μg [^{3}H]DMNA per kg body weight, the level of tritiated O^6-mdGuo in liver had been reported to be about 0.3 μmol/mol dGuo [28]. This is equivalent to about 60 adducts per 10^9 normal nucleosides measured as an incremental DNA damage. Our value of 200 adducts per 10^9 nucleosides, which represents the sum of background and dose-related increment, fits to the published data.

3.4. Outlook

For cancer risk assessment for DNA-reactive carcinogens, extrapolation to low dose is usually done by linear extrapolation, based on the hypothesis that the rate of adduct formation is proportional to dose as long as all enzymatic processes for metabolic activation and detoxication are below the concentration equivalent to the Michaelis constant. On this basis, there cannot be any low dose that would not be associated with some – perhaps negligible – risk. In view of the background DNA damage resulting from endogenous and unavoidable agents and actions it appears reasonable to think in terms of increments above background for the contribution of DNA adducts resulting from exposure to exogenous carcinogens to the spontaneous process of carcinogenesis. The method presented here provides a tool to do this for the three adducts chosen.

Acknowledgments

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