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Monitoring of dimethyl sulphate-induced N3-methyladenine, N7-methylguanine and O^6-methylguanine DNA adducts using reversed-phase high performance liquid chromatography and mass spectrometry

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ABSTRACT

This work describes the determination of N3-methyladenine, N7-methylguanine and O^6-methylguanine adducts in dimethyl sulphate-treated salmon-testes DNA employing reversed-phase high performance liquid chromatography (RP-HPLC) with UV–vis detection, followed by mass-spectrometric verification using electrospray ionisation in positive mode ESI(+). Within validation parameters, accuracy, precision, calibration parameters, limit of detection (LOD) and quantitation (LOQ) as well as stability of standard stock solutions were tested and presented for UV/vis detection. The limit of detection (LOD) was found to be 0.1 ng/mL for N3-methyladenine and 0.2 ng/mL for both N7-methylguanine and O^6-methylguanine (S/N = 3). The limit of quantitation (LOQ) was found to be 0.5 ng/mL for all measured compounds, (S/N = 10). Quantitative results were obtained for each substance based on eight-point calibration. Intra- and inter-day precisions were within 1.73–6.96 and 2.26–7.58%, respectively, and correlation coefficients of calibration curves (R^2) ranged from 0.9992 to 0.9997. Relative proportion of N7-methylguanine was accounted for 61.53 ± 2.97% (R.S.D. = 4.8), N3-methyladenine for 38.19 ± 2.99% (R.S.D. = 9.6) and O^6-methylguanine for 0.29 ± 0.02% (R.S.D. = 5.1), respectively. The application of the above-mentioned techniques provides a valuable contribution for simultaneous determination of methylated DNA adducts, and may represent a suitable approach for similar monitoring/screening studies.

1. Introduction

It is assumed that many chemical carcinogens initiate the complex process of mutagenesis and carcinogenesis by the interaction of their electrophilic moieties with critical nucleophilic sites in DNA (i.e. nitrogen and oxygen atoms in bases and the phosphate oxygen in the sugar–phosphate backbone). Very important group of base modifications are methylated purines that can be induced either by endogenous enzymatic systems (methylations by *S*-adenosylmethionine) or by exogenous reactive chemicals, such as large group of *N*-methyl-nitroso compounds [1]. These kinds of substances occur as environmental pollutants, components of tobacco smoke or engine exhaust. After metabolic activation, they become reactive and able to modify the DNA bases by two reaction mechanisms: monomolecular (S_N1) and bimolecular (S_N2) nucleophilic substitution [2]. Another modification is represented by the phosphate methyl-adducts, however, there is a scarce knowledge on their formation and removal [3].

Different agents result in different alkylation profiles and sensitive methods for detecting and quantifying of these DNA adducts are indispensable for comparative analysis of adducts data, induction of mutations, and mutational spectra in cells or tissues exposed to various alkylating (methylating) agents. Through such comparative analysis, information concerning the mechanisms of mutagenity and carcinogenity of individual methylated DNA adducts in different tissues or cells may be explored, and ultimately, relative cancer risk identified.

Dimethyl sulphate (DMS) (Fig. 1) is being used as a methylating agent in organic chemistry as well as a model compound in genetic and toxicological studies [4]. Its genetic effects include the induction of DNA damage, genetic duplications and phage induction in bacteria, mutations in fungi, plants and cultured mammalian cells, and cytogenetic alterations in plants and mam-

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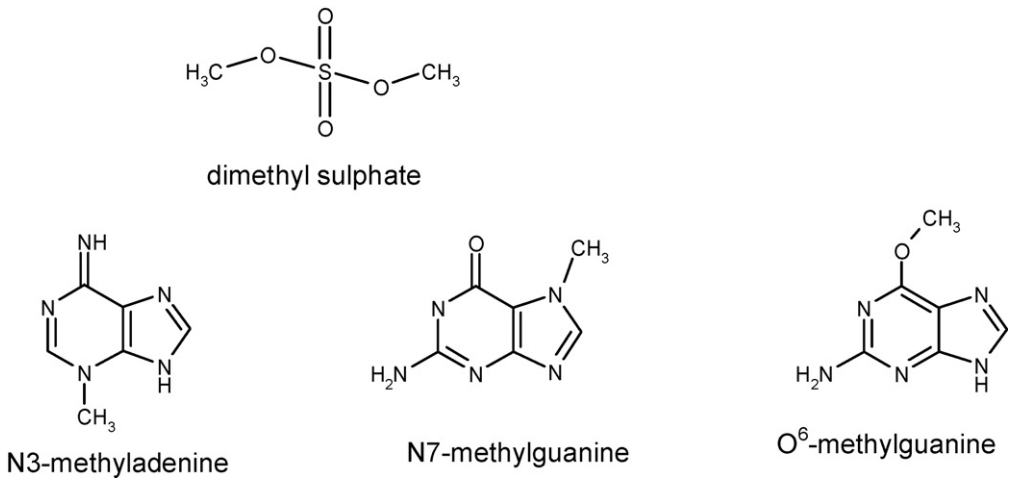

Fig. 1. The structure of the methylating agent (dimethyl sulphate) and of the monitored methylated adducts.

malian cells [5]. Dimethyl sulphate, a probable human carcinogen (group 2A) according to IARC (International Agency for Research on Cancer) [4], is a very strong methylating substance acting at nucleophilic spots of DNA. It results in the formation of methylated adducts predominantly at N7-position of guanine and N3-position of adenine through the S_N2 mechanism [2].

Many variations of analytical methods have been yet described for the determination of methylated DNA-adducts either spontaneously or hydrolytically released from DNA exposed to various methylating agents. These techniques involve immunological assays [6,7], fluorescence techniques [8], electrochemical detection [9,10], mass spectrometry [11–13] and ^{32}P-postlabelling [14–23]. Regarding mass spectrometry, Chao et al. [11] evaluated a quantitative determination of urinary N7-ethylguanine in smokers and non-smokers using an isotope dilution liquid chromatography coupled with tandem-mass spectrometry employing ESI(+). Additionally, Hua et al. evaluated negative and positive electrospray tandem mass spectrometry for the LC–MS/MS analysis of oxidized deoxynucleosides such as 8-hydroxy-2′-deoxyguanosine, 8-hydroxy-2′-deoxyadenosine and 2-hydroxy-2′-deoxyadenosine [13]. As mentioned above, ^{32}P-postlabelling technique was also widely used for investigating methyl DNA-adducts [14–20]. In this respect, Zhao et al. evaluated a capability of two-dimensional TLC and HPLC techniques in combination with ^{32}P-postlabelling, respectively [24]. Moreover, Vodicka et al. published various applications of ^{32}P-postlabelling technique for the determination of wide spectrum of DNA adducts caused by many types of reactive chemicals, with an employment of HPLC coupled with radioisotopic detector [21–23].

Regarding the stability of N3-methyladenine and N7-methylguanine adducts (Fig. 1) in DNA, both are rather unstable and readily undergo spontaneous release from the DNA chain, due to delocalized positive charge at the methylated ring [25,26]. Evidently, the rate of this cleavage depends on the substrate from which the adduct is released (double-stranded DNA, single-stranded DNA, etc.), pH conditions and surrounding temperature [27]. N3-methyladenine and N7-methylguanine are not considered promutagenic by themselves, but they are capable to induce secondary mutations through the formation of apurinic sites by the spontaneous depurination or through the creation of formamidopyrimidine (Fapy) products. Apurinic sites, as results of depurination of methylated adenine or guanine, cause misincorporation leading to mutations; N7-MeG to GC → TA transversion and N3-MeA to AT → TA transversion, respectively [28]. On the other hand, ring-opened N3-methyladenine or N7-methylguanine sites cause replication stop [29]. An acid-catalysed reaction depurinates such adducts and makes the corresponding bases free, whereas a base-catalysed reaction opens the imidazole ring and produces Fapy structures [30]. Above mentioned adducts, generated by methylating agents of clinical interest (i.e. temozolomide or decarbazine) or by compounds of exclusive experimental use (e.g. methylazoxymethanol (MAM), methane sulfonic acid methyl ester (MMS), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), or methyl-nitrosourea (MNU)), seem to have a similar genotoxic impact [31]. In fact, the *short patch* of base excision repair (BER) promptly repairs both N3-methyladenine and N7-methylguanine [32]. However, it has been established that N3-methyladenine, although produced in a relatively minor amount, can specifically induce sister chromatid exchange, chromatid and chromosome gaps and breaks, S phase arrest and apoptosis [33].

Despite being produced in low quantities [34], O^6-methylguanine, a relatively stable DNA adduct, is generally considered as the main cytotoxic and mutagenic lesion produced by methylating compounds [35]. If it is not correctly repaired by a highly specific O^6-methylguanine DNA methyltransferase (MGMT), it inappropriately pairs with thymine (leading to GC → AT transition) and triggers the intervention of the mismatch repair system (MMR). In the presence of MMR functional defects, the increased level of unrepaired O^6-methylguanine and O^6-methylguanine:thymine pair enhances the mutagenic potential and the chances of genetic defects, with consequent tumor onset [36]. In general, because of the wide spectrum of modified bases induced by methylating agents, it is difficult to correlate genotoxicity/carcinogenicity directly to any single methyl adducts [34].

In this study, dimethyl sulphate was used as a methylating reagent for *in vitro* experiments since it is able to methylate bases of DNA without any activation. The quantitation method by reversed-phase high performance liquid chromatography was applied for direct and simultaneous determination of concentration levels of N3-methyladenine, N7-methylguanine and O^6-methylguanine in a series of samples of enzymaticaly cleaved DNA treated *in vitro* with DMS. As a unique technique for the DNA adducts identity verification, mass spectrometry employing electrospray ionisation in positive mode was used.

2. Experimental

2.1. Caution

The DMS was handled in accordance with NIH (National Institute of Health, USA) guidelines for Laboratory Use of Chemical Carcinogens [37].

2.2. Reagents, solvents, enzymes, materials

Standard DNA from salmon testes, adenine, guanine, N3-methyladenine, N7-methylguanine, O^6-methylguanine, dimethyl sulphate, monobasic sodium phosphate, Trizma base, magnesium chloride, formic acid and ammonium formate (all of analytical grade), were purchased from Sigma Chemicals (Czech Republic), as well as all the enzymes used such as nuclease P1 (EC 3.1.30.1), alkaline phosphatase (EC 3.1.3.1), DNase I (EC 3.1.21.1) and snake venom phosphodiesterase (EC 3.1.4.1). All other chemicals, isopropanol, ethanol, methanol, sodium hydroxide, sodium acetate and hydrochloric acid were obtained from Penta Chemicals (Czech Republic). Ultrapure water was obtained from Milli Q Plus PF instrument (Millipore Corp., USA).

2.3. Preparation of calibration standard solutions (methylated bases)

Chromatographic method was calibrated by methanolic standard solutions of N3-methyladenine, N7-methylguanine and O^6-methylguanine in the calibration range 0.5–50 ng/mL, for each compound. Stock solution of N3-methyladenine was prepared at 50 ng/mL concentration level in the methanol: 0.1% formic acid aqueous solution, 9:1 (v/v). Stock solution of N7-methylguanine was prepared at the same concentration level in methanol containing 0.1% formic acid (v/v). Stock solution of O^6-methylguanine was prepared again at the same concentration in pure methanol. Series of standard calibration solutions at the appropriate concentration levels were obtained by sequential diluting of each standard stock solution with the corresponding solvent mixture for each standard substance. Internal standard (IS) working solutions (100 ng/mL of adenine used for N3-methyladenine and 50 ng/mL of guanine used for N7- and O^6-methylguanine analyses) were prepared by diluting the internal standard stock solutions with methanol. All standard solutions were stored at 4 °C until used for analysis.

2.4. Treatment of salmon testes DNA with DMS

2.4.1. Preparation and isolation of the N3-MeA and N7-MeG adducts

2 mg of standard DNA were dissolved in 1 mL of 0.5 mol/L sodium–phosphate buffer solution, pH 6.5 and 47 μL (500 μmol) of dimethyl sulphate were added. The mixture was left to react for 3.5 h with gentle mixing (Grand-bio rotator, P-Lab, Czech Republic) at ambient temperature. After the elapsed time, an excess of DMS was separated by centrifugation (500 × *g*, 5 min, ambient temperature) and methylated-DNA phase was transferred into a new vial. 500 μL of this fraction were collected to accomplish the depurination. This was achieved by 30 min boiling of the fractions in a water bath, followed by cooling down to room temperature. After centrifugation, the supernatant containing depurinated adducts was ten times concentrated using vacuum rotary evaporator (Centrivap Cold Trap, Labconco Corp., USA) and stored frozen until used for analysis. Before chromatographic analysis, the concentrated supernatant was dissolved in 1 mL of methanol and 5 μL of this solution was injected into column and analysed by HPLC-UV-MS.

2.4.2. Preparation and isolation of the O^6-MeG adduct

Dissolved methylated DNA, contained in the reaction mixture remaining after separation of N3-methyladenine and N7-methylguanine adducts, was precipitated by the addition of 200 μL of saturated sodium acetate and 500 μL of cold absolute ethanol. Resulting suspension was vortexed and kept at −20 °C for 2 h for precipitation. Following the precipitation DNA was centrifuged (11,000 × *g*, 5 min, 4 °C) and stored at −20 °C, as a methylated DNA free of depurinated adducts. This fraction was thawed at room temperature, washed by 70% ethanol and subsequently dissolved in 1.5 mL of 50 mmol/L Tris (pH 7.2) containing 1 mmol/L magnesium chloride. Resulting DNA solution was treated for 28 h at 37 °C with enzyme mixture containing nuclease P1 (24 U/mL), alkaline phosphatase (3 U/mL), DNase I (10 U/mL) and snake venom phosphodiesterase (0.3 U/mL) to obtain free O^6-MeG adduct, based on the procedure published by Koskinen et al. with small modifications [38]. After the elapsed time, reaction was stopped by keeping the mixture in a boiling water bath for 10 min and cooling down in an ice bath. Denatured proteins were spun down (1000 × *g*, 5 min, ambient temperature) and supernatant was transferred into a new vial. For analysis of the DNA cleavage products, vacuum rotary evaporator (Centrivap Cold Trap) was used for 10-fold concentration of the samples. 1 mL of methanol was added. Then 5 μL of the mixture was injected into HPLC column for separation and quantitation of the O^6-MeG adduct.

2.5. Chromatographic instrumentation

The HPLC equipment comprised of a solvent delivery module LC-6A (Shimadzu, Japan) coupled with UV–vis variable wavelength detector model SPD-2A (Shimadzu). HPLC instrument was equipped with a manual injector (ECOM Ltd., Czech Republic) and a 5 μL injection loop.

The RP-HPLC analyses were performed by using Synergi Polar-RP C_{18} column, 250 mm × 4.6 mm i.d., 4 μm particle size (Phenomenex, USA). The optimized chromatographic conditions were following: column temperature 20 °C, mobile phase 50 mmol/L ammonium formate with the addition of 2% isopropanol (v/v), pH 5.4, flow rate 1 mL/min, isocratic elution, UV detection at 254 nm.

For the data acquisition and evaluation, the Clarity Workstation Software (DataApex, Czech Republic) was employed.

2.6. Mass spectrometry

A RP-HPLC-MS was used for identification of investigated DNA-adducts formed by methylation of DNA with DMS. The ESI(+) mass spectra were obtained with the instrument Q-TOF Micro (Waters, USA). For the ESI(+) technique, the eluent from the column was splitted in front of MS, 100 μL/min was directed to MS and the rest to waste. The MS–ESI(+) instrumental parameters were used as follows: capillary voltage 2.7 kV; sample cone voltage 42 V; extraction cone voltage 1.0 V; source temperature 100 °C; desolvation gas temperature 250 °C, the flow rates of desolvation gas and cone gas (both nitrogen) were set at 50 and 250 L/h, respectively. Mass spectra were acquired in 100–1000 *m*/*z* range. Obtained data were processed with MassLynx, version 4.0 software (Waters, USA).

3. Results and discussion

In the presented study, dimethyl sulphate was used as a DNA-methylating reagent for *in vitro* experiments, as it is capable to directly methylate bases of DNA without any activation, particularly at N3 position of adenine, N7 and O^6 positions of guanine. Arising methylated DNA adducts were separated by HPLC-UV and identified using authentic standards of methylated DNA bases and

Table 1
Intra-day ($n = 6$) and inter-day ($n = 6$) precision and accuracy of the tested method

Added concentration (ng/mL)	Intra-day assay ($n = 6$)			Inter-day assay ($n = 6$)		
	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)
0.50	0.48 ± 0.0083	1.73	99.02	0.49 ± 0.0143	2.91	98.34
1.00	0.98 ± 0.0682	6.96	97.95	0.97 ± 0.0736	7.58	96.51
4.00	4.09 ± 0.1052	2.57	102.44	3.98 ± 0.0900	2.26	99.61

[a] Expressed as relative standard deviation.
[b] Expressed as [(mean observed concentration/nominal concentration) × 100].

mass spectrometry employing electrospray ionisation in positive mode.

Preparation and isolation of unstable methylated bases, N3-methyladenine and N7-methylguanine, required specific procedures and conditions as described in Section 2.4.1. The isolation of O^6-methylguanine, the stabile methylated base, necessitated different approach and enzymatic digestion was applied as described in detail in Section 2.4.2. It is well-known that the most important adducts after DNA methylation, i.e. N3-methyladenine, N7-methylguanine and O^6-methylguanine, are significantly related to increased risk of mutagenic and/or carcinogenic effects [25,26]. The data obtained within this study can provide a useful background for *in vivo* methylation studies on animals exposed to known carcinogens or on monitoring of individuals exposed to environmental/occupational methylating chemicals, both qualitative and quantitative.

3.1. LOD, LOQ, precision and accuracy of the chromatographic method

As an important step in the method development, validation parameters (LOD, LOQ, precision and accuracy) of the chromatographic method were determined with UV/vis detection. The limit of detection (LOD) was found to be 0.1 ng/mL for N3-methyladenine and 0.2 ng/mL for both N7-methylguanine and O^6-methylguanine, calculated as the concentration giving signal-to-noise ratio 3 (S/N = 3). The limit of quantitation (LOQ) was found to be 0.5 ng/mL for all measured compounds, (S/N = 10). To determine the intra-day precision of the method, three standards at concentration levels 0.50, 1.00 and 4.00 ng/mL were analyzed six times on the same day (Table 1). To determine the stability of the standard stock solutions expressed as inter-day precision, three standards at the concentration levels 0.50, 1.00 and 4.00 ng/mL were repeatedly run for 6 days (Table 1). The data on precision were expressed as the relative standard deviation (R.S.D.) and values on accuracy were calculated as follows (mean observed concentration of the standard substance/nominal concentration of the standard substance) × 100 (Table 1).

3.2. Evaluation of the calibration parameters

A method of internal standard (IS) calibration was employed for quantitation of the methylated bases. Calibration parameters were investigated by plotting the peak area ratios $A_{\text{methylated base}}/A_{\text{IS}}$, versus the nominal concentrations of the appropriate methylated base. Calibration parameters were obtained by analyzing the calibration standards prepared as described in Section 2.3. Each calibration curve for the appropriate methylated base was measured in triplicate ($n = 3$). The calibration curves were constructed at the concentration levels 0.5, 1, 2, 3, 5, 10, 20 and 50 ng/mL for N3-methyladenine, N7-methylguanine and O^6-methylguanine. Weighted linear regression analysis was used to determine the slope, y-intercept and correlation coefficient (R^2). For all calibration standards, the calibration curves were linear over the whole concentration range 0.5–50 ng/mL with the correlation coefficients R^2 equal to 0.9992 for N3-methyladenine, 0.9997 for N7-methylguanine and 0.9995 for O^6-methylguanine (Table 2).

3.3. Determination of methylated adducts N3-MeA, N7-MeG and O^6-MeG

In this study, the relevant DNA adducts, N3-methyladenine, N7-methylguanine and O^6-methylguanine, resulting from DMS methylation of DNA, were monitored and evaluated. N3-methyladenine and N7-methylguanine, the dominant peaks 1 and 2 in Fig. 2A (Fig. 2B represents untreated DNA after the depurination), were found in chromatogram of the depurinated fraction of the methylated DNA (Section 2.4.1). Both dominant N-methylated compounds, N3-methyladenine and N7-methylguanine, were identified by matching the retention times of the respective peaks with the authentic standards and also by mass spectrometry operating in a positive electrospray ionisation mode (Figs. 3 and 4, respectively). As shown in Fig. 3, N3-methyladenine provided pseudomolecular ion $[M+H]^+$ m/z 150.1 almost exclusively with no significant fragmentation. The fragmentation pattern of protonated N7-methylguanine observed in this study (Fig. 4) was consistent with data presented elsewhere [39]. In addition to pseudomolecular ion $[M+H]^+$ m/z 166.1, also characteristic fragment $[M\text{-}NH_3]^+$ m/z 149.1 was apparent in the ESI(+) spectrum. The third adduct resulting from DMS methylation, O^6-methylguanine, obtained by enzymatic cleavage (Section 2.4.2), was found in trace amount together with the parent bases of the monitored adducts, adenine and guanine, as shown in Fig. 5. The identity of all three compounds, i.e. adenine, guanine and O^6-methylguanine, were verified by the addition of the authentic standard substances into the sample.

Presented work is focused on the formation of the most frequent DNA adducts, N3-methyladenine, N7-methylguanine and O^6-methylguanine, and does not cover other minor methylation products. The discovered levels of methylations reflect a total sum of all three compounds mentioned above. Concerning the relative proportions of methylation, the most abundant methylation product in DNA can be ascribed to N7-methylguanine, determined at the level of 61.53 ± 2.97% (mean ± S.D.) with R.S.D. equal to 4.8, accounting for 2.01% of total guanine. The second most abundant adduct, N3-methyladenine, was found at the level of 38.19 ± 2.99%

Table 2
Calibration parameters of the method tested with standards, N3-methyladenine, N7-methylguanine and O^6-methylguanine

Substance	Calibration curve equation	R^2
N3-MeA	$y = 0.0621x + 0.0023$	0.9997
N7-MeG	$y = 0.0477x + 0.0037$	0.9992
O^6-MeG	$y = 0.0601x + 0.0737$	0.9995

Values of correlation coefficients, slopes and y-intercepts are summarized for each standard substance.

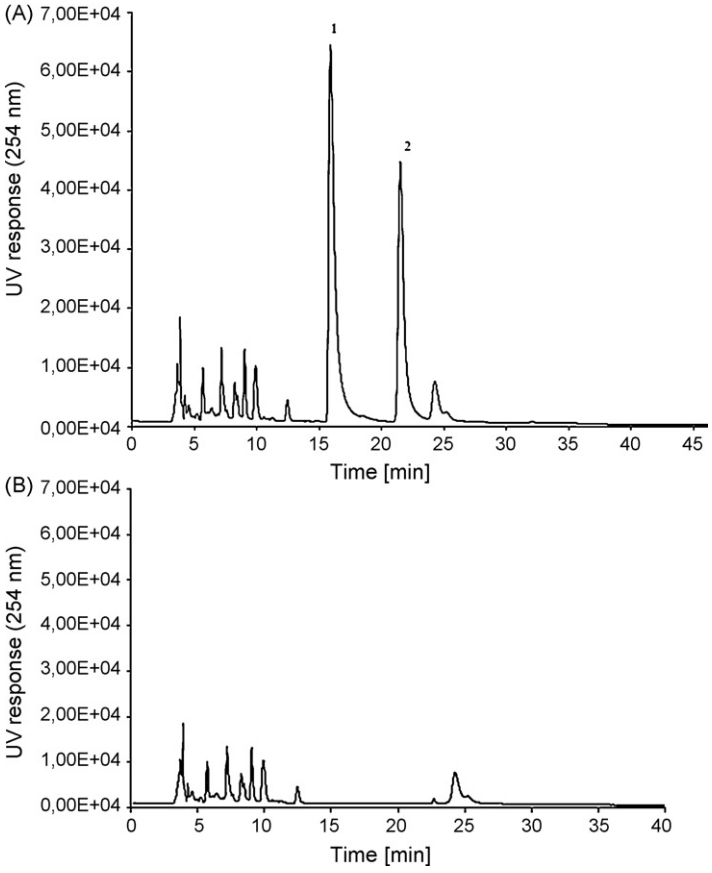

Fig. 2. (A) HPLC chromatogram of depurinated fraction. Peak 1 (RT 15.92 min) represents N3-methyladenine and peak 2 (RT 21.52 min) N7-methylguanine. Identity of both N3-methyladenine and N7-methylguanine was succesfully verified not only by retention times of authentic standards but also by MS (Figs. 3 and 4). (B) HPLC chromatogram of depurinated fraction from the untreated DNA samples.

with R.S.D. equal to 9.6, refering to 1.32% of total adenine. The minor lesion, O^6-methylguanine, was detected at levels of $0.29 \pm 0.02\%$ with R.S.D. equal to 5.1 that accounts for $9.42 \times 10^{-3}\%$ of total guanine, after *in vitro* methylation with DMS. Based on present results, the N7-/O^6-MeG ratio is equal to 212 for S_N2-methylating agent DMS. In comparison to another S_N2-methylating agent MMS, the ratio for DMS is a slightly higher than was found for MMS (N7-/O^6-MeG ratio = 200) [40]. The above ratio appear to depend on

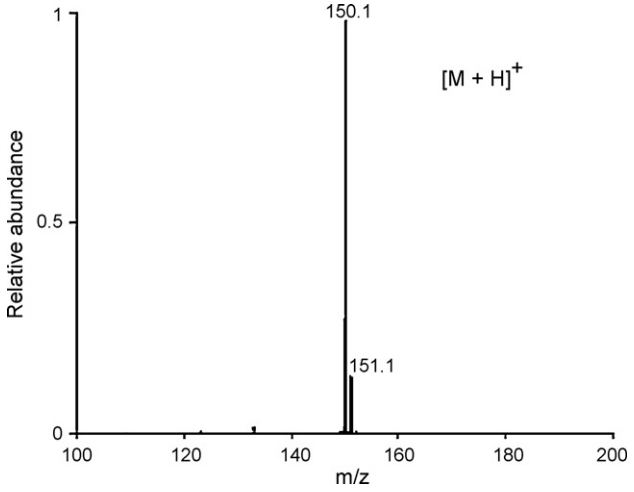

Fig. 3. ESI(+)–MS spectrum of N3-methyladenine.

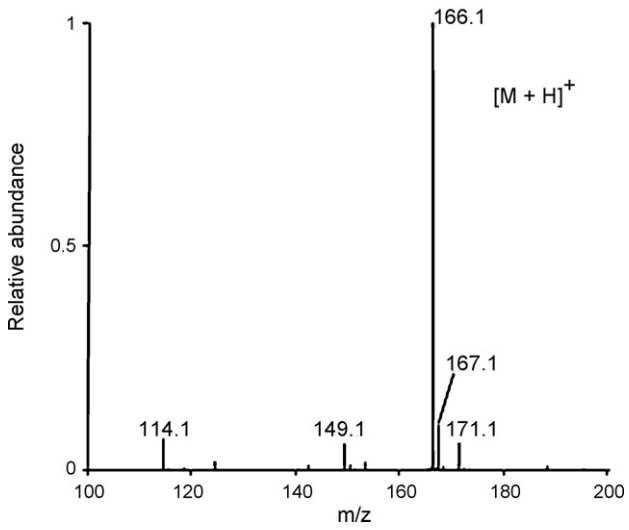

Fig. 4. ESI(+)–MS spectrum of N7-methylguanine.

particular reacting conditions (aqueous solution, pH, duration incubation), but not on the double- or single-strandedness of DNA [41,42].

Discussing the similar studies carried out in the past, it should be reminded that Zhang et al. reported a comprehensive LC–MS/MS analytical method employing ESI(+) for quantitation of both N7-methylguanine and O^6-methylguanine after treatment of DNA with MNU and MMS followed by DNA cleavage by acid hydrolysis. This approach led to release of the above mentioned adducts in one step [40]. Their study also considered the option to cleave DNA by enzymatic process. In this respect, it is difficult to perform enzymatic hydrolysis for both N7-methylguanine and O^6-methylguanine together since it requires long-time 37 °C digestion. It would imply a great loss of N7-methylguanine, the unstable adduct.

Based on our study, it is feasible to apply enzymatic cleavage. However, it is of a great importance to separate the spontaneously released adducts, N3-methyladenine and N7-methylguanine, as a depurinated fraction before subsequent enzymatic digestion. The remaining fraction, DNA free of the released unstable adducts, is suitable for cleavage by enzymes.

Furthemore, our study shows that DNA methylation can lead to generation of significant quantities of N3-methyladenine besides of prevailing N7-methylguanine. It was found that N3-

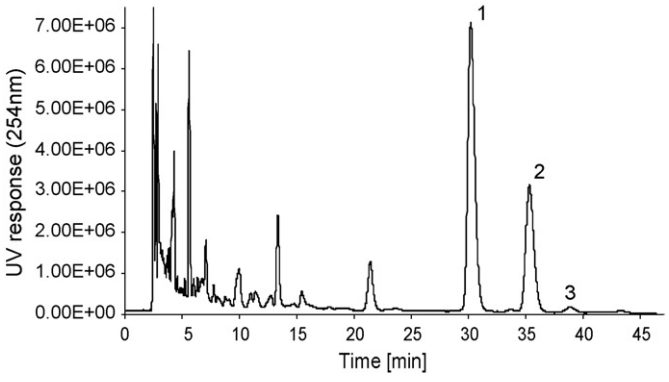

Fig. 5. HPLC chromatogram of enzymaticaly cleaved fraction containing parent bases of investigated adducts. Peak 1 (RT 30.28 min) represents adenine and peak 2 (RT 35.47 min) represents guanine, as they were acquired after cleavage. Peak 3 (RT 38.92 min) was established as O^6-methylguanine, the minor product upon DNA methylation by DMS.

methyladenine/N7-methylguanine ratio is about 0.65. It is very usefull to determine the two above mentioned adducts simultaneously since the both adducts have similar biological action and can lead to related mutagenic/carcinogenic effects in organisms [25,32]. For the purpose of human biomonitoring, the exact data on stabilities of N3-methyladenine and N7-methylguanine bound to the DNA chain are of substantial importance. Methylation at both N3 position of adenine and N7 position of guanine are known to be prone to depurination due to the delocalized plus charge formation at the heterocyclic ring, leading to N-glycosidic and phosphodiester bonds weakening [25,26].

Suprisingly, the N3-methyladenine adduct has not been determined simultaneously with N7-methylguanine and O^6-methylguanine after methylation yet. Only few authors studied similar systems and analysed one or two of the above mentioned methylated bases. For instance, Kopplin et al. has investigated urinary excretion of the N3-methyladenine in smokers after exposure to tobacco smoke [43]. Additionaly, Engelward et al. has appreciated biological consequences of N3-methyladenine in the mammalian genome after methylation by MMS in comparison with methylation by methyl-lexitropsin, a selectively methylating agent of N3 position of adenine [44].

Existing literature data on DNA methylation shows significant differences in methylating potency of different methylating agents. For instance, Hoffmann et al. compared potencies of induction of point mutations and genetic duplication of the methylating agents dimethyl sulphate (DMS, S_N2-methylating agent) versus methylazoxymethanol (MAM, S_N1-methylating agent) on bacterial DNA. The difference was reflected in the N7/O^6 ratio of methylated products of guanine [5]. Furthermore, the methylation power of MMS (S_N2-methylating agent) and MNU (S_N1-methylating agent) was reported [40]. Zhang et al. published approximately 200 times stronger effect in methylation of N7-position of guanine than in O^6-position upon MMS treatment. On the other hand MNU methylates N7-position of guanine only 6.5 times more abundantly than that of O^6-position of guanine. For illustration, the levels of 7-methylguanines, analyzed by ^{32}P-postlabelling method, were higher in laryngeal tumor samples than in blood leucocytes [45].

4. Conclusion

In summary, a new approach for monitoring of methylated DNA adducts has been established. The method involves: (a) simultaneous determination of spontaneously released N3-methyladenine and N7-methylguanine from DNA as products of exogenous methylation; (b) further enzymatic hydrolysis of remaining DNA free of released N-methylated adducts and (c) determination of O^6-methylguanine enzymaticaly released from methylated DNA.

The described method employes reversed-phase high performance liquid chromatography coupled with UV–vis and mass spectrometry with ESI(+) to detect, identify and quantify the most abundant products of DNA methylation. This approach can be useful in future studies aimed to the investigation of various types of alkylations of DNA and their relationship to mutagenity or carcinogenity.

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