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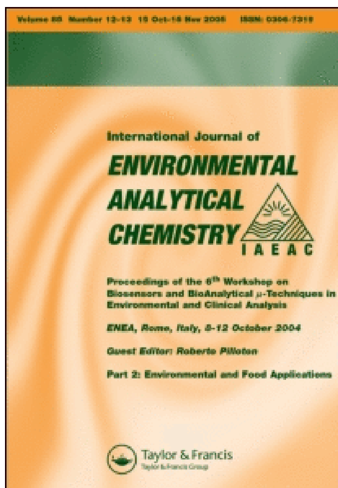

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Assessment of N-Nitrosodimethylamine Dna Adducts in Rat Hepatocytes by High Performance Liquid Chromatography and Immunosorbent Assay

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ASSESSMENT OF N-NITROSODIMETHYLAMINE DNA ADDUCTS IN RAT HEPATOCYTES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND IMMUNOSORBENT ASSAY

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A rapid and efficient enzyme-linked immunosorbent assay (ELISA) for quantification of DNA adducts was developed using affinity-purified, polyclonal antibodies directed against O^6-methylguanosine (O^6-meGuo) coupled to bovine serum albumin (BSA). The specificity of the antibodies was characterized by competitive inhibition assay using a number of nucleosides, nucleobases and their analogues. The O^6-methylguanine (O^6-meG) adduct was quantified in rat hepatocytes pretreated *in vitro* with N-nitrosodimethylamine (NDMA) by high performance liquid chromatography (HPLC) and compared to the data obtained by ELISA, using amplification by the avidin-biotin (AB) system. The low, 5 mM NDMA, dose induced a low cell cytotoxicity and the highest formation of the O^6-meG-DNA adduct. Thus, an inverse dose-response correlation was obtained by both methods for the cell viability determined as a function of NDMA concentration and subsequent formation of the O^6-meG-DNA adducts, reflecting possibly the involvement of active cell metabolism in enzymatic activation of NDMA. Quantitation of the adduct formation vs concentration of NDMA used for the incubation of cells, expressed in pg O^6-meG/µg DNA, showed a good correlation (r = 0.992) for both analytical methods.

KEY WORDS: N-nitrosodimethylamine, DNA adducts, ELISA, HPLC, rat hepatocytes.

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INTRODUCTION

N-nitroso compounds, including N-nitrosodimethylamine (NDMA), are ubiquitous pollutants encountered in the environment, particularly in numerous chemical, agricultural and consumer products. They represent a major class of important chemical mutagens, carcinogens, teratogens, and immunotoxic substances, which have been described as a serious hazard to human health[1-4]. The predominant but not directly mutagenic adduct is at the nucleophilic position 7 of guanine, whereas the minor alkylation adduct, O^6-methylguanine (O^6-meG), is a highly pro-mutagenic adduct[5-6]. A good correlation exists between the O^6-meG-DNA formation and induction of tumors in animals treated with NDMA or with N-methyl-N-nitrosourea[7]. Formation of O^6-meG-DNA causes a defect in the pairing of nucleic bases conducting to the erroneous incorporation of thymidine during the replication of DNA[8].

Sensitive immunoassays for the detection of different carcinogen adducts of DNA, have been developed by a number of investigators, as recently reviewed by Wild[9] and Santella[10]. These include competitive radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) using polyclonal and monoclonal antibodies. Other techniques involve HPLC, ^{32}P-adduct assay, immunohistochemical and immunocytochemical staining[9-11]. Application of immunoassays for quantification of carcinogen DNA adducts has, however, been difficult due to the relatively low sensitivity of direct ELISA assay and the relatively higher affinity of monoclonal antibodies to the modified base with the sugar moiety attached[9].

The objective of this study was to compare the HPLC and the direct ELISA immunoassay, amplified by the avidin-biotin (AB) system, used for quantification of DNA adducts in NDMA-exposed rat hepatocytes. For this purpose, we used an affinity-purified, polyvalent antibodies developed against O^6-methylguanosine-bovine serum albumin (O^6-meGuo-BSA) conjugate. Our data show that ELISA-AB can be applied as a useful, rapid and inexpensive assay for monitoring the exposure to genotoxic chemicals.

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MATERIALS AND METHODS

Rat hepatocytes

Rat hepatocytes were isolated from male Sprague-Dawley (200–250g) rats by collagenase perfusion[12]. Briefly, the procedure involved an *in situ* two-step perfusion of the liver through the portal vein. Preperfusion with Ca^{2+}-free Hanks' balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, N.Y.) was followed by perfusion with collagenase (60 U/ml, Gibco) in Ca^{2+}-containing HBSS. Liver cells were released in Williams' Medium E (WME) (Gibco) supplemented with 10% fetal calf serum (FCS) (Gibco) and 0.005% gentamycin. The cells were washed and counted. The suspensions of freshly isolated hepatocytes routinely contained >80% viable cells as estimated by trypan blue exclusion. Suspensions of 4.5×10^6 hepatocytes in 4.5 ml WME- 10% FCS were seeded in 25 cm^2 dishes and incubated for 2 hours at 37°C in an atmosphere of 95° 0_2 and 5% CO_2, to permit cell adherence. Then, the samples were washed for removing dead cells.

Treatment of cells with NDMA

NDMA (Sigma, St-Louis, MO) was used for the *in vitro* exposure of rat hepatocytes at four different concentrations: 5, 10, 15, and 20 mM, in WME supplemented with 1% FCS (Gibco). The cells were incubated for 2 hours at 37°C. Cell viability was determined in triplicate by the trypan blue exclusion test. Next, the cells were washed with HBSS, detached from the culture dish and placed in test tubes for subsequent DNA isolation.

Isolation of DNA

DNA was isolated from control and NDMA-treated hepatocytes by chloroform/isoamyl alcohol (24:1) (Sigma) extraction and purified by RNase (Sigma) treatment as previously described[12,13]. The purity of the DNA was confirmed by spectroscopic analysis; the A260/280 ratio was > 1.8. Next, the DNA samples were directly analyzed by enzyme-linked immunosorbent analysis (ELISA) or were hydrolyzed and O^6-meG was determined by high performance liquid chromatography (HPLC).

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Analysis of nucleic bases by HPLC

Rat hepatocyte DNA was hydrolyzed with 100 µl 0.1N HCl at 70°C for 30 min. The hydrolysate was then transferred to ultrafree-MC-Ependorf filters (0.45 µ) (Millipore, Mississauga, Ontario, Canada) and centrifuged for 5 min at 2200 g. After centrifugation, O^6-meG was separated by HPLC under conditions showing no interference with guanine, adenine, N-7 methylguanine (7-MeG) and other pyrimidine oligonucleotides[14]. The HPLC instrument was a Perkin-Elmer (series 3) chromatograph (Norwald, Connecticut) using a strong cation exchange column (Partisil—10 SCX, 25 cm × 4,6 mm i.d.) (Whatman Inc., Clifton, N. J.) and a guard column packed with Partisil 10 SCX absorbent (Whatman). For detection, the instrument was coupled to a spectroflow 980 fluorescence detector (ABI Analytical Inc., Kratos Division, N.J.). The excitation and emission wavelengths were set at 286 and 345 nm (filter) respectively. The mobile phase was a 0.2 M ammonium formate buffer, pH 7.2 (Fisher Scientific, Fair Lawn, N.J.) containing 3% acetonitrile (Caledon Laboratories Ltd., Georgetown, Ontario, Canada). It was filtered over a Millipore 0.45 µm filter and degassed with helium for 30 min before use. The flow rate was kept at 1.5 ml/min. The peaks were integrated with an integrator, model 3392A (Hewlett-Packard, Palo Alto, CA).

Antibody production and purification

O^6-Methylguanosine (O^6-meGuo) was synthesized from 6-chloro-2-aminopurine riboside according to Gerchman *et al.*[15] and its purity was found to be more than 99%, as confirmed by the HPLC assay. Next, O^6-meGuo was coupled to bovine serum albumin (BSA) by the

method of Erlanger and Beiser[16]. One hundred mg of O^6-meGuo and 100 mg of BSA were used in the conjugation. The coupling was confirmed by U.V. spectroscopy and the concentration of the O^6-meGuo-BSA conjugate was determined. Three to five albino New Zealand white rabbits (Institut Armand-Frappier, Laval-des-Rapides, Québec, Canada) were immunized with O^6-meGuo-BSA conjugate by five consecutive injections. The first inoculum consisted of 300 μg O^6-meGuo-BSA conjugate in phosphate-buffered saline and emulsified in complete Freund's adjuvant. The conjugate was injected intradermally at 20 sites on the back of each rabbit. Four booster i.m. injections were given in incomplete Freund's adjuvant. Blood samples were collected at 2,4,6 and 8 weeks after each injection and the serum titer was controlled at each interval. Antiserum obtained from the first blood sample after the last injection (i.e. after 8 weeks) was used in the present study. The O^6-meG specific-antibodies were affinity-purified on epoxy-activated Sepharose 6B (Pharmacia, Uppasala, Sweden) according to the method of Müller and Rajewsky[17]. Briefly, 30 mg of O^6-meG were incubated with 1.5 g of epoxy-activated Sepharose 6B, in 3 ml of 0.01 N NaOH for 20 h at 45°C. The gel was washed with water and incubated with 5 ml of anti-O^6-meGuo-BSA serum. After centrifugation, the specific antibody was eluted with 1 M acetic acid and dialyzed against phosphate-buffered saline (PBS). The antibodies were stored at – 20°C.

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Competitive inhibition of O^6-meG: antibody complexing by nucleotide analogues

The specificity of the affinity-purified, polyclonal anti-O^6-meG antibody was characterized by a competitive immunosorbent assay (ELISA) using ten different nucleotide analogues[17]. All nucleobases and nucleosides were obtained from Chemsyn Science Laboratories (Lenexa, Kansas, USA). Briefly, the competitive ELISA was performed in 96-well polystyrene microtiter plate (Becton Dickinson, Mississauga, Canada), using 1 μg per well of nucleoside-protein conjugate per ml of Tris-buffered saline (TBS). The remaining free binding sites were blocked with a solution of gelatin in TBS. Samples of 100 μl antibody were diluted hundred fold and this solution was premixed with either TBS or with the inhibitor, i.e. nucleobase- or nucleoside analogues. After incubation and washing, the bound antibody was detected by the addition of 100 μl horseradish peroxidase-coupled anti-rabbit lgG (Bio-Rad Laboratories, Richmond, CA). The enzyme activity was determined in triplicate using a Flow Titertek Multiscan spectrophotometer at 405 nm. The enzyme substrate was 2,3′-azino di-(3 ethyl-benzthiazolidine sulfonate (ABTS) with hydrogen peroxide (Kirkegaard and Perry Laboratory, Gaithersbourg, MD) as described previously by Fournier *et al.*[18].

Avidin-biotin immunosorbent assay (ELISA-AB)

The avidin-biotin enzyme-linked immunosorbent assay (ELISA-AB) was applied for the detection of O^6-meG adducts in DNA samples from NDMA-exposed rat hepatocytes. The O^6-meG was used to construct the standard curve and the linear range of the assay was

between 0.5 to 5.0 ng. As an antigen, 100 μl O^6-meGuo-BSA conjugate (5 μg/ml) or 2 μg DNA purified from control and NDMA-exposed cells (20 μg/ml) in TBS, pH 7.5, were added and the samples were kept for 1 h at room temperature and washed. Unoccupied binding sites were saturated by addition of 200 μl of a 0.5% (w/v) solution of gelatine in TBS; after 30 min, the wells were washed with TBS. All reagents were diluted in TBS containing 0.5% (w/v) gelatin. A stock solution of anti-O^6-meG antibody (34.4 μg/ml) was diluted 1:100 with TBS-gelatin and added to the microplates. After 2 hours of incubation, the wells were washed once with TBS-Tween (0.05%) and three times with TBS only. Bound antibody was detected either by addition of 100 μl of horseradish peroxidase-coupled anti-rabbit IgG (Bio-Rad) and classical ELISA procedure, as described previously[18], or by the avidin-biotin amplification system (ELISA-AB)[19]. Briefly, the bound antibody was detected by the addition of 100 μl biotin-labeled anti-rabbit IgG (Bio-Rad) to the wells. The mixture was incubated for 1 h and the plates were washed, followed by addition of 100 μl of avidin-horseradish peroxidase. The plates were washed again after 30 min and the enzyme activity was measured as described above.

Statistical analysis

Data obtained in each experiment are presented as the means ± standard error (S.E.). A linear regression analysis was performed to ascertain the correlation between the data obtained by the two different techniques (ELISA and HPLC)[20].

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RESULTS AND DISCUSSION

Specificity of affinity-purified, polyclonal anti-O^6-meG antibodies

The specificity of the anti-O^6-meG antibodies was examined by preincubation with increasing concentrations of nucleoside analogues, nucleobases and free bases followed by the competitive ELISA assay (Table 1). Thus, the data in Table 1 revealed a good cross-reaction of the antibody with the O^6-free base analogues. No cross-reaction of the antibodies with normal nucleosides was noted (not shown). The detection limit of the assay, expressed as a 20% inhibition of the competitive ELISA using O^6-meGuo-BSA conjugate, was 0.0015 μg for O^6-meG, O^6-meGuo and O^6-etG. For other analogues, such as 7-meG, 7-meA and 8-meA, the inhibition showed a much lower affinity, as compared to the O^6-analogues (Table 1). The relative amounts of nucleosides and nucleobases required to produce 50% inhibition of the antibody—antigen binding in the competitive ELISA assay are given in Table 1. Overall, the anti-O^6-meG antibodies primarily recognize adducts with O^6-methyl groups, with O^6-meG being an important determinant. In addition, the specificity of the antibodies was further validated *in situ*; an enzymatic digestion of DNA or RNA in NDMA- pretreated hepatocytes resulted in significant reduction of the protein A-gold immunochemical complexes[11].

Table 1 Competitive inhibition of the affinity-purified, polyclonal anti-O^6-meGuo-BSA antibody by nucleotide analogues.

Inhibitor[&]	*Abbreviation*	*µg of inhibitor required for 50% inhibition in ELISA*
O^6-Methyldeoxyguanosine	O^6-medGuo	0.004
O^6-Methylguanine	O^6-meG	0.008
O^6-Methylguanosine	O^6-meGuo	0.05
O^6-Ethylguanine	O^6-etG	0.125
7-Methylguanine	7-meG	>80
7-Methyladenine	7-meA	>80
3-Methyladenine	3-meA	>100
8-Methyladenine	8-meA	>100
O^4-Methylthymidine	O^4-meT	>100
O^2-Methylthymidine	O^2-meT	>100

[&] The nucleotide analogues are in order of decreasing specificity of the analogues to inhibit the O^6-meGuo-BSA—antibody complexing. The results are means of triplicates.

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The use of the avidin-biotin amplification system for the quantitative ELISA-AB immunoassay of the O^6-meG-DNA adduct was compared with the simple immunoassay (Table 2). The positive reaction of anti-O^6-meG antibody with either the O^6-meGuo-BSA conjugate or with the DNA isolated from the NDMA-treated hepatocytes was compared by the ELISA and the ELISA-AB. As shown in Table 2, an elevated background in the ELISA-AB amplification system was observed. This was, however, compensated by the substantially increased sensitivity of the assay, as compared to the standard ELISA.

Relationship between cell viability and O^6-meG-DNA adducts in NDMA-exposed rat hepatocytes

Rat hepatocytes were treated *in vitro* with four different NDMA concentrations: 5, 10, 15 and 20 mM NDMA in Williams' 1% FCS, to study the effect on cell viability and in parallel the amount of O^6-meG produced in the DNA of these cells. Cell viability was determined at the end of the treatment and the O^6-meG adduct was quantified both by the ELISA-AB and the HPLC assay for each NDMA concentration (Figure 1). Results show an inverse

Table 2 Determination of O^6-meG-DNA adducts by ELISA and ELISA-AB.

Antigen[&]	*ELISA@ O.D. 405 nm*	*ELISA-AB@ O.D. 405 nm*
BSA	0.042 ± 0.003	0.220 ± 0.047
O^6-meGuo-BSA	0.382 ± 0.009	1.940 ± 0.032
DNA	0.053 ± 0.003	0.370 ± 0.015
DNA/5mM NDMA	0.098 ± 0.009	1.630 ± 0.022

[&] BSA and DNA from control hepatocytes were the negative controls and the positive controls two different antigens were used: the O^6-meGuo-BSA and DNA extracted from the cells exposed *in vitro* to 5 mM NDMA.
@ The result are means of triplicates ± S.E.

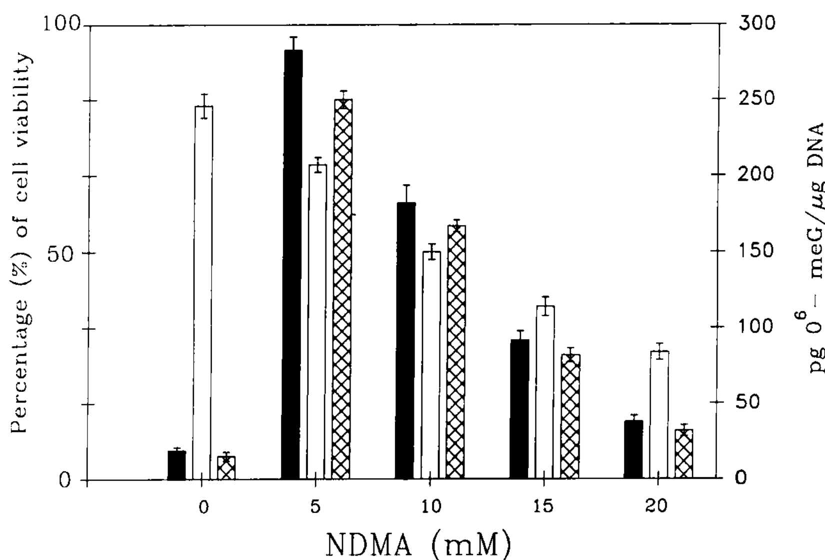

Figure 1 Correlation of the O^6-meG-DNA adducts formation, determined by ELISA-AB (double-crossed bars) and HPLC (black bars), and the cell viability (open bars) upon *in vitro* exposure of rat hepatocytes to NDMA.

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relationship between the NDMA concentration and the quantity of the O^6-meG-DNA adduct (Figure 1). There was, however, a good correlation between the viability of the NDMA-exposed rat hepatocytes and the concentration of the chemical. The lowest NDMA concentration used for incubation appeared to be the less cytotoxic for the hepatocytes (Figure 1).

Validation of the ELISA-AB assay by HPLC

The ELISA-AB immunoassay used to determine O^6-meG-DNA adduct formation in rat hepatoctyes exposed *in vitro* to NDMA was validated by HPLC. For this purpose, the samples of DNA isolated from NDMA-exposed hepatocytes were analyzed by both ELISA-AB and HPLC (Figures 1–3). As shown in Figure 2, analogous retention time was noted for the O^6-meG, detected in standard material, and in the hydrolysate of the DNA isolated from the hepatocytes exposed to 5 mM NDMA. Quantification of the O^6-meG nucleoside analogue by both methods is presented in the previously described Figure 1. The correlation coefficient (r) was calculated for the results of these assays (Figure 3). A good correlation (r) = 0.992, was obtained, based on calculations for all NDMA concentrations used for the incubation of cells, from five determinations analyzed by these two methods (Figure 3). Overall, validation of the ELISA-AB immunoassay with HPLC revealed a good agreement for these two methods, thereby suggesting applicability of both procedures for determination of O^6-meG DNA adducts.

High specificity of antibodies for the analysis of 0-alkylated deoxyguanosines is especially important in view of low levels of adducts resulting from environmental exposure of

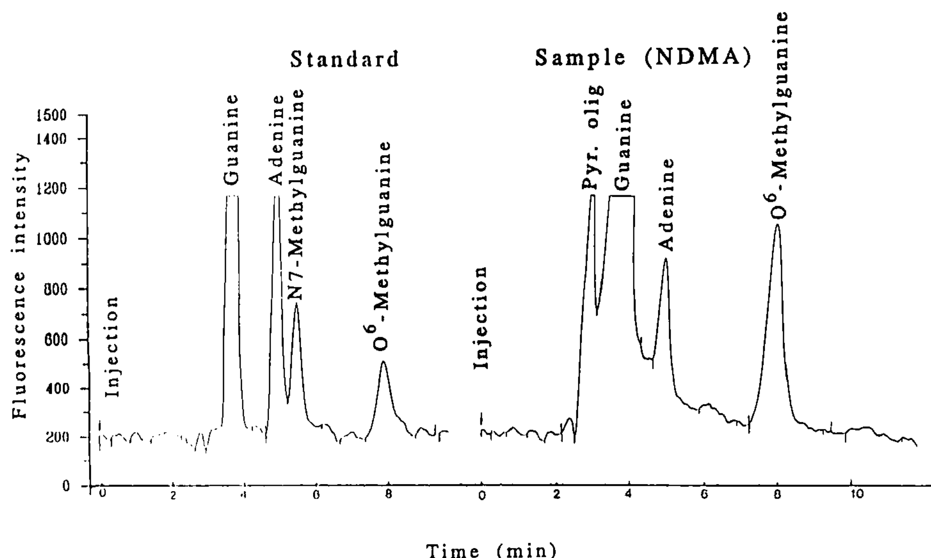

Figure 2 Separation of nucleobases and nucleobase analogues by HPLC.

man to alkylating agents. High sensitivity can be obtained by combination of enzymatic hydrolysis of DNA and purification of adducts by chromatography followed by RIA and ELISA, which have been applied mostly as competitive, equilibrium assays[9,10]. We show a high specificity of our affinity-purified, anti-O^6-meG polyclonal antibodies, which can be used for adduct detection by direct ELISA, amplified by the avidin-biotin (AB) system, in a 2 µg sample of unhydrolyzed DNA. Furthermore, enzymatic hydrolysis of DNA is required as a preparative step in the immunoassay for a majority of antibodies, due to the higher affinity of these antibodies for the sugar moiety attached to the modified base. Competitive ELISA analysis showed a good cross-reactivity of our antibodies with the free base, O^6-meG, which potentially permits an acid hydrolysis of DNA prior to the immunoassay. Such a good cross-reactivity was shown for two monoclonal antibodies[9]. Thus, both sensitivity and specificity of the assay is provided in our ELISA-AB system.

Another interesting observation in our studies is the inverse correlation between the quantity of O^6-meG adducts and cell viability after *in vitro* exposure of rat hepatocytes to different concentrations of NDMA. These results can be explained by the fact that high cytotoxic levels of NDMA possibly impaired its metabolic activation in hepatocytes, thus limiting the alkylation potential of the parent compound. The toxicity of nitrosamines was shown to be related to the activity microsomal amino-oxidases and cytochrome P-450[21]. Thus, intact metabolism, including the enzymatic activation of nitrosamines appears to be essential for DNA adduct formation. The *in vitro* cytotoxic effect of nitrosamines on primary hepatocyte cultures was postulated to be a potential indicator for the risk that these agents pose to man[22]. Our data, however, show that cell viability must be used with caution in such models, because when loss of hepatocyte viability increases, this parameter no longer adequately reflects the DNA-damaging potential of the nitrosamine.

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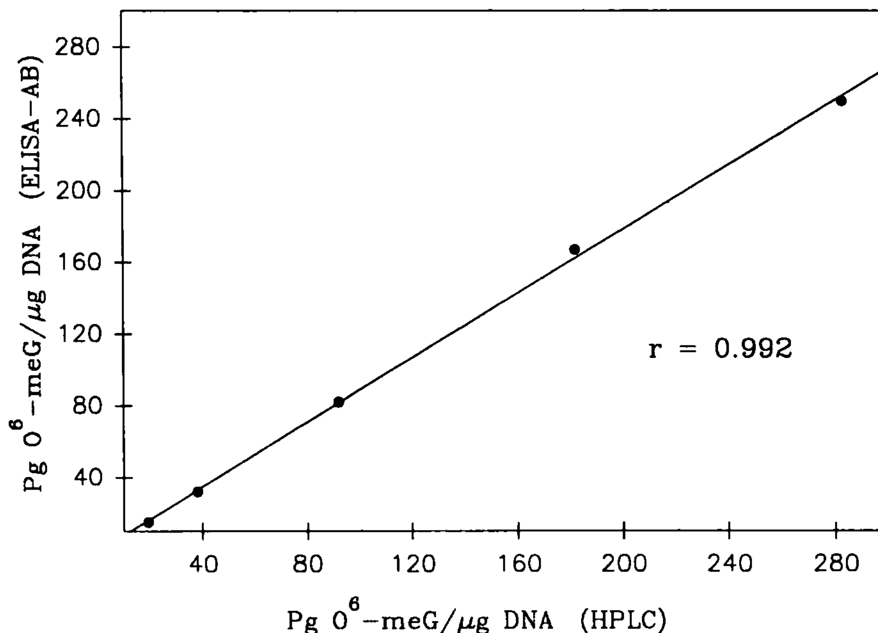

Figure 3 Comparative analysis and correlation coefficient for the quantification of O^6-meG-DNA adducts by the ELISA-AB and HPLC.

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Formation of O^6-meG and its persistence in cells has attracted the attention of many researchers, because of a correlation between the presence of this lesion and tumor induction[9,10]. Most chemical carcinogens appear to be genotoxic, and act via the covalent binding of either the carcinogen itself or its electrophilic metabolites to DNA. Thus, adduct quantification is considered as a specific and sensitive tool used for estimation of the internal dose at target macromolecular sites, as recently reviewed by several authors[9,10,23]. The ELISA-AB system that we developed in our laboratory provides such a tool with improved efficiency.

The sensitivity of an immunoassay of a specific, structurally modified DNA component mainly depends on the affinity and specificity of the antibodies directed against the respective purified product. Immunoassays are rapid, highly reproducible, and relatively inexpensive when compared to other assays, such as those requiring HPLC. By comparing the HPLC-fluorometric method described in this study to that of Genrke *et al.*[24], the former method is two times more sensitive than the latter. The described method is also six to twelve times more sensitive to that of Krstulovic *et al.* [25]. Moreover, the possibility of application of this method remains to be confirmed in humans. There is an advantage of HPLC-fluorometric method over HPLC-radioactive as far as the specificity for the quantification of O^6-meGua is concerned. But the former method is less sensitive than the latter. The described HPLC procedure is precise, accurate, and specific for the adduct O^6-meG. It compared favourably with a correlation coefficient of 0.992 with the ELISA-AB reported earlier. We recently demonstrated the applicability of the anti-O^6-meG antibody for ultramicroscopic localisation of NDMA-related DNA and RNA adducts in primary hepatocyte cultures[11].

In conclusion, NDMA-induced formation of O^6-meG-DNA adducts was quantified by an ELISA-AB system and validated by HPLC. The affinity-purified, polyclonal antibodies against O^6-meG-BSA conjugate appeared to be highly specific. O^6-meG was detectable by the ELISA-AB at a level of 0.2 pmole/µg DNA, which was confirmed by HPLC. Our ELISA-AB system allowed analysis of the correlation between cytotoxicity and genotoxic effects of NDMA in primary cultures of rat hepatocytes.

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