

Measurement of the malondialdehyde–2'-deoxy-guanosine adduct in human urine by immuno-extraction and liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry

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The major adduct of malondialdehyde with guanine, M₁G, was measured in human urine from non-smoking healthy individuals. M₁G is a mutagenic DNA lesion and a terminal product of lipid peroxidation *in vivo* that may be implicated in cancer related to lifestyle and diet. On the basis of a recently developed method for the quantification of M₁G as an excreted deoxynucleoside using immuno-extraction purification, chemical NaBH₄ reduction and liquid chromatography combined with atmospheric pressure chemical ionization tandem mass spectrometry, we demonstrate that the average 24 h excretion rate of M₁G-dR is about 12 ± 3.8 fmol kg⁻¹ (n = 5). Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: atmospheric pressure chemical ionization; liquid chromatography/tandem mass spectrometry; lipid peroxidation; biomarker; DNA adducts; malondialdehyde

INTRODUCTION

Oxidative stress has been widely implicated as a mechanism of disease, particularly arteriosclerosis and cancer. Non-specific oxidative injury of lipid membranes serves as an amplifier for initial free radical generation. Hence monitoring of specific lipid peroxidation (LPO) products may provide information about endogenous exposure to alkylating carcinogens and oxidative stress. Peroxidation of polyunsaturated lipids generates a range of reactive carbonyl compounds that possesses DNA-damaging potential, such as α,β -unsaturated aldehydes and ketoaldehydes.^{1,2} Malondialdehyde (MDA) is the most abundant product formed during LPO. MDA is mutagenic and carcinogenic in rodents.³ MDA can react with DNA bases to form stable adducts that are possible promutagenic lesions. The major product *in vitro* is the exocyclic pyrimido[1,2-a]purin-10(3H)-one, M₁G, formed after reaction of MDA with guanine.^{4,5} Base propenals, formed after deoxyribose oxidation, may be an important alternative route to endogenous M₁G formation.⁵

M₁G has been detected in cellular DNA from unexposed animals and humans.^{3,5–12} This measure provides information about steady-state levels expressed as a concentration of M₁G in the target tissue DNA. The M₁G lesion appears to be repaired by the nucleotide excision repair pathway.¹³ Measurement of the free modified nucleoside M₁G-dR excreted in urine may represent the whole-body-induced damage under steady-state conditions expressed as a rate of endogenous DNA damage. It follows that quantification of urinary M₁G-dR would provide a reliable and specific index of MDA exposure and thus be an important biomarker of lipid peroxidation *in vivo*. Measurement of excreted nucleobase in urine is regarded as less specific owing to contributions from RNA and possibly from diet.

Traditionally, MDA has been used as a biomarker for lipid peroxidation assessed by the reaction with thiobarbituric acid. This assay suffers from specificity problems when applied to body fluids and tissue samples.¹⁴ Measurement of F₂-isoprostanes, a group of prostaglandin-like peroxidation products of arachidonic acid, is a more reliable non-invasive alternative for assessment of lipid peroxidation *in vivo*.¹⁵ It seems, however, that isoprostane analysis is not straightforward owing to the occurrence of isomers.¹⁴

For assessment of LPO-associated DNA damage, it is important to use techniques that give specific chemical information about normal levels. Immunoaffinity purification in combination with tandem mass spectrometry (LC-MS/MS) provides specific identification and seems to meet the

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stringent sensitivity requirements. Recent methods did not provide a sufficient sensitivity to measure in rat urine, even after induction of lipid peroxidation.¹⁶ In this study we report an improved immuno-extraction liquid chromatography LC-/MS/MS method that is able efficiently to separate, concentrate and measure M₁G-dR in human urine. We also demonstrate that the use of atmospheric pressure chemical ionization (APCI), generally not considered useful for the analysis of nucleosides because of the labile glycosidic bond, is a highly sensitive alternative to the more frequently used electrospray technique.

EXPERIMENTAL

Materials

Acetonitrile was purchased from Fisher Chemicals (Leicester, UK), glacial acetic acid from Merck (Darmstadt, Germany) and methanol and 25% ammonia solution from J. T. Baker (Deventer, The Netherlands). Distilled water was purified through a Millipore ion-exchange system. All other solvents were of analytical grade. Sodium borohydride (99%) (NaBH₄) was purchased from Aldrich Chemical (Gillingham, UK) and ammonium sulfate ((NH₄)₂SO₄) from Merck. Oasis HLB C₁₈ solid-phase extraction columns were obtained from Waters (Wexford, Ireland). M₁G-dR and the stable isotope-labeled equivalent [¹³C,¹⁵N₂]M₁G-dR, used as an internal standard (ISTD), were synthesized as described previously.¹⁷ The concentrations of M₁G-dR standard and the stable isotope-labeled ISTD were determined by measuring the UV absorbance at 320 nm ($\epsilon = 5100 \text{ l mol}^{-1} \text{ cm}^{-1}$).

Urine samples

Human urine (24 h) was collected from six non-smoking healthy volunteers consuming free-of-choice diets. Urine collection was repeated after an interval of 1 week and aliquots were stored at -20°C prior to analysis. The volunteers were three males and three females aged 30–55 years. The urine was collected as part of another experiment approved by the local ethics committee.

Sample handling

ISTD (500 fmol) was added to 25 ml aliquots of urine. Protein was precipitated by the addition of one volume of a saturated ammonium sulfate solution followed by incubation on ice for 30 min. The precipitate was removed by centrifugation at 4000 rpm and 4°C for 10 min. Ammonium sulfate was removed on a pre-conditioned 200 mg C₁₈ solid-phase extraction column. The column was rinsed with 20 ml of water before M₁G-dR was eluted with 2 ml of methanol. The sample was dried in a vacuum centrifuge, dissolved in 100 μl of water and stored at -80°C until the next day. The concentrated urine sample was purified on Sepharose cross-linked with mono-clonal M₁G antibody specific against the exocyclic nucleobase moiety. Protocols for antibody preparation and immuno-extraction are given in detail elsewhere.^{10,12,17} The purified sample was chemically reduced with 10 μl of 10 mg ml⁻¹ NaBH₄.¹⁸ The reagent was removed on a 50 mg C₁₈ solid-phase extraction column, vacuum centrifuged to dryness and dissolved in a final

volume of 100 μl of 5 mM ammonium acetate buffer (pH 5.0) before LC/MS/MS analysis. The NaBH₄ reduction removed a double bond in the exocyclic ring of M₁G-dR and gave the 5,6-dihydro derivative (Fig. 1). This procedure consequently resulted in a 10-fold increase in LC/MS/MS sensitivity, hence the derivative was used for quantification.

Liquid chromatography

The Perkin-Elmer Series 200 HPLC system consisted of an autosampler two pumps and a vacuum degasser (Perkin-Elmer, Norwalk, CT, USA). The purified and concentrated urine sample was applied to a Zorbax Eclipse XBD-C18 column (50 \times 4.6 mm i.d., 3.5 μm film thickness) via the chromatographic system for fast-throughput isocratic elution in 5 mM ammonium acetate buffer (pH 5.0), with 3% acetonitrile delivered at a flow-rate of 0.8 ml min⁻¹. Aliquots of 50 μl , corresponding to 50% of the purified sample, were typically injected for LC/MS/MS analysis, with the derivative of M₁G-dR eluting after 3.06 min.

APCI-MS/MS

The LC system was coupled to an API 3000 triple-quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with a heated nebulizer ion source. Gas-phase ions were generated APCI with nitrogen as nebulizer, auxiliary and collision gas. The corona discharge needle was set at 3 μA , the source temperature was 475°C and the collision energy was 20 V. Data were collected in the positive ion mode using multiple reaction monitoring (MRM). Cleavage of the glycosidic linkage and neutral loss of the deoxyribose moiety from the MH⁺ ion generated the ion corresponding to the protonated nucleobase, BH₂⁺. The product ion spectrum of MH⁺ for the M₁G-dR derivative is displayed in Fig. 2, with the BH₂⁺ ion dominating. Characteristic MH⁺ \rightarrow BH₂⁺ transitions were used for quantification of the M₁G-dR derivative (m/z 306.05 \rightarrow 190) and the reduced form of the internal standard (m/z 309.05 \rightarrow 193) with dwell times set at 1000 and 250 ms, respectively. Standard solutions were chemically reduced in parallel to the purified urine samples and stored at -80°C prior to LC/MS/MS analysis. Quantification was calculated from the lower standard solutions made from 100 μl buffer aliquots spiked with 500 fmol of ISTD and M₁G-dR-standard at levels ranging from 0.1 to 25 nM. Since the levels subsequently found in urine were in the range 0–1.2 pM, a calibration curve for the range 0.1–10 pM is necessary, as is a rigorous quality control procedure, before the assay is used in, e.g., clinical trials and cross-sectional epidemiological studies.

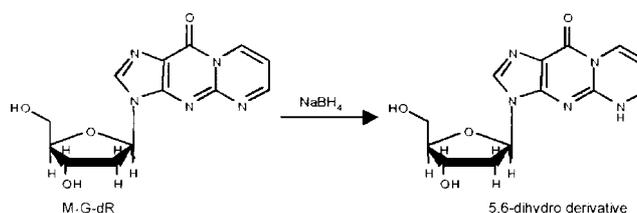


Figure 1. Chemical reduction of M₁G-dR to the 5,6-dihydro derivative.

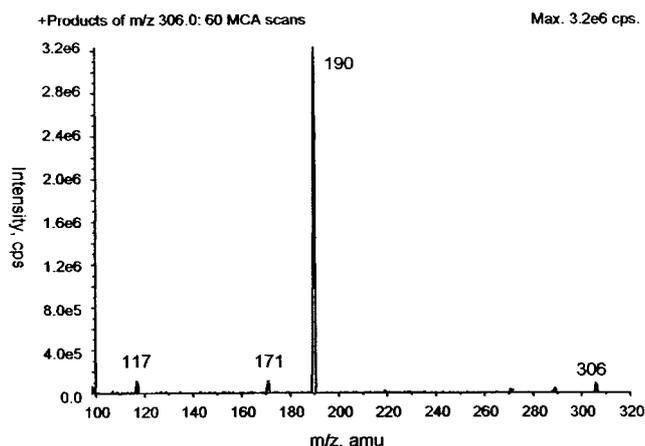


Figure 2. Product ion spectrum of the M_1G -dR derivative. The spectrum was achieved by collisional activation of m/z 306 (the MH^+ ion) and recorded over 60 scans. The major product ion at m/z 190 corresponding to loss of the deoxyribose moiety was used for quantification.

RESULTS

LC/APCI-MS/MS sensitivity

The sensitivity of the LC/APCI-MS/MS assay was determined by repeated flow injections of reduced M_1G -dR standard. Figure 3 shows the stable MRM signal from five injections of 5 μ l of 0.1 nM M_1G -dR standard in 5 mM ammonium acetate buffer (pH 5.0) containing 10% acetonitrile. Each peak represents 500 amol. The flow injection sensitivity test was routinely performed prior to sample analysis and clearly demonstrated the suitability of APCI for M_1G -dR monitoring. The high sensitivity was easily achieved and maintained for weeks without cleaning of the source and interface regions.

Assay evaluation

The sample concentrating procedure involved several steps, each one completed by vacuum centrifugation to dryness. To validate the purification procedure, blank urine was collected from one person, mixed and divided into 25 ml aliquots.

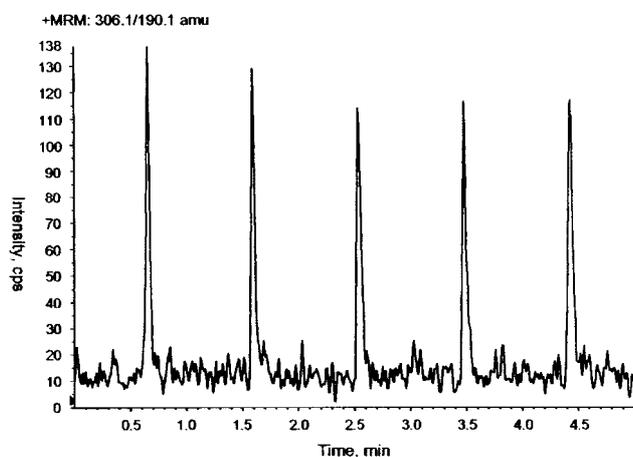


Figure 3. Flow injection analysis of chemically reduced M_1G -dR standard. Each injection corresponds to 500 amol of M_1G -dR.

Isotope-labeled standard, $[^{13}C,^{15}N_2]$ - M_1G -dR, which is not a natural component of urine, was added in increasing amounts (ranging from 1 to 120 pM) to urine samples in triplicate. The idea was to evaluate the recovery and reproducibility of the assay without response correction from the ISTD, and the limit of detection. Two blanks consisting of 25 ml of ammonium acetate buffer spiked at 120 pM with the $[^{13}C,^{15}N_2]$ - M_1G -dR ISTD were included. Samples were concentrated using the outlined multi-step procedure and processed from low to high concentration levels. In this particular experiment, the prepared immunoaffinity columns were single-use only.

Peak areas representing the reduced form of the ISTD, $[^{13}C,^{15}N_2]$ - M_1G -dR, were plotted against the spiked concentration of ISTD and showed a linear response–concentration correlation; hence the overall recovery was constant (about 60–70%) and the assay was reproducible. Unlabeled M_1G -dR was measured simultaneously and the ion chromatogram for the M_1G -dR derivative showed a response at the retention time for the ISTD in all samples. Later it proved necessary to clean the antibody-bound Sepharose gel with methanol since the antibody contained trace amounts of M_1G -dR. The whole purification procedure was carefully examined to eliminate further contamination problems and the internal ISTD standard was checked for any contribution of unlabeled M_1G -dR before use.

Determination of malondialdehyde–2'-deoxyguanosine adduct in human urine

To validate the immuno-extraction LC/APCI-MS/MS method, M_1G -dR was measured in 24 h urine collected from six non-smoking healthy volunteers. The collected urine volumes ranged from 625 to 3200 ml. To aliquots of 25 ml of urine were added 500 fmol of $[^{13}C,^{15}N_2]$ - M_1G -dR as ISTD and the samples were purified in duplicate. In parallel to the biological samples, two blanks (buffer spiked with ISTD) were also analyzed. The MRM profiles obtained from one of the purified urine samples are displayed in Fig. 4, where chromatogram A shows the peak corresponding to the derivative of M_1G -dR. Chromatogram B displays the MRM profile of the internal standard. Neither MRM profile obtained from blank samples showed any peaks. The M_1G -dR concentrations determined in the six human urine samples were in the range 0.23–0.96 pmol l^{-1} , corresponding to 1.13–16.3 fmol kg^{-1} per 24 h in excreted amounts. In one person the levels found were below the limit of quantification. The excreted amounts were about 1000-fold less than the levels reported for the oxidized guanosine adduct 8-oxo-2'-deoxyguanosine.¹⁹ The basal excretion rates given as fmol kg^{-1} body weight per 24 h are displayed in Fig. 5.

DISCUSSION

Basal M_1G levels have been measured in different types of cellular DNA from animals and humans as mentioned earlier. However, even though M_1G dR was reported in urine more than a decade ago, published data are scarce. The advances in LC/MS/MS have made it possible to develop an easier and more sensitive assay. With this

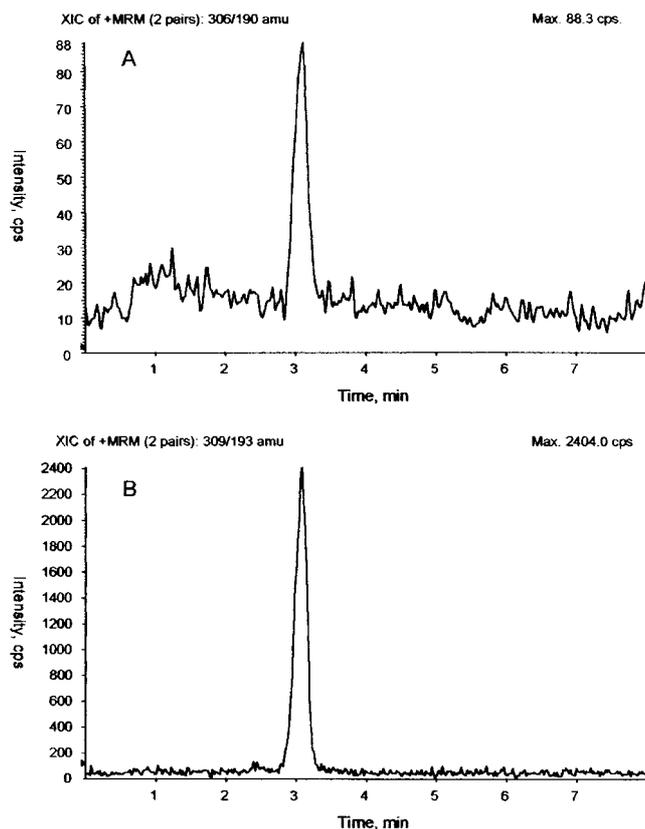


Figure 4. LC/APCI-MS/MS analysis of purified urine. Chromatogram A shows the MRM profile for the derivative of M_1G -dR (transition m/z 306 \rightarrow 190). Chromatogram B shows the MRM profile representing the ISTD (transition m/z 309 \rightarrow 193).

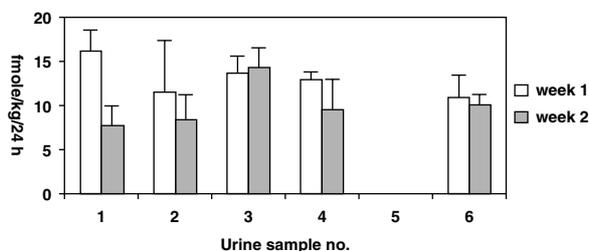


Figure 5. Bar plot showing the basal M_1G -dR excretion rates measured in urine from six human volunteers over 2 weeks. M_1G -dR levels measured from volunteer 5 were all below the limit of quantitation.

we demonstrate that the repair product of the mutagenic malondialdehyde-guanosine adduct, M_1G -dR, is present in human urine, and that the method has potential for quantitative analysis on a large scale since it can be automated with respect to the LC/MS/MS procedure. The sensitivity and specificity of LC/APCI-MS/MS were important for the quantification of M_1G -dR in urine. Attomole sensitivity was easily achieved by use of APCI as the ionization technique and we anticipate that this method will also be important for the measurement of other exocyclic DNA adducts. As an example, we achieved good sensitivity (in the low femtomole range) with the etheno adduct of 2'-deoxyadenosine (ϵ dA) using APCI, and obtained promising

results from ϵ dA determination in urine with on-line sample purification.²⁰ There is a general notion that APCI cannot be used for the analysis of nucleosides, but this appears to be possible in the two cases we have attempted.

The average M_1G -dR excretion rate in the present study was 12 ± 3.8 fmol kg^{-1} per 24 h ($n = 5$). M_1G -dR levels have earlier been assessed by HPLC with fluorescence detection to be 0.40 nmol kg^{-1} per 24 h.²¹ This result might be overestimated owing to a lower specificity of the analytical method. The low excretion level obtained with the immuno-extraction LC/APCI-MS/MS method, combined with the complexity of urine, have clearly demonstrated that detection and quantification of M_1G -dR in urine represent a major analytical challenge. This might explain the scarcity of data previously published on lipid peroxidation DNA adducts in humans, particularly urinary excretion.^{18,20,22} The measurement of M_1G -dR excretion rates in exposed individuals, e.g. induced peroxidation, has not been attempted so far.

Leukocyte M_1G level studies in one human intervention trial indicated that dietary fatty acid composition has a dramatic effect in women,²³ but further investigations of urinary M_1G -dR excretion rates with other markers of LPO *in vivo*, e.g. isoprostanes and etheno-DNA adducts, have to our knowledge not been performed. A homolog, PdG, was efficiently repaired by mammalian nucleotide excision repair complexes, suggesting that this repair system operates for M_1G .²⁴ The measurement of M_1G -dR levels in urine may underestimate the amount of M_1G -dR produced by DNA repair processes. The origin of urinary adducts is not fully established, nor has it been ruled out whether the M_1G lesion may be metabolized into a different form and thus escape analysis. The M_1G -dR adduct is presumably stable in urine, and most probably also in other body fluids. Therefore, M_1G -dR is probably not eliminated by other pathways. Therefore, it can be assumed that the formation rate is about 1000 times lower than, e.g., oxidative damage.¹⁹ Monitoring of repair products of specific endogenous DNA lesions in urine could turn out to be an important, non-invasive quantitative biomarker for lipid peroxidation-related DNA damage for future clinical and public health studies. However, the relative importance of these adducts in human carcinogenesis is not yet resolved or quantified.

At present, it is unknown whether the M_1G -dR is more biologically important for disease development than other DNA modifications, but it has the potential, being a pro-mutagenic lesion.³

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