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S. A. Yates, N. M. Dempster, M. F. Murphy and S. A. Moore*

School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF, UK

*Corresponding author. E-mail: s.a.moore@ljmu.ac.uk

Abstract

RATIONALE The lipid peroxidation product malondialdehyde forms M_1dG adducts with guanine bases in genomic DNA. The analysis of these adducts is important as a biomarker of lipid peroxidation, oxidative stress and inflammation which may be linked to disease risk or exposure to a range of chemicals.

METHODS Genomic DNA samples were subjected to acid hydrolysis to release the adducts in the base form (M_1G) alongside the other purines. A liquid chromatography-mass spectrometry method was optimised for the quantitation of the M_1G adducts in genomic DNA samples using product ion and multiple reaction mode scans.

RESULTS Product ion scans revealed four product ions from the precursor ion; *m/z* 188 → 160, 133, 106 and 79. The two smallest ions have not been observed previously and optimisation of the method revealed that these gave better sensitivity (LOQ *m/z* 79: 162 adducts per 10^7 nucleotides; *m/z* 106: 147 adducts per 10^7 nucleotides) than the other two ions. An MRM method gave similar sensitivity but the two smallest product ions gave better accuracy (94-95%). Genomic DNA treated with malondialdehyde showed a linear dose-response relationship.

CONCLUSION A fast reliable sample preparation method was used to release adducts in the base form rather than the nucleoside. The methods were optimised to selectively analyse the adducts in the presence of other DNA bases without the need for further sample clean-up. Analysis of genomic DNA gave results consistent with previous work and was applied to new samples. Thus, the method is suitable for the analysis of M_1(d)G adducts in biological samples.

Keywords: DNA adducts, M_1dG, malondialdehyde, mass spectrometry

Introduction

Malondialdehyde (MDA) is produced in vivo and has been linked to oxidative stress, inflammation and lipid peroxidation,[1, 2] and may arise from a number of endogenous or exogenous processes. It is known to be mutagenic in bacterial[3] and mammalian cells,[4] and enhances DNA damage caused by some chemicals.[5] Lifestyle factors such as smoking,[6-9] high fat diets[2, 10-12] and alcohol[1] have been associated with increased MDA levels and there is a vast body of evidence that links these to many types of cancer including, but not limited to, lung, breast and colorectal cancers.[13] Thus, MDA is widely used as a marker of oxidative stress and potential disease risk.[1, 2] Furthermore, MDA is known to form 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimidol[1,2-α]purin-10(3H)-one (M_1dG) adducts with deoxyguanosine in DNA.[14] M_1dG adducts have been positively associated with smoking, [15] alcohol.[10] and diets high in saturated fatty acids.[10, 12], . However, there is still ambiguity between studies with associations not always being found. For example, Leuratti *et al* found no association with smoking status. [15] Research has found that M_1dG influences the type of mutation that is formed depending upon where it is located in the DNA sequence,[16] and that some adducts can be linked to mutation hotspots for specific cancers.[17] Thus, M_1dG adducts have the potential to lead to cancer if repair mechanisms fail to repair either the adduct or the resulting mutation. Hence, it is desirable to be able to measure M_1dG adduct levels at biological levels in order to investigate the effects of exposure and the potential consequences for cancer development, prevention and treatment.

The analysis of M_1dG adducts in genomic DNA has been carried out by a number of methods, ranging from those involving antibodies and ^{32}P-post labelling[2, 18, 19] to coupled mass spectrometry methods.[20, 21] However, they all suffer from some disadvantages as well as advantages that have been reviewed elsewhere.[22] The main considerations are selectivity and sensitivity; techniques employing antibodies or isotope labelling are sensitive but can have specificity and reproducibility issues due to the multi-step nature of the methods[22] e.g. immunoslot blot assays (1 adduct per 10^8 nucleotides), ^{32}P-post-labelling (1 adduct per 10^{10} nucleotides) and accelerator mass spectrometry (1 adduct per 10^{12} nucleotides). These methods suffer from loss of structural information although they do have excellent sensitivity. Methods using mass spectrometry are widely used and the gold standard for adduct analysis is currently high pressure liquid chromatography-tandem mass spectrometry (LC-MS/MS)[23] which is very specific due to the combination of separation and mass selection and associated fragmentation patterns or reaction monitoring. Furthermore, the instrumentation is starting to approach other techniques in sensitivity although larger sample sizes are usually required.[20] Another benefit is that the analytes do not need to be labelled for analysis, thus, overcoming issues seen with other methods. One of the earliest methods for M_1dG analysis was by gas chromatography mass spectrometry (GC/MS) which used a acid hydrolysis to release the purines followed by derivatisation for volatility.[20] Low recoveries were found and later research has been based on LC-MS/MS methods with enzymatic hydrolysis including a recent example[24] and the earlier research by Rouzer *et al* who utilised a LC-MS/MS method to corroborate their data but do not appear to have used it for sample analysis.[20] LC-MS/MS has the advantage of not requiring the derivatisation step as the adducts are readily analysed in aqueous mobile phases. At the present time, no methods have been published for the direct analysis of the nucleobase, M_1G, by LC-

MS/MS without any additional preparation steps e.g. Jeong *et al* used an aldehye reactive probe derivatisation[25], whilst Ottender *et al* employed a reduction of the 6-oxo-M_1dG adduct in urine samples.[26] In all cases, the mass spectrometry methods were optimised to select the precursor ion for the adduct as its nucleoside form and detect product ions based primarily on the constant neutral loss (CNL) of the sugar moiety i.e. $[MH]^+ \rightarrow [MH - 116\ Da]^+$. This is a common approach in adduct analysis with very few examples of analysis of the bases rather than the nucleosides. In order to analyse adducts in genomic DNA samples, the initial step is usually to release the adducts from the DNA by a lengthy enzymatic hydrolysis to give the nucleosides with analysis by CNL as outlined above. In this research, we used a faster acid hydrolysis sample preparation method that we have used in other analyses[18] and then optimised the mass spectrometry for detection of the adduct as its base form i.e. M_1G (pyrimido[1,2-a]purin-10(3H)-one) rather than M_1dG. Harsher acid hydrolysis conditions have been used to release the nucleobases prior to derivatisation for GC/MS analysis but not for LC-MS/MS. We previously investigated acid hydrolysis conditions for HPLC analysis and found the methodology gave accurate data. [18] Hence, our aim was to utilise the fastest sample preparation methods in conjuction with LC-MS/MS analysis of the M_1G nucleobase. Monitoring of ions focussed upon identifying those specific to M_1G, instead of the CNL approach, thus offering greater specificity whilst maximising sensitivity.

Materials and methods

Materials

Ammonia solution, formic acid, guanine, iso-propanol, 1,1,3,3-tetramethoxypropane (TMP), concentrated hydrochloric acid (HCl), potassium hydroxide (KOH), 100% ethanol, calf thymus (CT) DNA, monobasic potassium dihydrogen phosphate (KH_2PO_4) and Supelclean ENVI-18 tubes and were purchased from Sigma-Aldrich, Gillingham (UK). Methanol HiPerSolv and water HiPerSolv were from VWR International Ltd, Lutterworth (UK).

Synthesis of malondialdehyde

A 100 mM stock solution of MDA was prepared with 0.1664 mL TMP in 4.69 mL HCl (0.1 M) and incubated at room temperature for 40 min. The solution was neutralised with 4.69 mL KOH (0.1M) and made up to 10 mL with water.[27]

Synthesis and purification of a pyrimido[1,2-*a*]purin-10(*3H*)-one standard

A procedure adapted from Singh *et al*[28] was used to produce M_1G: 0.1 g guanine (0.66 mmoles) was dissolved in 10 mL HCl (0.1M) at 40° C and reacted with 0.326 mL TMP (2 mmoles) for 2 h. Upon cooling and filtering, 0.0881 g yellow crystals were acquired (71% crude yield). Purification was carried out on SPE Supelclean ENVI-18 Tubes. 10 mg crude M_1G was dissolved in 1 mL ammonia solution and 9 mL water added. The pH was adjusted with formic acid to ~pH 5. A SPE cartridge was conditioned with 2 mL methanol followed by 2 mL ammonium formate buffer (50 mM, pH 5.4). The crude M_1G solution was added to the SPE cartridge and eluted with 1 mL aliquots of buffer/methanol with increasing methanol in 5% increments from 0–100%. 250 µL fractions were collected, cooled immediately, and frozen at -20° C. Fractions with pure M_1G peaks were identified by HPLC analysis, combined and freeze-dried (0.03 mg). The fractions were analysed on a Agilent 1200 series HPLC with PDA (254 nm) and FLD (excitation

360 nm and emission 500 nm) detectors, Phenomenex Gemini NX 3u C18 110A 100 x 2.00 mm column, column temperature 25° C, mobile phase 25:75 methanol/50 mM ammonium formate (pH 5.4), flow rate 0.2 mL/min and 20 µL injection volume. The SPE was repeated as above for additional purification. The identity was further confirmed by direct infusion MS and LC-MS in positive ion mode (MH^+ 188.2).

Optimisation of DNA sample preparation and M_1G stability

An acid hydrolysis procedure that has been used previously was optimised for DNA depurination and M_1G stability.[18] CT-DNA and M_1dG-DNA samples were initially analysed for DNA concentration by UV with detection at 260 nm. Aliquots were then subjected to ac id hydrolysis with formic acid (0.1 M) and incubated for 1 h at 70° C in a water bath. The samples were dried overnight at 40° C in a centrifugal dryer and redissolved in water, 1 mM formic acid, 100 mM formic acid or ammonium formate buffer (50 mM, pH 5.4). Samples were replicated with one set being sonicated for 2 mins to aid dissolution. Samples were analysed for guanine by HPLC-UV at 260 nm and the concentration of DNA calculated.

The stability of M_1G was investigated by subjecting aliquots of the standard to the same acid hydrolysis conditions and analysed by HPLC-FLD. Samples were prepared in triplicate.

Liquid Chromatography-Triple Quadrupole Mass Spectrometry

Analysis was carried out on a Waters Micromass Quattro Premier Triple Quadrupole Mass Spectrometer (Waters, UK), operated by MassLynx v. 4.1 software. Direct infusion of the M_1G standard was used to determine the optimum collision energy for analysis. ESI-MS full scan (+ ion mode) was measured in the *m/z* 145 to 300 range with a scan duration of 0.5 s, inter-scan delay 0.1 s and a cone voltage 50 V with 10 min data collection. In addition, two low energy collision dissociation tandem mass spectrometric analyses (CID-MS/MS) were used: 1) Product ion scan of *m/z* 188 with 28 eV collision energy and 50 V cone voltage; and 2) MRM scan monitoring the precursor → product ion transitions at 188 →79 *m/z* and 106 *m/z* with a 0.05 s dwell time. LC separations were carried out on a Waters 2690 HPLC separation module with a Waters 996 photodiode array detector (254 nm), Phenomenex Gemini NX 3u C18 110A 100 x 2.00 mm HPLC column, Phenomenex security guard cartridge guard column and A50-4288 4 mm L x 2.00 C18 guard cartridge, column temperature 25° C mobile phase 25:75 methanol/50 mM ammonium formate (pH 5.4), 0.15 mL/min flow rate and 10 µL injection volume. The flow was split between the UV and MS in a 2:1 ratio i.e. ~ 0.1 and 0.05 ml/min flow rate to the UV and MS respectively.

Treatment of DNA with malondialdehyde

CT-DNA was treated with MDA in a modified procedure.[19] 50 mg CT-DNA was dissolved in 10 mL KH_2PO_4 buffer (0.1 M) with heating at 60° C. 100 µL DNA aliquots (500 µg) were treated with MDA (0 – 50 mM) in a final volume of 500 µL, in triplicate for 5 days in the dark at 37 °C in a shaker incubator. The DNA was precipitated by cooling to 4° C, 500 µL cold iso-propanol added and inverted several times, centrifuged for 10 min at 12,000 g and the supernatant removed. The DNA pellet was washed with 500 µL cold 100% ethanol and centrifuged at 12,000 g for 10 min, then 500 µL cold 70% ethanol and centrifuged for 10 min at 12,000 g. The supernatant was removed and the DNA pellet used for adduct analysis.

Sample preparation for adduct analysis

The precipitated DNA pellets were re-suspended in 500 µL formic acid (0.1 M) and incubated for 1 h at 70° C in a water bath. The samples were then dried overnight at 40° C in a centrifugal dryer and stored at -20° C prior to analysis. Samples were warmed to room temperature, dissolved in 100 µL ammonium formate buffer (50 mM, pH 5.4) and analysed by LC-MS/MS within 24 h.

Analytical validation criteria

Specificity

ESI-MS full scan (+ ion mode), as described above, was carried out on 0.1 mg/mL guanine, 0.1 mg/mL adenine, M_1G standards and a hydrolysed M_1dG-DNA standard.[18] A M_1G standard (0.5 µg/mL) was spiked with 10-100 µg of guanine and analysed by LC-MS/MS in all modes.

Accuracy

Accuracy was determined by analysis of a M_1dG-DNA standard (21 pmol/µg DNA) that had been prepared and analysed independently.[18] 30 µg M_1dG-DNA was hydrolysed in formic acid (500 µL) as for the extracted DNA pellets and analysed by LC-MS/MS in all modes.

Precision

Peak precision was determined by calculating the % relative standard deviation (RSD) for the peak with the M_1G concentration closest to the limit of quantitation (LOQ). Precision was acceptable if < 0.5%.

Linearity

A M_1G standard (0.1 mg/mL; 535 µM) was prepared in buffer (50 mM ammonium formate, pH 5.4) with sonication for 20 min. The standard was diluted 10-15,000x in buffer to give standards in the range of 0.036 µM- 53.4 µM (7 pg/µL-10 ng/µL). Standard curves of peak intensity versus M_1G concentration were prepared for each of the product ions and the MRM scan. Correlation was assessed across the entire range and within the low range used for sensitivity calculations.

Limits of detection and quantitation

The limit of detection (LOD) and LOQ were determined using the calibration line method in the range of (0.03-0.5 uM). LOD = 3.3(standard error of regression line/gradient); LOQ = 10(standard error of regression line/gradient).

Results and Discussion

Stability of M_1G

It is known that M_1G needs to be kept in neutral or acidic solution during sample preparation and analysis procedures. Previous studies have shown that a high pH or the use of tris buffers for storage can cause ring-opening.[29, 30] Method development showed that M_1G was stable for a few days in aqueous solutions and hence standard solutions needed to be prepared daily or kept frozen for future use. The measurement of DNA concentration had been previously optimised by comparison of UV analysis followed by acid hydrolysis and analysis by HPLC-UV with comparable results (3.05 mg/ml and 3.01 mg/ml respectively).[18] During the current optimisation, the concentration of DNA samples was verified by UV and HPLC to ensure that the acid

hydrolysis procedure was effective. It was found that samples must be dried immediately after acid hydrolysis to remove all acid and ensure stability of the adduct until analysis. The samples were suspended in different solutions with both 10 mM formic acid and the LC buffer giving the highest guanine, and hence DNA, concentrations (96% and 97% of the UV concentration respectively). Furthermore, sonication of the samples for dissolution of the analyte for HPLC analysis gave DNA concentrations that were consistently higher. Thus samples should be suspended in a weak acid buffer (>10 mM formic acid or ammonium formate pH 5.4) with sonication immediately prior to analysis. This has proved to be of primary importance in the analysis of M_1G in DNA samples as the sample must be completely depurinated and then redissolved in the LC buffer for reliable results.

Previous research has shown that HCl (0.01-6 M) is not suitable for acid hydrolysis of M_1G due to degradation of the adduct and release of pyrimidines. Whereas, , 0.1M formic acid was shown to give a higher ratio of M_1G adducts than other acids and was stable under the conditions used for sample preparation.[18] Furthermore, a CT-DNA sample treated with MDA was then analysed using the same acid hydrolysis and HPLC conditions and compared it to data obtained elsewhere from a LC/MS analysis and the results were 17pmol/ug DNA and 18pmol/ug DNA respectively.[18] The same conditions were used herein and the stability of the adduct was verified. Standards subjected to the acid hydrolysis procedure showed no reduction in adduct levels after 60 mins compared with the original sample (peak area at t60 = 106% of the t0 sample). Acid hydrolysis followed by a derivatisation procedure has been used for sample preparation for analysis by GC-EC NCI/MS.[20] The stability of M_1G in a short oligonucleotide duplex was investigated and found to be stable. However, 0.7M formic acid was used confirming that the adduct is stable to harsher conditions than used in our studies. Therefore, consideration must be given as to whether the adduct is all released or if an under estimation of adducts is possible. In previous research the concentration of M_1G (and consequently M_1dG) was verified against independently prepared samples.[18] Thus, the acid hydrolysis procedure appears suitable for qunatitation of DNA and M_1G concentrations and this research sought to establish conditions suitable for LC-MS/MS analysis of the adduct as the base rather than the nucleobase. The ability of the current method to provide high quality data is a prime consideration herein.

Mass Spectrometry Method Development

Initial ESI-MS (+ ion mode) analysis of the M_1G standard by direct infusion showed the optimum cone voltage was 50 V that produced the intact protonated adduct of M_1G $[M_1G+H]^+$ at *m/z* 188. At lower cone voltages insufficient ions were generated, whereas higher cone voltages produced too much fragmentation with loss of the ions of interest. The optimum collision energy required to fragment the precursor ion of M_1G into product ions was found to be 28 eV. CID-fragmentation resulted in the formation of four product ions at *m/z* 160, 133, 106 and 79, as shown in Fig. 1. This can be explained by a loss of CO (28 Da) to give the largest product ion, and three other fragmentations with loss of CO plus multiples of HCN (27 Da) to give the three smaller product ions. The major product ion was at *m/z* 79 which can be explained by a 6-membered ring and is expected to be the most stable of the product ions. The two larger product ions have been identified by Szekely *et al*[31] during the analysis of the corresponding nucleoside, M_1dG, using multi-stage MS/MS analysis. M_1dG has an *m/z* of 304 and neutral loss of the deoxyribose unit (116 Da) occurred to give the protonated ion of M_1G (*m/z* 188) which is the starting point of our

analyses. They then observed the two larger ions seen in our analyses (*m/z* 160 and 133). However, the latter was seen under MS^4 analysis and explained by fragmentation of the 6-membered ring rather than the 5-membered ring of the purine moiety as suggested here. It is unclear as to whether their proposed fragmentation was confirmed but the ions observed in this work are best explained as shown in Fig. 1 with regard to the two additional ions identified. These ions were not observed by Szekely *et al* although their scan range was wide enough had they been formed. The difference in the mass spectra can be explained by the fact that their method was optimised for the nucleoside, M_1dG, and loss of the sugar, whereas our method is optimised to ionise and fragment the base, M_1G. The majority of published work on adduct analysis by mass spectrometry focusses on nucleosides rather than base analysis with the CNL of 116 as the main fragmentation as discussed earlier.

Liquid chromatography-mass spectrometry

Having established the mass spectrometry conditions, the LC method was optimised. Initially, the effect of eluant on the stability of M_1G was investigated in a number of acidic solutions as a high pH is known to cause ring-opening of the additional ring. This reaction occurs regardless of whether the adduct is in the base, nucleoside or nucleotide form so is a problem inherent to all analyses. Thus we investigated the use of formic acid, acetic acid and ammonium formate. Previous research has utilised ammonium formate in HPLC analyses [19], and this was also found to give the optimum sensitivity under our LC-MS/MS conditions. Isocratic and gradient methods were investigated but no advantage was gained in using a gradient method which resulted in longer run times due to the re-equilibriation time required. Several flow rates were tested with 0.15 mL/min being the optimum. Lower flow rates gave better peak separation; however, a reduction in sensitivity occurred which was undesirable. A higher flow rate (0.2 mL/min) was found to give the greatest sensitivity, but with a reduction in resolution. Thus, the optimum was 0.15 mL/min which gave better resolution with only a slight loss in sensitivity compared to the higher flow rate.

The mass spectrometry methods were run in conjunction with the optimised LC method. Two scans were run by LC-MS/MS, a products of *m/z* 188 scan and an MRM scan. An initial analysis showed that all ions gave good linearity. However, the two product ions of greatest intensity, *m/z* 79 and 106, were chosen for optimisation of the method in order to maximise sensitivity. The MRM scan was run of two reactions: *m/z* 188 → 79 and *m/z* 188 →106. Standards of M_1G, guanine and adenine were analysed by the optimised LC-MS/MS method. The data obtained for M_1G is shown in Fig. 2 where M_1G eluted at 2.9 min. An additional peak was seen on the UV chromatogram at 2.0 min (Fig. 2A) which was also the only peak present when a blank containing mobile phase was run, and was absent when water only was run. Fig. 2 shows that this peak was not present in any of the MS chromatograms for the standard, and absent in the blank MS chromatograms (Fig. 3A). A small peak corresponding to guanine (*m/z* 152) was detected at 2.3 min on the product ion TIC chromatogram Fig. 2C, with a relative intensity to the M_1G peak of 0.015% assuming the same response per mass of analyte. This was a problem throughout the analysis of M_1G using the product ion scan, although the guanine peak did not increase in size with increasing M_1G concentration. This indicates there may be some degradation of M_1G during the analysis and was not a standard purity issue. All parameters were optimised to minimise analyte degradation but could not be eliminated completely. The guanine peak was not present in

the MRM scans showing the improved specificity of the methods. Guanine and adenine were analysed by all methods to determine if they caused any interference with M_1G detection. In addition to the product ion and MRM scans, a full scan by LC MS was carried out for all ions within the range of *m/z* 70 – 1000. Guanine eluted at 2.1 min on the full scan chromatogram with *m/z* 151.86 as expected (data not shown). In addition to the product ion and MRM scans, a full scan by LC-MS was carried out for all ions within the range of *m/z* 70 – 1000. Guanine eluted at 2.1 min on the full scan. However, guanine was not detected by the product ion or the MRM scans demonstrating the selectivity of the methods. Adenine was not detected by any of the MS modes used but could be seen on the UV chromatogram at 2.7 min (data not shown). This peak was quite close to that of M_1G; however, as adenine was not detected by MS under these conditions, there was not expected to be any interference with M_1G detection. Ion suppression is a possibility which was not found to be problematic when the accuracy of the method was investigated.

The M_1G standard was spiked with different amounts of guanine to determine if there was any interference with M_1G detection due to signal suppression. Small differences could be seen in the peak intensities of the M_1G peak between the standards spiked with guanine and those not spiked, but not between those spiked with different amounts of guanine. The differences were < ±5% from the non-spiked M_1G standards and could be attributed to experimental and instrumental error.

A standard curve of M_1G was plotted of peak intensity versus M_1G concentration for the two smallest product ions and MRM, as shown in Fig. 4. All standard curves had good R^2 values (> 0.9833), with the MRM showing the best linear fit (0.9955). However, the curve for the smallest product ion (*m/z* 79) showed the steepest gradient. The MRM and *m/z* 106 curves were very similar to one another.

The sensitivity of the method was calculated by using the calibration line method (Table 1). All calibration lines gave R^2 >0.98 for the standards used. The lowest LOQs gave a %RSD value of 0.15%, which showed good peak precision. The closest peak on the calibration line to the LOD corresponded to 89 nM M_1G (167 pg M_1G per injection). The MRM and product ion chromatograms are shown in Fig. 3 (B).

The accuracy of the method was assessed by analysis of a M_1dG-DNA standard (21 pmol M_1G/µg DNA) that had been prepared and analysed independently. The chromatograms produced (Fig. 3C) were all similar to those of the standards. The levels of M_1G adduct in the M_1dG-DNA standard were calculated using the calibration equations of product ions *m/z* 79 and 106 and the MRM scan, as shown in Fig. 4. The injected sample concentrations were ~3 µM which is well above the LOQ of the methods. Table 1 shows the data for accuracy obtained by the LC-MS/MS methods. The amount of M_1G in the M_1dG DNA standard was comparable for both product ions *m/z* 79 and 106 with an accuracy of 94-95%. However, the MRM scan gave a much lower amount due to reduced ion intensity. The accuracy of both product ion scans is within the acceptable range of the previously determined value whereas the MRM analysis is very low despite the combined ion intensities of the two MRM acquisitions. However the calibration curve for this

scan of M_1G standard had a lower gradient than the others presumably due to the loss in sensitivity associated with MRM mode. These scans were replicated and gave similar results.

Analysis of M_1G Adducts in DNA

The methods were then applied to CT-DNA that had been treated with MDA to determine if the adducts could be quantitated in a more complex matrix comparable to biological samples. The same methods were applied and the data processed using the product ions of *m/z* 79 and 106 as these had been found to give the best accuracy. The M_1G concentrations are shown in Fig. 5 for each MDA concentration used. For both product ions, the concentration of M_1G increases with the concentration of MDA as expected, although the concentration is slightly lower for *m/z* 106 than *m/z* 79 in every sample. For *m/z* 79, there was a low concentration (0.16 µM) M_1G adducts in untreated DNA, which is also often found for many other adducts, however this is below the LOQ and just above the LOD, as shown in Table 1. For *m/z* 106, the untreated DNA and the DNA treated with 0.01 mM MDA have low negative values, which is not expected with samples below the LOQ. The levels of M_1G detected for the 0.1 mM MDA treatment were below the LOQs, but all other MDA concentrations gave adduct levels that were above the LOQ. Thus, levels of M_1G were not reliable at low concentrations of MDA (≤0.1 mM). However, a clear pattern can be seen whereby levels of M_1G increased with increasing MDA concentration demonstrating a dose-response relationship. Treatment with the highest concentration of MDA (50 mM) results in 1.95 µM and 1.74 µM M_1G adducts for *m/z* 79 and 106 respectively, which corresponds to 0.39 pmol/µg DNA and 0.35 pmol/µg DNA respectively (1205 adducts per 10^7 nucleotides and 1077 adducts per 10^7 nucleotides). Thus, it appears to be possible to detect an effect from 1 mM MDA upwards using a product ion scan which gives a M_1G concentration of 367 nM (*m/z* 79) or 438 nM (*m/z* 106) (227 and 271 adducts per 10^7 nucleotides respectively). However, the sensitivity could be improved by use of the most recent instrumentation where sensitivity can be improved by two orders of magnitude.[32] This does appear to be a dose-response relationship, as might be expected when treating DNA in solution with MDA and no other interfering factors, which suggests that the method would be reliable for measuring adducts in DNA from biological samples with appropriate sample clean-up.

Conclusion

In conclusion, we have established a reliable method to quantify M_1G adducts by LC-MS/MS that does not require lengthy expensive enzyme cleavage or derivatisation for the sample preparation stage. The method has >94% accuracy, with good precision, linearity and sensitivity in the pg range. Most notably, we have identified a fragmentation pattern that appears to be unique to M_1G. This could therefore be an important contribution to the field of adductomics where unique transitions are essential when identifying a range of adducts within biological samples. Precursor-product ion scans are more suited to adductomics studies than MRM scans as the latter require the precursors and products to be known.[33] The methods were applied to CT-DNA samples and treatment with MDA resulted in a dose-response relationship as expected. However, only DNA was involved and no cellular responses could have repaired these adducts. Future research should now focus upon identification of the M_1G adducts in biological samples from cells and human studies and potential future application to adductomics research.

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Table 1 The linearity and sensitivity of the M_1G standards (30-500 nM) and accuracy of genomic DNA analysis by LC-MS/MS

Ion/ mode	LOD		LOQ		R^2	Accuracy (%)
	nM	adducts/10^7 nucleotides	nM	adducts/10^7 nucleotides		
79	86	53	261	162	0.9923	94.03
106	78	48	238	147	0.9934	94.59
MRM	93	57	282	174	0.9587	73.79

Figures

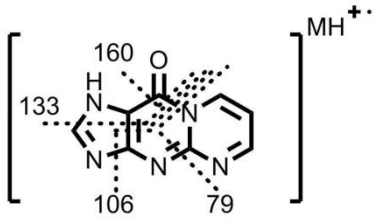

Figure 1 Proposed fragmentations for the product ions observed for M_1G

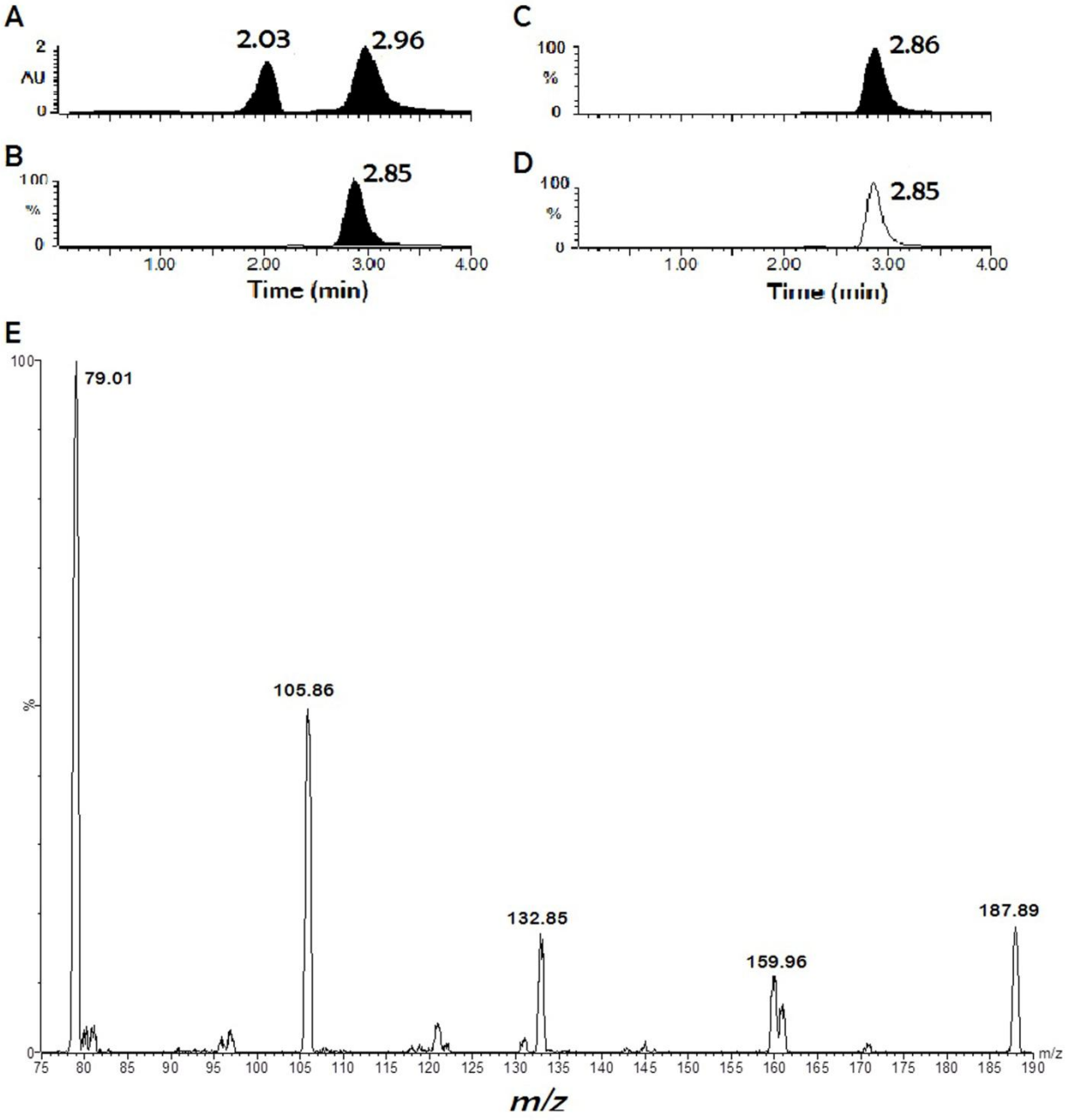

Figure 2 LC-MS/MS chromatograms and mass spectrum of M_1G standard (53 µM) - A) UV; M_1G = 2.96 min , B) MRM; M_1G = 2.85 min , C) product ion TIC; guanine = 2.30 min, M_1G = 2.86 min, D) product ion RIC; M_1G = 2.86 min, same obtained for *m/z* 79, 106, 133, 160 and 188 (E) Mass spectrum of product ion peak at 2.86 min with ions at *m/z* 79.01, 105.86, 132.85, 159.96 and 187.89.The flow is split after the column which accounts for the difference in RT between the UV and MS chromatograms.

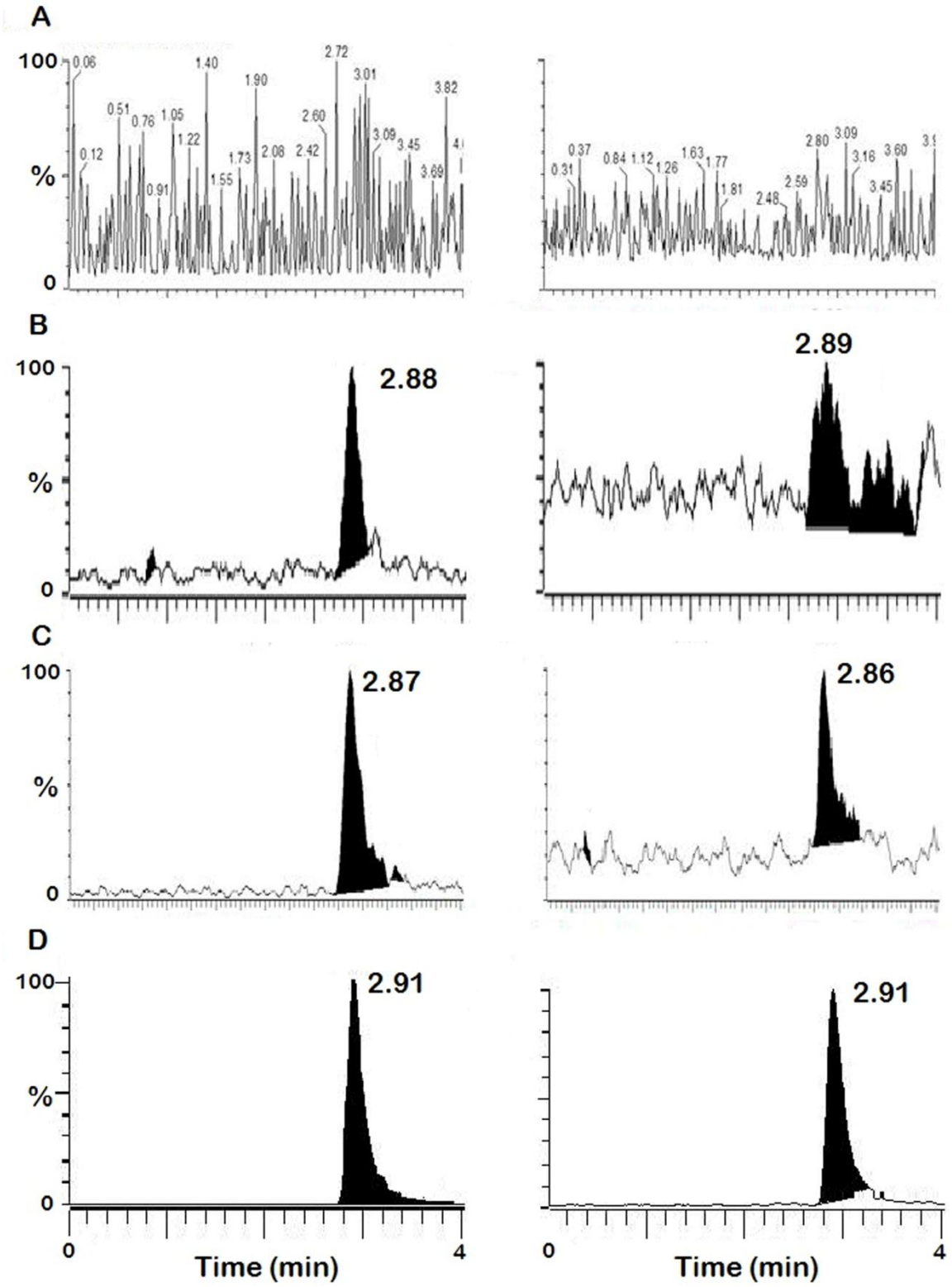

Figure 3 MRM (left) and product ion (right) chromatograms of A) blank, B) M_1G standard at the LOD (89 nM), C) M_1G standard at the LOQ (267 nM), and D) Hydrolysed M_1dG-DNA standard. The M_1G peak elutes at 2.9 min in the MRM and product ion but was not detected in the blank.

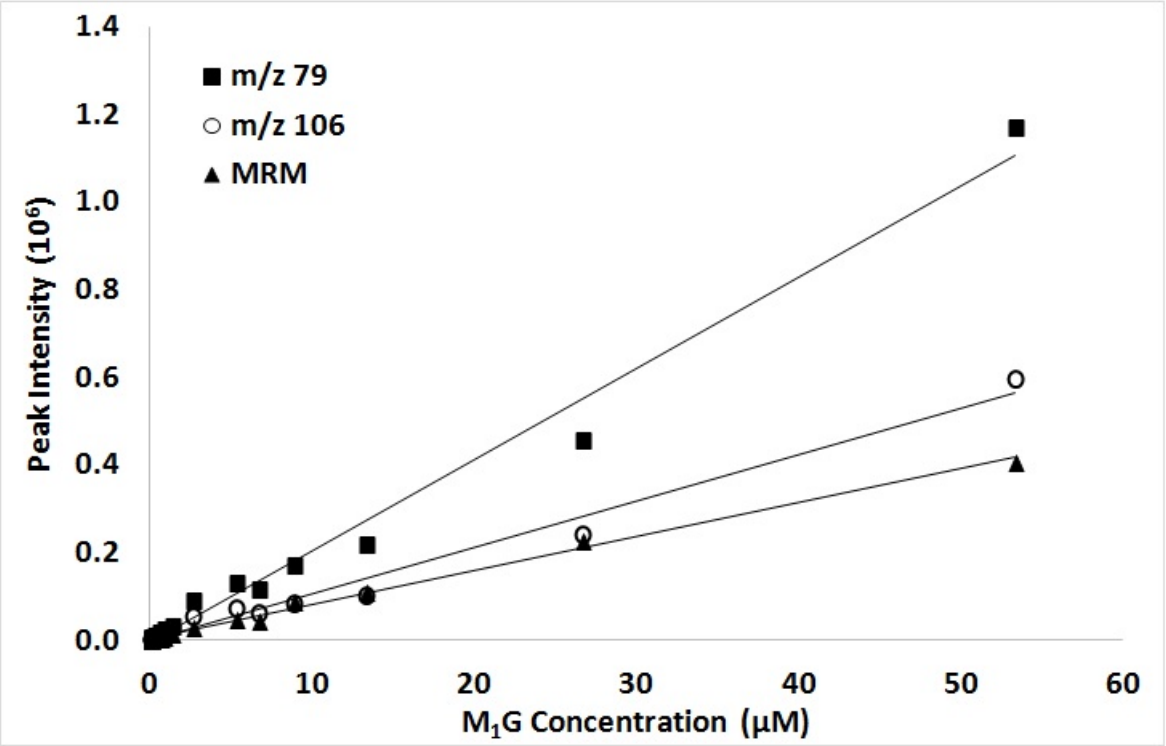

Figure 4 LC-MS/MS calibration curves for M_1G standards (0–53 μM) for product ion (*m/z* 79 and 106) and MRM (188 → 79 and 106) analyses. R^2 values: 0.9857 (*m/z* 79), 0.9853 (*m/z* 106) and 0.9955 (MRM).

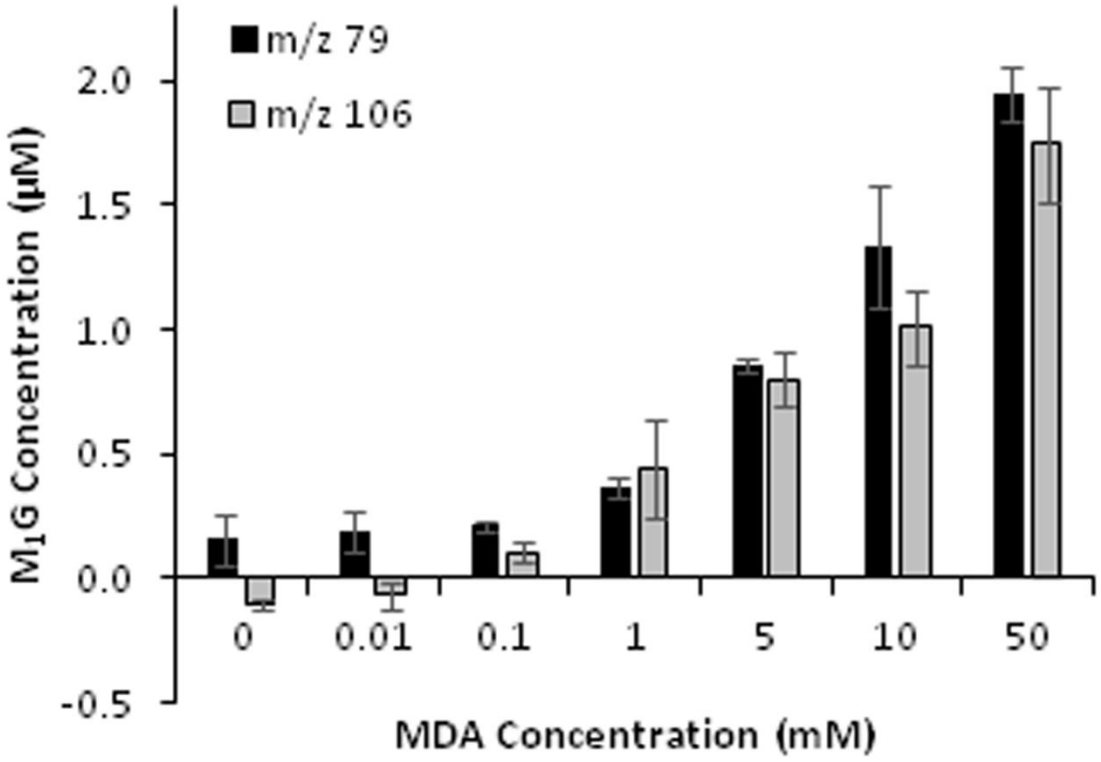

Figure 5 M_1G concentration in CT-DNA treated with 0 – 50 mM MDA analysed for product ions of M_1G (*m/z* 79 and 106). Error bars show ±1 SD from the mean (*n* = 3).