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Rapid Communications in Mass Spectrometry

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A Novel ^{14}C-Postlabeling Assay Using Accelerator Mass Spectrometry For the Detection of O^{6}-Methyldeoxyguanosine Adducts.

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Abstract.

Accelerator mass spectrometry (AMS) is currently one of the most sensitive methods available for the trace detection of DNA adducts and is particularly valuable for measuring adducts in humans or animal models. However, the standard approach requires administration of a radiolabeled compound. As an alternative, we have developed a preliminary ^{14}C-postlabeling assay for detection of the highly mutagenic O^6-MedG, by AMS. Procedures were developed for derivatizing O^6-MedG using unlabeled acetic anhydride. Using conventional LC-MS analysis, the limit of detection for the major product, triacetylated O^6-MedG, was 10 fmoles. On reaction with ^{14}C-acetic anhydride, using a specially designed enclosed system, the predominant product was ^{14}C-di-acetyl O^6-MedG. This change in reaction profile was due to a modification of the reaction procedure, introduced as a necessary safety precaution. The limit of detection for ^{14}C-di-acetyl O^6-MedG by AMS was determined as 79 attomoles, ~18,000 fold lower than that achievable by LSC. Although the assay has so far only been carried out with labeled standards, the degree of sensitivity obtained illustrates the potential of this assay for measuring O^6-MedG levels in humans.

Introduction

The frequent occurrence of methylated bases in DNA suggests that exposure to alkylating agents is a common event [1]. *N*-Methyl Nitroso compounds (NOCs) are a large group of chemicals which are known to methylate DNA and can be found in a number of environmental sources, such as the diet, through the consumption of smoke-cured fish, meat and beer [2], whilst *N*-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are present in cigarette smoke [3]. Relatively small population subgroups working in the chemical manufacturing industry suffer occupational exposure to weakly genotoxic methylating agents such as dimethyl sulphate (DMS) and methyl bromide, which is also used as a fumigant [1]. In addition, DNA continually suffers damage from endogenous sources of methylating compounds, with a possible candidate being S-adenosylmethionine [4]. Nitrosation products of naturally occurring compounds, such as poly- or monoamines and peptides [5, 6] are also known to introduce O^6-methyldeoxyguanosine (O^6-MedG) into DNA [6]. O^6-MedG is a pre-mutagenic lesion in both bacteria [7] and mammalian cells [8], as shown by the presence of mutations induced in cells treated with methylating agents, such as *N*-methyl-*N*-nitrosourea and *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. Studies utilizing plasmids containing a single site-specific adduct have confirmed that O^6-MedG is able to miscode, directing the introduction of the incorrect pyrimidine as its complementary base, when replicated in *E. coli* [9,10], resulting in the signature G → A transition mutation of alkylating damage [11]. Evidence that O^6-MedG may play an important role in the initiation of carcinogenesis comes from the frequent discovery of GC→AT mutations in the *ras* oncogene and *p53* tumour suppressor gene of tumours induced in rodents [12] by

methylating agents such as NDMA. If O^6-MedG is not repaired prior to replication, the resulting transition mutation may lead to cell death [13], sister chromatid exchanges [14], chromosomal aberrations [15] and malignant transformation [16].

A number of methods have been described for the detection and quantitation of O^6-MedG after exposure to various alkylating agents. Levels of O^6-MedG have been measured in DNA from rats treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and NDMA, using immunoassays [17] and the ATase repair assay [18], respectively. In humans, background levels of approximately 1 O^6-MedG/10^8 nucleotides have been detected in DNA of healthy individuals isolated from various tissues, using the repair assay [19, 20]. Whilst these two approaches are both highly specific, they are not particularly sensitive; therefore the development of an assay with improved sensitivity that retains the specificity of the current methods would be extremely valuable for the detection of O^6-MedG in human subjects.

Currently, accelerator mass spectrometry (AMS) is one of the most sensitive methods available for the detection of DNA adducts, typically offering at least 10-100 fold improvements in sensitivity over techniques such as ^{32}P-postlabeling [21]. Concentrations of long-lived, radioisotopes, such as ^{14}C, can be measured by AMS at attomole levels, which equates to the detection of as little as 1-10 DNA adducts/10^{12} nucleosides formed by a ^{14}C-labeled chemical [22]. The most common experimental approach utilising AMS in human studies has been to administer a ^{14}C-labeled compound of interest, then measure the extent of binding to DNA and protein, or the production of metabolites. This standard

approach is dependent on availability of ^{14}C-labeled compounds and obtaining the ethical and radiological approval to administer these to animals or humans, which may not always be possible.

The concept of ^{14}C-postlabeling as a means of adduct detection, would exploit the sensitivity of AMS without the need to administer a radioisotope to humans [23]. Development of ^{14}C-postlabeling assays for specific adducts could have a major impact in human biomonitoring, making it possible to conduct large population based studies to investigate the formation of low levels of adducts without prior administration of [^{14}C]-labeled chemicals. This would enable the assessment of individual variation and relationships between particular exposures and risk to be established. The general procedure involves isolation of specific adducts from DNA via enzymic digestion, followed by an enrichment step and subsequent addition of ^{14}C-labeled groups on to the adduct. ^{14}C-Labeled adducts can then be separated from other components of the reaction mixture by HPLC and quantified by AMS analysis. To date there is only a single reported study of AMS postlabeling, which describes the development of an assay for specifically quantifying benzo[*a*]pyrene DNA adducts formed *in vitro* [24]. In the current study, methods have been developed and optimised for ^{14}C-postlabeling O^6-MedG adducts and the limits of detection determined using AMS. Comparisons have also been made to more conventional adduct detection methods such as liquid scintillation counting (LSC) and liquid chromatography-mass spectrometry (LC-MS) analysis.

Materials and Methods

[1-^{14}C]-Acetic anhydride (185 MBq/mmol) was purchased from ICN pharmaceuticals (Basingstoke, UK), bottled solvents for use in the AMS laboratory were purchased from Fisher (Loughborough, UK) and all other chemicals were purchased from Sigma-Aldrich (Poole, UK).

General Instrumentation and Methods.

HPLC Analysis of Reaction Products

HPLC separation of compounds with UV detection was carried out on a Gilson instrument comprised of model 305 pumps, a 805 nanometric module and a 811b mixer (Anachem, Luton, Bedfordshire, UK). Compounds were injected using a 200 µl loop, onto a 250 x 4.6 mm, 5µ, Luna C_{18}(2) column (Phenomenex, Macclesfield, Cheshire, UK) operated at a flow rate of 1 ml/min and detected using a diode array detector, 1000s (Applied Biosystems, Warrington, Cheshire, UK). This HPLC system was specifically designated for radioactive applications, whilst a second HPLC system situated in the AMS laboratory was used solely for the separation of samples containing amounts of ^{14}C below the limit of detection for LSC (~ 25 dpm). This Jasco instrument was comprised of intelligent HPLC pumps (PY-1580) and dynamic mixer (HG-1580-32). Samples were placed in screw cap vials (as above) and injected by an intelligent sampler (AS-1555) with a 200 µl loop, onto a 250 x 4.6 mm, 5µ, Luna C_{18}(2) column at a flow rate of 1 ml/min. Compounds were detected using a multiwave detector (MD-1510, Jasco, Chelmsford, Essex, UK) set at 254 nm. ***Method 1:*** Reaction products were separated with a gradient of HPLC grade water (A) and MeOH (B) (0-5 min, 5% B, 5-30 min, 5-80% B, 30-35 min, 80-5% B, 35-45 min, 5% B) and UV absorbance was monitored at 254 nm.

Method 2: Reaction products were separated using a gradient of HPLC grade H_2O (A) and MeOH (B) (0-5 min, 5% B, 5-26 min, 5-62% B, 26-33 min, 62% B, 33-35 min, 62-70% B, 35-40 min, 70-5% B, 40-45 min, 5% B) and detected by UV at 254 nm on the system designated for radioactive use. ***Method 3:*** All samples processed for AMS analysis were separated on the Jasco HPLC system in the AMS laboratory using the conditions described in Method 2.

Mass Spectrometry Analysis of Reaction Products.

Positive ion electrospray analyses of products formed in the small-scale acetylation reactions were performed by continuous infusion of samples using a Harvard Apparatus model 22 syringe pump (Harvard Apparatus, Edenbridge, UK) into an Autospec Ultima-Q mass spectrometer (Micromass, Manchester, UK). Analysis by fast atom bombardment mass spectrometry was performed on a 70-SEQ mass spectrometer (Micromass, UK). A glycerol matrix was used and analyses were conducted in positive ionisation mode. LC-MS quantitation of reaction products was performed on a Quattro BioQ instrument (Micromass, UK) equipped with a pneumatically assisted electrospray source. The instrument was tuned using an O^6-MedG adduct standard, which was infused using a Harvard syringe pump at a rate of 10 µl/minute. Analysis was carried out in the positive ion mode with a source temperature of 110ºC. Samples were introduced after HPLC separation using a Varian 9012 LC pump (Varian, Walton-on-Thames, Surrey, UK) and a linear gradient of methanol in water (0 min, 20 % MeOH, 20 min, 50 % MeOH).

^{1}H-NMR Analysis.

Samples were redissolved in d_6-acetone prior to ^{1}H-NMR analysis, performed on a Bruker ARX 250 spectrometer (Bruker Biospin Ltd, Coventry, UK), operating at 250MHz.

Synthesis of tri-acetyl O^6-MedG standard.

A method was developed for the acetylation of O^6-MedG with acetic anhydride which was optimised to ensure consistent addition of the maximum number of acetyl groups onto the adduct, as shown in Figure 1. This standard was then used to establish the limits of detection for this adduct using UV and MS as the detection methods. This was a small-scale reaction (method A below), conducted using unlabeled acetic anhydride and it was hoped that this procedure could ultimately be employed in the ^{14}C-postlabeling assay. In comparison, a second method (B) is also described which was used to produce a synthetic standard of tri-acetyl O^6-MedG on a large-scale.

A. Small scale acetylation: O^6-MedG, (10 μg, 35.5 nmoles), was dissolved in anhydrous pyridine (20 μl) and reacted with acetic anhydride (5 μl, 53 μmoles) at 100 ºC for 1 h. The acetylated derivative was isolated by HPLC separation, using Method 1, and eluted at 27.2 min. ESI-MS analysis of the reaction product showed *m/z* values of 208, equivalent to [mono-acetyl O^6-MedG + H]$^+$ and 408, consistent with [tri-acetyl O^6-MedG + H]$^+$. To further confirm the structure of the tri-acetylated O^6-MedG, which was formed in > 90% yield, a synthetic standard was produced, for complete characterisation, using an alternative method (B).

B. Large-scale synthesis of tri-acetyl O^6-MedG: Tri-acetyl-dG, (640 nmoles), previously synthesised in this laboratory, based on a method by Gaffney *et al.* [25] was dried three times by evaporation with toluene, then dissolved in 50 ml dichloromethane (DCM) containing mesitylene sulphonyl chloride (500 mg), 4-dimethylaminopyridine (DMAP, 20 mg) and triethylamine (TEA, 500 µl) and reacted at room temperature until the solution turned yellow (~ 2 h). Quinuclidine (280 mg) was then added and the reaction continued for ~ 1 h until the solution darkened. Methanol (1.5 ml) and 1,8-diazabicyclo(5,4,0)undec-7-ene (600 µl), were added and the reaction was left at room temperature for a further 1 h. The production of tri-acetyl O^6-MedG was confirmed by thin-layer chromatography analysis of the reaction mixture, which was then concentrated. The residue was dissolved in 50 mM potassium phosphate buffer pH 6.5 (10 ml), extracted twice with DCM and purified by flash column chromatography on silica gel using a mobile phase of 30% MeOH in DCM. FAB-MS (*m/z* 208 and 408) and ^{1}H-NMR ($CDCl_3$) δ 2.2 (d, 6, 2 x COC**H$_3$**), 2.6 (m, 4, H$^{2'}$, NCOC**H$_3$**), 3.0 (m, 1, **H$^{2''}$**), 4.3 (s, 3, OC**H$_3$**), 4.4 (m, 3, **H$^{5'}$**, **H$^{5''}$**, **H$^{3'}$**), 5.5 (m, 1, **H$^{4'}$**), 6.4 (m, 1, **H$^{1'}$**), 8.0 (m, 2, N**H**, **H^8**) confirmed the product as tri-acetyl O^6-MedG. Fractions containing the desired product were combined and evaporated to dryness, giving 30 mg (11.5 %) yield.

Reaction of ^{14}C-acetic anhydride with O^6-MedG

^{14}C-Acetic anhydride is volatile; therefore care was taken during the acetylation procedure to guarantee that it was contained. For this purpose, a special drying system was developed to ensure the evaporation of reaction products was enclosed and any excess ^{14}C-acetic anhydride was contained. In order to synthesise a standard of ^{14}C-tri-

acetyl O^6-MedG in the highest yield possible, a small number of acetylation reactions were performed and the major products pooled, as this method was more efficient than a larger-scale reaction. ^{14}C-Acetic anhydride (18.5 MBq), was transferred from a breakseal vial (as supplied) into anhydrous pyridine (160 µl, dried over molecular sieves). Aliquots of O^6-MedG (8 x 1 µg), previously dried under nitrogen, were each redissolved in approximately of the 21 µl radioactive solution (12.5 µmoles acetic anhydride). The reactions were incubated in a heating block for 2.5 h at 100°C, cooled on ice, then quenched by the addition of 50% MeOH (40 µl). The reactions were pooled into two vials and dried in a diazomethane generator, which had been adapted into a drying chamber, with a cold trap and a succession of further traps joined to a vacuum pump, as illustrated in Figure 2. The contents of each vial were dissolved in 20% aqueous MeOH (1 ml) and analysed using HPLC Method 2. The fractions containing the major product from the reactions were collected, dried and quantitated by LSC.

Measurement of samples by LSC.

LSC was performed on a Beckman LS 6500 scintillation system (Beckman Coulter, Buckinghamshire, UK). Hydroflour scintillation fluid (5 ml) (National Diagnostics, Hull, UK) was added to each fraction and disintegrations per minute were counted for 10 min. The ambient background level was determined by measuring scintillation fluid alone, and was found to be ~25dpm (n = 3). This value was checked each time the instrument was operated.

Characterisation of ^{14}C-labeled product

According to HPLC analysis, the major product of the ^{14}C-acetylation reaction described above did not correspond to the tri-acetyl O^6-MedG produced in the unlabeled acetylation reaction. Therefore, in order to determine the identity of this product, a reaction was performed using the same transfer procedure as required for the ^{14}C-reaction but using unlabeled acetic anhydride, to produce a standard, which could then be characterised. Acetic anhydride was transferred into pyridine and O^6-MedG was redissolved using this mixture, then incubated at 100 ºC for 2.5 h. The reaction was stopped with 50% aqueous MeOH and dried under nitrogen. The reaction products were separated by HPLC, using Method 2 and the fraction containing the peak corresponding to the major product of the ^{14}C-postlabeling reaction was collected and freeze-dried. This was then analysed by FAB-MS (*m/z* 166 [O^6-methylguanine + H]$^+$ and 366 [di-acetyl O^6-MedG + H]$^+$) and ^{1}H-NMR (acetone-d_6) δ 2.0 (m, undistinguishable), 2.5 (m, 1, $\mathbf{H^{2'}}$), 3.1 (m, 1, $\mathbf{H^{2''}}$), 3.9 (s, 1, $OC\mathbf{H_3}$), 4.4 (m, 3, $\mathbf{H^{5'}}$, $\mathbf{H^{5''}}$, $\mathbf{H^{3'}}$), 5.4 (d, 1, $\mathbf{H^{4'}}$), 5.7 (s, 2, $N\mathbf{H_2}$), 6.3 (t, 1, $\mathbf{H^{1'}}$), 7.9 (s, 1, $\mathbf{H^8}$) and determined to be di-acetyl O^6-MedG. In addition to the major product, tri-acetyl O^6-MedG was also formed in approximately 40% yield, which is considerably lower than the proportion generated using the original acetylation method (method A above), indicating that incorporation of the transfer step accounts, at least in part, for the difference in reaction profile.

LC-MS assay for analysis of acetylated O^6-MedG.

Authentic standards of O^6-MedG (20 µl) were injected onto a 250 x 4.6 mm Luna C_{18}(2) column (Phenomenex) and detected by selected ion recording (SIR). To determine the

limit of detection of O^6-MedG adducts using LC-MS, aliquots of O^6-MedG (5 fmoles to 300 pmoles) were dried under nitrogen, redissolved in anhydrous pyridine (20 µl) and d_6-acetic anhydride ($C_4D_6O_3$, 5 µl), then incubated at 100°C for 1 h. Reactions were stopped by the addition of 50% aqueous MeOH (20 µl) and evaporated to dryness under nitrogen. The reactions were then redissolved in 20% MeOH (20 µl) and analysed directly by LC-MS using unlabeled tri-acetyl O^6-MedG as an internal standard. In addition, a standard of deuterated tri-acetyl O^6-MedG was analysed by ^{1}H-NMR: (D_2O), δ 2.7 (m, 1, $\mathbf{H^{2'}}$), 3.1 (q, 1, $\mathbf{H^{2''}}$), 4.05 (s, 3, OC$\mathbf{H_3}$), 4.3 (m, 3, $\mathbf{H^{5'}}$, $\mathbf{H^{5''}}$, $\mathbf{H^{3'}}$), 5.5 (s, 1, $\mathbf{H^{4'}}$), 6.5 (m, 1, $\mathbf{H^{1'}}$).

Limit of Detection of ^{14}C-Di-acetyl O^6-MedG by LSC.

To ascertain the limit of detection of isolated ^{14}C-acetylated adduct by LSC, a standard of ^{14}C-di-acetyl O^6-MedG (58 nmoles in 1 ml of H_2O) was diluted to provide 9 samples, spanning a concentration range covering four orders of magnitude. The dilutions were quantitated by LSC and were used to plot a calibration line (Figure 3). The same samples were also subject to HPLC separation (Method 2), and the fraction corresponding to ^{14}C-di-acetyl O^6-MedG collected and quantitated by LSC for each dilution. The dpm values were converted to fmoles of labeled adduct, and used to construct a curve for ^{14}C-di-acetyl O^6-MedG.

Limit of Detection of ^{14}C-Di-acetyl O^6-MedG by AMS

To determine the limits of detection of ^{14}C-di-acetyl O^6-MedG by AMS, samples of labeled adduct were diluted to produce concentrations below the limit of detection by LSC. Precautions were taken to prevent contamination of the AMS preparation laboratory

[26], therefore prior to analysis, samples were screened by LSC to ensure they did not contain measurable amounts of radioactivity above background levels. Duplicate samples of each dilution were subject to HPLC separation (Method 3) and one-minute fractions were collected throughout the entire run. The dilutions were injected from lowest to highest concentration to avoid cross contamination due to carry-over, with two blank injections of dH_2O in between samples. The HPLC fractions expected to contain the ^{14}C-di-acetyl O^6-MedG peak, eluting at 27 min, were submitted for AMS analysis, along with fractions immediately preceding the adduct (25 and 26 min). Analysis of a 28 min fraction revealed a low level of ^{14}C, similar to the 26 min fraction, confirming that the di-acetylated adduct elutes in the 27 min fraction.

Analysis of samples by AMS

AMS analysis of samples was carried out at Lawrence Livermore National Laboratory, according to the standard protocol [27]. Each sample was evaporated to dryness in a Speedvac then the residue was redissolved in 20% aqueous MeOH (1 ml). Half of each sample was added to a quartz tube with 100% tributyrin (1 µl), and was converted to graphite as described by Ognibene *et al.*, [28] then was analysed by AMS. Each graphite sample was analysed up to seven times for radiocarbon content by AMS or until the measurement variation was within ± 5%. The resulting data was converted from fraction modern to attomoles of O^6-MedG, taking into consideration the specific activity of the ^{14}C-acetic anhydride and the fraction labeled [29].

RESULTS

Acetylation of O^6-MedG.

Reaction of O^6-MedG with acetic anhydride yielded tri-acetyl O^6-MedG as the major reaction product (>90% yield), as indicated by ESI-MS analysis, which demonstrated the presence of quasi molecular ions at *m/z* 208 [mono-acetyl O^6-MeG + H]$^+$ and the major product, 408 [tri-acetyl O^6-MedG + H]$^+$, in addition to the corresponding Na^+ and K^+ adduct ions. A standard of this compound was produced in a larger-scale reaction and ^{1}H-NMR analysis confirmed the structure as having three acetyl groups on O^6-MedG, with one attached to the NH of guanine and the remaining two to the deoxyribose OH groups.

Limit of Detection of Tri-acetyl O^6-MedG by LC-MS.

To determine the limit of detection of tri-acetyl O^6-MedG by LC-MS, deuterated acetic anhydride was used to synthesise isotope-labeled derivatised adducts and the unlabeled tri-acetyl O^6-MedG standard was employed as an internal standard. The assay was conducted in this manner as a pure unlabeled tri-acetyl O^6-MedG standard had previously been synthesised, whereas a deuterated standard was not available at that time.

Initial LC-MS (SIR) analysis of the deuterated product from the acetylation reaction of 300 pmoles O^6-MedG did not reveal any ions at *m/z* of 417 and 439, which would be consistent with protonated d_9-tri-acetyl O^6-MedG and its sodiated ion. Therefore, a total ion scan of the reaction sample was carried out using tri-acetyl O^6-MedG as an internal standard. The resulting mass spectrum showed a sodiated molecular ion for tri-acetyl O^6-MedG with *m/z* 430, as expected, but also present were ions at *m/z* 418, 440 and 456, consistent with the addition of a proton, along with sodium and potassium ions

respectively, to the original predicted $[M+H]^+$ value (data not shown). This means that the compound has a molecular weight of 417, which is one mass unit higher than expected after the addition of three deuterated acetyl groups. ^{1}H-NMR analysis suggests that deuterium exchange has occurred at the H^8 position, since there is no peak visible for this proton in the spectrum (data not shown). This would account for the additional mass unit and consequently d_{10}-tri-acetyl O^6-MedG was utilised in this assay. Reactions were analysed by LC-MS using SIR detection, to ascertain the limit of detection. The ratio of peak areas of d_{10}-tri-acetyl O^6-MedG formed in reactions to unlabeled internal standard was used to construct a calibration line, as shown in Figure 3. The relationship between d_{10}-tri-acetyl O^6-MedG and the internal standard was linear, as expected, and the limit of detection was calculated as 10 fmoles of adduct, taking into account a signal to noise ratio of 1:5 ($n = 3$).

Acetylation of O^6-MedG using ^{14}C-acetic anhydride.

The use of ^{14}C-acetic anhydride as the labeling agent required that the acetylation method be altered as a transfer step had to be incorporated to remove the reagent from its supplied vial and an enclosed drying method was also necessary to prevent any ^{14}C-labeled material from escaping during evaporation. An unlabeled standard was prepared using this method, which had the same HPLC retention time as the major labeled reaction product and was characterised by FAB-MS and ^{1}H-NMR analysis. FAB-MS demonstrated that the major reaction product was di-acetyl O^6-MedG, as shown by the presence of quasi molecular ions at m/z 366 [di-acetyl O^6-MedG + H]$^+$ and m/z 166 [O^6-MeG + H]$^+$. This is further confirmed by ^{1}H-NMR, which indicates the two acetyl groups are present on the deoxyribose, due to the absence of OH protons in the NMR spectrum.

HPLC separation of the ^{14}C-acetylation reaction revealed that the major product was di-acetyl O^6-MedG (formed in 38 % yield), as the retention time was identical to that of the synthesised unlabeled standard. Importantly, no tri-acetyl O^6-MedG was detectable, and as the reaction of O^6-MedG with ^{14}C-acetic anhydride failed to generate any of the desired tri-acetylated derivative, di-acetyl O^6-MedG was consequently used in further development and application of the assay. The ^{14}C-di-acetyl O^6-MedG standard was collected by HPLC separation of the reaction products, concentrated under nitrogen, further purified by HPLC, and quantitated by LSC.

Limit of Detection of di-acetyl O^6-MedG by LSC after HPLC isolation

A linear relationship can be observed between the amount of ^{14}C-di-acetyl O^6-MedG and radioactivity contained in dilutions above a limit of detection of 745 fmoles, as measured by LSC. In comparison, further HPLC isolation of the adduct contained in these same dilutions followed by LSC quantitation increases the limit of detection to 1.4 pmoles, due to losses incurred during HPLC (Figure 4).

Limit of detection of di-acetyl O^6-MedG by AMS

For samples analysed by AMS, the total amount of ^{14}C contained in each submitted sample and control was calculated from the fraction Modern values, to construct a limit of detection curve for this adduct. The theoretical limit of detection, defined as the mean blank (solvent control) plus 3 times standard deviation of the mean (n = 5), is dependent on the background level of ^{14}C in the HPLC fractions collected from blank runs. As

shown in Figure 5, the limit of detection was equivalent to 79 attomoles of di-acetyl O^6-MedG in a 1 ml HPLC fraction. The limit of detection is calculated from the amount of ^{14}C present in the blank runs performed in between each sample run, which is influenced by any ^{14}C-labeled chemicals/ solvents injected onto the system.

Discussion

The ultimate aim of this study was to develop methodology for the derivatisation of O^6-MedG by ^{14}C-postlabeling, generating products that could be isolated and quantified by AMS. For comparison purposes the limits of detection attainable by more conventional methods for this adduct were also determined and the results clearly highlight the greater sensitivity of AMS.

The strategy taken was to establish a reaction protocol using unlabeled reagents for derivatising O^6-MedG by acetylation, then to adapt this for use with ^{14}C-acetic anhydride. The preferred reaction product should contain as many acetyl groups as possible, which based on initial reactions would be the tri-acetylated derivative, since for AMS analysis it is desirable to incorporate as many ^{14}C atoms as possible to maximise assay sensitivity. However, which specific acetylated derivative is chosen for quantitation purposes in the assay is not so important, providing that the reactions and yields of the derivative are consistent and the results reproducible, although derivatisation of two rather than three functional groups reduces the theoretical detection limits. This is particularly important with respect to assay efficiency, so as to allow losses due to acetylation and isolation of

the reaction products to be accounted for, and the level of adducts in a DNA sample accurately determined.

In a model reaction, LC-MS analysis was used to determine the limit of detection for deuterated tri-acetyl O^6-MedG. The fact that d_{10}-tri-acetyl O^6-MedG was the major product of the acetylation reaction with d_6-acetic anhydride appears to be caused by deuterium exchange at the H^8 position, as evidenced by ^{1}H-NMR analysis. Since acetic anhydride contains two stable methyl groups, the potential for donating a deuterium ion seems unlikely, although it is possible that acetic anhydride may tautomerise, thus releasing a deuterium ion available for exchange. It is also possible that the d_6-acetic anhydride purchased may contain impurities or degradation products, such as acetic acid, which could more easily donate a deuterium ion. The level of derivatised adducts measured for this assay resulted in a linear calibration line (Figure 3), and the limit of detection was determined as 10 fmoles.

Acetylation of O^6-MedG with ^{14}C-acetic anhydride yielded a change in the profile of reaction products, with di-acetyl O^6-MedG formed as the major product, as characterised by FAB-MS and ^{1}H-NMR using an unlabeled standard. Whilst the reason for this difference is not entirely clear, it is possible that since the newly incorporated transfer step was not performed under an anhydrous atmosphere, increased hydrolysis of the ^{14}C-acetic anhydride may account for the reduction in yield of the desired product. A further contributing factor could be the presence of impurities or unknown stabilising agents in the radiolabeled acetic anhydride that are not contained in the unlabeled reagent. As a

consequence of this finding and since this derivative was consistently demonstrated to be the major product, it was decided that this acetylated derivative would be used as the adduct form for quantitation by LSC and AMS in subsequent experiments.

The limit of detection of ^{14}C-di-acetyl O^{6}-MedG by LSC was found to be 745 fmoles, determined by quantifying the ^{14}C content in a series of diluted samples. Since in the final assay the labeled adduct will be purified by HPLC prior to AMS analysis, the effect of this step on the limit of detection was determined. On constructing a second calibration curve of ^{14}C-concentration measured by LSC in the same dilutions after HPLC separation and collection of the appropriate fractions the limit of detection was found to be 1.4 pmoles of adduct (Figure 4). This is approximately two-fold higher than the limit of detection achievable without HPLC. When dilutions containing less than 1.4 pmoles ^{14}C-di-acetyl O^{6}-MedG were analysed, the fractions contained more radioactivity than the ambient background level of 25 dpm and therefore were above the limit of detection, most likely caused by the background contamination level on the HPLC. In this range the relationship between amount of adduct present and ^{14}C measured was no longer proportional.

To determine the limit of detection by AMS, further serial dilutions of ^{14}C-di-acetyl O^{6}-MedG were prepared down to attomole levels and checked by LSC to ensure that only samples below a background level of 25 dpm are handled in the AMS lab, to prevent contamination. These steps were taken as it is recommended that no more than 10 dpm should be injected onto a HPLC system using a semi-prep column, in order to prevent

contamination [26]. AMS analysis of the HPLC purified ^{14}C-diacetyl-O^6-MedG fractions and random blank runs allowed the limit of detection to be determined. The amount of ^{14}C in each sample was converted into fmoles of di-acetyl O^6-MedG by taking into account the fraction labeling. The assay limit of detection, defined in this study as the mean background level of ^{14}C in blank fractions plus 3 times the standard deviation [30] was calculated as 79 attomoles of di-acetyl O^6-MedG, which is approximately 9500 fold more sensitive than LSC, without a HPLC step (Figure 6). In comparison, Goldman *et al.* attained a theoretical limit of detection of 100 attomoles for their acetylated BPDE adduct standard [24]. The maximum sensitivity attainable for this adduct should theoretically be greater because five ^{14}C-acetyl groups are added, compared to two acetyls reacted with O^6-MedG in this study. The chemistry employed in ^{14}C-labeling protocols requires that every assay has to be specifically developed and optimised for each individual DNA adduct, as potential sites of acetylation are dependent on adduct structure. Therefore, whilst the general steps undertaken in this assay are similar to those described by Goldman *et al*. [24], the details are inherently different. A particular feature of the assay developed in this study is the design and use of an enclosed drying system, which enables ^{14}C-postlabeling to performed in any designated fumehood, without the need for a specific radioactive laboratory, whilst ensuring all excess radioactivity is converted to a non-volatile form and safely contained.

Typical limits of detection for LC-MS are in the region of 1 adduct per 10^8 nucleosides [31, 32] but more sensitive assays have been developed, for example a limit of detection for BPDE-dG of 0.3 adducts in 10^8 normal nucleotides has been described [33], and as low

as 1-2 adducts per 10^{10} nucleotides for etheno dA and dC adducts has been reported [34], using LC-MS/MS with MRM analysis. As technologies improve, the limits of detection for DNA adduct analysis using conventional mass spectrometry will become increasingly more sensitive and may eventually be comparable to those currently achieved with AMS. Whilst ^{32}P-postlabeling is presently one of the most sensitive routinely used assays for the measurement of DNA adducts, and is generally more straightforward, than the ^{14}C-postlabeling assay presented here, the latter does offer several advantages: it is considerably more sensitive, does not suffer from artefacts since specific, known adducts are isolated and measured directly after labeling, and it is not limited by the half-life of ^{14}C, unlike the isotope ^{32}P. In addition, the ^{14}C-postlabeling assay should be easily adaptable for the measurement of any adduct regardless of size, including small alkyl adducts which can be difficult to detect by ^{32}P-postlabeling [35].

The limit of detection is the lowest amount of adduct that can be reliably quantified using the current methodology. In practice however, adduct recovery throughout isolation digestion, labeling and clean-up procedures prior to HPLC purification will not be 100%, therefore the limit of detection, relative to the amount of O^6-MedG in the original DNA sample, will actually be higher than this value. Typical recovery rates for the entire assay are in the order of 29 ± 10% and are relatively reproducible. Knowing the assay efficiency it is possible to more accurately estimate adduct levels in the starting sample.

To conclude, a preliminary ^{14}C-postlabeling assay has successfully been developed for the quantitation of O^6-MedG by AMS. The assay has been demonstrated to be several

orders of magnitude more sensitive for the detection of this adduct than current conventional methods. However, the assay still needs to be fully validated and applied to the measurement of O^6-MedG in DNA from exposed animals or humans to determine robustness and reproducibility. Initial studies indicate the assay should be sensitive enough to measure in the region of 1-10 O^6-MedG adducts per 10^{12} nucleotides (data not shown), which is four orders of magnitude lower than adduct levels previously reported in human tissue [19]. Ultimately this assay has enormous potential and with such improved sensitivity it should be possible to establish background levels of O^6-MedG in human populations as a result of endogenous or environmental exposures.

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Figure Legends

Figure 1. Proposed sites of O^6-MedG acetylation after reaction with acetic anhydride. On reaction with ^{14}C-acetic anhydride however, a di-acetylated derivative is the major product with acetyls at the 3'(C)O and 5'(C)O positions of the sugar moiety (*).

Figure 2. Containment apparatus designed to carry out ^{14}C-postlabeling reactions in a closed environment. The evaporated ^{14}C-acetic anhydride freezes onto the sides of the dry ice/acetone chamber [A], which on melting is safely collected [B]. Two additional traps are present as a further safety measure.

Figure 3. Typical LC-MS analysis of tri-acetyl O^6-MedG using SIR (**A**). d_{10}-Tri-acetyl O^6-MedG was quantified using unlabeled tri-acetyl O^6-MedG as the internal standard. The ratio of peak areas of the sodiated molecular ions (m/z = 440 and 430 for the deuterated and unlabeled tri-acetylated adduct respectively) was used to construct a calibration line as shown in (**B**), from which adduct levels could be determined. For each data point the mean of three different acetylation reactions was plotted. The limit of detection was determined to be 10 fmoles using this method.

Figure 4. HPLC separation of the ^{14}C-postlabeling reaction products is shown in (**A**): peaks 1 and 2 correspond to pyridine and unreacted O^6-MedG, 3 is ^{14}C-mono-acetyl O^6-MedG, 4 and 5 are ^{14}C-di-acetyl O^6-MedG derivatives. Peak 5 was collected as the major product and used to determine limits of detection of 0.75 and 1.4 pmoles for LSC and

HPLC separation with LSC, respectively as shown in (**B**). Each dilution was measured twice.

Figure 5. Relationship between the amount of adduct injected onto the HPLC system and that detected in the appropriate fraction by AMS (n =2). The limit of detection of ^{14}C-di-acetyl O^6-MedG (------) was determined to be 79 attomoles.

Figure 6. Comparison of the detection limits of ^{14}C-di-acetyl O^6-MedG by methods used in this study, illustrating the sensitivity of AMS relative to conventional methods.

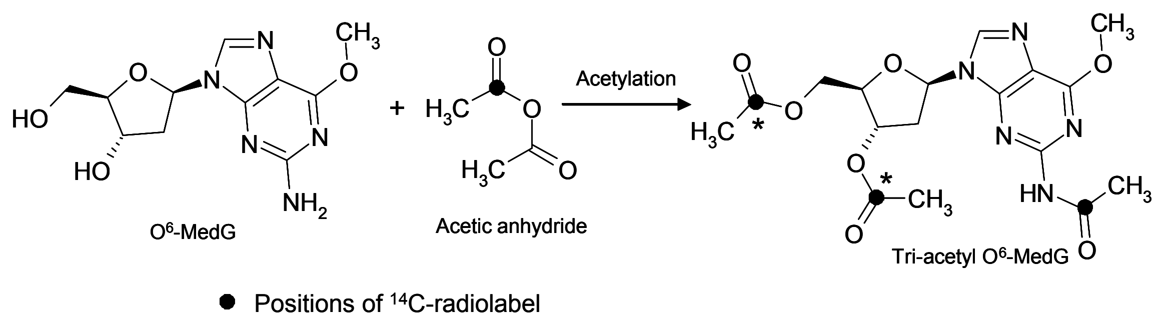

Figure 1.

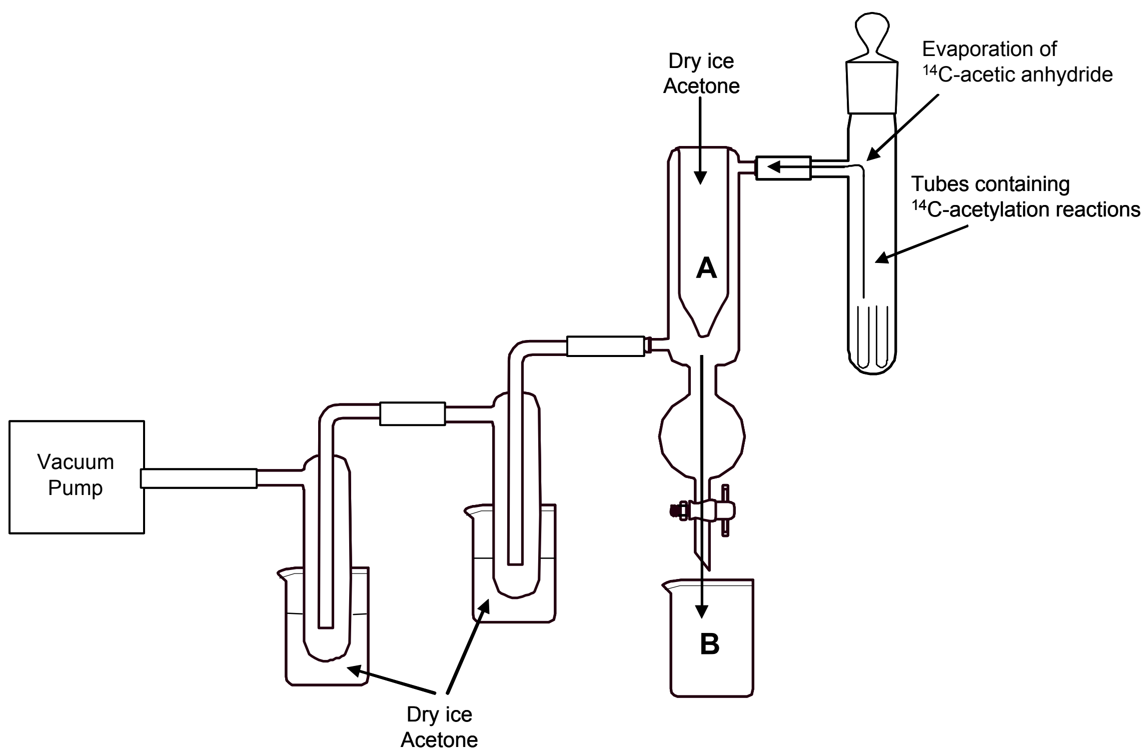

Figure 2.

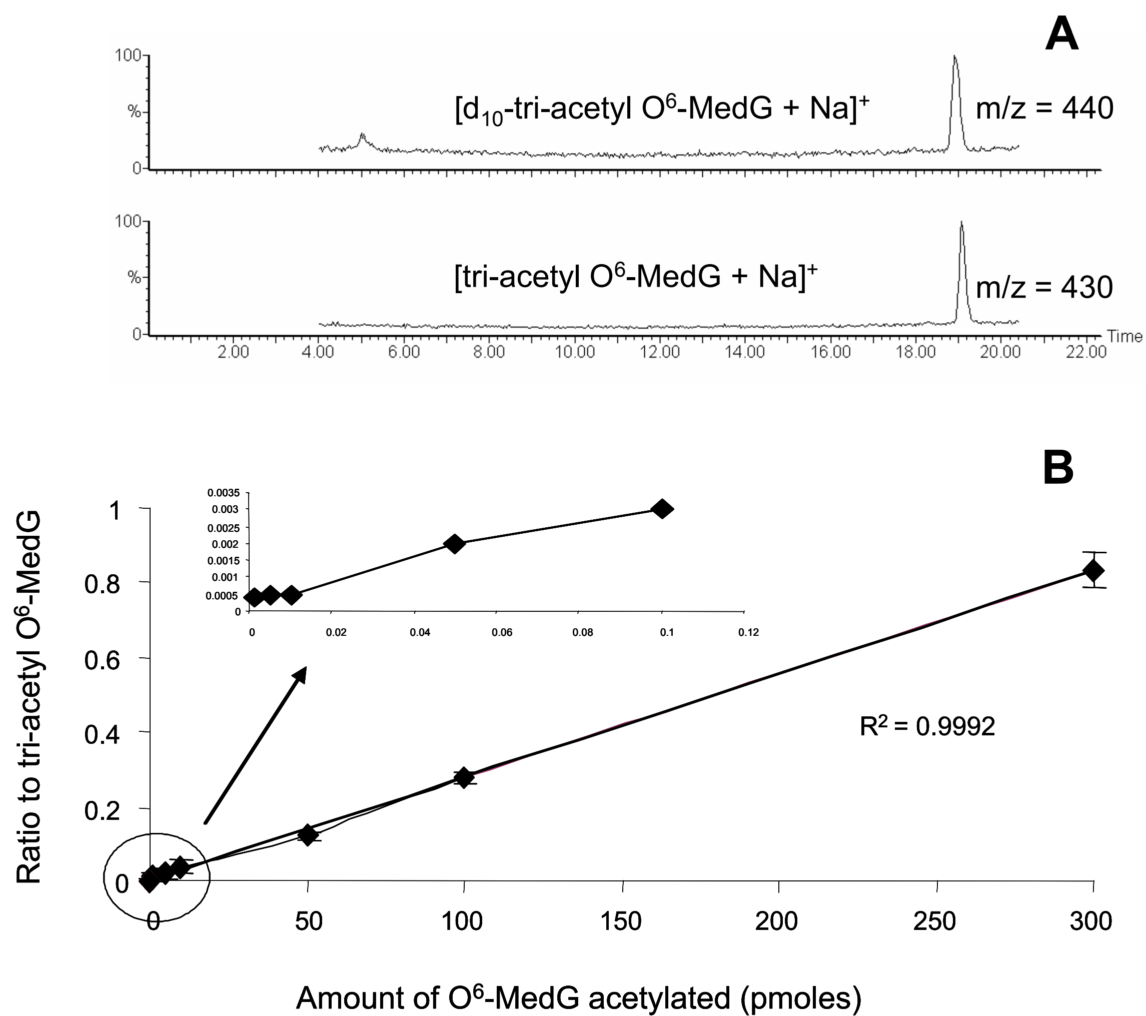

Figure 3.

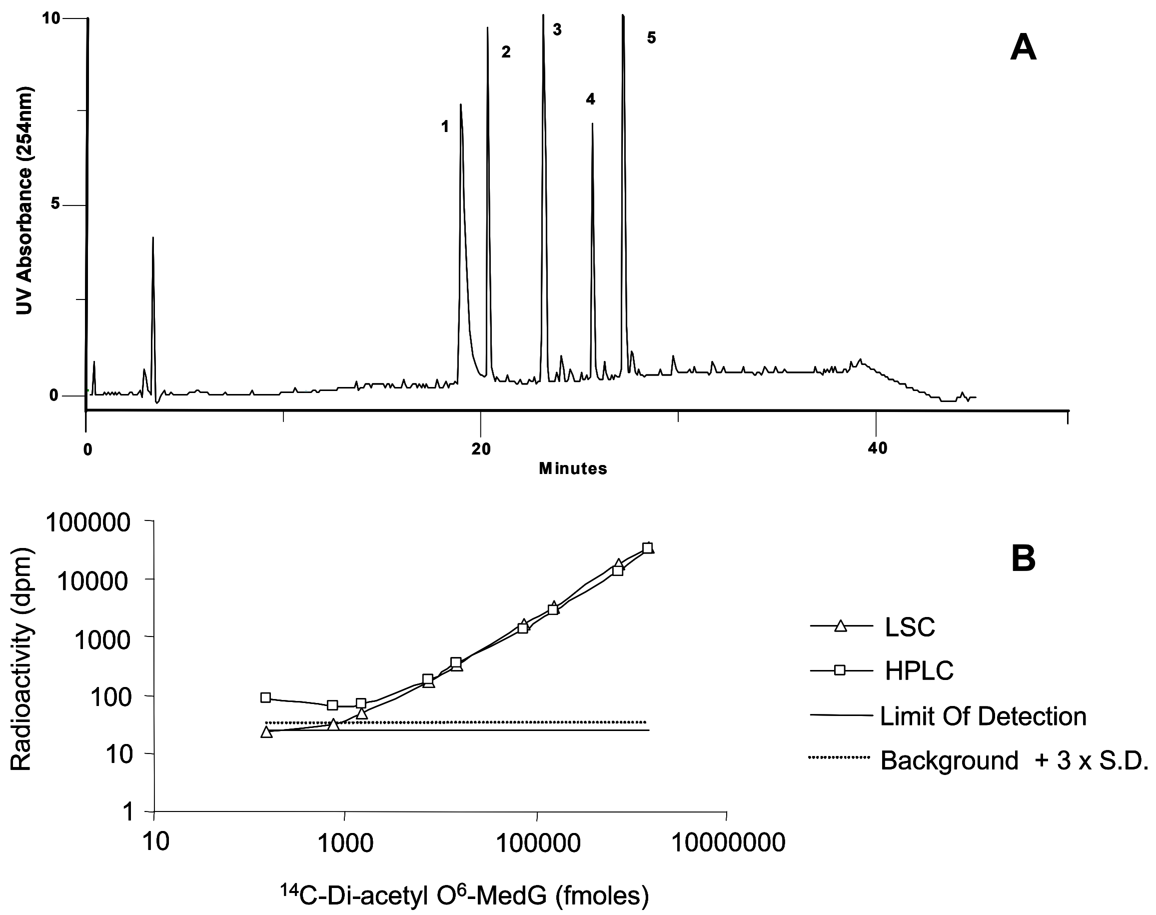

Figure 4.

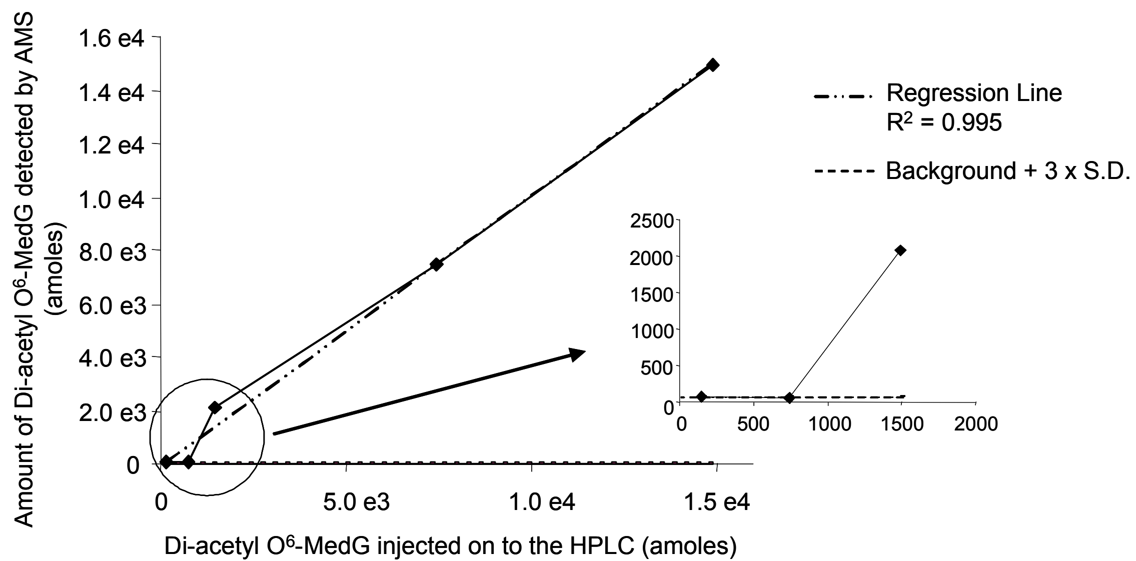

Figure 5.

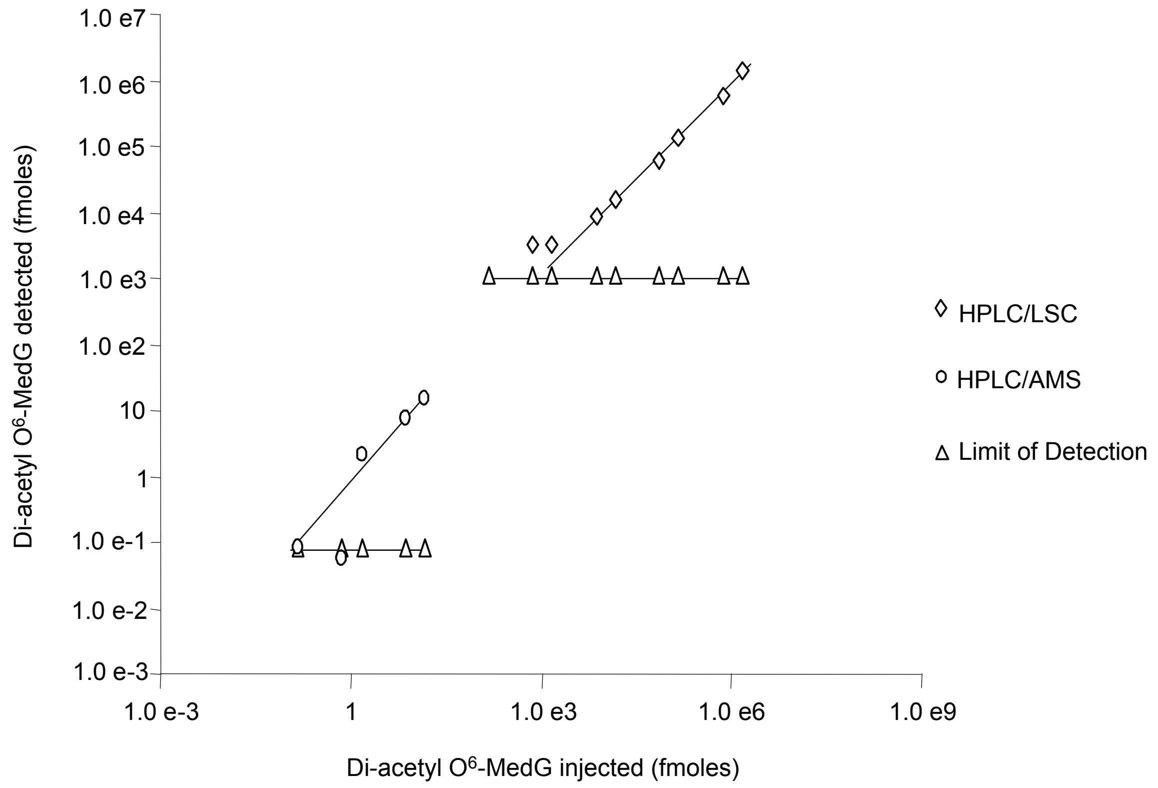

Figure 6.