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A Comparison of μLC/Electrospray Ionization-MS and GC/MS for the Measurement of Stable Isotope Enrichment from a [$^2H_2$]-Glucose Metabolic Probe in T-Cell Genomic DNA

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Measurement of the proliferation of lymphocytes and other high-turnover cell populations in vivo can be accomplished through the incorporation of an isotopically labeled DNA precursor into actively dividing cells and the subsequent determination of the isotope enrichment in the isolated genomic DNA from selected cell populations. Two published gas chromatography/mass spectrometry (GC/MS) methods were successfully modified by our laboratory whereby a postinjection methylation reaction, rather than silylation or acetylation, was used to form a volatile derivative of deoxyadenosine (dA). We also developed a second robust microcapillary liquid chromatography-electrospray ionization (μLC-ESI)/MS method that is faster and more sensitive than the GC/MS method and does not require sample derivatization. Following administration of [6,6-$^2H_2$]-glucose to human immunodeficiency virus-infected patients, peripheral blood was drawn; cells were obtained by lymphapheresis and fractionated. DNA was isolated from the desired cell subtypes and enzymatically hydrolyzed to the free deoxyribonucleosides. The digest was analyzed using both capillary GC/MS and μLC/ESI-MS to measure the levels of the dA and [$^2H_2$]-dA or their reaction products. Sample enrichments were calculated by comparison to standard curves prepared from dA and [$^2H_2$]-dA. The μLC/ESI-MS method required fewer cells, less sample preparation, shorter analysis times, and a single calibration curve. Overall, the μLC/ESI-MS method is superior to the GC/MS method in terms of precision and accuracy, while providing a 4-fold increase in sensitivity (from 20 pmol at 0.2% [$^2H_2$]-dA enrichment to 5 pmol at 0.1% [$^2H_2$]-dA enrichment).

Examining cell turnover in vivo can potentially provide important insights into the biology or pathophysiology of metabolic diseases such as acquired immunodeficiency syndrome (AIDS) and cancer. Direct measurement of proliferation in lymphocytes and other high-turnover cell populations in vivo has been used to infer the rate of cell division in human immunodeficiency virus (HIV)-infected patients with AIDS. One approach that is used to study lymphocyte kinetics is the administration of an isotopically labeled DNA precursor, which is then incorporated into actively dividing cells, and the subsequent determination of isotope enrichment in the isolated genomic DNA from the selected cell subpopulations.[1] In recent clinical studies, [6,6-$^2H_2$]-glucose has been used as a precursor to de novo nucleotide synthesis due to its benign nature (i.e., lack of radioactivity and toxicity). Following the intravenous administration of [6,6-$^2H_2$]-glucose to HIV-infected patients (or, for comparison, uninfected individuals) and its incorporation into the DNA of dividing cells, peripheral blood is drawn and fractionated. DNA is isolated from the desired cell subtypes and enzymatically hydrolyzed to the free deoxyribonucleosides.[1] Measurement of the isotopic enrichment of the DNA (2′-deoxyribonucleosides) is used to infer the rate of cell division and half-life using established mathematical models.[2]

Gas chromatography (GC) and liquid chromatography (LC) have both been used to separate and identify nucleosides from urine[3,4] and hydrolyzed DNA.[5,6,10−12] These two methods can be

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combined with on-line MS analysis to identify and quantify isotopically enriched deoxyribonucleosides (dRNs), which has proven to be essential for the study of T-cell proliferation in vivo.[7−9] Macallan et al. developed a GC/MS method for the quantification of isotopically labeled dRNs.[13] The first step described in this approach is the administration of glucose labeled with deuterium to the subject of interest (e.g., cell lines, rats, humans, etc.). After a specific time period, genomic DNA is isolated and hydrolyzed to give free dRNs that are derivatized with trimethylsilane and analyzed by GC/MS. The amount of incorporated deuterium indicates how much new DNA was synthesized (i.e., the rate of cell proliferation starting from the time that deuterated glucose is administered to the subject). This method, however, is limited in terms of yield, stability, sensitivity, and reproducibility and requires multidimensional abundance-corrected standard curves.[14]

A recent report from Neese et al.[14] introduced several improvements to the original GC/MS method that addressed these limitations. In this approach, the dA was isolated from the DNA hydrolysates using a reversed-phase solid-phase extraction (RP-SPE) column. Two derivatives were developed for analyzing the deoxyribose (dR) moiety of dA alone, with an aldonitrile−triacetate derivative, and with a reduced pentosetetraacetate (PTA) derivative. The PTA derivative showed greater stability and higher ion abundance compared to previous derivatives of dA. Therefore, the need for abundance-corrected standard curves was eliminated. This newer method, however, is more laborious and time-consuming.

The Macallan et al. approach was not reproducible in our laboratory, and the Neese et al. method was not robust enough for high-throughput analysis. Therefore, we modified the original GC/MS quantitative method by performing a simple permethylation step to derivatize dA prior to analysis. This modification results in a volatile, stable derivative that can be analyzed by GC/MS, is reproducible, and has been used successfully to study T-cell depletion turnover in HIV-1 disease.[15]

Although this modified GC/MS method gave good results, we recently designed a faster, more sensitive method based on the use of microcapillary liquid chromatography-electrospray ionization (μLC-ESI)/MS. A major advantage of this approach is that it does not require a derivatization step. In this report, we compare this new μLC/ESI-MS method with the modified GC/MS method that we previously developed for determining DNA synthesis in vivo through the quantitation of [$^2H_2$]-dA.

EXPERIMENTAL SECTION

Chemicals and Materials. 2′-Deoxyadenosine, anhydrous monobasic sodium phosphate, and anhydrous dibasic sodium phosphate were purchased from Sigma (St. Louis, MO), (5,5′-$^2H_2$)-2′-deoxyadenosine was from Cambridge Isotope Laboratories (Andover, MA), MethElute reagent was from Pierce (Rockford, IL), ammonium acetate and formic acid were from Fluka (Milwaukee, WI), LC-grade acetonitrile and methanol were from EM Science (Gibbstown, NJ), and ACS-grade *N*,*N*-dimethylformamide was from Fisher Scientific (Fairlawn, NJ). Deionized water was generated in-house using a NANOpure ultrapure water system from Barnstead (Dubuque, IA). An Oasis-HLB 96-well (solid-phase) extraction plate was purchased from Waters Corp. (Milford, MA), and 96-well (deep) polypropylene plates were from Marsh Bio-Products (Rochester, NY).

Sample Preparation. Following administration of the labeled glucose precursor to study volunteers, peripheral blood was drawn (or lymphapheresis was performed) and mononuclear cells were obtained by density gradient centrifugation.[15] The cells were further separated by fluorescent activated cell sorting or magnetic bead separation into lymphocyte subsets. Genomic DNA was isolated from the frozen pelleted cells using the Puregene kit (Gentra Systems, Minneapolis, MN) and hydrolyzed to free deoxyribonucleosides with DNase I, phosphodiesterase I, and bacterial alkaline phosphatase. Prior to analysis, the digest was cleaned by RP-SPE, using an Oasis-HLB (30 mg) 96-well extraction plate prepared as follows: 1 mL of methanol was aspirated through each well under vacuum followed by 1 mL of deionized water. Three-hundred microliters of 100 mmol L^{-1} phosphate buffer (pH 7.0) and 50 μL of the DNA enzymatic digest were then pipetted into each well. The sample solution was aspirated through the sorbent bed followed by 0.5 mL of deionized water and allowed to dry by aspirating air. Aspirating 0.5 mL of methanol effects elution of the dRNs. The eluate was collected, split into two equal volumes, and evaporated to dryness using a vacuum centrifuge (Jouan model RC10.10, Winchester, VA). For GC/MS analysis the residue was reconstituted in 20 μL of *N*,*N*-dimethylformamide to which an equal volume of MethElute reagent was added followed by agitation for 1 min on a vortex mixer. The solution was then transferred to a 200-μL glass autosampler vial and analyzed. For μLC/MS analysis, the residue was reconstituted in 50 μL of deionized water followed by agitation for 1 min on a vortex mixer, transferred to a 96-well (V-shaped) polypropylene microtiter plate, and analyzed by μLC/MS.

Instrumentation and Measurement of [$^2H_2$]-dA and dA. GC/MS Analysis. All analyses were carried out using a 6890 series GC system from Agilent Technology (Palo Alto, CA) connected to a 5973 series mass selective detector (Agilent Technology) equipped with a 7683 autosampler, a split/splitless sample injector, and a Hewlett-Packard Kayak PC that runs ChemStation (Agilent Technologies) software for data acquisition and instrument control. A 1-μL sample aliquot was injected into a 4-mm deactivated glass injection liner operating in the splitless mode. Gas chromatography was performed using a 15 m × 0.25 mm DB-5, 0.25-μm film fused-silica capillary column from J & W Scientific (Folsom, CA) with helium carrier gas at a flow rate of

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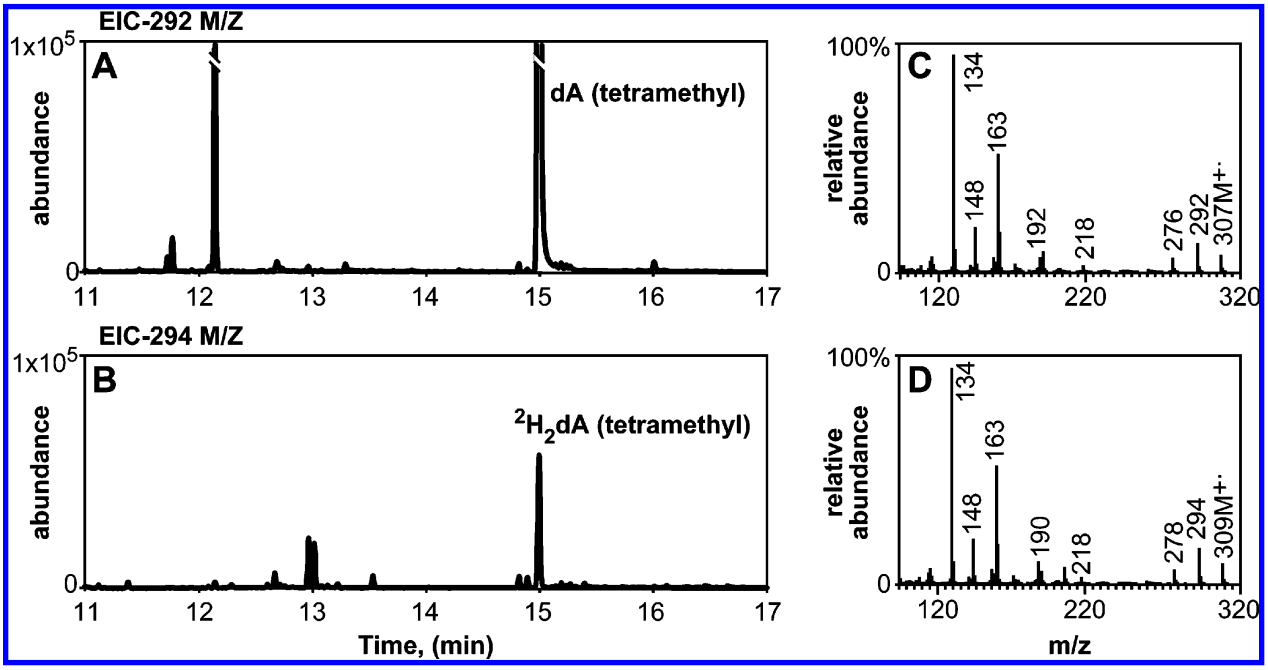

Figure 1. (left side) Extracted ion chromatograms from GC/MS-SIM analysis of a clinical DNA enzymatic digest reacted with MethElute: (A) ion profile for *m*/*z* 292 (tetramethyl-dA); (B) ion profile for *m*/*z* 294 (tetramethyl-[$^2H_2$]-dA). (right side) Full-scan electron-impact ionization mass spectra: (C) dA treated with MethElute showing the expected molecular radical cation at *m*/*z* 307 and loss of −(CH_3) at *m*/*z* 292; (D) [$^2H_2$]-dA treated with MethElute showing the expected molecular radical cation at *m*/*z* 309 and loss of −(CH_3) at *m*/*z* 294.

0.5 mL/min. The oven temperature was held at 60 °C for 3 min, then programmed to 275 °C using a gradient of 15 °C/min, and held at the final temperature for 4.4 min. Injector temperature and the MS transfer line were set at 250 and 280 °C, respectively. The mass selective detector was operated in electron-impact ionization/single-ion monitoring mode, recording ion currents for *m*/*z* 292 and 294, which corresponded to the loss of −(CH_3) from the permethylated [−(CH_3)$_4$] reaction products of deoxyadenosine (dA) and [$^2H_2$]-dA, respectively. Additional operating parameters were as follows: 6-min solvent delay, 100-ms dwell time, electron multiplier voltage of 1750 V, 230 °C source temperature, and 150 °C analyzer temperature. The ratio of the peak area of [$^2H_2$]-dA (*m*/*z* 294) to that of the total dA pool (*m*/*z* 292 + 294) was measured and compared to a concentration-dependent calibration curve generated by mixtures of [$^2H_2$]-dA and dA of known composition from 0 to 10% enrichment. Multiple linear regression analysis was used to correct for the effect of ion abundance (i.e., a line in two dimensions defined by the equation; $y = m_1x_1 + m_2x_2 + b$).

μLC/ESI-MS. All μLC/ESI-MS experiments were carried out using Agilent Technologies 1110 series instrumentation. The μLC system was connected on-line to an MSD/Trap equipped with an ESI source and a Hewlett-Packard Vectra PC that runs Chemstation software for data acquisition and instrument control. Chromatography was carried out using a 100 mm long × 0.5 mm i.d., glass-lined stainless steel (SGE, Austin, TX) microanalytical column packed in-house with Luna C-18 (5 μm) particles (Phenomenex, Torrance, CA). A sample volume of 1 μL was injected and eluted using a 15 μL/min mobile-phase gradient beginning at 3% (v/v) acetonitrile/97% 5 mM ammonium acetate, pH 5.0, for 1 min, increased linearly to 40% (v/v) acetonitrile over 6 min, and held for 3 min after which initial conditions were restored. ESI mass spectral data were acquired in positive ion mode using the following ion source conditions: drying gas temperature 250 °C at a flow of 7 L/min, nebulizer pressure at 15 psi, and capillary voltage at −3000 V. The ion trap settings used were as follows: accumulation time 100 ms, ion charge control target 30 000, scan range *m*/*z* 248−258. Extracted ion chromatograms were generated for *m*/*z* 252 and 254, respectively, corresponding to the protonated molecular ions $(M + H)^+$ of dA and [$^2H_2$]-dA. Quantitation was performed using a standard calibration curve. The ratio of the peak area of [$^2H_2$]-dA (*m*/*z* 252) to that of the total dA pool (*m*/*z* 252 + 254) was determined and compared to a concentration-independent calibration curve generated by mixtures of [$^2H_2$]-dA and dA of known composition from 0 to 10% enrichment. Multiple linear regression analysis was also performed for the purpose of direct comparison to GC/MS results.

RESULTS AND DISCUSSION

Modification of the existing GC/MS method to measure DNA synthesis[13] was carried out because the silylation of dA is prone to hydrolysis, and the modified method designed by Neese et al. is more labor-intensive and time-consuming, requiring purification of dA by SPE followed by derivative formation reactions and organic extraction.[14] Our work required a method that is sensitive, reproducible, and high throughput. A postinjection methylation reaction was used to form the tetramethyl derivative of dA, a volatile compound, which is a requirement for GC/MS analysis. The full-scan electron-impact ionization mass spectrum revealed that only high-mass ions contained the isotopic label (Figure 1C,D); therefore, the most abundant of these characteristic fragment ions were chosen for single ion monitoring (SIM) analysis, *m*/*z* 292 and 294 for tetramethyl dA and tetramethyl [$^2H_2$]-dA, respectively (Figure 1A,B). Calibration curves (Figure 2) were generated for 20 and 100 pmol of total dA, which covered deuterium enrichments of 0.1−10%. Samples of T-cell DNA

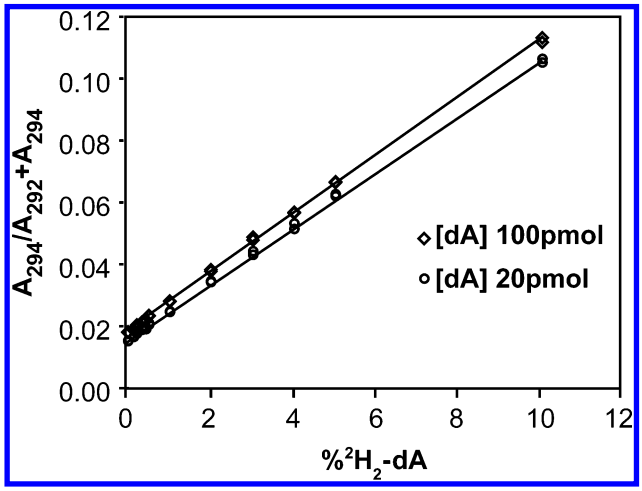

Figure 2. Typical GC/MS linear regression standard curves of [$^2H_2$]-dA enrichment vs peak area ratio for total dA amounts of 20 and 100 pmol ($n = 22$). The nonzero intercept corrects for the contribution of natural isotopic abundance of the A + 2 ion of *m*/*z* 292 to the peak area of *m*/*z* 294. The equation for the regression line of 100 pmol of dA is $y = 0.9375x + 1.871$ with an R^2 value of 0.9996. The equation for the regression line of 20 pmol of dA is $y = 0.8854x + 1.686$ with an R^2 value of 0.9986.

Table 1. Average Precision Data Comparison of Standards Analyzed by LC/MS and GC/MS and Expressed as Relative Standard Deviation (RSD)[a]

[$^2H_2$]-dA enrichment, %	LC/MS (RSD, %)		GC/MS (RSD, %)	
	100 pmol[b]	5 pmol	100 pmol	20 pmol
0.1	16.6	29.8	132.6	30.8
0.2	11.4	29.0	58.6	25.1
0.3	11.4	20.5	21.5	17.3
0.4	13.8	15.9	10.2	22.2
0.5	12.1	10.5	13.1	15.0
1	7.7	11.3	2.4	9.2
2	2.9	4.9	2.9	6.8
3	2.4	4.5	2.2	3.6
4	1.5	4.0	0.7	3.1
5	2.3	2.2	0.8	2.8
10	0.8	1.6	1.0	1.4

[a] Standards covering a range of isotopic enrichment from 0.1 to 10% are analyzed in duplicate at two levels of total dA and run on three consecutive days, resulting in a total of 12 data points for each enrichment standard. [b] Amount of total dA injected on-column

enzymatic hydrolysates were adjusted in volume so that the deoxyribonucleoside concentrations would be bracketed by the calibration curves. Three sets of 30 DNA digests of unknown isotopic enrichment obtained from a clinical study were analyzed in duplicate by GC/MS.[15] Precision (Table 1) and accuracy (Table 2) data were determined using standard calibration curves covering a range of 0.1−10% isotopic enrichment for two different amounts of dA run over three consecutive days. Precision expressed as relative standard deviation (RSD) varied from 0.7 to 133% with a mean of 17.4% ($n = 132$). Accuracy expressed as deviation of the experimental value from the actual value (DEV) varied from 1.8 to 54.9% with a mean of 16.3% ($n = 132$). In general, both the accuracy and precision of the measurements were inversely related to increasing enrichment. Precision for the DNA digests (Table 3) expressed as RSD varied from 0.1 to 108% with a mean of 13.3% ($n = 180$). The limit of quantitation for the GC/MS method was found to be 20 pmol of total dA (1 μL of a 20 μmol L^{-1} solution), which would still allow for the measurement of 0.1% $^2H_2$ enrichment. However, with relative error as high as 108%, in practical terms only 0.2% enrichment could be measured reliably.

Table 2. Average Accuracy Data Comparison of Standards Run on LC/MS and GC/MS and Expressed as Percent Deviation (% DEV) of the Experimental Value from the Actual Value[a]

[$^2H_2$]-dA enrichment, %	LC/MS (DEV, %)		GC/MS (DEV, %)	
	100 pmol[b]	5 pmol	100 pmol	20 pmol
0.1	31.3	23.2	34.6	42.5
0.2	9.5	25.2	54.9	39.3
0.3	11.3	17.1	38.7	22.0
0.4	11.5	14.4	24.1	16.4
0.5	9.7	10.0	24.3	14.5
1	6.6	10.5	9.7	8.8
2	2.6	3.9	3.2	6.3
3	3.1	4.4	2.2	3.6
4	1.4	3.1	1.8	3.6
5	2.8	1.8	2.2	2.9
10	0.9	1.2	2.0	1.9

[a] Standards covering a range of isotopic enrichment from 0.1 to 10% were analyzed in duplicate at two levels of total dA and run on three consecutive days resulting in a total of 12 data points for each enrichment standard. [b] Amount of total dA injected on-column.

Table 3. Precision Data Comparison of $^2H_2$ Enrichment Measured in DNA Digests Analyzed by LC/MS and GC/MS(dA) and Expressed as Relative Standard Deviation (RSD)[a]

group	LC/MS (RSD, %)		GC/MS (RSD, %)	
	mean	range	mean	range
set 1 ($n = 60$)	9.6	0.4−38.3	15.0	0.5−47.8
set 2 ($n = 60$)	7.0	0.1−42.5	13.7	0.4−108
set 3 ($n = 60$)	6.0	0.2−39.3	11.1	0.1−52.9
overall ($n = 180$)	7.5	0.1−42.5	13.3	0.1−108

[a] Mean and range of precision are displayed for three sets of (30) samples analyzed in duplicate ($n = 60$).

This modified GC/MS procedure, however, still has several disadvantages. Due to the flash derivatization kinetics, the method is concentration dependent, and in order to get meaningful and accurate quantitative isotopic enrichment data, two calibration curves that bracket the expected concentration range of dA need to be generated for each experiment, as shown in Figure 2. In addition, the number of cells required to isolate enough DNA to produce a solution of 20 μmol L^{-1} dA (the limit of quantitation) is $\sim 5 \times 10^5$, which can be a difficult task for certain cell populations. Another weakness of the method is that the use of a relatively harsh ionization technique, such as electron impact, results in the generation of multiple fragments that may interfere with the quantitation of dA and deuterated dA.

Due to the aforementioned reasons, a quantitative method whereby derivatization and multiple calibration curves could be avoided was designed. The method that met our requirements combined μLC and ESI-MS. Tandem MS spectra of [$^2H_2$]-dA acquired by μLC-MS/MS (data not shown) revealed no product

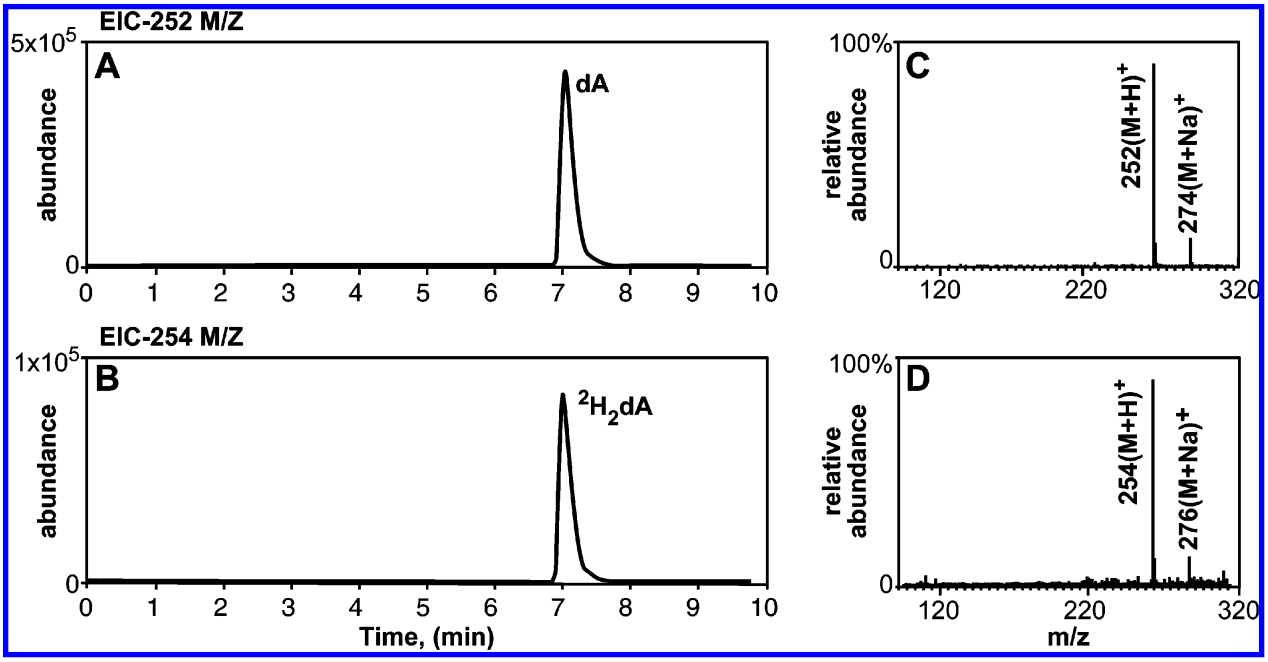

Figure 3. (left side) μLC/ESI-MS extracted ion chromatograms of a clinical DNA enzymatic digest: (A) ion profile for *m*/*z* 252 (dA) and (B) ion profile for *m*/*z* 254 ([$^2H_2$]-dA). (right side) Full-scan electrospray ionization mass spectra of (C) dA showing the expected protonated molecular cation at *m*/*z* 252 and (D) [$^2H_2$]-dA showing the expected protonated molecular cation at *m*/*z* 254.

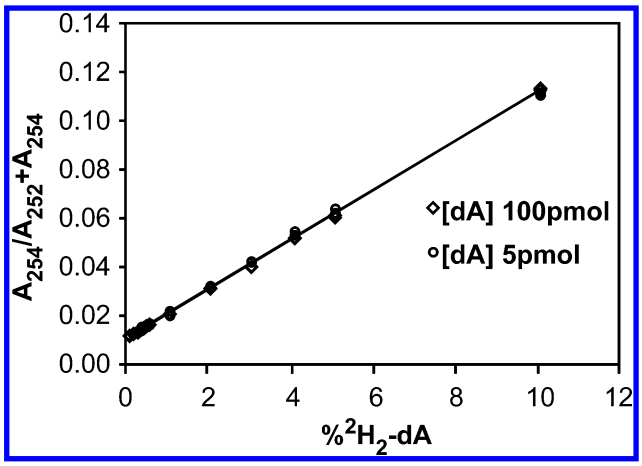

Figure 4. Typical μLC/MS linear regression standard curves of [$^2H_2$]-dA enrichment vs peak area ratio for total dA amounts of 5 and 100 pmol ($n = 22$). The nonzero intercept corrects for the contribution of natural isotopic abundance. Note that both regression lines are overlapping. The equation for the regression line of 100 pmol of dA is $y = 0.0101x + 0.0114$ with an R^2 value of 0.9997. The equation for the regression line of 5 of pmol dA is $y = 0.0101x + 0.0118$ with an R^2 value of 0.9989.

ions that retained the isotopic label; therefore, quantitation by μLC-MS was accomplished using ions extracted from the primary mass spectrum (Figure 3C,D) that correspond to the protonated molecular ions of dA (*m*/*z* 252) and [$^2H_2$]-dA (*m*/*z* 254) (Figure 3A,B). Calibration curves (Figure 4) were generated for 5 and 100 pmol of total dA, which covered deuterium enrichments of 0.1−10%. The same three sets of 30 DNA digests were analyzed as before, and the overall precision data and accuracy data for the calibration standards are summarized in Table 1 and Table 2, respectively. The precision observed ranged between 0.8 and 29.8% RSD with a mean of 9.9% RSD ($n = 132$). The accuracy observed ranged from 0.9 to 31.3% DEV with a mean of 9.3% DEV ($n = 132$). Precision for the DNA digests (Table 3) expressed as RSD varied from 0.1 to 42.5% with a mean of 7.5% ($n = 180$). The limit of quantitation for the LC/MS method was found to be 5 pmol of total dA (1 μL of a 5 μmol L^{-1} solution), which would still allow for the measurement of 0.1% $^2H_2$ enrichment.

A marked improvement can be seen in μLC/MS over GC/MS for both precision and accuracy, particularly when low enrichment values (<1% [$^2H_2$]-dA) are being measured, which happens to be the enrichment range for the majority of kinetics time points in clinical T-cell studies. Furthermore, the two calibration curves were superimposable (i.e., not concentration dependent), which means that a single calibration curve, in the specified concentration range, is sufficient for the quantitative measurements. Because the μLC/MS method uses a soft ionization technique (i.e., ESI) that does not produce fragmentation, lower detection limits can be achieved (0.1% enrichment versus 0.2% enrichment by GC/MS). The use of a more selective MS technique shifts the burden of selectivity from the chromatographic process, resulting in shorter run times and higher sample throughput. We also found that DNA digests can be directly analyzed by μLC/MS (data not shown), greatly streamlining the analysis process by performing DNA isolation, digestion, and sample analysis in a single 96-well plate.

To test the validity of the μLC/ESI-MS method, the same DNA digests that were analyzed by the GC/MS method were also analyzed by the μLC-ESI MS method. A plot of the results (Figure 5), gave a linear relationship with a slope of 1.005 that showed that both procedures gave comparable quantitative data. In addition, both methods were tested in terms of precision and accuracy. The results are given in Tables 1−3, which show that the μLC-ESI MS method was superior to the GC/MS method, especially at the low levels of dA enrichment. In addition to the higher selectivity, the μLC-ESI MS method is twice as fast as the

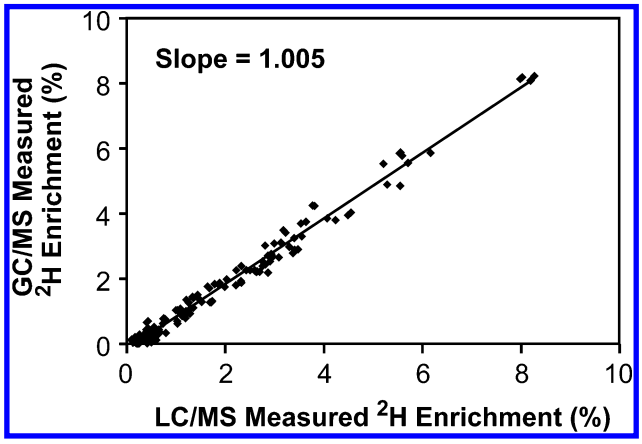

Figure 5. Linear regression plot of μLC/MS vs GC/MS [$^2H_2$]-dA enrichment results of clinical DNA enzymatic digests. The slope of the line is 1.005 with an R^2 value of 0.985. (n = 180).

GC/MS method since it does not require sample derivatization or the generation of multiple linear regression calibration curves. Overall, the μLC-ESI MS method provides shorter analysis time, less sample preparation, and a 4-fold increase in sensitivity, which translates into fewer cells being required (approximately 1.25×10^5 vs 5×10^5).

CONCLUSIONS

We developed a GC/MS method whereby a postinjection methylation reaction is used to form the volatile tetramethyl derivative of dA. While this method is reliable, it has several limitations related to sensitivity, concentration dependence, and overall robustness. To address these limitations, we also developed a μLC/ESI-MS method that does not require sample derivatization or cleanup and is more sensitive, accurate, and precise. The capillary GC/MS method measured the intensities of ions corresponding to the permethylated [$-(CH_3)_4$] derivative of dA and [$^2H_2$]-dA. The area ratio of [$^2H_2$]-dA to that of the total dA pool was determined and compared to a concentration-dependent calibration curve. The μLC/ESI-MS method measured intensities of ions corresponding to the protonated molecular ions of (dA) and [$^2H_2$]-dA. Quantitation by μLC/MS/MS was accomplished by measuring the peak area of the ions at m/z 252 and 254. Overall, the μLC/MS method provides shorter analysis times, less sample preparation, a 4-fold increase in sensitivity, and lower detection limits, 0.1 versus 0.2% enrichment by the GC/MS method.

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