

# Comparison of UV, Fluorescence, and Electrochemical Detectors for the Analysis of Formaldehyde-Induced DNA Adducts

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## Abstract

A high-performance liquid chromatography (HPLC) method for normal and formaldehyde-modified deoxynucleosides after hydrolysis of exposed and unexposed human placental DNA was compared with ultraviolet (UV), fluorescence (FL), and electrochemical (EC) detectors. The lower quantifiable limits (LQL) for UV detection at 254 nm were 10–22 pmol for N<sup>6</sup>-hydroxymethyldeoxyadenosine (N<sup>6</sup>-dA), N<sup>2</sup>-hydroxymethyldeoxyguanosine (N<sup>2</sup>-dG), and N<sup>4</sup>-hydroxymethyldeoxycytidine (N<sup>4</sup>-dC), with N<sup>4</sup>-dC having the highest LQL and the 2 purines the lowest LQL. Similarly, LQLs using FL (excitation: 264 nm; emission: 340 nm) were 14–30 pmol, with N<sup>2</sup>-dG having the lowest LQL and N<sup>6</sup>-dA the highest. The LQL for N<sup>2</sup>-dG by EC detection at +1.10 V was 27 pmol, over 50-fold greater sensitivity than for the other hydroxymethyl deoxynucleosides; deoxyguanosine was similarly detected more sensitively than the other normal deoxynucleosides. Percent relative standard deviations ranged between 6 and 13% for both intra- and interrun assays for all detectors. HPLC–UV allows all the deoxynucleosides to be detected without the flow cell washing and use of fresh solutions necessary for the more selective FL detection, the latter not having enzyme blank interferences. EC allows only deoxyguanosine and N<sup>2</sup>-dG to be detected at pmol levels with no blank interferences. HPLC–UV allowed more sensitive detection of N<sup>2</sup>-dG and N<sup>6</sup>-dA than the other techniques and is recommended. The UV, FL, and EC properties of the hydroxymethyl deoxynucleosides of dA, dG, and dC are reported for the first time.

## Introduction

Formaldehyde (FA) as its formalin solution is widely used in hospital pathology laboratories and in industry (1,2). Several studies indicate that FA causes nasal squamous cell carcinomas in rats and mice (3–5). Two Danish studies found relative risks of 3.0 for nasopharyngeal cancer in male workers exposed to

≥ 1 ppm FA for at least 10 years (6,7). Vaughan et al. (8) also reported that occupational exposure to FA in the United States increased the risk for nasopharyngeal cancer at five cancer registries (odds ratio = 3.0 for people exposed to ≥ 1.1 ppm for over 10 years). IARC (9) considers FA to be a probable human nasal carcinogen (Group A2). The biological effects of FA arise from its interactions with proteins and nucleic acids (10).

FA can react to form hydroxymethyl derivatives at DNA bases with primary amino groups (11,12). DNA adducts are potential dosimeters and may also be predictive markers of effect for carcinogens (11). High-performance liquid chromatography (HPLC) has been shown to be an efficient technique for separating normal and some modified deoxynucleosides in hydrolyzed DNA adduct samples (11,12). The main difficulties for the detection of FA-modified DNA *in vivo* are analytical obstacles for the measurement of very low amounts (pmol) of adducts. Because the amount of tissue for analysis is also limited, sensitive detectors are required for DNA adduct assay.

Our research group has previously reported (13) on the optimization of the chromatographic resolution of the normal deoxynucleosides and their hydroxymethyl deoxynucleosides from one another using HPLC and ultraviolet (UV) detection at 254 nm, in addition to the optimization of the hydrolysis conditions of human placental DNA to its constituent deoxynucleosides, and the synthesis of the hydroxymethyl deoxynucleosides. The next step was to determine which relatively inexpensive HPLC detectors were suitable for picomole analysis of normal and modified deoxynucleosides. The present paper, therefore, describes comparative studies using separation with reversed-phase (RP)-HPLC followed by UV, fluorescence (FL), and electrochemical (EC) detection to determine which detector is most sensitive and selective for hydroxymethyldeoxynucleosides.

## Experimental

### Apparatus

Formalin [37% (w/w) FA in 10% methanolic aqueous solution] was purchased from Aldrich (Milwaukee, WI). Its FA con-

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tent was verified by sodium sulfite titration as detailed elsewhere (13). Deoxyadenosine (dA), deoxyguanosine (dG), deoxycytidine (dC), thymidine (dT), human placental DNA (Type XIII), and alkaline phosphatase (Type XVII from human placenta) were purchased from Sigma (St. Louis, MO). DNase I (from bovine pancreas) and phosphodiesterase I (from *Crotalus adamanteus*) were obtained from ICN (Costa Mesa, CA). Bis-Tris buffer, disodium EDTA, methanol (Optima), and ammonium acetate (HPLC grade) were purchased from Fisher Scientific (Tustin, CA).

The HPLC system consisted of a Hewlett-Packard (HP) 1090 LC. The detectors included an HP 1050 UV diode-array variable wavelength detector, a Waters 470 scanning FL detector, and an HP 1049A programmable EC detector. Peak area chromatograms were produced with an HP 3396 series II integrating recorder. A 10- $\mu$ L manual injection loop was loaded with 100  $\mu$ L. The C<sub>18</sub> RP analytical column (250 mm  $\times$  4.6-mm i.d.) was obtained from Beckman (Fullerton, CA).

UV/visible (VIS) spectra between 190 and 820 nm were measured on an HP 8452 diode-array spectrophotometer coupled to an HP 89500 UV/VIS ChemStation. Matched Suprasil™ cells of 1.0-cm path length were used. Molar absorptivities for normal deoxynucleosides were calculated from the Beer's Law plots. FL intensities of deoxynucleosides were measured by a Perkin-Elmer 650-40 FL spectrophotometer over the range 220–830 nm at a slit width of 10 nm. The Suprasil cuvet had a path length of 1.0 cm. Maximum wavelengths for excitation (Ex) and emission (Em) were determined.

A voltammogram was obtained by scanning the solution in the HPLC flow cell from 0.60 V to 1.20 V in the amperometric (constant current) mode and plotting peak area versus working electrode potential (voltage) for each compound at stop-flow conditions. The working electrode was a glassy carbon disk (–0.40 V to +1.40 V). The reference electrode was AgCl/KCl. All measurements were performed at least in triplicate.

#### Preparation of hydroxymethyldeoxynucleoside standards

No commercial hydroxymethyldeoxynucleoside standards are available. Details of optimization of conditions to synthesize standards are provided elsewhere (13).

Standards were synthesized optimally by reacting deoxynucleosides with FA in 5mM Bis-Tris buffer/0.1mM disodium EDTA buffer. Each deoxynucleoside and the mixture (0, 0.5, 1.0, 2.0, and 4.0 nmol/mL) were exposed to 100 ppm FA (1.0 mL 10% formalin) at 37°C for 6 h. The deoxynucleosides were resolved by HPLC with a C<sub>18</sub> RP analytical column. The gradient elution began with pH 6.0 5mM ammonium acetate at 0.3 mL/min for 0–20 min, a gradient of 0 to 6% methanol at a flow rate of 0.3 to 1.4 mL/min between 20 and 30 min, and finally isocratic conditions of 6% methanol/5mM ammonium acetate at 1.4 mL/min between 30 and 50 min. The latter isocratic condition was not necessary for standards but was for DNA hydrolysates to clean the column.

Quantitation of normal deoxynucleosides was performed by the method of external standards. The hydroxymethyldeoxynucleosides identified were N<sup>6</sup>-hydroxymethyl-deoxyadenosine (N<sup>6</sup>-dA), N<sup>2</sup>-hydroxymethyldeoxyguanosine (N<sup>2</sup>-dG), and N<sup>4</sup>-hydroxymethyldeoxycytidine (N<sup>4</sup>-dC). Each corresponding

hydroxymethyldeoxynucleoside eluted soon after its unmodified deoxynucleoside, but dT did not react (13). The moles of hydroxymethyldeoxynucleoside produced were calculated by subtracting the moles of normal deoxynucleoside after reaction from the original moles of normal deoxynucleoside and assuming one reaction product for each deoxynucleoside apart from dT. The standard curves were then utilized for the deoxynucleoside mixture and DNA hydrolysate determinations. All concentrations were evaluated at least in triplicate.

Pure N<sup>6</sup>-dA and N<sup>2</sup>-dG were also collected separately directly from the HPLC column using a methanol/water gradient of 5–10% over 20 min and 2–8% over 20 min, respectively. The eluates were collected at –10°C with storage at –20°C. The excess water and methanol were evaporated at 40°C until constant weight. The normal deoxynucleoside was also quantified directly to obtain the purity of the collected solid. Standard curves were then constructed using ammonium acetate buffer as described to compare response factors (slopes). Stability studies over 6 months were also conducted. The results of the stability investigations are provided elsewhere (13).

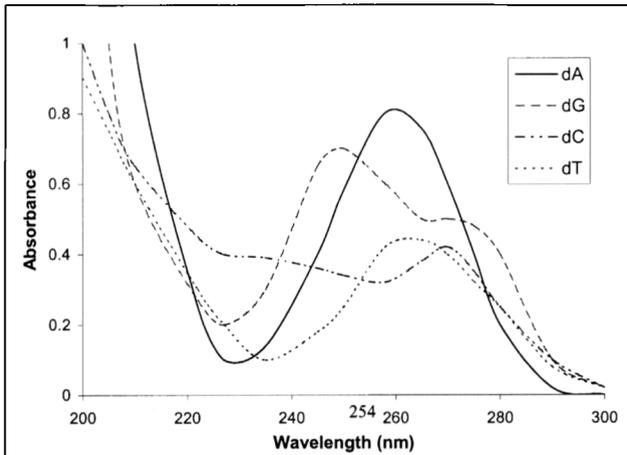
#### Preparation of human placental DNA samples

The optimization of the analysis of human placental DNA is given elsewhere (13). The optimized procedure follows.

A 1.0 mL solution containing 80  $\mu$ g human placental DNA and 3.34mM FA (1.0  $\mu$ L of 10% formalin) in pH 4.5 40mM sodium acetate buffer was incubated for 20 h at 37°C. The DNA was precipitated by the addition of 30  $\mu$ L of 1M sodium chloride followed by 800  $\mu$ L ice-cold ethanol. The DNA was recovered by centrifugation (14,000  $\times g$  for 10 min) and dissolved in 1.0 mL of 5mM Bis-Tris/0.1mM disodium EDTA buffer (pH 7.1). The reagent blank sample had 10% methanolic water added instead of 37% FA. The DNA solutions were hydrolyzed by incubation with DNase I (10 U) in 10mM magnesium chloride at 37°C for 30 min. This was followed by addition of 75  $\mu$ L 5mM Bis-Tris buffer, human placental alkaline phosphatase (0.1 U), and snake venom phosphodiesterase (0.1 U) at 37°C for 30 min to liberate the deoxynucleosides. The solution was centrifuged at 20,000  $\times g$  for 10 min to remove precipitated material. Aliquots of the supernatant were injected into the HPLC, and the same gradient elution program as for the deoxynucleosides was used. At least triplicate samples of DNA and reagent blanks were processed and analyzed. The supernatants were stored at –20°C.

#### Statistical analysis

Average area versus moles injected plots were generated for each exposure condition from the appropriate data. The linear portion of each curve was fitted by a least-squares regression line using Excel. Intercepts, slopes, their standard deviations, correlation coefficients ( $r$ ), and  $p$  values were calculated. The slopes and their standard deviations were compared by analysis of variance (ANOVA) and Student  $t$ -tests;  $p$  values of  $\leq 0.05$  were considered to be significant. Detection limits (DL) were defined as 3 times the signal-to-noise ratio, and lower quantifiable limits (LQL) were 10 times the signal-to-noise ratio, the latter being equivalent to a maximum percent relative standard deviation of 10%.



**Figure 1.** Averaged UV absorption spectra ( $n = 3$ ) for normal deoxynucleosides ( $10^{-3}$ M) in 5mM ammonium acetate with 6% methanol (pH 6.5) in a 1-cm pathlength Suprasil cuvet. dA: deoxyadenosine; dG: deoxyguanosine; dC: deoxycytidine; and dT: thymidine. All percent relative standard deviations were  $\leq 10\%$  and are not shown for clarity.

**Table I. The LQs (pmol) of Normal and Modified Deoxynucleosides for a 10- $\mu$ L Injection for UV, FL, and EC Detectors at the Same HPLC Chromatographic Conditions\***

|                           | dA     | dG  | dC     | dT     | N <sup>6</sup> -dA | N <sup>2</sup> -dG | N <sup>4</sup> -dC |
|---------------------------|--------|-----|--------|--------|--------------------|--------------------|--------------------|
| UV detection <sup>†</sup> | 11     | 7.6 | 12     | 15     | 10                 | 10                 | 22                 |
| FL detection <sup>‡</sup> | 13     | 4.4 | 4.9    | 5.0    | 30                 | 14                 | 23                 |
| EC detection <sup>§</sup> | > 1100 | 22  | > 1100 | > 1100 | > 1100             | 27                 | > 1100             |

\* By definition, the percent relative standard deviation for each LQL is 10% ( $n = 3$ ).

<sup>†</sup> At 254 nm.

<sup>‡</sup> Excitation: 264 nm and emission: 340 nm.

<sup>§</sup> At +1.10 V.

**Table II. The Fluorescence Intensities of  $10^{-6}$ M Normal Deoxynucleosides in (pH 6.5, 5.0mM) Ammonium Acetate/6% Methanol in a 1-cm Suprasil Cell at Different Excitation and Emission Wavelengths\***

| Excitation (nm) | Emission (nm) | Fluorescence Intensity <sup>†</sup> |                 |                 |                 |
|-----------------|---------------|-------------------------------------|-----------------|-----------------|-----------------|
|                 |               | dA                                  | dG              | dC              | dT              |
| 254             | 330           | 1.99 $\pm$ 0.08                     | 1.84 $\pm$ 0.09 | 2.01 $\pm$ 0.10 | 2.71 $\pm$ 0.05 |
|                 | 340           | 2.04 $\pm$ 0.10                     | 1.89 $\pm$ 0.08 | 2.08 $\pm$ 0.04 | 2.80 $\pm$ 0.09 |
|                 | 350           | 1.42 $\pm$ 0.09                     | 1.38 $\pm$ 0.07 | 1.41 $\pm$ 0.06 | 1.14 $\pm$ 0.05 |
| 264             | 330           | 2.09 $\pm$ 0.04                     | 1.78 $\pm$ 0.07 | 1.88 $\pm$ 0.04 | 2.51 $\pm$ 0.03 |
|                 | 340           | 2.36 $\pm$ 0.11                     | 2.01 $\pm$ 0.04 | 2.19 $\pm$ 0.10 | 2.95 $\pm$ 0.09 |
|                 | 350           | 2.10 $\pm$ 0.04                     | 1.74 $\pm$ 0.08 | 1.89 $\pm$ 0.07 | 2.60 $\pm$ 0.09 |
| 274             | 330           | 1.54 $\pm$ 0.08                     | 1.10 $\pm$ 0.06 | 1.24 $\pm$ 0.04 | 2.42 $\pm$ 0.05 |
|                 | 340           | 1.73 $\pm$ 0.04                     | 1.23 $\pm$ 0.05 | 1.35 $\pm$ 0.08 | 2.60 $\pm$ 0.09 |
|                 | 350           | 1.68 $\pm$ 0.07                     | 1.16 $\pm$ 0.04 | 1.28 $\pm$ 0.07 | 2.47 $\pm$ 0.03 |

\* The data are solvent blank subtracted. The arithmetic means and standard deviations were calculated from data using interrun samples ( $n = 3$ ) and parallel reagent blanks ( $n = 3$ ).

<sup>†</sup> Arbitrary units.

## Results

### UV detection

To establish the conditions for UV detection of deoxynucleosides, the optimal UV wavelength of the detector had to be determined first. Figure 1 shows the UV spectra of deoxynucleosides. The  $\lambda_{\max}$  are < 215 nm and 260 nm for dA; < 205 nm, 252 nm, and 273 nm for dG; < 200 nm and 262 nm for dT; and < 200 nm, 235 nm, and 272 for dC.

A wavelength of 254 nm was chosen as the compromise optimum wavelength in the long wavelength band because it is the  $\lambda_{\max}$  for dT and dA and close to the  $\lambda_{\max}$  for dG. The dC absorbance at 254 nm is about 70% of the absorbance at its long wavelength  $\lambda_{\max}$  of 272 nm. Another reason for choosing 254 nm was to accommodate UV filter detectors. DLs between 3 and 6 pmol were calculated for deoxynucleosides on HPLC-UV. The LQLs for dA, dG, and dT were 8–12 pmol/10  $\mu$ L, but dC had a higher LQL at 22 pmol/10  $\mu$ L (Table I). The hydroxymethyl deoxynucleoside always had a higher LQL than its original deoxynucleoside except for dA and N<sup>6</sup>-dA that were about equal.

Although absorbances were greater than 254 nm at wavelengths from 215 nm to 190 nm for all deoxynucleosides, HPLC baseline instability of samples and blanks increased greatly during gradient elution to such an extent that there was no advantage relative to 254 nm analyses.

The slopes obtained by the indirect method of quantitation were not statistically different at  $p \leq 0.05$  (Student  $t$ ) from those for the corresponding hydroxymethyldeoxynucleoside collected directly from the HPLC column. Only one hydroxymethyldeoxynucleoside was detected for each of dA, dG, and dC. All samples analyzed immediately after thawing contained no cross-linked modified deoxynucleosides (13).

### FL detection

Table II shows the FL spectrophotometer optimization data for the normal deoxynucleosides. For excitation wavelengths of 254, 264, and 274 nm, the emission  $\lambda_{\max}$  was 340 nm for all normal deoxynucleosides. Excitation at 264 nm elicited the highest FL emission.

The LQLs of normal deoxynucleosides (dA excluded) measured by HPLC-FL at excitation wavelength 264 nm and emission wavelength 340 nm were about 4–5 pmol, 2–3-fold lower than that of HPLC-UV at 254 nm (Table I). The LQL for each modified deoxynucleoside was higher than that of its unmodified deoxynucleoside: N<sup>6</sup>-dA was 2.3 times higher than dA; N<sup>2</sup>-dG was 3.2 times greater than dG; and N<sup>4</sup>-dC was 4.7 times higher than dC. Whereas N<sup>2</sup>-dG had the lowest LQL, N<sup>6</sup>-dA had the highest (Table I) with that for N<sup>4</sup>-dC intermediate, unlike the order for UV (Table I).

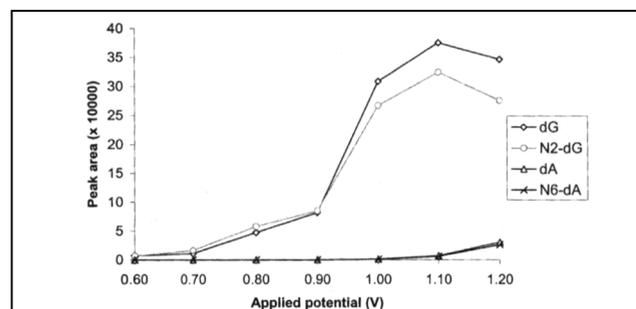
### EC detection

The area versus voltage plots used to optimize the voltage selected for EC are shown in Figure 2. An oxidation potential of +1.10 V was

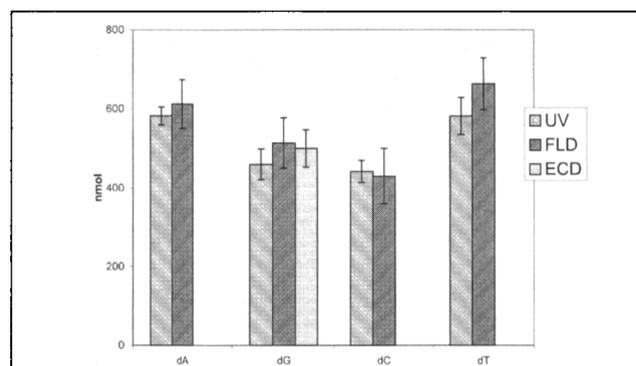
chosen to achieve optimal balance between maximum response and minimum background noise. dG and N<sup>2</sup>-dG were electrochemically active at higher than +0.80 V with their optimum at +1.10 V (Figure 2). dG and N<sup>2</sup>-dG were at least 50 times more sensitive than the other deoxynucleosides (Table I). dA, N<sup>6</sup>-dA, dC, N<sup>4</sup>-dC, and dT had much less electrochemical activity between +0.60 and +1.20 V. LQLs of 22–27 pmol were calculated for dG and N<sup>2</sup>-dG, being linear in the picomole range only and with dG having the lowest LQL. The EC was thus very selective for guanine-containing deoxynucleosides.

### Analysis of human placental DNA

The amount of normal and modified deoxynucleosides obtained by UV, FL, and EC after 20 h incubation of human placental DNA with 100 ppm FA at 37°C, are shown in Figures 3 and 4, respectively. Apart from the insensitivity of EC detection to dA, dC, dT, and their hydroxymethyl derivatives, the concentrations of normal and modified deoxynucleosides determined in DNA by the three detectors were not significantly different at  $p \leq 0.05$  by ANOVA and Student *t* testing (Figures 3 and 4, respectively). This gives added confidence that no systematic error has occurred. Furthermore, percent relative standard deviations ranged between 6 and 13% for both intra- and interrun assays for all detectors, attesting to the precision of the data.

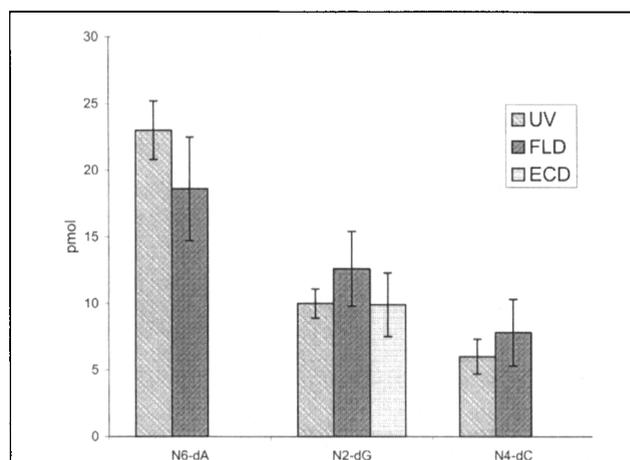


**Figure 2.** Averaged voltammograms (peak area versus voltage) of normal and hydroxymethyl deoxynucleosides (100 pmol) in the HPLC flow cell ( $n = 3$ ). All percent relative standard deviations were  $\leq 10\%$  and are not shown for clarity.

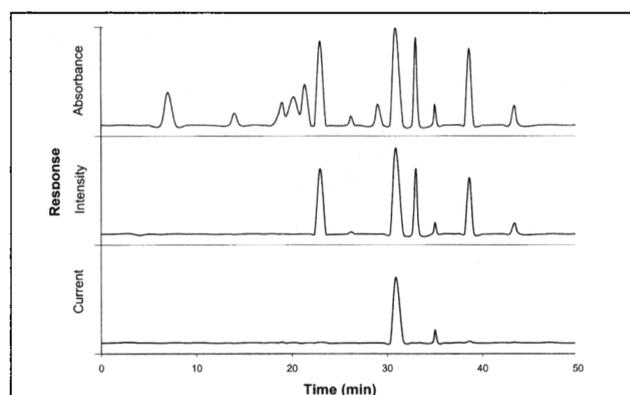


**Figure 3.** Amounts (nmol) of normal deoxynucleosides in 80 µg human placental DNA incubated with 100 ppm FA analyzed by UV detection (UV) at 254 nm, fluorescence detection (FLD) at 264 nm excitation/340 nm emission, and electrochemical detection (ECD) at +1.1 V. Gradient: 5mM ammonium acetate at 0.3 mL/min for the first 20 min, 6% methanol and 1.4 mL/min at 30 min for 30–50 min. The standard deviations are  $1\sigma$  for  $n = 3$ .

Typical chromatograms with UV, FL, and EC detection of modified and unmodified deoxynucleosides in human placental DNA after reaction with FA are depicted on the same time scale in Figure 5. Four normal deoxynucleosides and three modified deoxynucleosides showed baseline separation. The FL method showed much less interference from reagents than did the UV detector at 254 nm and so is more selective than the UV detector. Measurement of all normal and modified deoxynucleosides was possible with the FL detector as with the UV detector. HPLC–EC shows only 2 large peaks at the retention time of dG (31 min) and N<sup>2</sup>-dG (35 min). Several other much smaller EC peaks occurred at the expected retention times of the other normal and modified deoxynucleosides.



**Figure 4.** Amount (pmol/10 µL) of modified deoxynucleosides in 80 µg human placental DNA incubated with 100 ppm FA analyzed by UV detection (UV) at 254 nm, fluorescence detection (FLD) at 264 nm excitation/340 nm emission, and electrochemical detection (ECD) at +1.1 V. Gradient: 5mM ammonium acetate at 0.3 mL/min for the first 20 min, 6% methanol and 1.4 mL/min at 30 min for 30–50 min. The standard deviations are  $1\sigma$  for  $n = 3$ .



**Figure 5.** Representative chromatograms of the DNA hydrolysate (80 µg human placental DNA incubated with 100 ppm FA) with ultraviolet UV (Absorbance), fluorescence FLD (Intensity), and electrochemical ECD (Current) detectors. Gradient elution program: 5mM ammonium acetate at 0.3 mL/min for the first 20 min, 6% methanol and 1.4 mL/min at 30 min for 30–50 min. UV was at 254 nm, FLD was at excitation 264 nm/emission 340 nm, and ECD was at +1.10 V. Peak retention times: dC, 22.5 min; N<sup>4</sup>-dC, 26 min; dG, 31 min; dT, 33 min; N<sup>2</sup>-dG, 35 min; dA, 38 min; and N<sup>6</sup>-dA, 43 min. The other UV peaks are from background.

## Discussion

This is the first investigation to compare directly the sensitivities, selectivities, precision, and accuracies of three different HPLC detectors for the same commercially available human DNA treated with formaldehyde and hydrolyzed to its constituent deoxynucleosides. Such a comparison for unmodified DNA is also a necessary quality assurance/quality control step for analysis of a human DNA reference material, and the three techniques give results that are not statistically different at  $p \leq 0.05$  by ANOVA for those deoxynucleosides that are detected sensitively. As expected, the molar concentrations for dA and dT are equivalent at  $p \leq 0.05$  (Student *t*), as are those for dC and dG in unmodified human placental DNA (Figure 3).

The UV spectra of the deoxynucleosides measured are similar to those of the DNA free bases (14). The deoxynucleosides have characteristic peaks between 240 and 280 nm. The molar absorptivities at 254 nm in the present work in  $M^{-1}cm^{-1}$  were  $13,800 \pm 400$  for dA;  $13,600 \pm 500$  for dG;  $6900 \pm 200$  for dC; and  $7000 \pm 300$  for dT. Surprisingly, there are no literature spectra or molar absorptivities published for comparison of these deoxynucleosides, although such data do exist for the free bases, nucleosides, and the nucleotides. The HPLC–UV assay at 254 nm has been used to determine normal deoxynucleosides, deoxynucleotides, and DNA/RNA bases (15–17).

The HPLC–UV assay at 254 nm had also been utilized to detect hydroxymethyldeoxy-nucleosides liberated by DNA hydrolysis after DNA reaction with FA (11,12), but, because resolution from interferences of unknown sources was not achieved (12), the technique was qualitative but not quantitative. Quantification was only recently accomplished by our research group through resolution of the analyte peaks from enzyme blank sample interferences and judicious selection of the enzyme sources used in the DNA hydrolysis (13).

Cheng et al. (18) found FA cross-linked deoxyguanosine under their reaction conditions that differed greatly from those used in the present work. Long incubation times of excess FA and dG favored cross-linked dimer production at the expense of  $N^2$ -dG. Under our conditions of reaction, no cross-linked deoxynucleotides were detected when DNA samples were analyzed immediately upon thawing (13). Feldman (19) reviewed the early data on hydroxymethyl derivatives of DNA and RNA bases, nucleosides, and nucleotides, including the conditions to achieve cross-linking.

The sensitivity of HPLC–FL was lower than that of HPLC–UV for  $N^6$ -dA and  $N^2$ -dG, but higher for dG, dC, and dT, and about the same for dA and  $N^4$ -dC (Table I). The LQLs of FA-modified deoxynucleosides by FL are higher than those of their normal deoxynucleosides signifying less ring electron density. Thus, substitution of a hydroxymethyl group for one hydrogen on the nitrogen generally decreases FL in these compounds at these wavelengths. The FL data for the hydroxymethyl deoxynucleosides in Table II are similar to those of the normal deoxynucleosides (20–23). The present study is the first report of the FL characteristics of the hydroxymethyl deoxynucleosides.

HPLC–FL detection was relatively insensitive to flow rate change under isocratic conditions. However, much noisier baselines occurred during gradient elution. This caused inaccuracy

in integrations and limited sensitivity. The UV absorbing response also remained constant for at least 6 months (13) when solutions were stored at  $-20^\circ C$ , but consistent FL responses required fresh solutions on each analysis day. The flow cell wall of the FL detector required regular weekly cleaning by flushing with 30% nitric acid. Such cleaning was not necessary for the UV absorption flow cell. Thus, even though the FL technique was more selective in that the enzyme blank interferences were not detected, the sensitivities for  $N^6$ -dA and  $N^2$ -dG were lower than for UV detection, fresh standard solutions had to be utilized for each FL analysis, and in addition, more flow cell washing was necessary than for the UV flow cell.

The UV and FL detectors are relatively inexpensive. EC detection is another relatively inexpensive detector. Only bases with low oxidation potential can be measured by EC. The electrochemical activities of normal deoxynucleosides in the present work are similar to those previously reported, where the DL for dG was 1.0 mmol/L or 10 pmol/10  $\mu L$  (24), with the other normal deoxynucleosides not detected sensitively. Not only was dG detected sensitively in the present study, but  $N^2$ -dG was detected between 0.8 and 1.2 V as well. However, hydroxymethylation causes a small but statistically significant decrease in sensitivity relative to dG (Table I). The present study is the first report of the electrochemical sensitivity of  $N^2$ -dG. dC and dT do not respond up to +1.00 V (24,25). There has to be at least one hydrogen on a purine ring amino group for the deoxynucleoside to be electrochemically active. The order of the electron-donor capacity is therefore  $dG > N^2$ -dG  $\gg$  dA  $> N^6$ -dA  $> dC, N^4$ -dC, dT.

Although EC and FL detection offer a marked advantage over UV detection in terms of producing an extremely clean enzyme blank and sample background (Figure 5), the flow rate change in the chromatographic solvent gradient step for EC made the baseline unstable. Much greater sensitivity could be measured at isocratic conditions. The spacer and electrode surfaces also required daily cleaning with acetone and methanol. Although selectivity for dG and  $N^2$ -dG can be desirable when such bases are the dominant research interest, simultaneous detection of the other deoxynucleosides in the same sample is often required too. For such cases, the UV and FL detectors are recommended, with UV detection preferred because it requires less flow-cell cleaning, no requirement for fresh standard solutions and samples, and higher sensitivities for  $N^6$ -dA and  $N^2$ -dG.

These three detection methods are certainly more simple and inexpensive than are HPLC–MS (26,27), immunoassays (28,29), and  $^{32}P$ -postlabeling techniques (25) for quantitation of DNA adducts.  $^{32}P$ -Postlabeling cannot distinguish adducts of low molecular weight agents like FA from unreacted DNA (30). LC–MS–MS is an expensive detector but should be investigated if more sensitivity is required because femtomole quantities of some modified deoxynucleosides can be detected (31). In any case, chromatographic optimization with these inexpensive detectors may avoid difficulties with the more sensitive and expensive detectors when use of the latter is ultimately essential.

## Conclusions

The comparison of three relatively inexpensive HPLC detec-

tors showed each had different strengths and weaknesses. The EC detector was specific for dG and its hydroxymethyl derivative, and produced clear chromatograms at the picomole level. The FL method also did not detect interferences from the hydrolysate blank unlike the UV technique, but the purine hydroxymethyl deoxynucleosides were detected with less pmol sensitivity than by UV. Both UV and FL detection allowed all normal deoxynucleosides to be detected, as well as all their hydroxymethyl derivatives. UV detection also allowed less flow cell cleaning and less reliance on fresh solutions, these being necessary in FL detection.

## Acknowledgments

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