Development and validation of RP-HPLC method for analysis and stability study of 8-carboxyphenyl-2'-deoxyguanosine and 8-bromo-2'-deoxyguanosine

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Abstract
Modifications on C8 position of purine base have been studied for their effects on B-DNA to Z-DNA conversion. The 8-bromo and 8-carboxyphenyl adduct are among the most potent Z-DNA facilitators and hence are aimed to be utilized for Z-DNA based probe for binding study between Z-DNA and potential Z-DNA binding proteins. However, modification of the purine may also lead to destabilization of the nucleoside that will limit its practicality to be used for Z-DNA probe construction. In this study, the method development and validation for 2'-deoxyguanosine (dG), 8-bromo-2'-deoxyguanosine (8-BrdG), and 8-carboxy-2'-deoxyguanosine (8-CpdG) determinations were performed with InertSustain® C18 (4.6 x 150 mm, 5 µm) column. A mixture of acetonitrile and ammonium acetate buffer pH 6.0 (92:8) was used as mobile phase with flow rate at 1 mL/min. A novel, simple, sensitive, precise, and accurate isocratic RP-HPLC method was obtained and used for stability evaluation of dG, 8-BrdG, and 8-CpdG. The first-order kinetic result obtained with the validated method indicate that 8-bromo and 8-carboxyphenyl adducts destabilized nucleoside unit and increased deglycosylation rate of dG, while 8-BrdG was more stable than 8-CpdG and was a suitable selection for Z-DNA probe development.

Keywords: RP-HPLC, Z-DNA, 8-carboxy-2'-deoxyguanosine, 8-bromo-2'-deoxyguanosine, Method validation, Stability

Introduction
Nucleic acids have been known for their capability to form various conformations, which in turn may possess different biological activities. Study of nucleic acids structure is empirical to understand biological function that may lead to the discovery of novel drug target. The left-handed Z-DNA was discovered for thirty years [1] and it is among the current nucleic acids research interest due to the possibility that it may play important biological role. Interaction between Z-DNA and Z-DNA binding proteins is believed to be the fundamental event behind the recent discovered Z-DNA activities such as gene regulation of CSF-1 [2] and vaccinia virus pathogenesis [3]. Lab-on-chip such as immobilized DNA probe on solid surface is a recent trend to study interaction between macromolecules [4,5] due to its versatile application on
several detection and characterization methods. Modifications on C8 position of guanine base, for instance, methylation [6], bromination [7], and arylation [8] that facilitate the formation of Z-DNA have potential to be utilized on Z-DNA probe. 8-Bromo and 8-carboxyphenyl guanine adducts are very effective Z-DNA facilitators [7, 8] due to the effects of both adducts to aid B to Z conversion at near-physiological condition compared to other modifications that require high concentration of cation (e.g. Na+, K+, Mg2+) to induce transition of B-DNA to Z-DNA. Hence, they are most likely to be used to construct Z-DNA probe. On the other hand, modification on guanine base may increase susceptibility for hydrolysis of glycosidic bond of nucleosides and nucleic acids [9,10]. Therefore, stability effect of C8 modified guanine adduct on nucleoside should also be considered to ensure usability of the modified adduct on Z-DNA probe. Stability indicating analysis method for 8-bromo-2′-deoxyguanosine (8-BrdG, Figure 1) and 8-carboxyphenyl-2′-deoxyguanosine (8-CpdG, Figure 1) is necessary to evaluate stability of the modified nucleoside. Several chromatographic methods for detection of 2′-deoxyguanosine (dG) have been described previously [11,12] but without validation to confirm reliability of the methods. Moreover, none have been reported on analytical method for 8-BrdG and 8-CpdG detection. Our study objective is to develop a simple, sensitive, precise, and accurate analytical method that can be used to evaluate stability of the C8 modified dG.

**Materials and Methods**

A nucleoside, dG, was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The modified nucleosides, 8-BrdG and the 8-CpdG were synthesized, purified, and characterized according to previous report [13]. All chemicals and reagents used in this study were analytical grade. Ammonium acetate was purchased from Rankem (Faridabad, India). Ammonia solution was purchased from Lobachemie (Mumbai, India). Glacial acetic acid was purchased from Baker (Pennsylvania, USA). Hydrochloric acid was purchased from Emsure (Darmstadt, Germany). Chromatographic grade acetonitrile and methanol were obtained from Burdick & Jackson (Pennsylvania, USA). Milli-Q® water purification system was used to prepare all necessary reagents in this study.

Chromatography was performed on HPLC LC10 system (Shimadzu, Japan) that contains photodiode array detector, temperature controlled column chamber, and autosampler. Command control and chromatographic data processing were performed using LC solution program (Shimadzu, Japan). Weighing of samples and chemicals were carried out on analytical balance (Mettler Toledo, USA). Potentiometer (Beckman Coulter, Inc, USA) was used for pH measurement. The ultrasonic bath RK 103H (Bandelin, Germany) was used during sample preparation.

**Chromatographic Condition:** A single isocratic chromatographic condition was developed for analysis of all nucleosides used in this study. The binary mobile phase system composed of acetonitrile and buffer solution (acetonitrile:buffer, 92:8) was used. The buffer solution was prepared by dissolving 6.55 g of ammonium acetate, adjusting pH to 6.0 with glacial acetic acid, and purified water was added to the final volume of 1000 mL. InertSustain® C18 (4.6 x 150 mm, 5 μm) column was used as a stationary phase. Chromatography was performed with flow rate equal to 1.0 mL/min with the injection volume of 20 μL. The total run time for each run was 12 min and the signals of analytes were detected at 253 nm.

**Standard Solution Preparation:** All stock standard and standard solutions were prepared with mobile phase as diluent. The stock standard solution was prepared according to the following procedure. Accurately weigh 20 mg of dG, 8-BrdG or 8-CpdG to 200 mL volumetric flask. About 150 mL mobile phase was added to the flask and the solution was sonicated with an ultrasonic bath for 10 min. The prepared solution was allowed to cool down to room temperature and was adjusted to final volume with mobile phase. Various concentrations of nucleoside standard solutions were prepared by serial dilution from stock standard solution.

**Validation Procedure:** To ensure the reliability and suitability of the method to determine the amount of dG, 8-BrdG, and 8-CpdG, a full method validation has been

![Figure 1](https://example.com/figure1.png)
performed according to the current version of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline [14] and The Association of Analytical Communities (AOAC) guideline [15] for analytical method validation. Evaluation of specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ) were conducted in this study with the acceptance criteria from ICH and AOAC guidelines.

Specificity: The purpose of specificity test is to demonstrate that the analytical procedure can be used to determine analytes in the presence of their impurities without interference. Due to the lack of authentic degradation products of the modified nucleosides, forced degradation of nucleoside solutions were used as a source of impurities. Specificity of the test method has been demonstrated with stress testing under acid and thermal stresses that are relevant to hydrolysis of nucleosides. For acid stress condition, dG, 8-BrdG, and 8-CpdG (1.5 µg mL\(^{-1}\)) were exposed to 0.25N HCl at room temperature, while thermal stress condition of dG, 8-BrdG, and 8-CpdG (10 µg mL\(^{-1}\)) were heated at 80 °C. The stress samples were neutralized with mobile phase and were brought to neutral pH with 0.1N NaOH if necessary. The samples were then analyzed with the developed analytical method. Resolution among analyte peaks and peak purity were recorded and evaluated to demonstrate specificity of the analytical method for dG and the modified dG.

Linearity: The objective of linearity study is to demonstrate the linear relationship between detector response and analyte concentration in a given range. System linearity has been conducted by injection of six serial dilutions of nucleoside stock solution with nominal concentration range from 0.5-40 µg mL\(^{-1}\). The solutions have been injected in triplicate into the HPLC system and a calibration curves have been plotted between peak area and corresponding concentration to show linearity of the analytical method. Regression analysis has been performed using least square method. Linear equation and correlation coefficient (r) were used to evaluate linearity of the method.

Precision: Precision of the method can be demonstrated by repeatability. Triplicate analysis of three concentration levels of each nucleoside (5, 10, and 20 µg mL\(^{-1}\)) were performed. Relative standard deviation (RSD) was determined to evaluate the closeness among series of measurement.

Accuracy: The accuracy of the method was demonstrated by mean of standard addition method. Various amounts of dG, 8-BrdG, or 8-CpdG have been spiked into mobile phase. Triplicate analysis of three concentration levels of each nucleoside (5, 10, and 20 µg mL\(^{-1}\)) were performed. Recovery was determined by comparison between amount of standard added and found in the sample.

LOD and LOQ Determination: LOD and LOQ studies have been conducted to determine sensitivity of the method. Injections of four serial dilutions of stock solutions of dG (10-71 ng mL\(^{-1}\)), 8-BrdG (30-145 ng mL\(^{-1}\)), and 8-CpdG (50-260 ng mL\(^{-1}\)) have been performed. The minimum concentration that gives signal to noise ratio more than 10 is LOQ, while LOD is the minimum concentration that gives signal to noise ratio more than 3.

Stability Study Procedure and Kinetic Measurements: Stability of dG, 8-BrdG, and 8-CpdG were evaluated under thermal stress according to the working condition during annealing process of DNA that requires incubation of DNA sample at high temperature. Each nucleoside solution with concentration of 10 µg mL\(^{-1}\) was exposed to heat in temperature controlled water bath at 80 °C. The stressed dG solutions were collected at initial and after 12, 24, 36, 48, 54, and 72 hr incubations. In case of 8-BrdG and 8-CpdG, the stressed solutions were collected at initial and after 2, 4, 6, 8, and 10 hr incubations. The stressed samples were then analyzed with the validated method to determine the remaining amount of nucleosides. The first-order kinetics plots of the unmodified and modified dG degradations were obtained from the chromatographic results.

Results

Method Development: The aim of study is to develop a single novel analytical method that can be used to study degradation and stability of dG, 8-BrdG and 8-CpdG. Due to chromophoric purine on dG that absorbs UV light,
RP-HPLC with UV detection was initiated for method development. An optimum pH of aqueous acetonitrile mobile phase has been studied and selected based on pKa of dG (pKa₁ = 1.6, pKa₂ = 9.2, and pKa₃ = 12.3) [16]. A mobile phase at pH 6.0 was selected for the reason that nucleoside analytes were in single form. As a result, the retention time of dG, 8-BrdG, and 8-CpdG were observed at 2.9 min, 9.3 min, and 4.7 min respectively. The suitability of developed method has been tested with nucleoside standard solutions. The system suitability results from dG, 8-BrdG and 8-CpdG standard solution (Table 1) indicates that the method was suitable to further perform method validation.

Method Validation: To demonstrate that the analytical procedure can be used to selectively determine dG, 8-BrdG and 8-CpdG in the presence of their degraded product without interference, the stressed samples that contain nucleosides and their degradation products were analyzed with the developed method. Hydrolysis of the unmodified and modified dG to form guanine and C8-modified guanine bases were expected after the nucleosides exposed to acid or high temperature (Figure 2). The resolution of the dG, 8-BrdG, and 8-CpdG peaks were above 2.0 which indicates that the nucleosides were well resolved from their degradation products (Figure 3). The result from peak purity calculation also confirmed that there was no interference from impurities for dG, 8-BrdG and 8-CpdG detections. Regression analysis of the calibration curves of dG, 8-BrdG and 8-CpdG from system linearity study indicates the linear response of detector with the nucleoside concentration ranged from 0.5 to 41 µg mL⁻¹ with the r of the calibration curves more than 0.995 for all nucleosides (Table 2).
The precision of the method was shown by low % RSD of peak area measurement from triplicate of three sample solutions (from Table 3, 0.11-0.67 % RSD for dG, 0.22-0.91 % RSD for 8-BrdG, and 0.21-1.06 % RSD for 8-CpdG). The accuracy of the method was determined from standard addition study in all three concentrations of nucleosides have satisfied the acceptance criteria of 90-107% recovery based on AOAC guideline [15] as shown in Table 3. The sensitivity of the method to detect and quantify dG, 8-BrdG, and 8-CpdG has been determined based on the observed signal to noise of the nucleoside peaks. The LOD and LOQ of dG detection were 30 ng mL$^{-1}$ and 71 ng mL$^{-1}$ respectively. In case of the modified nucleosides, the LOD and LOQ of 8-BrdG were 29 ng mL$^{-1}$ and 145 ng mL$^{-1}$ respectively, while the LOD and LOQ of 8-CpdG were 52 ng mL$^{-1}$ and 208 ng mL$^{-1}$ respectively. Based on the method validation results, the developed method for dG, 8-BrdG, and 8-CpdG detections is precise, accurate, sensitive, and suitable for the nucleoside stability study.

**Table 1. System suitability results**

<table>
<thead>
<tr>
<th>System suitability parameters</th>
<th>dG</th>
<th>8-BrdG</th>
<th>8-CpdG</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plate number</td>
<td>3517</td>
<td>6166</td>
<td>3769</td>
<td>NLT 2000</td>
</tr>
<tr>
<td>Resolution</td>
<td>4.3</td>
<td>16.3</td>
<td>5.2</td>
<td>NLT 2.0</td>
</tr>
<tr>
<td>%RSD of peak response</td>
<td>0.15</td>
<td>0.17</td>
<td>0.17</td>
<td>NMT 2.0</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.14</td>
<td>0.99</td>
<td>1.04</td>
<td>NMT 1.5</td>
</tr>
</tbody>
</table>

The precision and accuracy results

<table>
<thead>
<tr>
<th>Parameters</th>
<th>dG (µg)</th>
<th>8-BrdG (µg)</th>
<th>8-CpdG (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Added (µg)</td>
<td>5.155</td>
<td>10.310</td>
<td>20.620</td>
</tr>
<tr>
<td>Found (µg)</td>
<td>5.112</td>
<td>10.434</td>
<td>21.022</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>99.18</td>
<td>101.20</td>
<td>101.95</td>
</tr>
<tr>
<td>Bias (%)</td>
<td>-0.82</td>
<td>1.2</td>
<td>1.95</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.33</td>
<td>0.67</td>
<td>0.11</td>
</tr>
</tbody>
</table>

**Stability of the unmodified and modified nucleosides:** For stability comparison of dG, 8-BrdG, and 8-CpdG, rate kinetic study of nucleoside hydrolysis was conducted with aqueous solution of nucleosides at 80°C. With the newly validated stability indicating RP-HPLC method, deglycosylation of the substituted and unsubstituted dG were observed. Injection of guanine standard gave peak eluted at the same retention time as hydrolysis product of dG. Hence, the identity of hydrolysis product of dG was confirmed to be guanine. Due to similar chemistry of nucleosides, the same deglycosylation process should occur to the modified nucleosides. Therefore, the C8 substituted guanines were expected to be hydrolysis products of 8-BrdG and 8-CpdG. The first order kinetic data including rate constant (k) and half-life (t½) of dG, 8-BrdG, and 8-CpdG were observed from this study (Table 4). Hydrolysis rate of both C8 modified dG at 80°C were higher than dG with 8-BrdG hydrolyzed at 23.4 time faster than dG, while 8-CpdG underwent hydrolysis ~50 times faster than dG. The estimated half-life of dG in aqueous solution at 80°C was 60.6 hr, while the presence of 8-bromo and 8-carboxyphenyl adducts have greatly

**Table 3 Regression equation parameters from linearity study**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>dG</th>
<th>8-BrdG</th>
<th>8-CpdG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration range (µg mL$^{-1}$)</td>
<td>0.52 - 41.2</td>
<td>0.50 - 40.0</td>
<td>0.50 – 39.6</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>1.0000</td>
<td>0.9997</td>
<td>0.9997</td>
</tr>
<tr>
<td>Slope</td>
<td>5.728 x 10$^4$</td>
<td>5.047 x 10$^4$</td>
<td>2.433 x 10$^4$</td>
</tr>
<tr>
<td>Intercept</td>
<td>-5.231 x 10$^3$</td>
<td>3.191 x 10$^3$</td>
<td>2.898 x 10$^3$</td>
</tr>
</tbody>
</table>
Table 4 First order kinetic data of dG, 8-BrdG, and 8-CpdG hydrolysates obtained at 80°C

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>k</th>
<th>t½ (hr)</th>
<th>k/k(dG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dG</td>
<td>0.011 ± 5.8 x 10⁻⁴</td>
<td>60.6</td>
<td>-</td>
</tr>
<tr>
<td>8-BrdG</td>
<td>0.268 ± 3.1 x 10⁻⁴</td>
<td>2.6</td>
<td>23.4</td>
</tr>
<tr>
<td>8-CpdG</td>
<td>0.568 ± 2.5 x 10⁻³</td>
<td>1.2</td>
<td>49.7</td>
</tr>
</tbody>
</table>

1 k/k(dG) is a ratio between first order rate constant of the modified dG and dG

reduced half-life of dG to 2.6 and 1.2 hr respectively. The bulky 8-bromo and 8-carboxyphenyl adducts may increase deglycosylation rate due to the relief of strain in the activated complex [17]. Higher degree of steric from 8-carboxyphenyl aduct may explains greater destabilization from the aryl adduct compared to 8-bromo adduct. From kinetic data, 8-BrdG has been found to be more stable than 8-CpdG and may prove to be more practical to be used to promote Z-DNA formation in DNA probe.

Conclusion

A novel, simple, sensitive, precise, and accurate isocratic RP-HPLC method for dG, 8-BrdG, and 8-CpdG analysis was obtained from this study. Stability of the C8 modified deoxyguanosines, 8-BrdG and 8-CpdG, were evaluated by utilizing the validated analytical method. The modifications of dG with 8-bromo or 8-carboxyphenyl adduct destabilized nucleosides such that the modified dG were more susceptible to hydrolysis than the unmodified nucleoside. The result from stability study indicates that 8-BrdG was more stable than 8-CpdG. Hence, 8-BrdG is a more suitable alternative for Z-DNA probe construction.

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References