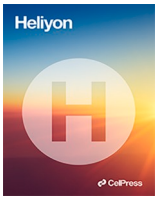
Heliyon 10 (2024) e38610

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Ultra high-performance liquid chromatography tandem mass spectrometry: Development and validation of an analytical method for N2-Ethyl-2'-deoxyguanosine in dried blood spot

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ARTICLE INFO

Keywords:
Acetaldehyde
DNA adduct
Dried blood spot
Ethanol
N2-Ethyl-2'-deoxyguanosine (N2-Et-dG)
UPLC-MS/MS

ABSTRACT

In the body, ethanol is metabolized to acetaldehyde by alcohol dehydrogenase and CYP2E1. Acetaldehyde is the main carcinogen that forms DNA adducts, N2-Ethylidene-2'-deoxyguanosine (N2-Ethylidene-dG), which can cause DNA damage and lead to cancer. However, N2-Ethylidene-dG is an unstable form at the nucleoside level and is difficult to measure. So it needs to be reduced using $NaBH_4$ to become N2-Ethyl-2'-deoxyguanosine (N2-Et-dG). This study aims to obtain a selective, sensitive, and validated analytical method to determine N2-Et-dG using ultra-high-performance liquid chromatography-tandem mass spectrometry with allopurinol as the internal standard. The N2-Et-dG analysis was performed using a C_{18} Acquity® Bridged Ethylene Hybrid column of (1.7 μm, 100 mm × 2.1 mm). The sample matrix used was dried blood spots, and then the DNA sample was extracted using the QIAamp DNA Mini Kit. The optimum analysis conditions were obtained in a combination eluent of 0.1 % acetic acid and methanol (60:40 v/v) with a flow rate of 0.1 mL/min and eluted isocratically for 5 min. Quantification analysis was performed using triple quadrupole mass spectrometry with electrospray ionization in positive mode, with detection at *m/z* 296.16 > 180.16 for N2-Et-dG and 137.03 > 110.05 for allopurinol, respectively. The validated analytical method follows the 2018 USA Food and Drug Administration guidelines with a linear concentration range of 5–200 ng/mL.

1. Introduction

Approximately two billion adults worldwide consume alcoholic beverages, with an average daily consumption of 13 g of ethanol [8,9]. Ethanol undergoes metabolism to acetaldehyde through processes involving alcohol dehydrogenase (ADH), CYP2E1, and catalase. Acetaldehyde is primarily oxidized to acetic acid by acetaldehyde dehydrogenase (ALDH). During this process, there is a reduction of NAD^+ to NADH, altering the NAD^+/NADH ratio and increasing reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to the formation of DNA and protein adducts. These adducts being referred to are primarily oxidative adducts and contribute to the initiation and progression of cancer [10].

In individuals with normal ALDH, acetaldehyde can be further metabolized into acetic acid, undergoing conjugate metabolism and being excreted in feces. However, a significant number of East Asians exhibit ALDH enzyme polymorphisms resulting in poor enzyme

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https://doi.org/10.1016/j.heliyon.2024.e38610
Received 16 April 2024; Received in revised form 26 September 2024; Accepted 26 September 2024
Available online 27 September 2024

activity (poor metabolism). This leads to elevated acetaldehyde levels in the body, posing a much higher risk of cancer compared to individuals with normal ALDH [8–10].

Research on the polymorphism of ALDH has been conducted by Yukawa et al., [3]. In this study, it was found that ALDH polymorphism directly contributes to the accumulation of acetaldehyde levels in the body. The high accumulation of acetaldehyde, coupled with the presence of polymorphisms, leads to the formation of adducts. Acetaldehyde can form adducts with proteins and DNA. However, based on its reactivity, acetaldehyde is more reactive with DNA than proteins because DNA molecules, especially those in the N7 and N2 groups, are more nucleophilic than proteins found in the body. Consequently, acetaldehyde forms more adducts with DNA compared to protein adducts [3,14].

DNA adducts can induce polymerization errors and mutations in critical genes, activating oncogenes and inactivating tumor suppressor genes [7]. DNA adducts can serve as biomarkers for the early detection of cancer before it reaches an advanced stage. The type of DNA adduct formed by acetaldehyde depends on the deoxyribonucleoside used. The interaction of acetaldehyde with deoxyguanosine (dG), the most reactive, results in the main DNA adduct N2-Ethylidene-2′-deoxyguanosine (N2-Ethylidene-dG), which is very stable in DNA. Although N2-Ethylidene-dG is abundant, it is unstable at the nucleoside level and challenging to measure. To measure N2-Ethylidene-dG, $NaBH_3CN$ or $NaBH_4$ is added to convert it to N2-Ethyl-2'-deoxyguanosine (N2-Et-dG), which is stable and quantifiable or analyzable [21,22]. DNA adduct formation can be a biomarker of exposure to carcinogens and is used for early cancer detection in the body [11]. The following is the chemical reaction (Fig. 1).

Cited from (Johnson, C. H. et al., 2021, has been reprocessed using ChemDraw Professional software.):

The analytical method to determine N2-Et-dG was investigated by Singh et al., in 2012 [15], involving venipuncture of 10 mL of blood samples in each subject collected into heparinized venoject tubes, followed by DNA extraction using the liquid-liquid extraction method. This method utilizes toxic reagents in large volumes, and the eluate obtained is less purified compared to using kits. The reducing agent $NaBH_4$ is used to reduce the N2- Ethylidene-dG adduct formed in the body. Furthermore, Singh et al. [15,16], conducted various validation parameters, including LLOQ, calibration curve, intra- and inter-day precision accuracy, and recovery, without specific reference to other FDA requirements. The research [15] evaluated the potential for DNA damage from exposure to marijuana cigarette smoke using the adduct biomarker N2-Et-dG with KCKUT-SM/SM ESI+ with m/z 296 > 180 for N2-Et-dG and m/z 301 > 185 for [$^{15}N_5$]N2-Et-dG (as an internal standard).

In this study, several considerations were taken into account to ensure subject convenience in the biosampling technique. Challenges such as the preparation of blood collection and transportation, issues related to green chemistry, and the use of extraction methods requiring large volumes of organic solvents were addressed. Consequently, a more effective sampling method was developed utilizing dried blood spot (DBS) and the DNA Mini Kit QIAamp Mini Kit [18] for extraction. The choice of DBS was motivated by its

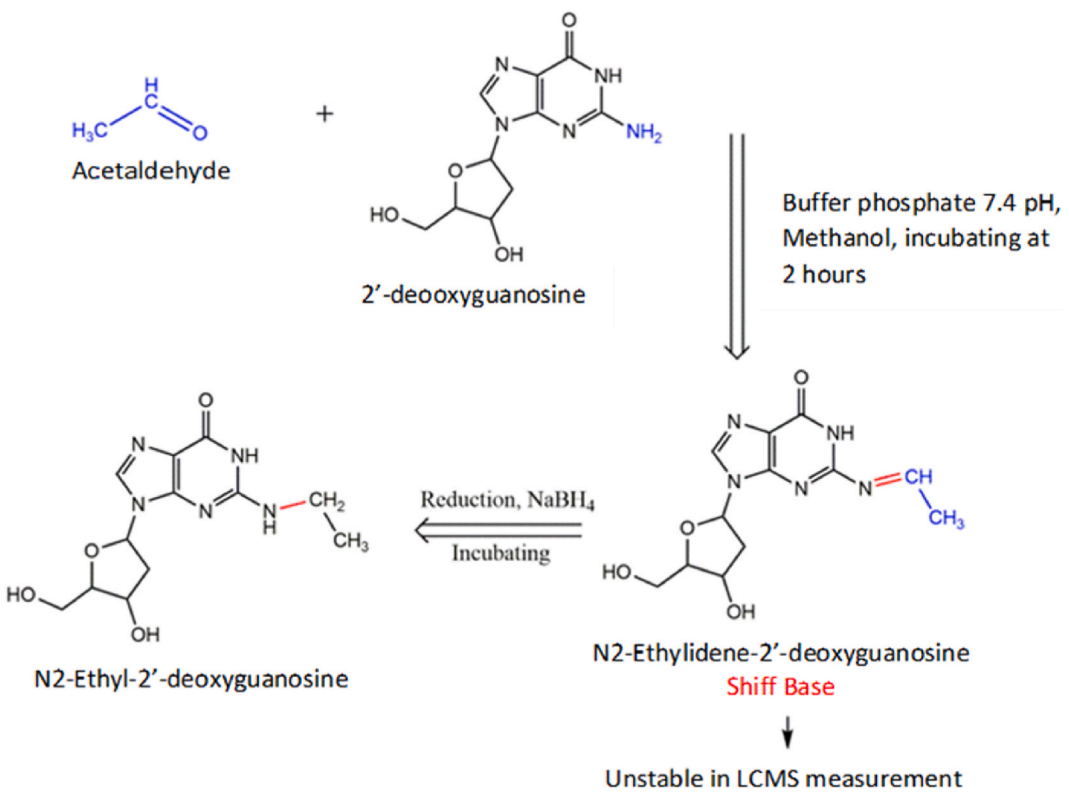

Fig. 1. Reduction of DNA adduct using sodium borohydride ($NaBH_4$).

advantages, including a collection volume of only 30–100 μL [23], enhanced patient comfort due to finger prick blood sampling, ease of storage, and a smaller sample volume collected (green chemistry).

The internal standard (IS) selected for analysis must have a structure and physicochemical properties similar to or analogous to the analyte to be tested. In previous studies, the internal standard used was stable isotope labeled (SIL) [$^{15}N_5$] N2-Et-dG, which is a radioisotope of N2-Et-dG [15,16]. SIL is quite difficult to obtain, so this study uses an internal standard of allopurinol, which has a similar structure to the guanine base core of the test compound [17]. The validated method parameters used in this study refer to the 2018 US Food and Drug Administration (FDA) guidelines. The parameters tested in the method validation include calibration curve, selectivity, sensitivity, namely determining the lowest quantitative limit, lower limit of quantitation (LLOQ), carryover, accuracy, precision, dilution integration, recovery, matrix effect, and stability of the analyte in the matrix [4]. The results of the study can be used as a basis for selecting biosampling and bioanalysis methods to see the relationship between alcoholic lifestyles and the potential for the formation of N2-Et-dG, which is an adduct of acetaldehyde in the blood.

2. Materials and method

Chemical and reagents. N2-Ethyl-2'-deoxyguanosine (Sigma Aldrich, Singapore); Acetaldehyde (Sigma Aldrich, Singapore); allopurinol as an internal standard (Jiangsu Yew Pharm, China); $NaBH_4$ (Loba Chemie, Mumbai); formic acid, acetic acid, acetonitrile, methanol, and ethanol (HPLC-grade, Merck, Germany); human blood (Indonesian Red Cross); blood collection tubes (1.5 mL; Stardec, Indonesia); DBS Whatman 903 paper (Sigma Aldrich, Singapore); QIAamp DNA mini kit (Qiagen, Australia); ultrapure water (treated with Arium® Pro Ultrapure System, Sartorius Arium Pro, USA).

UPLC-MS/MS conditions. The experiment was performed on an ACQUITYTM UPLC system (Waters Corp., Milford, MA, USA) and a Xevo TQD triple quadrupole mass spectrometer (Waters Corp., Manchester, UK) equipped with positive electrospray ionization (ESI+). All data were acquired in centroid mode by the MasslynxTM NT4.1 software and analyzed by the QuanLynxTM program (Waters Corp., Milford, MA, USA). The analyte was separated on an Acquity® UPLC BEH C18 column (1.7 μm, 100 mm × 2.1 mm, Waters, Milford, MA, USA). The eluent used had three different compositions to determine the best eluent consisting of 0.2 % formic acid in water with acetonitrile, 0.1 % acetic acid in water with methanol, and 0.1 % acetic acid in water with acetonitrile; flow rates were also carried out in several combinations to determine the best flow rate, namely 0.1 mL/min, 0.15 mL/min, and 0.2 mL/min; autosampler temperature 8 °C; and injection volume 10 μL. The isocratic elution was used within 5 min. The mass spectrometric detector parameters were optimized and set as follows: nitrogen desolvation temperature of 350 °C with a flow rate of 650 L/h and capillary voltage of 2.98 kV. The cone voltage was 16 V for N2-Et-dG and 44 V for allopurinol as IS, and the collision energy was 10 V for N2-Et-dG and 12 V for allopurinol as IS. The detector was performed in positive ion mode, obtained by the positive mode of electrospray ionization (ESI+) technique, and quantification was acquired with multiple reaction monitoring (MRM) with an ion transition at 296.16 > 180.16 for N2-Et-dG and 137.03 > 110.05 for allopurinol, respectively.

Preparation of stock and working standard solution. A stock solution of N2-Et-dG was prepared at 5.0 mg/5.0 mL and diluted with methanol to obtain a stock solution of N2-Et-dG with a concentration of 1000 μg/mL. The stock solution of allopurinol as an internal standard was prepared at 5.0 mg/5.0 mL and diluted with ultrapure water to obtain the stock solution with a concentration of 1000 μg/mL. All solutions were stored at −20 °C and brought to room temperature before use.

Calibration standards and quality control (QC). The standard solution of the calibration curve is made by diluting the N2-Et-dG parent solution with a concentration of 1000 μg/mL using ultrapure water to obtain concentrations of 100 ng/mL to 5000 ng/mL. Subsequently, each solution is diluted until a concentration range of 5–200 ng/mL is reached. The volume is equal to 50 μL. Next, an internal solution containing 5 μg/mL of allopurinol is made.

The quality control solution is produced by diluting the N2-Et-dG master solution with a concentration of 1000 μg/mL using ultrapure water to obtain three concentrations, namely QCH (highest concentration), QCM (medium concentration), and QCL (lowest concentrations). QCH with a minimum requirement of 75 % maximum limit of calibration curve (ULOQ = 200 ng/mL) of 150 ng/mL.

a. QCM with a requirement about 30–50 % calibrations range of 100 ng/mL.
b. QCL with a maximum requirement 3 times the minimum limit of the calibrating curve (LLOQ = 5 ng/mL), of 15 ng/mL.

Optimization of Dried Blood Spot (DBS) sample preparation. Blood samples containing N2-Et-dG as a positive control were prepared by mixing 10 μL of N2-Et-dG solution with a concentration of 500 μg/mL with 990 μL of whole blood to obtain a mixture with a concentration of 5 μg/mL. In dried blood spots, the drying time and blood spotting volume were optimized. The optimization of drying time for DBS samples was carried out with variations of 60, 120, and 180 min and then dried at 25 °C. Based on the determined peak area, the DBS drying time optimization was selected. The DBS sample spotting volume was optimized when the optimal DBS drying time was established. Throughout the optimization process, variations of 30 μL, 40 μL, and 50 μL were employed. Samples were optimized to the highest degree possible in order to facilitate DNA extraction. The chosen DBS sample spot volume optimization was based on the measured peak area.

Preparation of DNA Extract. Following the instructions from the QIAamp DNA Mini and Blood Mini Handbook [18]. Using DBS, the DNA extraction process was carried out on spiked blood samples. The following protocol was followed in order to prepare the samples: 1. Spotting blood-containing analyte onto DBS paper under optimal conditions, then allowing it to dry. Cut the DBS paper and insert it to the microcentrifugation tube; 2. Fill the tube with 180 μL of ATL buffer (lysis buffer) and incubate for 10 min at 85 °C. The liquid or droplets that might have remained within the lid were then removed by centrifuging it for a minute at 8000 rpm; 3. Add 20 mL of the proteinase K stock solution, vortex, and incubate for 1 h at 56 °C. It was then centrifuged for 1 min at 8000 rpm to remove the

liquid and droplets from the lid. 4. A second round of incubation was carried out to optimize temperature and time of reduction of N2-Ethylidene-dG to N2-Et-dG using NaBH4; 5. Include 200 μL of 96 % ethanol and vortex. After that, it was centrifuged for a minute at 8000 rpm to get rid of any liquid or droplets still on the lid; 6. After being moved to a QIAamp small spin column, the mixture was covered and centrifuged for 1 min at 8000 rpm. The material was moved to a 2 mL collecting tube once the preceding centrifugation's tube was emptied. Note: To avoid aerosol production, the QIAamp tiny spin column must to be tightly closed; 7. Add 500 μL of buffer AW1, shut the cover, and centrifuge for a minute at 8000 rpm. Then, after discarding the tube from the earlier centrifugation, transferred to a 2 mL collecting tube; 8. Added 500 μL of AW2 buffer and centrifuged for 3 min at 14,000 rpm. Then, after discarding the tube from the earlier centrifugation, transferred to a 2 mL collecting tube; 9. After incubating the QIAamp small spin column for 1 min at room temperature (15–25 °C), insert it into a clean 1.5 mL collection tube microcentrifuge, add 150 μL of AE buffer, and centrifuge for 3 min at 8000 rpm. Note: The sample preparation procedure is done between 15 and 25 °C, or room temperature. In addition, a thorough technique validation was carried out in accordance with the 2018 FDA recommendations.

Optimization DNA Reduction Using $NaBH_4$. DNA reduction was carried out by preparing the extraction eluate using the QIAamp mini kit procedure [18]. Then the eluate was reacted with acetaldehyde in phosphate buffer pH 7.4, and methanol was incubated for 2 h to form Schiff base (N2-Etileden-dG) [19]. The addition of $NaBH_4$ was carried out at the 4th point in the preparation of DNA extract process, and then optimization was carried out by reacting Schiff's base using $NaBH_4$ with five combination optimizations as follows:

After that, the sample was kept at room temperature so that it could be used for the upcoming analysis (DNA extract preparation). The results of the analysis are then compared and the most optimum analysis conditions are selected based on the shape of the chromatogram, peak retention time, and the resulting analyte area.

Selectivity. Selectivity tests were carried out with six blood sources, with different variations prepared according to the selected method. Then each blank and concentration was made into two replicates. Next, the interference from the matrix on the retention time of the analyte and internal standard was calculated. Interference still meets the requirements if the peak area of the analyte in the blank matrix obtained is not more than ±20 % of the LLOQ peak area and not more than 5 % in the internal standard [4].

Linearity. Calibration standards were prepared and analyzed by plotting the peak area ratios of the analyte to the IS versus the nominal concentration using a linearly weighted regression method in triplicate. The bias of the calculated concentrations was within ±15 % of the nominal concentrations, except for the LLOQ, which had an allowed deviation of ±20 % [4].

Lower limit of quantification. LLOQ was established by analyzing blank blood samples spiked with ½ or ¼ of the lowest concentration of N2-Et-dG in the sample. The analyte response should be identifiable, discrete, and reproducible with acceptable precision and accuracy (less than 20 % for each criterion) [4].

Accuracy and precision. Accuracy and precision were evaluated by assessing five replicates of the QC samples at four concentration levels (LLOQ, low, medium, and high) on three consecutive validation days. Intra-day and inter-day precision required % coefficient of variance (%CV) not to exceed 15 %, and accuracy (%diff) should be within ±15 % and except the LLOQ with an allowed deviation of ±20 % [4].

Recovery. Recovery values (%) were calculated at three QC levels (QCL, QCM, and QCH) by comparing the peak areas of the regularly processed QC samples with those of spiked post-extraction samples. The % coefficient of variance (%CV) of the recovery values should be less than 15 % [4].

Carry over. Carry-over was assessed by injecting blank blood samples after the calibration standard at the upper limit of quantification. The measured peak area should not be greater than 20 % of the peak area of the analyte at the lower limit of quantification (LLOQ) and 5 % of the peak area of the internal standard, respectively [4].

Matrix effect. Matrix effect testing was carried out to determine the differences resulting between the analyte in a biological sample and the analyte in a standard solution and at the same concentration. The matrix effect was calculated by comparing the ratio of the peak area of N2-Et-dG and standard in allopurinol in the matrix with the peak area of N2-Et-dG and standard in allopurinol in the standard solution. Matrix effect testing was carried out from 6 different matrix sources with 2 replicas for each source to obtain QCL and QCH concentrations. The requirement for the matrix effect is that the %KV does not exceed ±15 % [4]. The standardized matrix factor (MF) values with the internal standard should obtain the acceptance range of 0.80–1.20 [4,12].

Stability. Stock solution stability of N2-Et-dG and allopurinol was evaluated in the short term at 0, 6, and 24 h in a refrigerator at 8 °C) and in the in the long term at 1, 7, 15, and 30 days at storage at −20 °C). The test was performed in two replicates, and the %diff value should not be more than 10 % [1,20]. Sample stability was tested by analyzing the QCL and QCH after short-term storage (kept in the refrigerator for 0, 6, and 24 h) and long-term storage (at −20 °C in the freezer in the freezer for days 1, 10, and 15). Autosampler stability (kept at autosampler temperature for 0 and 24 h). The test is performed in three replicates, and the %diff and %CV values should not be more than 15 % [4].

3. Result and discussion

This study described the development and validation of the UPLC-MS/MS method for quantitative analysis of N2-Et-dG in dried blood spots. The UPLC-MS/MS is currently considered the best choice for supporting bioanalytical studies due to high specificity, sensitivity, and rapidity.

4. Method development

Selection of Internal Standard. In UPLC-MS/MS, an internal standard (IS) should ideally be an isotopically labeled compound or possess physiochemical properties as similar to the target analyte as possible. Additionally, it should elute as closely as possible to the

target analyte [17]. Allopurinol was selected as an internal standard due to its similar characteristics and structural analogy to the guanine base found in N2-Et-dG.

Optimization of mass condition. To optimize ESI conditions with the MRM analysis method, it was chosen to achieve optimal conditions for identifying compounds by inputting precursor ions and daughter ions, providing data on the cutoff collision energy and voltage required to obtain the best conditions for both the parent ion and daughter ion. Additionally, the MRM method offers the advantage of a better signal-to-noise (S/N) ratio compared to the SIM method [6,13].

Based on the optimization results, the detection with the best response for the parent ion and daughter ion had *m/z* values of 296.16 > 180.16 for N2-Et-dG (Fig. 2a) and 137.03 > 110.05 for allopurinol respectively (Fig. 2b). The resulting fragmentation follows the theory of using the ESI (+) ionization method, involving the formation of multivalent ions with the resulting fragmentation pattern $(M + nH)^n+$.

The positive ionization is related to their basic properties. The ESI (+) ionization mode was chosen because DNA compounds are basic, so the mechanism that occurs is ionization with the addition of H^+ ions [6].

Optimization of Eluent. Three eluent combinations were tested: 0.2 % formic acid in water with acetonitrile, 0.1 % acetic acid in water with methanol, and 0.1 % acetic acid in water with acetonitrile. Using a 70:30 ratio of aqueous phase to organic solvent, 0.1 % acetic acid in water with methanol was selected for producing the best chromatogram with the largest area. Further testing showed the 60:40 mixture yielded the greatest area. Adjusting methanol concentration sped up retention times, and higher pH stabilized the eluent for better ionization in ESI (+) usage. However, too much organic solvent could cause chromatogram tailing and analyte degradation, reducing the response area.

Optimization of Flow Rate. Three flow rates were tested: 0.1 mL/min, 0.15 mL/min, and 0.2 mL/min. A flow rate of 0.1 mL/min was chosen because it produced the best chromatogram with a large area and an optimal retention time. Higher flow rates resulted in sharper peaks and faster retention times, speeding up analysis but reducing the resulting area and increasing column pressure, which could damage the column.

System Suitability Test. After obtaining optimal analysis conditions (acetic acid 0.1 % in water (A) and methanol (B); 60:40 v/v; 0.1 mL/min), a system suitability test was conducted to ensure accurate data production. The percent CV of the area for N2-Et-dG and allopurinol was 4.08 % and 3.48 %, respectively, while the percent CV of the retention time was 0.01 % and 0.00 %. These results suggest that the system is functioning well, meeting the %CV requirements (not exceeding 6 %) [2]. The process continues with the analysis using validated and optimized methods, followed by the evaluation and publication of reports and journals [5].

Optimization of Dried Blood Spot (DBS) Sample Preparation. DBS samples were tested, and an optimal drying time of 120 min was determined, providing the greatest peak area response compared to 60 min and 180 min. A drying time that is too fast results in a small response due to incomplete blood drying, while excessively long drying times produce smaller responses because prolonged

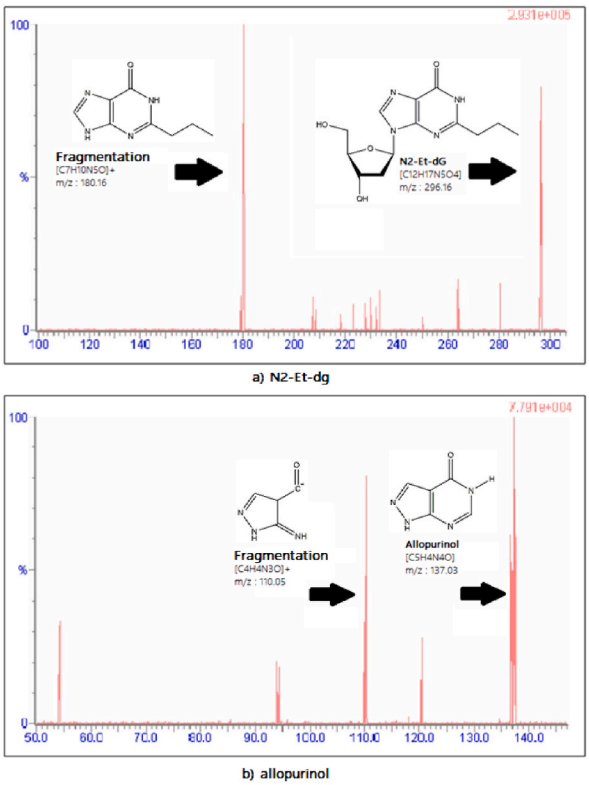

Fig. 2. Fragmentation spectrum of (a) N2-Et-dG, (b) allopurinol as IS.

drying strengthens analyte absorption into the DBS, making extraction difficult.

After figuring out the ideal drying time, evaluating spotting volumes was the next step in optimization. After testing three different concentrations of 30 μL, 40 μL, and 50 μL, the greatest peak area response was obtained with a spotting volume of 50 μL. The amount of analyte absorbed in DBS is directly correlated with the volume of blood spots. Response area values at 30 μL and 40 μL penetration volumes are significantly smaller than those at 50 μL penetration volumes [23].

Optimization DNA Reduction Using $NaBH_4$. In this study, optimization of the addition of $NaBH_4$ was carried out after the preparation of DNA extract with 5 conditions (A, B, C, D, and E) in Table 1. Based on the experimental results, the conditions chosen to reduce acetaldehyde with $NaBH_4$ were carried out in the D condition because it produced the best response and area readings. Condition D: The first incubation for 10 min (temperature 70 °C) added 2.0 mg of $NaBH_4$, the second incubation for 60 min (temperature 40 °C) added 2.0 mg of $NaBH_4$, the third incubation for 90 min (temperature 40 °C) added $NaBH_4$ 2.0 mg, and the final incubation for 30 min (temperature 40 °C) added 4.0 mg of $NaBH_4$.

DNA reduction involved preparing the extraction eluate using the QIAamp mini kit, followed by reacting the eluate with acetaldehyde in phosphate buffer pH 7.4 and methanol incubation for 2 h to form Schiff base (N2-Ethyliden-dG). $NaBH_4$ was added after incubation with AL buffer to reduce debris, as adding AL buffer significantly reduces DNA debris, minimizing contamination. N2-Ethyliden-dG was obtained by reacting the extraction eluate with an average of around 1500–3000 ng of DNA, followed by digestion with acetaldehyde in phosphate buffer pH 7.4 and methanol incubation for 2 h to achieve optimal conditions in condition D.

Method Validation. Validation of the quantitative UPLC-MS/MS method was assessed, including selectivity, linearity, lower limit of quantitation (LLOQ), accuracy and precision, recovery, carryover, matrix effect, and stability of the analytes in a biological matrix, according to the FDA guidelines on the bioanalytical method validation committee for medicinal products for human use [4].

In accordance with the FDA guidelines on bioanalytical method validation, the developed method demonstrated acceptability in terms of linearity, lower limit of quantification, selectivity, accuracy and precision, recovery, carryover, matrix effect, and stability.

Lower limit of quantification. The LLOQ was 5.00 ng/mL for N2-Et-dG, with %CV and %diff of back-calculated concentrations of LLOQ of 4.19 % and <20 % for N2-Et-dG, respectively (Table 2). Based on 2018, FDA regulations state that LLOQ concentrations are necessary [4].

Selectivity. The representative chromatograms resulting from the UPLC-MS/MS analysis of 500 μL of analyte from the blank blood sample and spiked LLOQ of N2-Et-dG and allopurinol. 0–16.67 % for N2-Et-dG and 0–4.86 % for allopurinol matrix components and impurities, respectively. Fig. 3(a, b) shows that there are no significant interfering peaks due to endogenous components or reagents.

Accuracy and precision. The intra- and inter-day accuracy and precision were shown in Table 3. The data demonstrate that the accuracy and precision values are within the acceptable criteria.

Recovery. The mean extraction recoveries of N2-Et-dG were 86.57 %, 86.72 %, and 89.27 % (n = 3) at the concentration of QCL, QCM, and QCH, with %CV values of 0.14 %, 0.19 %, and 0.83 %, respectively. While the IS was 91.22 % at the concentration of 500 ng/mL, the present CV value was 3.74 %.

Carry over. The measured peak area of the blank blood sample injected after calibration standard at the ULOQ (200 ng/mL) was 15.66 % of the peak area of the analyte at LLOQ for N2-Et-dG, respectively; 3.44 % of the peak area of the IS, respectively.

Matrix effect. The mean MEs of N2-Et-dG were 89.25 % and 89.86 at the concentrations of QCL and QCH, with %CV of 2.92 % and 1.73 %, respectively. While for the IS, 91.73 % and 90.40 at the concentration of QCL and QCH, with %CV of 5.20 % and 2.98 %, respectively, with an MF average of 0.974, are required [4,12]. These data indicate that the ME (ion suppression) from dried blood spots was negligible under the current conditions.

Stability. Storage of stock solutions of N2-Et-dG in methanol and allopurinol in ultrapure water at room temperature for 24 h in the refrigerator (8 °C) and in the freezer (−20 °C) for 1 month did not alter the analyte of N2-Et-dG and allopurinol. The stability test results of N2-Et-dG and allopurinol in dried blood spots are given in Table 4. The data indicate that N2-Et-dG and allopurinol are stable during sample preparation and storage conditions.

Linearity. The calibration curve over the concentration range of 5–200 ng/mL for N2-Et-dG was linear and acceptable. The calibration equation obtained had a correlation coefficient (r) > 0.98, and the %diff of the calculated concentrations was acceptable. Data on the inter-day calibration curve of N2-Et-dG are shown in Table 5.

5. Conclusion

In conclusion, the development and validation of the UPLC-ESI-MS/MS technique for the quantitative analysis of N2-Et-dG in dried blood spots using allopurinol as an internal reference were accomplished with success. N2-Et-dG concentrations can be measured with extreme speed, sensitivity, and specificity with this approach. The study's LLOQ for N2-Et-dG was 5 ng/mL, acquired by first preparing

Table 1
Optimization of the addition of $NaBH_4$ as a reducing agent for N2-Ethylidene-dG.

Condition	Incubating for10 min at 70°C	Incubating for 60 min at 40°C	Incubating for 90 min at 40°C	Incubating for 30 min at 40°C
A	1,5 mg	1,5 mg	1,5 mg	–
B	1,5 mg	1,5 mg	1,5 mg	3,0 mg
C	2,0 mg	2,0 mg	2,0 mg	–
D	2,0 mg	2,0 mg	2,0 mg	4,0 mg
E	3,0 mg	3,0 mg	3,0 mg	6,0 mg

Table 2
The accuracy and precision from LLOQ of N2-Et-dG.

LLOQ conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (%diff)	Precision (%CV)
5.00	5.43	8.61	4.19
	5.14	2.84	
	5.14	2.89	
	4.88	−2.37	
	4.95	−1.05	

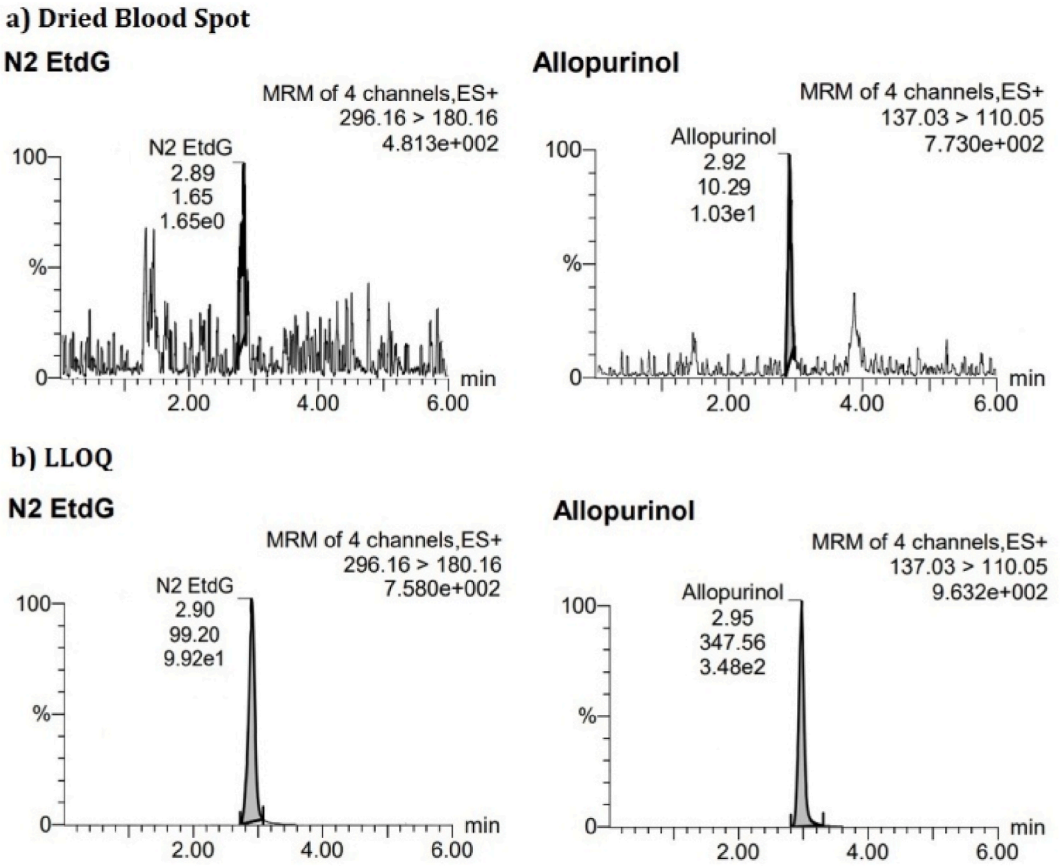

Fig. 3. Representative UPLC-MS/MS chromatograms of N2-Et-dG and Allopurinol (a) Dried blood spot; (b) Dried blood spot with analyte at LLOQ.

Table 3
The intra-day and inter-day accuracy and precision of N2-Et-dG.

Nominal conc. (ng/mL)	Intra-day		Inter-day	
	Mean accuracy (%diff)	Precision (%CV)	Mean accuracy (%diff)	Precision (%CV)
5.00	−2.03 to 4.35	2.97	−0.42 to 17.08	5.81
15.00	−3.67 to −1.98	0.77	−14.92 to 8.41	9.20
150.00	−3.60 to −1.25	0.91	−3.60 to 11.21	5.63
200.00	−1.48 to 2.17	0.45	−3.86 to 3.17	1.39

the sample using a dried blood spot, then extracting the DNA using the QIAamp Mini Kit, and finally decreasing the adduct N2-Ethylidene-dG under the D condition: first incubation (10 min 70 °C added 2.0 mg of $NaBH_4$), (60 min 40 °C added 2.0 mg of $NaBH_4$), (90 min 40 °C added $NaBH_4$ 2.0 mg), and the final incubation (30 min 40 °C added 4.0 mg $NaBH_4$). As an internal standard, allopurinol can regulate matrix effects and shares structural analog qualities with the guanine base present in N2-Et-dG.

Table 4
The stability test results N2-Et-dG and allopurinol in dried blood spot.

Stability experiments	Stable to-	
	N2-Et-dG	Allopurinol
Short-term storage (24 h, 8°C)	24 h	24 h
Long-term storage (freezer -20°C)	30 days	30 days
Autosampler (24 h)	18 h	18 h

Table 5
Data of inter-day calibration curve of N2-Et-dG.

Replica inter-day	R	Slope (b)	Intercept (a)
1	0.9994	0.0344	0.1062
2	0.9987	0.0363	0.0756
3	0.9980	0.0324	0.0724
Mean	0.9987	0.0344	0.0847

Ethical approval

This study has obtained an ethical review from the Human Research Ethics Committee of Universitas Indonesia with the number KET-230/UN2.F1/ETIK/PPM.00.02/2022 which complies to the Declaration of Helsinki.

Informed consent

All subjects signed the informed consent as a form of willingness to participate in the study. All subjects filled out a form containing the subject's identity, smoking habits, and alcohol consumption habits.

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Dimas Prayuda: Writing – original draft, Formal analysis. **Yahdiana Harahap:** Writing – review & editing, Methodology, Conceptualization. **Bantari Wisynu Kusuma Wardhani:** Writing – review & editing, Validation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Directorate of Research and Community Services (DRPM) Universitas Indonesia, Depok, Indonesia.

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