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Development and validation of assays for the quantification of β-D-N⁴-hydroxycytidine in human plasma and β-D-N⁴-hydroxycytidine-triphosphate in peripheral blood mononuclear cell lysates

Teresa L. Parsons a, Lindsay A. Kryszak a, Mark A. Marzinke a, b, *

a Department of Medicine, Johns Hopkins University, 5200 Eastern Ave, Mason F. Lord Tower, Suite 6000, Baltimore, MD 21224, USA
b Department of Pathology, Johns Hopkins University, 1800 Orleans St., Sheikh Zayed Tower, Baltimore MD 21287, USA

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ABSTRACT

The novel antiviral prodrug molnupiravir is under evaluation for the treatment of SARS-CoV-2. Molnupiravir is converted to β-D-N⁴-hydroxycytidine (NHC), which is the primary form found in systemic circulation. β-D-N⁴-hydroxycytidine-triphosphate (NHCtp) is the bioactive anabolite produced intracellularly. Sensitive and accurate bioanalytical methods are required to characterize NHC and NHCtp pharmacokinetics in clinical trials. Human K₂EDTA plasma or peripheral blood mononuclear cell (PBMC) lysates were spiked with NHC (plasma) or NHCtp (PBMC), respectively. Following the addition of isotopically-labeled internal standards and sample extraction via protein precipitation or lysate dilution, respectively, samples were subjected to liquid chromatographic-tandem mass spectrometric (LC-MS/MS) analysis. Methods were validated in accordance with FDA Bioanalytical Method Validation recommendations. NHC can be quantified in plasma with a lower limit of quantification (LLOQ) of 1 ng/mL; the primary linearity of the assay is 1–5000 ng/mL. Assay precision and accuracy were ≤ 6.40% and ≤ ± 6.37%, respectively. NHC is unstable in whole blood and has limited stability in plasma at room temperature. The calibration range for NHCtp in PBMC lysates is 1–1500 pmol/sample, and the assay has an LLOQ of 1 pmol/sample. Assay precision and accuracy were ≤ 11.8% and ≤ ± 11.2%. Ion suppression was observed for both analytes; isotopically-labeled internal standards showed comparable ion suppression, resulting in negligible (<5%) relative matrix effects. Sensitive, specific, and dynamic LC-MS/MS assays have been developed and validated for the quantification of NHC in plasma and NHCtp in PBMC lysates. The described methods are appropriate for use in clinical trials.

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pathogen, which is a single-stranded RNA virus, causes COVID-19 [1]. COVID-19 is associated with a heavy disease burden and can severely impact several organ systems, including the lungs, kidneys, liver, muscles, and nervous system [2]. With the global spread of SARS-CoV-2 beginning in early 2020, significant efforts have been made in both vaccine research for the prevention of COVID-19, as well as in the identification and utilization of novel or repurposed therapeutics for the management of those with symptomatic COVID-19. To date, ten agents have received emergency use authorization (EUA) by the US Food and Drug Administration (US FDA) for the management of patients with COVID, including one antiviral agent, the adenosine nucleoside analog remdesivir [3]. Other investigational agents are also being evaluated in clinical trials, including the ribonucleoside analog molnupiravir, also known as EIDD-2801 or MK-4482 [4,5].

Molnupiravir is an orally bioavailable isopropylester prodrug of β-D-N⁴-hydroxycytidine (NHC; EIDD-1931), which has broad antiviral activity against a number of RNA viruses based on in vitro and animal studies [6–9]. In a pre-clinical Venezuelan equine encephalitis virus mouse model, NHC was taken up by target tissues and anabolized to β-D-N⁴-hydroxycytidine-triphosphate (NHCtp; EIDD-2061), the bioactive anabolite of NHC [10]. NHCtp is a viral RNA polymerase substrate,

* Corresponding Author at: DABCC, Departments of Medicine and Pathology, Johns Hopkins University, 1800 Orleans St., Sheikh Zayed Tower, B1020F, Baltimore, MD 21287, USA.
E-mail address: mmarzin1@jhmi.edu (M.A. Marzinke).

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leading to the induction of lethal viral mutagenesis and impairment of viral fitness [4,6]. Studies in primary human cultures have shown that NHC is active against several coronaviruses, including SARS-CoV, SARS-CoV-2, and Middle East respiratory syndrome coronavirus (MERS-CoV) [11]. Molnupiravir treatment in an immunocompromised mouse model demonstrated inhibition of SARS-CoV-2 replication in vivo, reinforcing the potential for the compound in the management of COVID-19 [12]. At the 2021 Conference on Retroviruses and Opportunistic Infections (CROI), data from a phase 2a clinical trial showed that treatment with molnupiravir resulted in a significant reduction in time to negativity of infectious virus from nasopharyngeal swabs as compared to placebo in participants with COVID-19 [5].

As molnupiravir continues to be evaluated for the treatment of SARS-CoV-2, a more thorough understanding of drug pharmacology will be required, especially as several studies include dose escalations to assess drug safety and tolerability. Thus, bioanalytical assays will be needed to measure the primary form in systemic circulation (NHC) as well as its active intracellular anabolite (NHCtp). Previous studies refer to the measurement of NHC and its anabolites via liquid chromatography-tandem mass spectrometry (LC-MS/MS); however, these assays were chiefly employed for the determination of drug concentrations in preclinical trials, and specific validation metrics were not described [10,13]. In a recent phase 1 study, which assessed the tolerability, safety and pharmacokinetics of molnupiravir, molnupiravir and NHC concentrations in human plasma were quantified using assays with lower limits of quantification (LLOQs) of 5 ng/mL; NHCtp was not measured in that study [4]. In order to support ongoing and future work focused on molnupiravir, herein we describe the development and validation of rapid bioanalytical methods for the quantification of NHC in human plasma and intracellular NHCtp in peripheral blood mononuclear cells (PBMC) lysates.

## 2. Experimental

### 2.1. Chemicals

NHC and NHCtp, as well as their respective internal standards, β-D-N°-hydroxycytidine-[13C₅] (NHC-IS) and β-D-N°'-hydroxycytidine-triphosphate-[15C₅] (NHCtp-IS), were generously provided by Ridgeback Therapeutics (Miami, FL) through an agreement with the Emory Institute for Drug Development (Atlanta, GA). All compounds arrived with certificates of analysis. Purities were determined by HPLC-UV and ¹H NMR; purities of NHC and NHC-IS were 99.7% and 96.1%, respectively; purities of NHCtp and NHCtp-IS were 97.2% and 90.2%, respectively. Chemical structures for NHC and NHCtp are shown in Fig. 1. Drug-free human K₂EDTA plasma and isolated human PBMC at a final concentration of approximately 100 × 10⁶ cells per donor were acquired from BioIVT (Hicksville, NY). PBMC were lysed in 70% methanol to achieve a final lysate concentration of 2 × 10⁶ cells/mL.

Optima™ LC/MS-grade water, methanol, acetonitrile, water with 0.1% formic acid, acetic acid, and reagent-grade 14.8 M ammonium hydroxide were acquired from Fisher Scientific (Fair Lawn, NJ). Reagent-grade formic acid and 10 M ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO).

### 2.2. Preparation of standards and quality controls

For the quantification of NHC in K₂EDTA plasma, standards were prepared at final concentrations of 1, 5, 25, 100, 500, 1000, 2500, and 5000 ng/mL. QCs in K₂EDTA plasma were prepared at the following levels: 1 (lower limit of quantification, LLOQ), 3 (Low), 250 (Mid), 4000 (High), and 15000 ng/mL (Dilution QC). The NHC-IS used was prepared at a final stock concentration of 50 ng/mL.

For the quantification of NHCtp in PBMC lysate, calibrator and QC stocks were prepared in 70:30 acetonitrile:water. Calibration standards were prepared by spiking blank PBMC lysate at 2x10⁵ cells/mL with appropriate volumes of working stock solutions. Calibrator and QC concentrations were assigned as pmol/sample, to reflect the volume of sample (0.1 mL) analyzed for intracellular anabolite quantification. Final NHCtp concentrations of calibration standards in PBMC lysate were 1, 5, 25, 100, 500, 1000, and 1500 pmol/sample. NHCtp QC materials in PBMC lysate were prepared at 1, 3, 250, 1200, and 6000 pmol/sample, and assigned at LLOQ, low, mid, high and dilution QC levels, respectively. NHCtp-IS was dissolved separately in 70:30 acetonitrile:water for a final concentration of 2000 pmol/mL.

### 2.3. Sample preparation

For quantification of NHC in human plasma, sample preparation was carried out via protein precipitation using a 96-well Captiva 0.45 µm protein precipitation filtration plate (Agilent Technologies, Santa Clara, CA). Briefly, 50 µL of sample was pipetted into the Captiva plate wells, followed by 50 µL of NHC-IS solution. Next, 0.5 mL of acetonitrile was added to each well and samples were incubated for 5 min. Samples were eluted via vacuum filtration, evaporated to dryness, and reconstituted in 0.6 mL of 0.1% formic acid.

For quantification of NHCtp in PBMC lysate, 100 µL of sample was pipetted into a 96-well collection plate (Waters Corporation, Milford, MA), followed by 25 µL of NHCtp-IS. Samples were evaporated to dryness under nitrogen, and reconstituted in 100 µL water.

![Fig. 1. Structures and molecular weights of (A) NHC (C₉H₁₅N₃O₅) and (B) NHCtp (C₁₀H₁₆N₃O₅P₃). The asterisk (*) denotes position of isotopically labeled atoms for internal standards.](image-url)
2.4. Analyte separation and detection

For NHC quantification in human plasma, 10 µL of reconstituted material was introduced into a Waters Acquity UPLC system interfaced with an API 5000 mass analyzer with an electrospray ionization (ESI) source (SCIEX, Redwood City, CA). Chromatographic separation occurred using a Zorbax Eclipse Plus C18, narrow bore, 2.1 × 50 mm UPLC column with a 3.5 µm particle size (Agilent Technologies) with a mobile phase system of 0.1% formic acid in water (mobile phase A; MPA) and 0.1% formic acid in acetonitrile (mobile phase B; MPB). NHC was separated via a gradient elution at a flow rate of 0.4 mL/min. The full LC gradient is provided in Supplemental Table 1. NHC was monitored in positive ionization and selective reaction monitoring (SRM) modes. Ion transitions monitored for NHC and NHC-IS were 260.200 → 128.000 m/z and 265.300 → 128.100 m/z, respectively. The entire run time of the assay was 2.50 min; NHC eluted at 0.63 min.

For quantification of NHCtp in PBMC, 20 µL of reconstituted material was introduced into a Shimadzu Nexera X2 HPLC system (Shimadzu Corporation, Kyoto, Japan) interfaced with a QTRAP 6500 mass analyzer with an ESI source (SCIEX). Chromatographic separation occurred using a Scherzo SM-C18, 3 × 50 mm column with a 3.0 µm particle size (Imtakt USA, Portland, OR) with a mobile phase system of 50 mM ammonium formate: 5 mM ammonium hydroxide (MPA) and 80 mM ammonium formate: 8 mM ammonium hydroxide in [80:20] water: acetonitrile (MPB). Analytes were eluted under a gradient; the assay was operated at a flow rate of 0.75 mL/min. The full LC gradient is provided in Supplemental Table 1. NHCtp was monitored in multiplexed SRM and negative ionization modes. NHCtp and its internal standard were monitored at the following transitions: NHCtp: 497.600 → 502.800 and 265.300 → 260.200 m/z, respectively. The entire run time of the assay was 3.00 min; NHCtp eluted at 0.33 min.

2.5. Data analysis

Data were acquired using Analyst® 1.7.1 (SCIEX). Calculations for validation assessment, which included precision, accuracy, stability, and matrix effects, were performed using Microsoft Office Excel 2016. Outliers were defined using the Grubb’s Outlier Test.

2.6. Method validation

The LC-MS/MS assays for the quantification of NHC in human plasma and NHCtp in PBMC lysate were validated in accordance with the United States Food and Drug Administration (FDA) Guidance for Industry, Bioanalytical Method Validation recommendations [14]. Intra- and inter-assay precision and accuracy, linearity, dilutional integrity, selectivity, stability, matrix effects, and carryover were all evaluated.

2.6.1. Precision and accuracy

Precision and accuracy studies were conducted by evaluating QC materials prepared at the LLOQ, low, mid and high QC concentrations. Replicates of 6 at each level were evaluated to assess intra-assay precision and accuracy; inter-assay precision and accuracy were evaluated over 3 (NHCtp in PBMC) or 4 (NHC in human plasma) independent runs. Observed means, standard deviations (SDs) and coefficients of variation (%CV) were calculated for all QC levels. Precision was determined as the %CV of the peak area ratio of NHC or NHCtp to their respective internal standards. Accuracy was characterized by the percent deviation (%DEV), which is a measure of the difference between mean observed and theoretical QC concentrations divided by the theoretical concentration and multiplied by 100.

2.6.2. Linearity, dilution Integrity, and carryover assessment

Calibration standards were analyzed at the beginning and end of each analytical run. Standard curves for NHC (plasma) and NHCtp (PBMC) were calculated using the analyte to internal standard peak area ratio and determined using quadratic (plasma) or linear (PBMC) regression with 1/x² weighting. Extended linearity was evaluated through the preparation of dilution QCs at concentrations three times above the upper limit of quantification (ULOQ) for NHC in plasma (15000 ng/mL) and at four times the ULOQ for NHCtp in PBMC (6000 pmol/sample). For the plasma assay, the dilution QC was diluted up to 500-fold with blank K2EDTA human plasma. For the PBMC assay, the dilution QC was diluted up to 50-fold with 70:30 methanol: water. Additionally, two- and five-fold dilutions of mid and high QCs were prepared for both matrices. Precision and accuracy were assessed by setting theoretical values at calculated diluted concentrations. Carryover was determined by analyzing four consecutive samples at the ULOQ and evaluating post-injection blanks to ensure that the observed peak areas observed was < 20% of the LLOQ.

2.6.3. Stability challenges

Sample matrix stability was assessed by incubating QC-spiked human plasma and PBMC lysate at room temperature for 24 h and 72 h (plasma) or 77 h (PBMC lysate) prior to extraction and analysis. For the plasma assay, an independent set of QCs spiked in whole blood were maintained at room temperature for 2, 4, and 24 h prior to processing and storage at ≤ -70 °C. An additional set was also maintained on ice for 4 h. Plasma from treated samples was compared to those of whole blood samples which were immediately processed post-spiking. For all stability challenges, acceptability was determined as %DEV ≤ 15% ((treated – untreated) / untreated)*100 or as %DEV ((observed – theoretical/theoretical)*100).

Post-preparative stability was assessed by storing extracted low, mid, and high QC samples (n = 6) in 0.1 % formic acid (plasma) or water (PBMC lysate) at 2–8 °C for 72 h (plasma) and 137 h (PBMC lysate) and then injecting with a fresh calibration curve. Re-injection stability was assessed by preparing extracted QC samples in 0.1 % formic acid (plasma) or water (PBMC lysate) at the low, mid, and high concentrations (n = 6). They were injected immediately and then left at 10 °C for 72 h (plasma) or 15 °C for 52 h (PBMC lysate) and re-injected.

Freeze-thaw stability studies were performed using QC samples in replicates of four (plasma) or six (PBMC lysate) that were frozen and thawed, for approximately 2 h (plasma) or 6 h (PBMC lysate), through three cycles. Stability was evaluated by comparing observed values to freshly thawed and analyzed samples. Long-term stability was assessed by analyzing QC materials stored at ≤ -70 °C for up to 260 days (plasma) or 148 days (PBMC lysate) off of a freshly prepared standard curve and was measured as %DEV.

2.6.4. Selectivity

Selectivity was performed by analyzing 6 independent lots of drug-free human plasma or PBMC. Samples were prepared as described above. Chromatographic review was performed to monitor for potentially interfering substances at the expected retention times and ion transitions. For NHC and NHCtp, blank sample peak areas were required to be less than 20% of the LLOQ. For internal standards, the blank sample peaks areas were required to be less than 5% of the IS response.

2.6.5. Matrix effects and extraction efficiency

For human plasma and PBMC lysates, the effects of ion suppression or enhancement were assessed for NHC and NHC-IS or NHCtp, and NHCtp-IS, respectively, following experiments previously described by Matuszewski and colleagues [15]. Un-extracted materials were prepared at low, mid, and high QC concentrations in the absence of sample matrices. Post-extracted materials were prepared by spiking post-extracted matrix samples with NHC and NHC-IS, or NHCtp and NHCtp-IS, respectively, at low, mid, and high QC concentrations. For the human plasma assay, pre-extracted sets were analyzed following the previously described sample preparation conditions. For the PBMC lysate assay, the Matuszewski approach could not be used to measure...
recovery or process efficiency, as the procedure utilizes direct analysis in lieu of an extraction method. Therefore, recovery was defined as the average of the inter-assay mean concentrations divided by the theoretical concentration times 100 across the analytical measuring range of the assay. Pre- (plasma) and post-extracted (plasma and PBMC lysate) sets were evaluated using independent lots of human plasma (n = 6) or PBMC lysate (n = 6). Peak areas for analytes and internal standards were used to determine overall matrix effects, extraction efficiency, and processing efficiency.

2.6.6. PBMC concentration

Cell concentration experiments were performed to determine the effect of PBMC concentration on NHCtp recovery. PBMC lysates were prepared at 0.2, 2, 10, and \(2 \times 10^6\) cells/mL. The \(2 \times 10^6\) PBMC lysate condition was assigned the control as this is the concentration at which calibrators and QCs were prepared. Effect of cell concentration on NHCtp was measured as %DIF from control.

3. Results

3.1. Method development

The analytes monitored in the described assays are similar in terms of their mass to charge ratios as well as their structural characteristics. Therefore, chromatographic and mass spectrometric conditions were assessed to optimize for separation, detection, and quantification. For analytes in PBMC lysate, several C18 and HILIC columns were tested without success prior to evaluation of the Scherzo SM-C18 column. Mobile phases evaluated varied in base concentrations; optimal separation and ionization was observed by using 50 mM ammonium formate: 5 mM ammonium hydroxide (MPA) and 80 mM ammonium formate: 8 mM ammonium hydroxide in [80:20] water: acetonitrile (MPB). This combination, with a gradient elution to 50% MPB, resulted in reproducible separation and elution for each analyte. Representative chromatograms of NHC isolated from plasma and NHCtp isolated from PBMC lysates at the LLOQ, along with their internal standards, are presented in Fig. 2.

3.2. Method validation

3.2.1. Precision and accuracy

Intra- and inter-assay precision and accuracy of plasma NHC and PBMC NHCtp were assessed at LLOQ, low, mid, and high QC concentrations. Across the analytical measuring range of the assay, intra-assay precision and accuracy ranged from 1.73 to 5.71% and –6.27 to –3.23% for plasma NHC and 2.72 to 7.71% and –8.83 to 8.69% for PBMC NHCtp, respectively. Across three (PBMC lysate) or four (plasma) independent runs, inter-assay precision and accuracy ranged from 3.43 to 6.40% and –6.37 to –5.22% for plasma NHC and 1.94 to 11.8% and –11.2 to 8.77% for PBMC NHCtp, respectively. Precision and accuracy results were within FDA bioanalytical recommended thresholds; a summary of intra- and inter-assay precision and accuracy is provided in Table 1.

3.2.2. Linearity, Dilution, and carryover analysis

Calibration curves were generated using weighted \(1/x^2\) quadratic (plasma) or linear (PBMC lysate) regression of analyte-to-internal peak area ratios. The analytical measuring ranges of the assays were 1–5000 ng/mL and 1–1500 pmol/sample for plasma NHC and PBMC NHCtp, respectively. Representative calibration curves for NHC in human plasma and PBMC NHCtp are shown in Fig. 2.
plasma and NHCtp in PBMC lysate are illustrated in Supplemental Fig. 1.

Dilution studies were performed both within and above the analytical measuring range of the assays to characterize the ability to analyze samples that are volume-limited or that exceed each assay’s ULOQ. For partial volume analyses, %CVs and %DEVs ranged from 2.12 to 4.05% and ≤±5.11% for plasma NHC and ≤±4.15% and ≤±5.60% for PBMC NHCtp. Minimal carryover was observed for all analytes across both specimen sources; following four ULOQ injections, peak areas were < 20% of the LLOQ signal, indicating negligible carryover within the analytical measuring range of the assay.

3.2.3. Stability challenges

Analyte stability for both the human plasma and PBMC assays was assessed under a variety of conditions, including in-matrix, post-preparative, freeze–thaw, and long-term storage. Whole blood stability was also performed for the plasma assay. Stability was assessed by comparing treated and freshly prepared or thawed QC samples.

In human plasma, NHC demonstrated acceptable room temperature stability for 24 h (%DIF: ≤±12.4%), but a %DIF was observed ranging from −7.95 to 19.9% at 48 h, exceeding acceptability thresholds. NHC was also unstable in whole blood when stored at room temperature for 2–24 h (%DIF: −70.1 to 1.12%); however, NHC is stable in whole blood when stored on ice for up to 4 h (%DIF: −2.58 to −0.88%). NHC in plasma was stable for up to 3 freeze/thaw cycles, with a %DIF ranging from −3.58 to 1.27%. Further, NHC was stable in extract matrix (0.1% formic acid) and exhibited re-injection stability at 4–8 °C for up to 72 h, demonstrating %DIF of 0.631 to 1.09% and %DEV of −7.41 to −4.68%, respectively. Lastly, long-term stability assessment of NHC in plasma at ≤−70 °C demonstrated stability for up to 260 days, with %DEV ranging from −8.03% to −6.77%.

NHC in PBMC exhibited acceptable stability under a number of conditions. The anabolite is stable in PBMC lysate at room temperature for up to 77 h, with %DIF ranging from −2.44% to 0.871% (NHCtp). NHCtp is stable for up to 3 freeze/thaw cycles, with %DIF ranging from −5.06 to −0.391%. NHCtp also exhibited stability in extract matrix (water) for up to 137 h when stored between 2 and 8 °C, with %DIF ranging from −4.11 to 3.23% and demonstrated re-injection stability for 52 h, with %DEV ranging from −9.17 to 9.74. Lastly, long-term stability assessments show that NHCtp is stable in PBMC lysate at ≤−70 °C for up to 148 days, with %DEV ranging from −6.21% to 5.79%. A summary of stability studies is included in Table 2.

3.2.4. Selectivity & matrix effects

Assay selectivity was evaluated by analyzing 6 blank lots of plasma and PBMC lysate. Representative chromatograms for NHC and NHCtp at each assay’s LLOQ are overlaid with chromatograms of blank matrix (Fig. 3). Peak areas for all analytes in both specimen types were

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### Table 1

| Control          | Mean (SD) | %CV | %DEV | Mean (SD) | %CV | %DEV |
|------------------|-----------|-----|------|-----------|-----|------|
| LLOQ (1.00 ng/mL)| 0.945 (0.013) | 3.31 | −5.46 | 0.946 (0.016) | 6.40 | −5.44 |
| Low (3.00 ng/mL) | 2.90 (0.156) | 5.71 | −3.23 | 2.83 (0.127) | 4.48 | −5.73 |
| Mid (250 ng/mL) | 234 (3.32) | 1.42 | −6.27 | 237 (8.1) | 3.43 | −5.22 |
| High (4000 ng/mL)| 3754 (64.9) | 1.73 | −6.16 | 3745 (130) | 3.47 | −6.37 |
| NHCtp-Plasma    | 1.09 (0.128) | 7.71 | 5.17 | 1.05 (0.081) | 11.8 | 8.77 |
| Low (3.00 pmol/sample) | 2.66 (0.117) | 3.85 | −8.83 | 2.74 (0.105) | 4.39 | −11.2 |
| Mid (250 pmol/sample) | 246 (6.53) | 3.40 | −1.11 | 247 (8.41) | 2.65 | −1.62 |
| High (1200 pmol/sample) | 1301 (25.2) | 2.72 | 8.69 | 1304 (35.5) | 1.94 | 8.42 |

### Table 2

| Freeze-Thaw Stability | Control Mean (%CV) | Challenged Mean (%CV) | %DIF | Sample Matrix Stability (RT)** | Control Mean (%CV) | Challenged Mean (%CV) | %DIF | Extract Matrix Stability† | Control Mean (%CV) | Challenged Mean (%CV) | %DIF |
|-----------------------|--------------------|-----------------------|------|---------------------------------|--------------------|-----------------------|------|---------------------------|--------------------|-----------------------|------|
|                        | NHC-Plasma         |                       |      |                                 |                    |                       |      |                           |                    |                       |      |
| Low (3.00 ng/mL)      | 2.97 (1.60)        | 2.86 (1.62)           | −3.58| 2.97 (1.60)                     | 2.60 (1.63)        | −12.4                 | 2.89 (3.03) | 2.92 (6.22) | 1.09 |
| Mid (250 ng/mL)       | 242 (2.21)         | 245 (4.56)            | 1.27 | 242 (2.21)                      | 216 (2.39)         | −10.7                 | 239 (2.91) | 241 (1.78) | 1.04 |
| High (4000 ng/mL)     | 3556 (3.37)        | 3550 (4.34)           | −0.169| 3556 (3.37)                     | 3446 (1.79)        | −3.09                 | 3805 (4.35) | 3829 (4.46) | 0.631|
| NHCtp-PBMC            |                    |                       |      |                                 |                    |                       |      |                           |                    |                       |      |
| Low (3.00 pmol/sample) | 3.25 (6.59)        | 3.09 (5.87)           | −5.06| 3.25 (6.59)                     | 3.28 (8.89)        | 0.871                 | 3.45 (5.24) | 3.31 (2.82) | −4.11|
| Mid (250 pmol/sample) | 243 (2.54)         | 242 (1.45)            | −0.391| 243 (2.54)                      | 239 (1.72)         | −1.64                 | 260 (3.91) | 252 (7.13) | −3.13|
| High (1200 pmol/sample)| 1141 (2.50)        | 1097 (2.22)           | −3.78| 1141 (2.50)                     | 1113 (1.66)        | −2.44                 | 1155 (2.83) | 1192 (2.50) | 3.23|

a Replicates of 6 from a single analytical run.
b Replicates of 4 (plasma NHC) or 6 (PBMC NHCtp).
c Replicates of 6 for all analytes.

data represent three (PBMC NHC, NHCtp) or four (plasma NHC) independent runs.
sample-specific matrix effect. Table 3 summarizes plasma NHC and PBMC NHCP matrix effects, recovery efficiency, and processing efficiency, as applicable.

### 3.2.5. Cell concentration

PBMC concentrations of 0.2, 2, 10, and $2 \times 10^6$ cells/mL were spiked with NHCP for recovery comparison. %DIF from the control ($2 \times 10^6$ cells/mL) ranged from $-2.98$ to $10.7\%$ (data not shown). Additionally, the $2 \times 10^6$ cells/mL condition produced chromatographic peak broadening (Supplemental Fig. 2).

### 4. Discussion

In this report, we detail the development and validation of novel bioanalytical assays for the quantification of the antiviral NHC and its intracellular bioactive anabolite. LC-MS/MS assays for the quantification of NHC in human plasma and intracellular NHCP in PBMC lysate were validated in accordance with FDA bioanalytical guidelines; assays met all analytical acceptance characteristics for use in clinical trials. Of particular note, the methods utilize low sample volumes, have efficient specimen preparation workflows, and have short run times. Additionally, we also validated the measurement of NHC in PBMC (data not shown). However, as NHC may freely diffuse from PBMC during cellular isolation and processing, while intracellular NHC is monitored, it is not reported. Two distinct methods were used for the quantification of NHC in human plasma and NHCP in PBMC. This was attributed to an inability to achieve reproducible separation, elution, and quantification of NHCP using the mobile phase system and analytical column employed for NHC in plasma. In order to achieve optimal NHCP separation and elution, the Scherzo SM-C18 octodecyl silica-based column with anion and cation ligands was required; further, separation was achieved using a mobile phase system at a pH of 8.6. While this system would be amenable for NHC quantification, we elected to use a more commonly available reversed phase column and operate in positive ionization mode for NHC measurements in human plasma.

NHC is administered as the pro-drug molnupiravir and is currently in phase 2/3 clinical trials for the treatment of patients with symptomatic COVID-19. Consequently, the availability of methods to measure primary systemic and bioactive forms of molnupiravir, plasma NHC and intracellular NHCP, respectively, will aid in characterizing therapeutic efficacy and drug pharmacokinetics-pharmacodynamics. Based on recent findings from a phase 1, first in-human, dose-escalation study, NHC geometric mean maximum concentrations (Cmax) ranged from 223 ng/mL to 6350 ng/mL in participants treated with a single dose of 50 mg to 1600 mg molnupiravir, respectively [4]. The analytical measuring range of the described plasma NHC assay (1–5000 ng/mL) is sufficient to characterize NHC over the evaluated range of administered doses. Of note, we did not develop a bioanalytical assay for molnupiravir quantification, as it is rapidly converted to NHC in vivo. Based on work by Painter et al., molnupiravir was only quantifiable in participants receiving higher doses of molnupiravir, and was not measurable beyond 1.5 h post-drug administration [4].

### Table 3

Summary of matrix effects, recovery and processing efficiency.

|                  | % Matrix Effects Analyte | % Matrix Effects Internal Standard | % Recovery Efficiency Analyte | % Recovery Efficiency Internal Standard | % Processing Efficiency Analyte | % Processing Efficiency Internal Standard |
|------------------|-------------------------|-----------------------------------|-------------------------------|----------------------------------------|-------------------------------|------------------------------------------|
| **NHC-Plasma**   |                         |                                   |                               |                                        |                               |                                          |
| Low (3.00 ng/mL) | 72.1                    | 74.6                              | 74.2                          | 75.2                                   | 52.1                          | 56.1                                     |
| Mid (250 ng/mL)  | 69.2                    | 70.8                              | 73.4                          | 74.0                                   | 50.8                          | 52.4                                     |
| High (4000 ng/mL)| 71.1                    | 72.2                              | 76.2                          | 73.8                                   | 54.1                          | 53.3                                     |
| **NHCP-PBMC**    |                         |                                   |                               |                                        |                               |                                          |
| Low (3.00 pmol/sample) | 86.3                  | 100                               | N/A                           | N/A                                    | N/A                           | N/A                                      |
| Mid (250 pmol/sample) | 62.7                   | 62.3                              | N/A                           | N/A                                    | N/A                           | N/A                                      |
| High (1200 pmol/sample) | 35.6                  | 36.6                              | N/A                           | N/A                                    | N/A                           | N/A                                      |

* Recovery efficiency and processing efficiency not determined as NHC and NHCP measurements were performed by direct dilution and injection.
While the human plasma NHC assay met all acceptance criteria, we did characterize important pre-analytical considerations required for specimen collection and processing. NHC is unstable in whole blood. Incubation of NHC-spiked whole blood from 2 to 24 h demonstrated a significant negative bias of −21.5% as early as 2 h. However, placement of whole blood on ice mitigated the loss of NHC. NHC also exhibited limited stability in plasma; 24 h plasma stability of NHC when maintained at room temperature was acceptable, but storage at 48 h resulted in a mean negative bias of −19.2% across the measuring range of the assay. These important pre-analytical observations should be considered during sample collection, and specimen processing should be expedited in order to ensure accurate results.

The LC-MS/MS assay developed and validated for NHChp quantitation in PBMC lysates mitigated the need for enzymatic dephosphorylation of negatively charged NHChp. This is distinct from other antiviral anabolite methods, which enzymatically convert the anabolite to the parent molecule for quantification and require significant sample manipulation [16]. NHChp is eluted quickly; this is likely attributed to the highly polar nature of the compound; while additional efforts were made to increase anabolite retention on the column, the validated method provided the required responses for reproducible quantification. In this method, direct anabolite separation and mass spectrometric detection was achieved via the use of a basic mobile phase system at pH 8.6, and ion monitoring in negative ionization mode. The basic pH used in the mobile phase system limits the number of samples and injections that can be analyzed on a column. During the validation of the assay, we observed that each column accommodated approximately 600 injections. This may be a consideration for studies in which a large volume of laboratory testing is required.

Calibration standards and QCs for NHChp PBMC assay were assigned as pmol/sample. As the method is designed to quantify intracellular concentrations and the number of isolated PBMC may vary both within and between individuals, results should be normalized to cell count for appropriate interpretation. We observed chromatographic changes in NHC and NHChp, including peak broadening at cell concentrations exceeding 1 × 10^6 cells/mL (Supplemental Fig. 2). This may be attributed to the one-step sample preparation and an increased density of cellular material introduced into the column. Cell counts should be taken into consideration during sample analysis. To date, there are no published reports of NHChp from human PBMC; however in vitro studies show efficient intracellular conversion of NHC to NHChp in several cell lines [17]. In HepG2 cells exposed to 20 μM NHC, peak NHChp concentrations were observed 6 h post-treatment, with maximum concentrations of 733 pmol/10^6 cells. These data suggest that our intracellular NHChp assay is sufficiently sensitive to measure NHChp in human PBMC [17].

While we report the development and validation of sensitive and reproducible assays for the quantification of NHC in human plasma and NHChp in PBMC, there are limitations. Chiefly, given the novelty of the compound as an investigational COVID-19 treatment, we are unable to describe drug pharmacokinetics in this report. However, the analytical measuring ranges of the assays are appropriate for the molnupiravir doses under evaluation. The PBMC method described is currently being applied to several phase 2/3 clinical trials to evaluate intracellular concentrations in PBMC lysates were evaluated post-lysis in 70% methanol; it is noted that continued metabolism may occur post-collection. In order to ensure accurate NHChp measurements, it is recommended that samples are processed and lysed rapidly (typically within 60 min of collection). Lastly, while anabolite assessments in PBMC may provide additional information on molnupiravir efficacy, the overall relevance of NHChp concentrations in PBMC as opposed to target cells and tissues important in SARS-CoV-2 pathogenesis is not known. There may be a future need to develop and validate NHChp methods in other cellular populations or tissues.

5. Conclusions

We have described sensitive and reproducible bioanalytical assays that facilitate the rapid quantification of NHC in plasma and NHChp in PBMC. We characterized all validation parameters for drug quantification, and detailed important pre-analytical considerations required for accurate NHC and intracellular anabolite measurements. The described assay will be applied to clinical studies to better understand PK-PD relationships of molnupiravir in the treatment of COVID-19.

CRediT authorship contribution statement

Teresa L. Parsons: Conceptualization, Validation, Data curation, Formal analysis, Writing – review & editing. Lindsay A. Kryszak: Validation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. Mark A. Marzinke: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122921.

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