

# LC-MS Approach for Comprehensive Monophosphoramidite Prodrug Monitoring in Human Plasma

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## ABSTRACT

Current research initiatives constitute a multifaceted approach to address the complexities of the pandemic, involving the study of an antiviral drug known as Mono Phosphoramidite pro drug, pharmacological investigations, and advanced diagnostics. Analytical methods for detecting and quantifying antiviral compounds have also evolved over the past decade, with relevance to COVID-19 treatment. This research critically assesses sample pre-treatment and extraction methods, detection and quantification techniques, and methods with preliminary separation steps. The major goal of this study is to contribute scientific solutions against SARS-CoV-2 and mitigate its detrimental effects. The goal is to reliably detect Mono Phosphoramidite pro drug at low concentrations in human plasma, facilitating its quantification in therapeutic drug tracking and pharmacokinetic investigation. The subsequent validation process will assess the method's accuracy, precision, linearity, sensitivity, selectivity, and stability, adhering to international regulatory guidelines for quantitative analysis in clinical and research settings. Successful development and validation of this LC-MS/MS method will significantly contribute to ongoing research and clinical use of mono phosphoramidite pro drug (Remdesivir). It will enable the determination of pharmacokinetic parameters, aid in dose optimization, and support safe and effective patient administration. This robust bioanalytical method will ultimately enhance our understanding of the Mono Phosphoramidite pro drug's pharmacological properties and improve its therapeutic application of infectious diseases.

**Keywords:** Mono Phosphoramidite, bioanalytical, linearity, human plasma

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## INTRODUCTION

Coronavirus Disease 2019 (COVID-19) refers to a global pandemic brought on by the severe to acute respiratory syndrome coronavirus 2 (SARS-CoV-2). By end of 2019, this brand-new coronavirus had disseminated quickly and reached almost every country on the planet. When an infected subject coughs, sneezes or speaks, the discharged droplets are considered as the main mode of transmission. It can also be transferred by contacting contaminated surfaces. From mild to severe symptoms, COVID-19's clinical presentation spans a range of manifestations.<sup>1</sup> Fever, coughing, tiredness, dyspnea, anosmia (loss of smell), and ageusia (loss of taste) are typical symptoms. In most of the chronic infective cases, the illness may turn as pneumonia and acute respiratory distress syndrome (ARDS), which increases the risk of mortality, especially in elderly people and people with pre-existing medical conditions. The global response to this pandemic has been multifaceted and has included widespread diagnostic testing, social distancing measures, mandates for mask usage, temporary lockdowns, and ambitious vaccination campaigns.<sup>2,3</sup> Vaccines have played a pivotal role in

mitigating disease severity and transmission<sup>4</sup>, but treatment options have also been explored to alleviate symptoms and improve patient outcomes.<sup>5</sup> This multifaceted approach underscores the complexity of managing the global health crisis posed by COVID-19. Scientific research and public health strategies continually evolve in pursuit of more effective disease management and prevention.<sup>6</sup> The goal is to develop a detection method that, even at low plasma concentrations, can quantitatively measure Mono Phosphoramidite pro drug for pharmacokinetic studies and therapeutic medication monitoring.<sup>7</sup> After the method has been built, a rigorous validation procedure will be used to assess its performance features.<sup>8</sup> As a part of the validation, Mono Phosphoramidite pro drug's stability in human plasma under various storage conditions, linearity over a wide dynamic range, selectivity to ensure little interference from endogenous substances, and accuracy and precision at various concentration levels will be evaluated for the assay. Mono Phosphoramidite pro drug is currently being explored<sup>9</sup>, and its therapeutic use will be greatly impacted by the development and validation of a sensitive and specific LC-MS/MS method for measuring it in human

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plasma.<sup>10</sup> Overall, this study aims to provide a robust bio analytical method that will enable researchers and clinicians to accurately measure Mono Phosphoramidite pro drug concentrations in human plasma, advancing our understanding of its pharmacological properties<sup>11</sup> and ultimately enhancing its therapeutic application in the treatment of infectious diseases.

## MATERIALS AND METHODS

### Materials

The LCMS method used acetonitrile, water (Milli Q), triethylamine, and methanol (all of LCMS grade) obtained from reputable manufacturers.

### Instruments

The bioanalytical analysis was performed using a combination of various instruments, including HPLC

(Waters Alliance e 2695 with Empower software 2.0), LCMS/MS (QTRAP 5500 triple quadrupole), pH meter (Eutech 2700), weighing balance (Sartorius TE214S), ultrasonicator (Unichrome UCA 701), pump (Isocratic model), centrifuge (Remi R-8C), and cyclo mixer (Remi CM-101). Emedisivir.

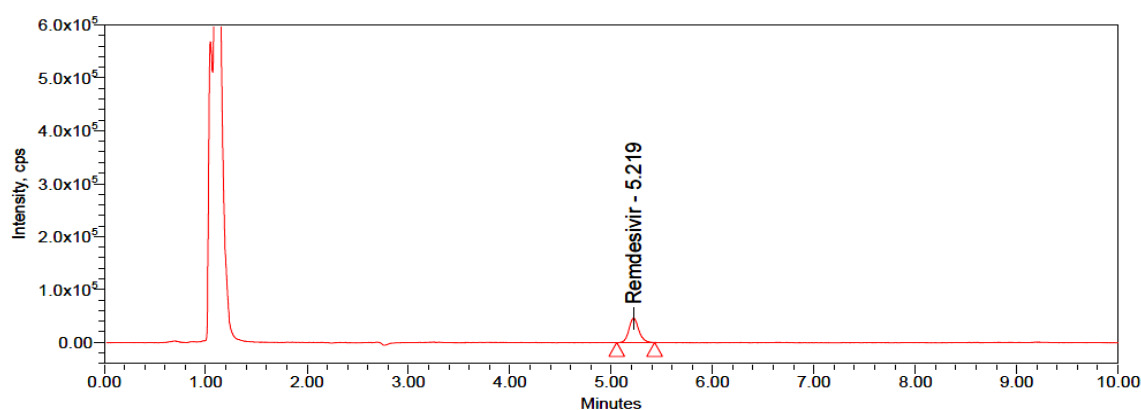
10 milliliters of acetonitrile were used to dissolve 5 milligrams of the medication and the internal standard, D4-Remdesivir, which was then diluted to yield 40ng/ml stock solutions. 200 µl of plasma, 300 µl of acetonitrile, 500 µl of internal standard, and various drug stock solutions (Table 1) were combined to create spiked plasma samples. The mixture was vortexed for 10 minutes, after which methanol was added to precipitate proteins. Following a further five minutes of vortexing and ten minutes of centrifugation, LC-MS/MS was used to examine the supernatant.

**Table 1: Mono Phosphoramidite pro drug working solution for stock prepared for a standard curve**

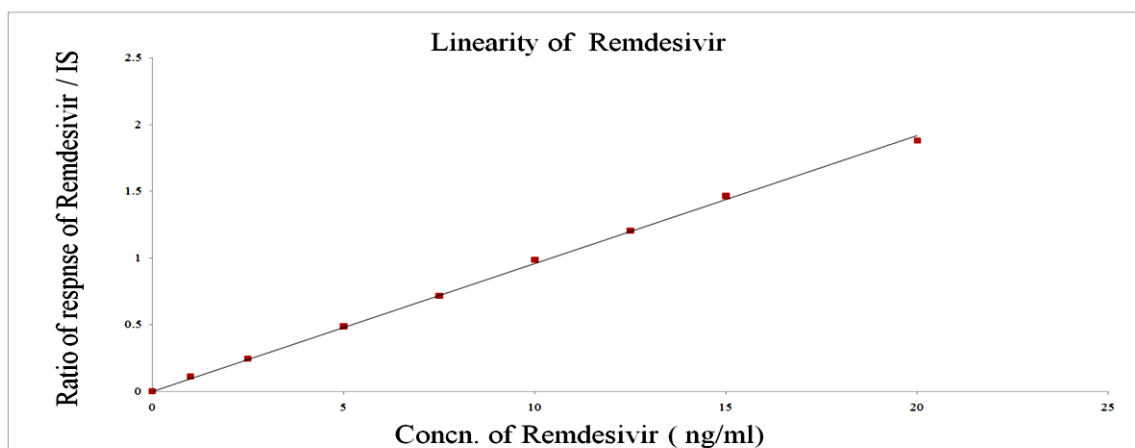
| Linearity      | Plasma (µl) | ACN (µl) | Std Stock (µl) | IS (µl) | MP added (µl) | Analyte (ng/ml) | Entrect response | Area res ratio |
|----------------|-------------|----------|----------------|---------|---------------|-----------------|------------------|----------------|
| Linearity-1    | 200         | 300      | 50             | 500     | 1450          | 1.00            | 0.388            | 0.111          |
| Linearity-2    | 200         | 300      | 125            | 500     | 1375          | 2.50            | 0.849            | 0.244          |
| Linearity-3    | 200         | 300      | 250            | 500     | 1250          | 5.00            | 1.688            | 0.486          |
| Linearity-4    | 200         | 300      | 375            | 500     | 1125          | 7.50            | 2.463            | 0.714          |
| Linearity-5    | 200         | 300      | 500            | 500     | 1000          | 10.00           | 3.429            | 0.986          |
| Linearity-6    | 200         | 300      | 625            | 500     | 875           | 12.50           | 4.163            | 1.203          |
| Linearity-7    | 200         | 300      | 750            | 500     | 750           | 15.00           | 5.058            | 1.463          |
| Linearity-8    | 200         | 300      | 1000           | 500     | 500           | 20.00           | 6.529            | 1.877          |
| Slope          |             |          |                |         |               | 0.0940          |                  |                |
| Intercept      |             |          |                |         |               | 0.01637         |                  |                |
| R <sup>2</sup> |             |          |                |         |               | 0.99935         |                  |                |

**Table 2: Mono Phosphoramidite Prodrug System Suitability**

| Name of Sample | Area of analyte       | RT of analyte (min) | Area of ISTD          | RT of ISTD (min) | Area Ratio |
|----------------|-----------------------|---------------------|-----------------------|------------------|------------|
| MQC            | 3.428x10 <sup>5</sup> | 5.225               | 3.485x10 <sup>5</sup> | 5.222            | 0.9836     |
| MQC            | 3.462x10 <sup>5</sup> | 5.224               | 3.481x10 <sup>5</sup> | 5.236            | 0.9941     |
| MQC            | 3.479x10 <sup>5</sup> | 5.221               | 3.476x10 <sup>5</sup> | 5.227            | 1.0009     |
| MQC            | 3.466x10 <sup>5</sup> | 5.223               | 3.449x10 <sup>5</sup> | 5.231            | 1.0049     |
| MQC            | 3.458x10 <sup>5</sup> | 5.227               | 3.478x10 <sup>5</sup> | 5.235            | 0.9942     |
| MQC            | 3.487x10 <sup>5</sup> | 5.229               | 3.461x10 <sup>5</sup> | 5.230            | 1.0075     |
| Mean           | 3.463x10 <sup>5</sup> | 5.225               | 3.474x10 <sup>5</sup> | 5.230            | 0.9980     |
| SD             | 0.02045               | 0.00286             | 0.0198                | 0.00519          | 0.00874    |
| %RSD           | 0.59                  | 0.05                | 0.62                  | 0.10             | 0.88       |



**Figure 1: Human plasma spiked with analyte at LLOQ and IS**



**Figure 2:** Calibration plot for concentration v/s Area ratio of mono phosphoramidite pro drug  
*Sensitivity*

**Table 3: Sensitivity Results**

| Replicate Number | LLOQ                               |
|------------------|------------------------------------|
|                  | Nominal Concentration(ng/ml)       |
|                  | 1.154                              |
|                  | Nominal Concentration Range(ng/ml) |
|                  | (1.023-1.241)                      |
|                  | Area of Analyte                    |
| 1                | 0.342x105                          |
| 2                | 0.336x105                          |
| 3                | 0.357x105                          |
| 4                | 0.312x105                          |
| 5                | 0.328x105                          |
| 6                | 0.364x105                          |
| N                | 6                                  |
| Mean             | 0.340x105                          |
| SD               | 0.01904                            |
| %RSD             | 5.6                                |
| % Mean Accuracy  | 98.18%                             |

**Table 4:** Precision and accuracy obtained for mono phosphoramidite pro drug (n= 6)

| Sample    | Spiked concentration<br>(ng/ml) | Mean<br>(ng/ml) | SD     | Accuracy (%) | (%) RSD |
|-----------|---------------------------------|-----------------|--------|--------------|---------|
| Intra-day |                                 |                 |        |              |         |
| LLOQ      | 0.3865x105                      | 0.3841 x105     | 0.0157 | 96.32        | 0.82    |
| LQC       | 1.6724x105                      | 1.6711x105      | 0.0326 | 98.28        | 0.16    |
| MQC       | 3.4625x105                      | 3.4628x105      | 0.0458 | 100.05       | 0.33    |
| HQC       | 5.0637x105                      | 5.0664x105      | 0.0269 | 99.89        | 0.08    |
| Inter-day |                                 |                 |        |              |         |
| LLOQ      | 0.3851 x105                     | 0.3836 x105     | 0.0126 | 95.63        | 0.76    |
| LQC       | 1.6739 x105                     | 1.6759 x105     | 0.0364 | 97.46        | 0.27    |
| MQC       | 3.4696 x105                     | 3.4665 x105     | 0.0428 | 99.58        | 0.26    |
| HQC       | 5.0619 x105                     | 5.0643 x105     | 0.0238 | 97.42        | 0.14    |

**Table 5:** Recovery of mono phosphoramidite pro drug

| Replicate Number | HQC       |              | MQC       |              | LQC       |              |
|------------------|-----------|--------------|-----------|--------------|-----------|--------------|
|                  | Extracted | Un Extracted | Extracted | Un Extracted | Extracted | Un Extracted |
| Mean             | 5.059x105 | 5.631x105    | 3.343x105 | 3.846x105    | 1.645x105 | 2.142x105    |
| SD               | 0.00753   | 0.00971      | 0.02156   | 0.01620      | 0.02532   | 0.01317      |

|                  |        |         |        |         |        |         |
|------------------|--------|---------|--------|---------|--------|---------|
| %RSD             | 0.15   | 0.17    | 0.63   | 0.42    | 1.54   | 0.61    |
| %Mean Recovery   | 96.72  | 101.22% | 95.87% | 101.47% | 94.35% | 100.01% |
| Overall Recovery | 98.27% |         |        |         |        |         |
| Overall SD       | 3.0167 |         |        |         |        |         |
| Overall %RSD     | 3.07   |         |        |         |        |         |

**Table 6: Matrix effect**

| S.No.           | Plasma Lot No. | HQC                                | LQC           |
|-----------------|----------------|------------------------------------|---------------|
|                 |                | Nominal Concentration(ng/ml)       |               |
|                 |                | 15.341                             | 5.369         |
|                 |                | Nominal Concentration Range(ng/ml) |               |
|                 |                | (15.269-15.517)                    | (5.206-5.578) |
|                 |                | Calculated Concentration(ng/ml)    |               |
| 1.              | Lot 1          | 5.056x105                          | 1.683x105     |
|                 |                | 5.047x105                          | 1.657x105     |
|                 |                | 5.052x105                          | 1.658x105     |
| 2.              | Lot 2          | 5.055x105                          | 1.625x105     |
|                 |                | 5.026x105                          | 1.556x105     |
|                 |                | 5.047x105                          | 1.586x105     |
| 3.              | Lot 3          | 5.033x105                          | 1.574x105     |
|                 |                | 5.029x105                          | 1.536x105     |
|                 |                | 5.026x105                          | 1.527x105     |
| 4.              | Lot 4          | 5.038x105                          | 1.529x105     |
|                 |                | 5.047x105                          | 1.533x105     |
|                 |                | 5.022x105                          | 1.547x105     |
| 5.              | Lot 5          | 5.057 x105                         | 1.549x105     |
|                 |                | 5.053 x105                         | 1.558x105     |
|                 |                | 5.022 x105                         | 1.542x105     |
| 6.              | Lot 6          | 5.036x105                          | 1.531x105     |
|                 |                | 5.018x105                          | 1.574x105     |
|                 |                | 5.045x105                          | 1.529x105     |
| n               |                | 18                                 | 18            |
| Mean            |                | 5.039x105                          | 1.572x105     |
| SD              |                | 0.01320                            | 0.05011       |
| %CV             |                | 0.26                               | 3.19          |
| % Mean Accuracy |                | 97.01%                             | 90.81%        |

**Table 7: Stability studies of mono phosphoramidite pro drug**

| Stability levels   | Concentration level | Mean       | SD      | %CV  | % Mean Stability |
|--------------------|---------------------|------------|---------|------|------------------|
| <b>Autosampler</b> | HQC                 | 5.056 x105 | 0.02122 | 0.42 | 97.3             |
|                    | MQC                 | 3.458 x105 | 0.02709 | 0.78 | 99.9             |
|                    | LQC                 | 1.649 x105 | 0.02307 | 1.4  | 95.23            |
| <b>Wet Extract</b> | HQC                 | 5.046x105  | 0.01725 | 0.34 | 97.14            |
|                    | MQC                 | 1.655x105  | 0.02205 | 1.33 | 95.58            |
|                    | LQC                 | 3.462x105  | 0.02034 | 0.59 | 99.97            |
| <b>Freeze-thaw</b> | HQC                 | 5.018x105  | 0.01395 | 0.28 | 96.6             |
|                    | MQC                 | 1.632x105  | 0.01136 | 0.7  | 94.25            |
|                    | LQC                 | 3.419x105  | 0.01015 | 0.3  | 98.72            |
| <b>Bench Top</b>   | HQC                 | 5.025x105  | 0.01218 | 0.24 | 96.73            |
|                    | MQC                 | 1.64x105   | 0.01098 | 0.67 | 94.71            |
|                    | LQC                 | 3.412x105  | 0.00781 | 0.23 | 98.53            |

|                    |     |           |         |      |       |
|--------------------|-----|-----------|---------|------|-------|
| <b>Dry Extract</b> | HQC | 5.037x105 | 0.01401 | 0.28 | 96.96 |
|                    | MQC | 1.631x105 | 0.01236 | 0.76 | 94.19 |
|                    | LQC | 3.390x105 | 0.00578 | 0.71 | 97.89 |
| <b>Short term</b>  | HQC | 4.908x105 | 0.00662 | 0.13 | 94.48 |
|                    | MQC | 1.562x105 | 0.02127 | 1.36 | 90.21 |
|                    | LQC | 3.307x105 | 0.00423 | 0.13 | 95.5  |
| <b>Long term</b>   | HQC | 4.629x105 | 0.00672 | 0.15 | 89.11 |
|                    | MQC | 1.488x105 | 0.00504 | 0.34 | 85.93 |
|                    | LQC | 3.110x105 | 0.00701 | 0.23 | 89.8  |

### Conditions of chromatography

The **establishment** of the appropriate chromatographic **circumstances** involved several **experiments** using different buffers and mobile phases.<sup>12</sup> The optimized mobile phase was a mixture of 0.1% TEA and acetonitrile measuring in the ratio of 50:50. The chromatographic column used was Inertsil ODS (250mm, 4.6mm, 5 $\mu$ ), which provided suitable separation and peak resolution for mono phosphoramidite pro drug and the internal standard (IS).

### Sample Extraction

The liquid-liquid extraction technique was employed for extracting the plasma samples. Mono Phosphoramidite prodrug 10ng/ml and IS was 10ng/ml were extracted from 200  $\mu$ l of plasma using 300  $\mu$ l of acetonitrile then 500  $\mu$ l of diluent was added, vortexed for 5 minutes then followed by centrifugation for 20 min under 5000rpm. The extracted supernatant was then analyzed by injecting it into the HPLC.

### Method Validation

The system's suitability was also checked to ensure the instrument's proper functioning.<sup>13</sup> The QC samples at different concentrations (LQC, MQC, HQC) was utilized to determine on varied days.<sup>14</sup> Using LCMS, the developed bio-analytical method for Mono phosphoramidite prodrug in human plasma was evaluated for specificity, selectivity, linearity, sensitivity, precision, accuracy, recovery, and matrix effect.<sup>15</sup> The stability of the drug in plasma was evaluated under various conditions, including freeze-thaw, bench-top, wet extract, and auto-sampler stability. The procedure was verified in compliance with regulatory requirements, making it suitable for pharmacokinetic and pharmacodynamics studies of drug in spiked human plasma.<sup>16</sup>

## RESULTS AND DISCUSSION

### Method Validation

#### Specificity and Selectivity

The specificity of the bioanalytical method was evaluated by analyzing blank human plasma and plasma spiked with Mono Phosphoramidite pro drug and the IS. The chromatograms obtained from the analysis showed that there were no interfering peaks at the retention times of the Mono Phosphoramidite pro drug and the IS, indicating excellent specificity (Figure 1). This observation suggests that the developed method can accurately detect and

quantify Mono Phosphoramidite prodrug in the presence of endogenous **materials** in human plasma.

#### System suitability

Six sequential shots of the analyte as well as an internal standard conjunction in the MQC, (Table 2) concentrations were applied to the sample during processing. During the technique validation, the system's suitability was assessed every day. Analyte and the ISTD area ratio were shown to have %RSDs of 0.59 and 0.88%, respectively. hence it passed the system suitability. The findings are outlined in Table 2.

#### Linearity and Calibration Curve

To assess the linearity of the method, calibration standards of mono phosphoramidite pro drug at different strengths (from 1 ng/ml to 20 ng/ml) were prepared and analyzed using LC-MS. To create the calibration curve, peak area proportions of mono phosphoramidite pro drug to the IS against the aligned concentrations. The calibration curve displayed good linearity (Figure 2) over the concentration range tested, with a correlation coefficient ( $r^2$ ) of 0.99. This indicates that the method can accurately quantify mono phosphoramidite pro drug over a wide dynamic range.

#### Sensitivity and Lower Limit of Quantification (LLOQ)

It has been determined by assessing the limit of quantification. The LOQ, characterized as the least amount of mono phosphoramidite pro drug that can be quantified with acceptable accuracy and precision, was found to be 1 ng/ml. This demonstrates the method's ability to detect and quantify analytes at low concentrations in human plasma, making it suitable for pharmacokinetic studies and efficacy evaluations. The %RSD for mono phosphoramidite pro drug was found to be 5.6%. Hence it passed the sensitivity Table 3.

#### Precision and Accuracy

The assessment of both intra-day and inter-day precision was employed to evaluate the precision of the bioanalytical method. Six replicates of the drug at three dissimilar concentration levels (low, medium, and high) were examined during same day in order to test for intra-day precision. Inter-day precision was assessed by examining under same concentration levels over three consecutive days. The results demonstrated that the relative standard deviation (RSD%) for both intra-day and inter-day precision was within acceptable limits, Table 4 indicating good repeatability and reproducibility of the method.

Additionally, the accuracy of the method, expressed as percentage relative error (%RE), was within  $\pm 15\%$ , further confirming the reliability of the method for quantifying mono phosphoramidite pro drug in human plasma.

#### *Recovery and matrix effect*

By comparing the peak regions generated by extracted plasma samples with those obtained from reference solutions for mono phosphoramidite pro drug with the IS at similar concentrations, the extraction of the mono phosphoramidite pro drug with the IS was evaluated. The method exhibited good recovery rates, with extraction efficiencies close to 90% for both analytes. This indicates that the extraction process effectively recovers analyte from human plasma and enhances the method's sensitivity Table 5. Additionally, the matrix effect, which refers to the potential interference of endogenous substances on the ionization of analytes, was evaluated. The results shown in Table 6 minimal matrix effects on the quantification of mono phosphoramidite pro drug further confirming the method's selectivity.

#### **Stability Studies**

To assess the stability of mono phosphoramidite pro drug in human plasma under various conditions. The analyte's stability was evaluated under benchtop, auto-sampler, short-term, long-term, and freeze-thaw cycles at  $-80^{\circ}\text{C}$ . Wet extract and dry stability **were evaluated by storing spiked quality control samples for eighteen hours at room temperature.** Results indicated that mono phosphoramidite pro drug remained stable in human plasma under these conditions, with no significant degradation observed shown in Table 7. This ensures the reliability of the method during sample handling and storage.

#### *Comparison with Literature Data*

To validate the accuracy of the developed method, the obtained results were compared with literature data, if available. The comparison showed good agreement between the developed method and previously reported methods for mono phosphoramidite pro-drug quantification in plasma. This further supports the reliability of the method and its potential application in preclinical and clinical studies.

#### **CONCLUSION**

The integration of Therapeutic Drug Monitoring (TDM) into the realm of COVID-19 treatment deserves unequivocal recognition and consideration. We have meticulously examined the existing body of evidence about Mono Phosphoramidite prodrugs that have received the coveted approvals of official agencies, such as European Medicines Agency (EMA) and the Food and Drug Administration (FDA). This comprehensive analysis represents a pivotal step toward ensuring the precision and efficacy of COVID-19 treatment regimens. To realize this vision, we propose a strategic framework aimed at enhancing the feasibility and widespread acceptance of TDM in clinical practice, thus elevating the standards of care delivery. Stability studies confirmed the selected prodrug stability in human plasma under various conditions, validating the method's reliability during sample handling and storage. Overall, currently validated

LC-MS/MS method serves as an important tool for accurate quantification of Mono Phosphoramidite pro drug in human plasma, facilitating pharmacokinetic and pharmacodynamics studies, therapeutic drug monitoring, and advancing the effective and safe clinical application of it in treating viral infections.

#### **DECLARATIONS**

##### **AVAILABILITY OF DATA: NA**

**COMPETING INTERESTS:** The authors state unequivocally that no known competitive economic objective or interpersonal relationship could have had any influence on any of the work presented in this study.

##### **AUTHORS CONTRIBUTION**

Mr. Kiran Kumar Ganta, a research scholar made a significant practical contribution to the research and manuscript. I have made intellectual contribution to the research work and manuscript until its completion. Dr. Raja Sundararajan had verified the drafted manuscript, proof reading and edited the manuscript.

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