Validated Stability-Indicating RP-HPLC Method for Daclatasvir in Tablets

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ABSTRACT

Objectives: The current study goal was to create a precise, sensitive, and validated reverse phase-high performance liquid chromatography (RP-HPLC) method for assessing the direct-acting antiviral daclatasvir (DCV) as well as to evaluate the stability of DCV in both drug and tablet formulations. The current investigation was to display stability indicating methods under different stress conditions, including hydrolysis (acidic, basic, and neutral), oxidation, and photolysis.

Materials and Methods: All experiments were performed on HPLC Agilent 1100 with a stainless steel Hypersil C8 column having a particle size of 5 µm and a dimension of 4.6 x 250 mm. The mobile phase was acetonitrile: 0.05% orthophosphoric acid (50:50 v/v) in isocratic mode with 0.7 mL/min flow rate and wavelength 315 nm was selected for detection.

Results: This method was validated for linearity and range, accuracy, precision, limit of detection, limit of quantification, and robustness in accordance with International Council for Harmonisation (ICH) requirements. The results were satisfactory. It was observed that retention time (tR) was 3.760 ± 0.01 min. In acidic conditions, DCV degradants show tR at 3.863, 4.121, and 4.783 min and tandem mass spectrometry (MS/MS) spectra scans having m/z 294, 339, 561.2 fragment ions. In basic condition, DCV degradants show tR at 5.188, 5.469 min and MS/MS spectra scans having m/z 294, 339, 505.2, 527.2 fragment ions. In oxidation conditions, DCV degradants shows tR at 4.038 min and MS/MS spectra scans having m/z 301.1 and 3391 fragment ions were observed.

Conclusion: All the mass fragments exhibited additional degradation observed for different stress conditions. This will help to identify the structure of the degradant and its pathways. No degradation was observed in neutral and photolytic conditions.

Key words: Daclatasvir, RP-HPLC, tablets, validation, stability-indicating method

INTRODUCTION

Millions of people suffer from hepatitis C virus (HCV) infections. Daclatasvir (DCV) permits once-daily oral treatment, has been shown in in vitro experiments to have a very potent antiviral activity against several HCV genotypes.1,2 Since, direct acting antivirals (DAAs) for HCV have become available, any possible medication interactions between antiretrovirals and DAAs must be assessed before co-therapy.3 Cirrhosis and hepatocellular cancer have been linked to chronic HCV in various parts of the world.4 DCV is an effective, new, and non-selective structural protein inhibitor, formerly known as BMS-790052.5 NSSA, the multifunctional protein which is a crucial part of the HCV replication complex, is inhibited by DCV. It inhibits viral RNA replication and virion development. Data from computer models and in vitro investigations point to an interaction between DCV and the protein’s N-terminus in domain 1 that may result in structural alterations that reduce NS5A activity.6–8 Chemically, DCV is methyl ((1S)-1-((2S)-2-[5-(4′-((2S)-1-((2S)-2-(((methoxycarbonyl)amino)-3-methylbutanoyl)-2-pyrrolidinyl)-1H-imidazol-5-yl)-4-biphenyl)yl)-1H-imidazol-2-yl)-1-pyrrolidinyl)carbonyl)-2-methylpropyl) carbamate (Figure 1). The molecular formula of DCV is C79H57N5O5 and the molecular weight is 738.89 g/mol.9 Nimje and Deodhar10,11 developed stability indicating methods for DCV by high-performance
thin-layer chromatography and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Some bioanalytical methods such as ultra-performance liquid chromatography (UPLC)-MS/MS for DCV in human plasma and high performance liquid chromatography (HPLC) with ultraviolet (UV) detector were reported by Rezk et al. and Nannetti et al. respectively. By using UHPLC-MS/MS and LC-MS, Ariaudo et al. and Jiang et al. demonstrated the measurement of several direct antiviral drugs in human blood plasma. All of these techniques are effective for detecting DCV in human blood plasma; however, they are not appropriate for routine assays to detect DCV in medication and tablet dosage forms. Several HPLC methods have been reported for determining DCV single and its combination with other drugs under different conditions according to a review of the literature. As regulatory guidelines, it is essential to ascertain the ability of assay method to quantify the drug component in presence of its degradation products. The mentioned above reported HPLC methods have not ascertained the ability of the assay method to quantify DCV in presence of its degradation products. Hassib et al. demonstrated the reverse phase (RP)-HPLC method for determining DCV and the behavior of its degradation under diverse circumstances. This study used acetonitrile and potassium dihydrogen phosphate buffer as mobile phase, which is driven through column C_{18} at a rate of 2 mL/min. The flow rate in the above reported method is on higher side, which can adversely affect the quality of the chromatography not giving the analyst sufficient time to interact with the stationary phase. Moreover, ability of the method to quantify DCV in presence of its degradation product was not evaluated. The stability indicating HPLC-UV was developed by Zaman and Hassan for characterizing forced degradation products and quantifying DCV. In the above method, the retention time (t_r) of DCV is 16.4 min making it a time consuming method for assay of formulations containing DCV. The method can be preferred for quantification of known impurities in DCV formulations. The preferred t_r in HPLC assay method is 3 to 6 min. Baker et al. presented a stability-indicating HPLC-diode array detector method utilising a Waters C_{18} column with isocratic elution of the mobile phase made up of a 75:25 v/v combination of acetonitrile and phosphate buffer with pH of 2.5. While a novel QDa mass detector for DCV was developed by Jagadabi et al. to determine the mass of possible contaminants, stability-indicating UPLC method was reported. However, the above method is primarily used for estimation of the process-related and known impurities in DCV. Considering limitations of the reported methods such as high flow rate, high t_r and stability indicating property of assay method being not evaluated, it was thought worthwhile to develop and validate stability indicating RP-HPLC method for DCV formulations, which is economical and less time consuming without affecting the quality of the method as per parameters given in International Council for Harmonisation (ICH) guidelines. In this research work, economical solvents and a simple procedure were used for the estimation of DCV by RP-HPLC. To evaluate the stability of DCV in drugs and their drug derivatives, force degradation research was conducted. This information helps to determine the shelf life of DCV under various settings. The quantitative examination of DCV following exposure to various stability-indicating experiments is described in the current piece of work. The approach was validated in accordance with International Council for Harmonisation (ICH) standards, demonstrating accuracy, precision, and reliability. Therefore, this method can be applied to the routine study of DCV stability under various conditions.

MATERIALS AND METHODS

Chemicals and reagents

Mylan Laboratories Ltd. (Hyderabad, India) kindly provided of DCV pure drug sample to us. All HPLC grade solvents were used throughout the analysis including water, methanol, and acetonitrile (Fisher Scientific, India). Analytical grade reagents such as hydrogen peroxide, hydrochloric acid, sodium hydroxide, and α-phosphoric acid were used throughout the analysis. DCV tablets “MyDekaTM60” and “DACLACURE 60” were purchased from a local market drug store. They were manufactured by Mylan Laboratories Limited and Emcure Pharmaceutical Industry, respectively. Each tablet contained 60 mg of DCV.

Instrumentation and chromatographic conditions

HPLC Agilent 1100 with variable detector (G1314A) and (G13104) pump was used for all the studies. The solutions were degassed using a DGU-20A3 prominence degasser. The data was gathered and processed using ChemStation software. The material employed was a stainless steel Hypersil C_{18} that was 4.6 x 250 mm in size, 5 µm particle size, filled with octadecyl silane stationary phase and had ligands bound to silica surface, and column temperature was set at 40 °C. For this research, a humidity chamber from Newtronic and a Rolex ultrasonicator were employed. An examination into photodegradation was conducted using a UV lamp (6 w, Vilber Lourmat, France) and photostability chamber. The light output was 200 Wh/m² and its intensity was 1.2 million lux h. A weighing balance (AX200, Shimadzu Corporation Japan) with minimum 0.1 mg and maximum 200 g capacity was employed throughout the analytical procedure. The isocratic mobile phase was used with acetonitrile and 0.05% α-phosphoric acid (50:50 v/v). Before being used in the study, the mobile phase was filtered through a membrane filter of size 0.45 μm and using an ultrasonic sonicator for degassing the solvents for 10 min. The flow rate of the mobile phase was 0.7 mL/min and the injection had a 10 μL volume. A variable wavelength detector was used to perform a wavelength scan and a wavelength of 315 nm was chosen for investigation.
Preparation of standard stock and tablet solution
The standard stock solution was made by placing a quantity of 10 mg of DCV standard in a 10 mL volumetric flask with mobile phase, shaking well and adding mobile phase to achieve a concentration of 1000 μg/mL. The stock solution was diluted, mixed well, and used to test all DCV validation parameters with required concentrations. After 15 min of sonication, the mixture was filtered through a membrane filter paper of size 0.45 μm. Twenty DCV tablets (MyDekla™ and DACLACURE 60) were weighed and the average weight was calculated. The tablet was triturated in a dry spotless mortar to create a fine powder. A quantity of tablet fine powder containing 10 mg of DCV was accurately taken, dissolved in minimum mobile phase, thoroughly mixed, and stirred for 5 min in 10 mL volumetric flask. To create a volume of 1000 μg/mL concentrated solution, up to 10 mL of mobile phase solvent was added. Sample solutions from the above solution were adequately diluted for future research examination. All the solutions were filtered using the membrane filter paper with a pore size of 0.45 μm.

Validation of proposed method
Linearity and range
Different series of DCV solutions were made from standard stock solutions and diluted with diluent for linearity test. Concentration range of 10.00-50.00 μg/mL was identified and five concentrations (10, 20, 30, 40, and 50 μg/mL) were chosen. In order to evaluate linearity, peak area and peak height were subjected to least squares regression analysis to produce a calibration equation with slope, Y-intercepts, and correlation coefficient (r²).

Accuracy
In order to confirm the method accuracy for quantifying DCV, standard addition and recovery tests were carried out. Recovery studies employ a typical addition procedure to a synthetic mixture created in lab to assess the accuracy of the method in triplicate. Three different concentration levels of known amounts of DCV samples were put to a pure drug with a consistent weight of 10 μg/mL (80, 100, and 120% of label claim) in triplicates. To make up the volume of diluted samples, mobile phase was used. The recovery was calculated using the peak area.

Precision
For determination of DCV, repeatability (intraday) and intermediate precision (interday) of the procedure were used to establish its precision for each six samples. The intraday precision was conducted on the same day with the same sample at certain time. The same sample solution was used on one consecutive day to accomplish the interday precision. To determine the method accuracy, the results were obtained and the relative standard deviation (RSD) % was computed. For accuracy, RSD% of DCV peak areas was measured.

Sensitivity
Limit of detection (LOD) and limit of quantification (LOQ) estimates were used to determine the measurement of DCV sensitivity. DCV was determined at signal-to-noise (S/N) ratios of 3:1 and 10:1 for LOD and LOQ, respectively.2b

Robustness
Robustness analysis was used to examine the impact of small deliberate adjustments on the ideal chromatographic settings created for DCV. A 10% change in flow rate, a 2 nm shift in wavelength, and a 2% change in the composition of mobile phase were among the other modifications. Three samples of DCV (30 μg/mL) were evaluated using it in all the aforementioned circumstances. All system suitability traits and conditional changes were compared under all altered settings.

Stability-indicating analytical method (ICH Q1A)
A crucial step in the process of developing drug medicine is stability testing. The purpose of stability testing is to demonstrate how product quantity fluctuates over time and in different environmental contexts. Recommendations can be made for shelf life period based on factors including temperature, humidity, and light. The assay of the active ingredient and the degradation products produced during stability tests are two key aspects of the drug product that are crucial in determining the shelf life duration. The conditions such as 0.1 N HCl, 0.1 N NaOH, 30% H₂O₂, humidity, hydrolysis, UV light, and temperature were used in the forced degradation investigation of DCV. 20 mL of 0.1 N HCl, 0.1 N NaOH, and water were used to treat 20 mg of DCV, which is the comparable amount. For four hours the solutions were refluxed at 60 °C. H₂O₂ solution (30%) was employed for the oxidation investigation, which was refluxed at 60 °C for 6 h. After filtering the solution appropriate amount of dilution was added. For 10 days, a layer of dry solid DCV powder was placed in direct sunlight to perform photolytic testing. On the platform, a petridish held the medication. For 10 days, visible light at 1.2 million lux h and UV light at 200 Wh/m² were used in degradation experiment. All these conditions were applied continually until significant deterioration was attained. All degradation research samples were gathered and diluted to the proper concentration before analysis. This solution was put into HPLC instrument for testing. The standard untreated drug was compared to DCV assay of stressed samples. The conclusion drawn from the RP-HPLC analysis of all stressed samples is that the suggested analytical strategy can identify all degraded products, demonstrating the methods potential to indicate stability.

RESULTS
Method development of daclatasvir
The aim of this study was to develop and validate RP-HPLC method for the degradant separation using RP-HPLC data to clarify the structure. The chromatographic method was created to look into several important contaminants detected in DCVs. For method development, DCV was injected into HPLC simultaneously with all stress samples. To accomplish the effective separation, different ratios of various solvent combinations were explored. Using a C₁₈ column as the stationary phase and an acetonitrile: 0.05% OPA in water
(50:50 v/v) mobile phase with a flow rate of 0.7 mL/min, a wavelength of 315 nm, and a column temperature of 40 °C with a total run time of 10 min, the best separation of DCV from its related substance was observed. The measured DCV $t_R$ was 3.760 ± 0.01 min. The chromatogram of DCV is depicted in Figure 2.

**Method validation**

The HPLC instrument met the analysis criteria for the system suitability test for DCV chromatogram and is presented in Table 1.

**Linearity and range**

By injecting five different level concentrations of pure DCV ranging from 10 to 50 µg/mL, the analytical method linearity was examined. Plotting peak area versus concentration yielded the slope, $Y$-intercepts, and correlation coefficient of each DCV concentration. Data generated from linearity studies displayed a correlation between peak area and concentration. DCV method was found to be linear (10 to 50 µg/mL) concentration ranges with regression coefficients of 0.9998. DCV linearity curve is depicted in Figure 3.

**Accuracy**

The correctness of an analytical procedure is determined by the agreement between true and experimental values. To ascertain the accuracy, three concentrations (18, 20, and 22 µg/mL) from various ranges of DCV standard curves were chosen. DCV recovery was examined in triplicate for concentrations of the drug sample at 80, 100, and 120%. Recovery experiments were used to examine the effects of excipients, which are frequently used in pharmaceutical formulations of drugs. A good level of quantitative skill was demonstrated by the recovery of DCV in the sample. Table 1 displays the accuracy results.

**Precision**

According to ICH regulations, the devised HPLC technology was evaluated for precision of the study. Intermediate precision (intraday precision) and percent RSD from six replicates of DCV samples were acquired. DCV samples were seen to have intraday precision on the same day. Three separate days were used to investigate DCV for inter-day precision for the repeatability study from the six replicates of DCV. The results are presented in Table 1.
Quantitative aspects

S/N ratios of 3 and 10 were used to assess LOD and LOQ for DCV. LOD of 0.0416 µg/mL and LOQ of 0.1261 µg/mL were obtained after DCV injection, indicating enhanced sensitivity for detection and quantification.

Robustness

All the analyses were sufficiently resolved and order of elution was unaltered, when the chromatographic parameters such as flow rate, mobile phase composition, and wavelength were purposefully adjusted. Changes were made to the flow rate (0.2 mL/min), mobile phase composition (10%), and wavelength (2 nm). \( t_r \), observed peak area, and RSD were reported in accordance with acceptable bounds. Table 2 presents the robustness results.

Degradation behavior of the drug and characterization of degradants

DCV dramatically degrades in alkaline, acidic, and oxidative environments, but is found to be stable in neutral and photolytic situations. Table 3 includes the degradation study observation table for all conditions. According to data from mass spectra and HPLC chromatograms seen in various stress settings, some drug product impurities or derivatives were observed as D1, D2, and D3 under various stress conditions including alkaline, acidic, and oxidative. All of the HPLC chromatograms, mass spectra, and ion fragmentation peaks are shown in Figure 4. To examine if any excipients were discovered, blank samples were firstly put through the same arduous testing as DCV samples. HPLC chromatograms and mass spectra of commercial tablets and blank samples were contrasted. In \([M + H]^+\) ESI positive mode, the peak of DCV mass spectra was discovered to be at \(m/z\) 739.3, which was further broken down into \(m/z\) 370.1, \(m/z\) 513.2, and \(m/z\) 565.2. Considering that those are the \(m/z\) peaks most typical of pure DCV drug.

Acidic condition

For the acidic condition, DCV was refluxed with 0.1 N HCl for 4 h (1 mg/mL). Then, this acidic solution (20 µg/mL) was added to the HPLC instrument while maintaining the same chromatographic conditions. The measured DCV \( t_r \) was 3.762 min. The other peaks named as D1, D2, and D3 were seen at \( t_r \) of 3.863, 4.121, and 4.783 min. The mass spectrometer \([M + H]^+\) ESI mode employed the same sample. The fragmentation of additional peaks, which were not visible in pure DCV, was noticed as \(m/z\) 339.1 and \(m/z\) 561.2 in the mass spectrum shown in Figure 4. This shows that degradant is in acidic medium.

Table 2. Evaluation data for robustness study of daclatasvir

<table>
<thead>
<tr>
<th>Robustness parameters</th>
<th>( t_r ) minute</th>
<th>Peak area*</th>
<th>Assay % ± SD</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate (0.6 mL/min)</td>
<td>3.762</td>
<td>5237.72</td>
<td>100.63 ± 1.0443</td>
<td>1.0377</td>
</tr>
<tr>
<td>Flow rate (0.7 mL/min)</td>
<td>3.788</td>
<td>5257.81</td>
<td>101.05 ± 0.5272</td>
<td>0.5217</td>
</tr>
<tr>
<td>Flow rate (0.8 mL/min)</td>
<td>3.779</td>
<td>5263.78</td>
<td>101.13 ± 1.1720</td>
<td>1.1588</td>
</tr>
<tr>
<td>Mobile phase composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetonitrile: 0.05% o-phosphoric acid (4:9:1 v/v)</td>
<td>3.752</td>
<td>5207.84</td>
<td>100.05 ± 1.1663</td>
<td>1.1657</td>
</tr>
<tr>
<td>Acetonitrile: 0.05% o-phosphoric acid (5:5:0 v/v)</td>
<td>3.788</td>
<td>5238.12</td>
<td>100.63 ± 1.0078</td>
<td>1.0014</td>
</tr>
<tr>
<td>Acetonitrile: 0.05% o-phosphoric acid (5:1:49 v/v)</td>
<td>3.761</td>
<td>5220.25</td>
<td>100.29 ± 1.1329</td>
<td>1.1296</td>
</tr>
<tr>
<td>Wavelength</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wavelength (314 nm)</td>
<td>3.721</td>
<td>5256.88</td>
<td>100.99 ± 0.5778</td>
<td>0.5721</td>
</tr>
<tr>
<td>Wavelength (315 nm)</td>
<td>3.742</td>
<td>5262.27</td>
<td>100.90 ± 0.7735</td>
<td>0.7804</td>
</tr>
<tr>
<td>Wavelength (316 nm)</td>
<td>3.761</td>
<td>5236.80</td>
<td>100.79 ± 0.8431</td>
<td>0.8498</td>
</tr>
</tbody>
</table>

*Results of three replicates, \( t_r \): Retention time, SD: Standard deviation, RSD: Relative standard deviation

Table 3. Results of degradation products for DCV obtained under stress conditions

<table>
<thead>
<tr>
<th>Serial no stress condition</th>
<th>Name of degradation product</th>
<th>Retention time (min)</th>
<th>Major peak of fragmentation pattern (m/z)</th>
<th>Percentage of degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1N HCl</td>
<td>D1, D2, D3</td>
<td>3.863, 4.121, 4.783</td>
<td>339.1, 561.2</td>
<td>72%</td>
</tr>
<tr>
<td>0.1N NaOH</td>
<td>D1, D2</td>
<td>5.188, 5.469</td>
<td>294.1, 339.1, 505.2, 527.2</td>
<td>97%</td>
</tr>
<tr>
<td>(H_2O) (30%)</td>
<td>D3</td>
<td>4.038</td>
<td>301.1, 339.1</td>
<td>28%</td>
</tr>
<tr>
<td>(H_2O)</td>
<td></td>
<td></td>
<td>370, 513, 565, 739</td>
<td>No degradation</td>
</tr>
</tbody>
</table>

DCV: Daclatasvir
Alkaline condition
In the alkaline condition, DCV was refluxed with 0.1 N NaOH for 4 h at 60 °C (1 mg/mL). After that, this alkaline solution was added to the HPLC instrument, while still maintaining the same chromatographic conditions. The measured DCV \( t_r \) was the same as 3.648 min, while other chromatographic peaks were observed at \( t_r \), 5.188 min and 5.469 min, another peak was named as D1 and D2, respectively. The other mass fragmented ions such as \( m/z \) 294.1, \( m/z \) 339.1, \( m/z \) 505.2, and \( m/z \) 527.2 in the mass spectrum \([M + H]^+\), which were not seen in pure DCV, are displayed in Figure 4. The same solution was used in the ESI mode of mass spectrometer.

Oxidation condition
In the oxidation condition, DCV was refluxed with 3% hydrogen peroxide at 60 °C for 4 h (1 mg/mL). From this oxidized solution, a 20 µg/mL solution was made and put into the HPLC device with the same chromatographic settings. The measured DCV \( t_r \) was 3.690 min, while one more additional peak named D1 at \( t_r \), 4.038 min was observed. The mass spectrometer \([M + H]^+\) ESI mode employed for the same sample. The mass spectrum shown in Figure 4 contains degradans fragments with \( m/z \) 301.1 and 339.1, which are absent in pure DCV drug sample.

Figure 4. HPLC chromatograms and total ion MS/MS spectra scans relative to DCV stressed conditions. HPLC chromatogram of DCV exposed to 0.1N HCl, 60 °C for 4 h, degradant \( t_r \) at 3.863, 4.121, 4.783 min, and MS/MS spectra scans having \( m/z \) 339.1, 561.2 fragment ions observed (A, B). HPLC chromatogram of DCV exposed to 0.1 N NaOH, 60 °C for 4 h, degradant \( t_r \) at 5.188, 5.469 min, and MS/MS spectra scans having \( m/z \) 294.1, 339.1, 505.2, and 527.2 fragment ions observed (C, D). HPLC chromatogram of DCV exposed to 30% H\(_2\)O\(_2\), 60 °C for 4 h, degradant \( t_r \) at 4.038 min and MS/MS spectra scans having \( m/z \) 301, 3391 fragment ions observed (E, F). HPLC chromatogram of DCV exposed to water, 60 °C for 8 h (G, H).

HPLC: High performance liquid chromatography, MS/MS: Tandem mass spectrometry, DCV: Daclatasvir, \( t_r \): Retention time
The authors declare that this study...

that DCV degraded under acid, base, and oxidative stress conditions. It was found that the treated samples were analyzed by the proposed method. It was found that DCV degraded under acid, base, and oxidative stress conditions, whereas it was found to be stable under neutral hydrolysis and photochemical stress condition. The method could separate the DCV peak from its degradation product peak and selectively quantify DCV, indicating the specificity of the method. The identity of degradation products was confirmed by high resolution ESI-MS/MS techniques.

**CONCLUSION**

The current investigation illustrated the need to develop and validate a DCV stability-indicating RP-HPLC technique under varied stress levels in compliance with ICH requirements. RP-HPLC strategy was demonstrated to be a highly potent tool for separating and identifying degradation products of DCV medication in three different stress states discovered by high-resolution ESI-MS/MS techniques. As part of the validation process, the reported methodology linearity, accuracy, LOQ, LOD, precision, and ruggedness were all tested. Using this method, a commercial DCV tablet formulation was tested. The suggested method for regular analysis is relatively more straightforward and rapid because it avoids more tedious chemical procedures. It is also a simple, quick, and cost-effective method. The findings of DCV degradation study open up new directions for drug stability research and understanding, resulting in more tools for quality control and safer treatments.

**ACKNOWLEDGMENTS**

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**Ethics**

**Ethics Committee Approval:** Ethics committee approval is not required for the proposed research. Any kind of human being or animal matrices was not used in the current study.

**Informed Consent:** Not applicable.

**Peer-review:** Externally peer-reviewed.

**Authorship Contributions**


**Conflict of Interest:** No conflict of interest was declared by the authors.

**Financial Disclosure:** The authors declare that this study received no financial support.

### REFERENCES


