Hydroxychloroquine and Management of COVID-19: What is behind its Accurate Detection in its Pharmaceutical Products?
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Background: Hydroxychloroquine Sulphate, is an antimalarial drug used in treatment and prophylaxis of malaria, and other conditions like rheumatoid arthritis, and tried as a prophylaxis of venous thromboembolism. It is one of the important choices in COVID-19 management, as shown by about 80% improvement in pneumonia, and prevention of further disease progression to severe conditions upon following Hydroxychloroquine therapeutic regimen. Aim: The aim of this work was to develop an economical, precise, accurate and specific analytical method for quantitative estimation of Hydroxychloroquine sulphate in pharmaceutical product for the purpose of using it as a quality control tool for testing Hydroxychloroquine sulphate products pre-market and post-market distribution. Insuring the presence of labelled drug amount in the dosage from. Methods: Determination of HQC in commercial pharmaceutical formulations dispensed in hospitals and community pharmacies and administered by patients, by in house development of a validated, selective and sensitive High Performance Liquid Chromatography (HPLC) test method, where, the procedure used is in accordance with published literature data, pharmacopeia, and international guidelines. Results: The method is specific and selective, and showed linearity R²>0.999 within concentration range of 25-300 µg/mL, accuracy results within the range of 98% - 102%, precision CV% less than 2%. The assayed tablets mean recovery are 99.045%. Moreover, dissolution results are fulfilling the required limit of 70% percent dissolution within 60 minutes. Conclusion: The in-house developed analytical method is easy, and cost effective for use in quantification of HQC.

Keywords: Hydroxychloroquine; Analytical method; Corona virus 2019; pharmacopeia, Dissolution, Validation.

INTRODUCTION
Corona virus 2019 a widely spreading pandemic caused by SARS-CoV-2. The main infection spreading pathways reported to be through large respiratory droplets. Also SARS-CoV-2 has been found in stool and urine of affected individuals [1]. The disease severity has varied from mild self-limiting flu-like illness to severe pneumonia infection, acute respiratory distress syndrome and death [2]. There were 19,462,112 confirmed cases of COVID-19 worldwide with a death rate of 3.7% according to the situation report of World Health Organization on August 9, 2020 [3]. Africa showed 884,990 confirmed cases with a mortality rate of 1.85%. Europe showed a 3,562,774 confirmed cases with a mortality rate of 3.07%, USA showed a 10,447,261 confirmed cases with a mortality rate of 3.68% till 9th August, 2020 [3].

On the other hand, Hydroxychloroquine Sulphate (HCQ) is used in the treatment and prophylaxis of malaria, and has main role in management of systemic and discoid lupus erythematosus, rheumatoid arthritis, porphyria cutanea tarda, sarcoidosis, and various skin disorders, and for prophylaxis of venous thromboembolism [4].

Recently, Hydroxychloroquine Sulphate (HQC) showed a promising results in treatment of patients with Corona virus (COVID-19), as shown in a trial for 62 patients infected with SARS-CoV-2, 46.8% were male and 53.2% were female, where, the findings showed that Time To Clinical Recovery (TTCR), recovery time of body temperature and the cough remission time were remarkably reduced in those patients taking HCQ treatment. Moreover, pneumonia had been improved in a large percentage of the HCQ treatment group patients 80.6% in comparison with control group patients 54.8%. There are four patients
their conditions exacerbated and encountered severe illness, those patients are found to be in the control group. Two subjects suffered from a mild adverse reaction in the HCQ treatment group patients [5].

Another trial on COVID-19 included twenty patients that received HCQ treatment protocol. The results showed a noticeable viral carriage reduction at the Sixth day on HCQ treatment compared to control group, and the average viral carrying time was much lower than that registered for those who do not receive therapy. Upon addition of azithromycin to HQC, they showed noticeable more efficient viral elimination [6]. Moreover, the preliminary results from a trial of HQC for COVID-19 indicate that when adding HCQ at the start of a standard treatment for patients hospitalized with mild illness a quicker recovery occurred in comparison with placebo. The results also indicate that Hydroxychloroquine might contribute in protection against exacerbating COVID-19 illness cases [7].

Research studies including 100 COVID-19 patients enrolled from 10 hospitals which were suffering from pneumonia, showed that upon administration of HCQ for the management of covid-19, inhibition of worsening of pneumonia were superior to the control group. Also an improvement in lung imaging results, and reduction in disease time course were noticed [8].

Another investigation included 36 patients treated with HQC were evaluated, the findings reported that 6 showed no symptoms, 22 throat symptoms complain and 8 showed pneumonia. At day 6 of treatment 70% of the patients in the HCQ-treated group showed complete viral eradication compared with 12.5% in the control group [9]. Hydroxychloroquine sulphate is available in the form of tablets under the trade name Plaquenil® tablets [10], the adult dosing 200–400 mg per day, maximum of 6.5 mg/kg per day [11].

A Literature survey has revealed some methods for analysis of Hydroxychloroquine sulphate in biological and non-biological fluid of different pharmaceutical formulations as HPLC with mass detector [12], HPLC with UV variable wavelength detector [13–20], HPLC with fluorescence detector [21, 22] and electrochemical [23] detection.

In this work, the proposed validated in house developed method is economical, precise, accurate and specific analytical method for Hydroxychloroquine sulphate determination in pharmaceutical dosage form, and in dissolution testing. Validation items are performed as per United States Pharmacopeia (USP), ICH and FDA guidelines. [24,25,26] Assay testing, invitro dissolution procedures and criteria are performed in accordance with FDA Biowaiver / Biopharmaceutics classification system Guidelines [27].

Performing in vitro testing like assay, uniformity of dosage units, and dissolution are important step in determining the validity of dosage form for intended use. Moreover, predicting dosage form behaviour before conducting bioequivalence studies if required. Some research institutes are working on In vivo-In vitro correlation to predict pharmacokinetic behaviour, and bioequivalence of generic drug products from invitro dissolution results. Drug assay should be within the limit of 93 to 107% of the labelled claim, and dissolution limit should be not less than 70% of the drug dissolved within 1 hour in order to ensure valid and safe for administration of Hydroxychloroquine tablets, to obtain an effective therapeutic outcome.

Routine random drug samples should be selected from community and hospital pharmacies and subjected for quality control testing in order to ensure that the dosage form are keeping its integrity and the labelled claim of active ingredient is in compliance with the required specification after they are exposed to market transportation conditions and shelf-storage in pharmacies. Also, absence of any drug degradative or toxic product is important and should be checked.

Finally, accurate investigations of HQC in pharmaceutical products could not be relied upon unless a valid analytical method is well-developed for drug determination. The aim of this routine check is to ensure that the patients are administering a valid and safe therapeutic product to obtain the desired therapeutic outcomes and avoid the incidence of drug toxicity or adverse events.

**MATERIALS AND METHOD**

Routine random drug samples are selected from community pharmacies and hospital pharmacies. Quality control testing like Assay testing and dissolution testing are performed on those samples to ensure that the dosage form is preserving its integrity with absence of any degradative products, and the labelled claim of active ingredient is in compliance with the required specification after they are exposed to market transportation conditions, and shelf-storage in pharmacies. The aim is to ensure that the patients are administering safe and valid drug product to attain required therapeutic efficacy and avoid any potential drug toxicity or side effects.

**MATERIALS**

Hydroxychloroquine sulphate standard were obtained from CHINOIN Pharmaceutical and Chemical Works Private Co. Ltd. – a Sanofi Company, Hungary. All solvents were of the HPLC grade and were...
purchased from Merck (Germany). Rest of chemical agents used were of analytical reagent (AR) grade and were purchased from Scharlau (Spain).

**Analytical Methods**

**Instrumentation**

The analysis was performed by using the analytical balance Sartorius, pH meter portable BOECO, the HPLC used is of Thermo Spectra System 4000 HPLC system, equipped with Spectra system P4000 Gradient Pump, Spectra system Auto sampler fitted with a 100 µl loop and Spectra system P1000 Ultra-Violet Detector was used. The output signal was monitored and processed using a Chromquest 4.2 Software. The chromatographic column used was a 250 mm x 4.6 mm, Agilent (HC-2) RP-C18 with 5µm particles.

Before use, the mobile phase was vacuum-filtered through a 0.45 µm membrane filter and degassed with Sonication. The water was distilled and then purified by a ELGAPURE water purification system (England).

**Chromatography Conditions**

The mobile phase consisted of 0.25% phosphoric acid (0.55mM Na-1-Pentane sulfonate): Acetonitrile 85:15 (V/V). Mobile phase flow rate was 1 mL/min. Peaks were monitored at 343 nm. Analysis performed at room temperature, the volume of solution injected onto the column was 1µL.

**Preparation of stock and standard solutions**

A master solution of HCQ sulfate (500 µg/ml) was prepared by accurately weighing an equivalent amount to 50 mg of Hydroxychloroquine sulfate into 100 ml volumetric flask and dissolved in 70 ml of methanol and volume completed with methanol. Aliquots of the standard master solutions of Hydroxychloroquine sulfate were transferred using A-grade bulb pipettes into 10 ml volumetric flasks and solutions were made up to the volume with mobile phase to give the final concentrations of 25 to 300 µg/ml.

**Method Validation**

The in-house developed HPLC method validation was performed as per ICH guidelines.

**Specificity**

It is the capability of the analytical method to determine target analyte in presence of all potential impurities. Stress study were performed at concentration of 50 µg/mL Hydroxychloroquine in active pharmaceutical ingredients (API) and formulated tablet samples to indicate stability and specificity of the developed analytical method. Intentional drug degradation was performed under stress conditions of heat (Exposed at 85°C for 1 hr), acid (1N HCl for 1 hr at 85°C), and base (1N NaOH for 1 hr at 85°C).

**Linearity**

Linearity was studied by preparing standard solution at eight concentration levels from 80 to 120% of the target analyte concentrations i.e. Concentrations ranging from 25-300 µg. These analyses were performed in triplicate [25, 26].

**Precision**

It is an assessment of intra-day variability in results obtained at three concentrations, with nine determinations in one laboratory, on the same day. Calculated %RSD is used to express precision [25, 26].

**Lower Limit of detection (LLOD) / Lower limit of quantitation (LLOQ):**

Can be defined as the concentration of analyte that would yield signal-to-noise ratios of 3 for LLOD and 10 for LLOQ respectively. LLOD and LLOQ were determined by standard deviation of y-intercepts of regression lines and slope of calibration. [25, 26]

**Estimation of Hydroxychloroquine in pharmaceutical dosage form**

To determine the content of Hydroxychloroquine in tablets (label claim: 200 mg Hydroxychloroquine Sulphate) Weigh and grind not less than twenty tablets. Transfer an accurately weighed portion of powdered tablets equivalent to 200 mg Hydroxychloroquine sulphate into 200ml volumetric flask and add 150ml (Methanol :Water 1:1 V/V). Sonicate with intermittent shaking for 15 minutes and cool to room temperature, complete to volume with (Water: Methanol 1:1 V/V). 0.45 µm nylon filter were used to filter solutions. Suitable aliquot of the filtrate was added to a volumetric flask and volume completed with mobile phase to obtain a concentration of 50 µg/ml. Sample solution was injected into HPLC with injection volume 1µl, three times, under the predetermined validated chromatographic conditions. Drug chromatographic responses was determined at 343 nm and concentrations in the samples were determined by comparing sample chromatographic response with that of the standard.

**Estimation of Hydroxychloroquine in In-Vitro Dissolution testing of pharmaceutical dosage form**

Dissolution testing procedures were applied on twelve dosage units (Tablets) under dissolution media water, pH1.2, pH4.5, pH6.8 using UPS Type II device at 50 rpm for water medium, and 75 rpm for pH1.2, 4.5, and 6.8 media as follows:

1. The above mentioned dissolution conditions were applied and performed by placing six Film Coated Tablets in six vessels (one Tablet in each vessel). Five ml of each sample was withdrawn after 10, 15, 20, 30, 45 and 60
minutes of dissolution where, 5ml of blank (dissolution media) was added to replace this withdrawn volume and achieve constant volume of Dissolution media (900ml).

2. The withdrawn 5ml at each sampling interval was added in a coded labelled test tube and then filtered through syringe membrane filter (PTFE 0.45µm).

3. The previously mentioned procedures were repeated on another six Film Coated Tablets.

4. The filtered withdrawn samples were then analysed by HPLC-UV apparatus for drug detection and quantification at 343 nm.

**RESULTS**

Importance of Hydroxychloroquine has been showed in many research and experimental studies in treatment of COVID-19, and prevention of progression to critical illness, and decreasing mortality rates. Study results reported an improvement in pneumonia by 80.6% for those patients treated with Hydroxychloroquine compared to 54% for those patients how didn’t administer Hydroxychloroquine [5].

A remarkable reduction in viral carriage after 6 days on HCQ treatment was reported in which 70% of patients showed complete viral eradication. Moreover, by addition azithromycin to HQC, a noticeable more efficient viral elimination occurred [6, 9].

Also trials indicated that when adding HCQ at the start of the therapeutic protocol lead to quicker recovery of mild illness cases, indicating that HCQ has a main contribution in protection against exacerbating COVID-19 illness cases, and improvement in lung imaging results [7, 8].

Determination of HCQ was carried out by RP-HPLC using Mobile phase having a composition of 0.25% phosphoric acid (0.55mM Na-1-Pentane sulfonate) : Acetonitrile 85:15 (V/V). Then finally filtered using 0.45µ nylon membrane filter and degassed in sonicator for 10 minutes. The column used was C18 Agilent (HC-2) 250X4.6 mm p.s. 5um. Flow rate of Mobile phase was 1.0 ml/min, System suitability parameters such as theoretical plates were above 3400, and tailing factor less than 1.6.

**Method Validation**

After development of the analytical method it was subjected to method validation according to ICH and FDA guidelines. [25, 26] The aim of validation is to demonstrate whether the method is acceptable for its required application or not. A standard procedure is followed to evaluate required validation items (specificity, linearity, accuracy, precision, Lower limit of detection and Lower limit of quantitation, and system suitability).

**Specificity**

Blank samples containing solvent were injected and showed no drug detected. The drug was unstable under acidic and basic stress conditions (Figures 3 and 4). The drug was degraded approximately to 31%. But it was more stable in neutral conditions when the drug was refluxed with water for 1 h, and was degraded approximately to 83.5% (Figure 4) in basic conditions. Due to specificity of UV wave length used, no degradation was shown in chromatograms. The stability of stock solution under conditions of (2 °C to 8 °C) was determined by quantitation of Hydroxychloroquine and comparison to freshly prepared standard (Figures 1 and 2). No significant differences between stock and freshly prepared standard solution was found.

All forced degradation samples were analysed with the aforementioned HPLC conditions using a UV detector to monitor the homogeneity and purity of the Hydroxychloroquine peak. Individual related substances, placebo, and Hydroxychloroquine showed no interference, thus providing a specific analytical method.
Fig-3: Chromatogram of HCQ Stress HCL

Fig-4: Chromatogram of HCQ Stress NaOH
Linearity

Hydroxychloroquine showed linearity from 25 to 300 µg/ml ($r^2 = 0.999$) for HPLC. Linearity was evaluated by determining eight standard working solutions in the range of 25-300 µg/ml in triplicate using water, pH 1.2, pH 4.5, and pH 6.8 media as a solvent. Peak areas of HCQ were plotted versus HCQ concentration (µg/ml) with linear regression analysis performed on the results (Table-1). High value Correlation Coefficient ($r^2$) and low value intercept CV% (less than 5%) indicates validation of the analytical method linearity adherence of the system to Beer's law. The resulted chromatogram showed a sharp, symmetrical, and well separated peak at Retention time of 4.2 min (Figure-1).

Table-1: Results of Analytical Method Validation (Linearity) at different dissolution media

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Hydroxychloroquine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Range</strong></td>
<td>25-300 µg/mL</td>
</tr>
<tr>
<td><strong>Linearity correlation equation</strong></td>
<td></td>
</tr>
<tr>
<td>At Dissolution medium (Water)</td>
<td>$Y = 1787.580X - 12661.941$</td>
</tr>
<tr>
<td>At Dissolution medium (pH1.2)</td>
<td>$Y = 1872.992X + 1067.921$</td>
</tr>
<tr>
<td>At Dissolution medium (pH4.5)</td>
<td>$Y = 1519.711X - 9962.548$</td>
</tr>
<tr>
<td>At Dissolution medium (pH6.8)</td>
<td>$Y = 1315.647X - 10840.741$</td>
</tr>
<tr>
<td><strong>Linearity correlation coefficient $R^2$</strong></td>
<td></td>
</tr>
<tr>
<td>At Dissolution medium (Water)</td>
<td>0.999702</td>
</tr>
<tr>
<td>At Dissolution medium (pH1.2)</td>
<td>0.999596</td>
</tr>
<tr>
<td>At Dissolution medium (pH4.5)</td>
<td>0.999657</td>
</tr>
<tr>
<td>At Dissolution medium (pH6.8)</td>
<td>0.999751</td>
</tr>
<tr>
<td><strong>Mean Slope ±SD</strong></td>
<td></td>
</tr>
<tr>
<td>At Dissolution medium (Water)</td>
<td>1787.580±1.323</td>
</tr>
<tr>
<td>At Dissolution medium (pH1.2)</td>
<td>1872.992±2.295</td>
</tr>
<tr>
<td>At Dissolution medium (pH4.5)</td>
<td>1519.711±2.164</td>
</tr>
<tr>
<td>At Dissolution medium (pH6.8)</td>
<td>1315.647±2.918</td>
</tr>
<tr>
<td><strong>Mean Intercept ±SD</strong></td>
<td></td>
</tr>
<tr>
<td>At Dissolution medium (Water)</td>
<td>– 12661.941±230.805</td>
</tr>
<tr>
<td>At Dissolution medium (pH1.2)</td>
<td>1067.921±183.274</td>
</tr>
<tr>
<td>At Dissolution medium (pH4.5)</td>
<td>– 9962.548±76.314</td>
</tr>
<tr>
<td>At Dissolution medium (pH6.8)</td>
<td>– 10840.741±371.172</td>
</tr>
<tr>
<td><strong>Standard error of slope</strong></td>
<td></td>
</tr>
<tr>
<td>At Dissolution medium (Water)</td>
<td>0.764</td>
</tr>
<tr>
<td>At Dissolution medium (pH1.2)</td>
<td>1.325</td>
</tr>
<tr>
<td>At Dissolution medium (pH4.5)</td>
<td>1.249</td>
</tr>
<tr>
<td>At Dissolution medium (pH6.8)</td>
<td>1.685</td>
</tr>
<tr>
<td><strong>Standard error of intercept</strong></td>
<td></td>
</tr>
<tr>
<td>At Dissolution medium (Water)</td>
<td>133.255</td>
</tr>
<tr>
<td>At Dissolution medium (pH1.2)</td>
<td>105.813</td>
</tr>
<tr>
<td>At Dissolution medium (pH4.5)</td>
<td>44.060</td>
</tr>
<tr>
<td>At Dissolution medium (pH6.8)</td>
<td>214.296</td>
</tr>
</tbody>
</table>

Where, $n=3$, average of three determinations, SD (±): standard deviation.

Precision

The intra-day variations can be demonstrated in terms of % RSD values. The %RSD values in dissolution medium USP (Water), pH1.2, pH4.5, and pH6.8 showed to be less than or equal to 2 %, indicating a good precision. It is acceptable according to acceptance limit of these parameters. The mean RSD% in medium USP (Water), pH1.2, pH4.5, and pH6.8 were 0.065%, 0.276%, 0.723%, 0.239%.

Lower Limit of Detection (LLOD) and Lower Limit of Quantification (LLOQ)

The LLOD and LLOQ of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The LLOD is the lowest analyte concentration that gives a measurable response. The LLOD for Hydroxychloroquine was found to be 0.426µg/mL, 0.323µg/mL, 0.166µg/mL, and 0.931µg/mL for dissolution media Water, pH 1.2, pH 4.5, and pH 6.8 respectively. The LLOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified. The LLOQ was 1.291µg/ml, 0.979µg/ml, 0.502µg/ml, and 2.821µg/ml for dissolution media Water, pH 1.2, pH 4.5, and pH 6.8 respectively.
Assay (Potency) of Hydroxychloroquine

The developed and validated method was applied for the assay of HCQ 200mg tablet, where, the obtained mean recovery percent was found to be 99.045% which indicate the selectivity of the analytical method for detection of HCQ without interference with inactive ingredients.

Dissolution of Hydroxychloroquine

The developed and validated method was applied for dissolution testing of HCQ Tablets and the results were reported as the dissolution profile of the mean percentage dissolved of the reference HCQ tablets which was in compliance with the FDA Biowaiver / Biopharmaceutics classification system Guidelines [27] and the United States Pharmacopeia (USP) [24].

The results of dissolution percent of HCQ 200mg tablet in water (USP medium) after 10, 15, 20, 30, 45, and 60 minutes were 54.756%, 83.188%, 92.282%, 94.511%, 95.256%, 97.056% respectively, and in medium pH1.2 were 64.707%, 82.002%, 87.086%, 89.306%, 91.035%, 93.822% respectively.

Additionally, the results of dissolution percent in medium pH4.5 after 10, 15, 20, 30, 45, and 60 minutes were 74.150%, 86.057%, 91.225%, 93.474%, 96.133%, 97.905% respectively and in medium pH6.8 after 10, 15, 20, 30, 45, and 60 minutes was 57.237%, 78.649%, 84.629%, 88.104%, 89.970%, 91.844% respectively.

The previous results of analytical method validation, assay testing, and dissolution testing indicate that the method validation and pharmaceutical drug product is in compliance with the required specifications, and thereby will provide the required therapeutic effect in COVID-19 treatment.

DISCUSSION

Hydroxychloroquine showed a promising results in management of COVID-19, prevention of progression to critical illness, and decreasing mortality rates which was obvious in high percentage of rapid improvement in pneumonia [5] with a remarkable reduction in viral carriage and complete viral eradication after nearly less than one week of treatment [6]. Early treatment with HCQ resulted in quicker recovery of mild illness cases and improvement in lung imaging results, thus, indicating the importance of HCQ treatment in therapeutic protocols of COVID-19 [7, 8].

It is worthy to mention that, the estimation and assaying of HCQ in pharmaceutical products including tablets and evaluation of its in vitro behaviours including dissolution with a validated analytical method is important to ensure the clinical efficacy of HCQ.

As shown previously, the clinical importance of HCQ sulphate as an antimalarial drug in the treatment and prophylaxis of malaria and its main role in the management of systemic and discoid lupus erythematosus and rheumatoid arthritis [4], besides, its recent promising clinical results in the treatment of patients with Corona virus (COVID-19) [5-7].

The analytical method developed and validated in this work is nearly in compliance with some reported validated methods [24, 28], the calibration curves in different dissolution media, with different pH, as water, pH 1.2, pH 4.5, and pH 6.8 were linear over the range of 25 to 300 µg/ml, r² was equal to 0.999, accuracy of the results was in the limit of 98% - 102%, and RSD% were less than 2% which is in accordance with ICH and FDA guidelines [25, 26]. The standard deviation for intercept value was less than 5%, system suitability parameters as theoretical plates were above 3400, tailing factor less than 1.6 and so it could be used for determination of HCQ in bulk and in pharmaceutical products.

The analytical method applied in this study was simple, of excellent sensitivity, specificity, precision and accuracy, where, the materials and reagents used in analysis are common, convenient and available, including, C18 column 250mm X 4.6mm, phosphoric acid, ion pairing agents (Na-l-Pentane sulfonate) and acetonitrile. Moreover, the mobile phase pumped in an isocratic mood and total run time was 5.5 minutes which permit analysis of up to 250 samples per 24 hours.

Finally, the developed validated analytical method is appropriate for use in preliminary and routine quality control check on distributed Hydroxychloroquine products in the market to ensure their compliance under the different storage conditions in pharmacies and hospitals with specifications, deterioration and reduction of the active ingredient (HCQ) into inactive degradation product in the final dosage form [28] administered and handled by patients and thus assuring its therapeutic and clinical effect.

CONCLUSION

The HPLC analytical method developed for determination of HCQ in bulk and marketing products, and dissolution samples as revealed by the validation data enables specific, accurate, and precise analysis of the drug. The developed analytical method showed enough sensitivity for HCQ quantification in drug products and thus can be used for routine analysis, quality control, and for stability studies of pharmaceutical preparations and consequently assuring to some extent the efficacy and safety of HCQ in the management of several diseases including COVID-19.
Author Contributions
Conceptualization, N.A.S.; methodology, M.A.R.; S.A.R. validation, S.A.R. and E.M.S.; formal analysis, N.A.S., and E.M.S.; investigation, N.A.S.; data curation, M.A.R. and E.M.S.; writing—original draft preparation, E.M.S.; writing—review and editing, N.A.S., and M.A.R.; visualization, All authors; supervision, N.A.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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