A comparative study of HPLC and UV spectrophotometric methods for oseltamivir quantification in pharmaceutical formulations

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ABSTRACT
Oseltamivir is an antiviral drug and is used in the treatment of all influenza viruses. It is the most effective antiviral option against all influenza viruses that can infect humans. UV and LC methods have been developed and validated according to ICH guidelines for various parameters like selectivity, linearity, accuracy, precision, LOD and LOQ, robustness for the quantitative determination of oseltamivir in pharmaceutical formulations. LC method has been performed using reverse phase technique on a C-18 column with a mobile phase consisting of 20 mM potassium dihydrogen phosphate solution and acetonitrile (60:40, v/v) at 25 °C. The mobile phase flow rate was 1.2 mL min⁻¹. For the determination of oseltamivir, UV spectrum has been recorded between 200 and 800 nm using methanol as solvent and the wavelength of 215 nm has been selected. Both methods have demonstrated good linearity, precision and recovery. No spectral and chromatographic interferences from the capsule excipients were found in UV and LC methods. In both methods, correlation coefficients were greater than 0.999 within a concentration range of 10–60 mg mL⁻¹ using UV and LC. Intra-day and inter-day precision with low relative standard deviation values were observed. The accuracy of these methods was within the range 99.85–100.17% for LC and from 99.26 to 100.70% for UV. Therefore UV and LC methods gave the most reliable outcomes for the determination of oseltamivir in pharmaceutical formulation.

KEYWORDS
oseltamivir, Covid19, UV, LC, methods

INTRODUCTION
COVID-19 has infected millions of people and Coronavirus disease has spread around the World since December 2019. As the number of people infected grows day by day. The World Health Organization (WHO) has declared it as a pandemic. As of April 15, 2021, a total (worldwide) of approximately 137,866,311 coronavirus cases with 2,965,707 deaths have been reported [1]. It has been effectively spread through multiple routes, such as airborne transmission, direct contact, oral ingestion, and fomite.

The sudden emergence of COVID-19 has had a profound impact on human health and life as well as the global economy. After Severe Acute Respiratory Syndrome and Middle East Respiratory Syndrome, COVID-19 is the third member of the coronavirus family that has caused human diseases in recent decades [1, 2].

COVID-19 infection causes a wide range of symptoms, including a mild cough, a simple fever, weakness, pneumonia, hemoptysis, diarrhea, and multiple organ failure, as well as death [3, 4]. COVID-19 is especially dangerous to older people with cardiovascular or
respiratory diseases, and they are at a higher risk of severe health complications [5, 6]. Coronavirus patients are generally treated with antiretroviral, anti-inflammatory, antiparasitic, antibiotic drugs and oxygen-assisted plasma therapy [7–11].

An effective therapeutic agent or antiviral drug is not yet available for the treatment of COVID-19. Therefore, COVID-19 disease could not be controlled with high mortality rates and has become a major problem on a global scale. As a result, it is critical to look for reliable and effective treatment methods for coronavirus patients. Current antiviral drugs meet an unmet medical need to treat coronavirus patients. There is an urgent need to develop a treatment method to reduce the coronavirus epidemic and its effects on humans.

Various drugs such as Remdesivir, Hydroxychloroquine, Chloroquine, Favipiravir, Ritonavir, Lopinavir, Ruxolitinib, Darunavir, Baricitinib, Cobicistat, Tocilizumab [12], Azithromycin, Interferon beta-1b [13], Corticosteroids, and Ribavirin are being investigated for Coronavirus treatment.

An Antiviral drug oseltamivir is used to treat all influenza viruses. It is resistant against all antiviral option against all influenza viruses that can infect humans, including pandemic influenza viruses. It was developed by Gilead Sciences (Fig. 1). It has also been used as anti-SARS-CoV-2 therapy in the treatment and treatment of anti-MERS-CoV in Korea [14, 15]. It has been shown that when used in combination with antibacterial therapy, early administration of oseltamivir will minimize the period of fever and the time from peak to decline in outpatients with suspected COVID-19 [16].

For quantification of oseltamivir in pharmaceutical form or biological fluids, a number of analytical procedures have been published. These include spectrophotometric methods [17–19], spectrophotometric method [20, 21] high performance liquid chromatographic methods [22–25], high performance thin layer chromatographic method [26], capillary electrophoresis [27], and liquid chromatographic-tandem mass spectrometric methods [28–29]. Some of these methods are complex. These methods require expensive instruments, a large amounts of organic solvents and special reagents. Analysis times are long.

Unlike complex analytical techniques, HPLC and UV-spectrophotometric techniques for oseltamivir quantification are low-cost, effective, easy-to-use, and on-site. In order to ensure the safety and effectiveness of drugs in various matrices, qualitative and quantitative analysis is crucial. The LC is a more commonly used tool in quality control laboratories due to its high sensitivity and accuracy. The UV method is very simple because it does not require any reagents, pH adjustments, or extraction techniques.

As a result, UV-spectrophotometric and LC-chromatographic methods for quantifying oseltamivir in pharmaceutical preparations were developed and validated. The results of these methods have been compared statistically using variance analysis. We also evaluated the effectiveness and applicability of the methods, focusing on Quality Control Research.

**Fig. 1. Molecular structure (oseltamivir)**

### MATERIALS AND METHODS

#### Experimental equipments

Chromatographic analysis was carried out on an Agilent 1,260 series liquid chromatograph equipped with an UV-Vis detector, a quaternary pump, a vacuum degasser, a column oven, and Chemstation software. The present study also utilized a Mettler-Toledo electronic balance (Mettler-Toledo, Switzerland), a Milli-Q water purification system (Millipore, USA). A Shimadzu UV-1800 spectrophotometer with a double beam using 1.0 cm quartz cells and UV-Probe software was used to record the spectrophotometric data (Shimadzu UV-1800 spectrophotometer, Japan).

#### Chemicals

All chemical compounds were analytical and hplc grade. Potassium dihydrogen phosphate (99.9%), methanol (≥99.9%), and acetonitrile (≥99.9%), were bought from Sigma-Aldrich. Ultra pure water was produced using a Milli-Q system (Millipore). Pure oseltamivir was kindly provided by Atabay Pharmaceuticals Inc. (Istanbul, Turkey). Enfluvir (50 mg per capsules) was obtained from local pharmacy.

#### Standard solutions

For the creation of the calibration curve, stock standard solution of oseltamivir (500 μg mL⁻¹) was prepared in methanol. The subsequent stock solution has been sonicated and filtered through a 0.22 μm filter. Further, stock standard solution was diluted with methanol to obtain standard solutions at concentrations in the range (10–60 μg mL⁻¹) prior to analyses.

#### Sample solution

The contents of the 10 Enfluvir capsules were accurately weighed and emptied into a clean and dry mortar, then it was homogenized. Then, the capsule powder equivalent to 50 mg of oseltamivir was transferred to a 100 ml calibrated volumetric flask and dissolved in 30 mL of methanol. Volume was completed to 100 ml with methanol to give a concentration of 500 μg mL⁻¹. This flask was connected to a shaker for 10 minutes to completely disperse the components and then the final solution was filtered using a Whatman filter paper (No. 42).
Determination of $\lambda_{\text{max}}$

First, the spectrophotometer was calibrated to zero. Then the maximum absorption wavelength of oseltamivir solution (50 $\mu$g mL$^{-1}$) was determined by scanning in the range of 200 and 400 nm (Fig. 2).

Conditions

Chromatographic analysis were carried out on a liquid chromatograph (Agilent 1,260) with a UV–vis detector. Oseltamivir was analyzed at a flow rate of 1.2 mL min$^{-1}$ using a mobile phase composed of 20 mM potassium dihydrogen phosphate solution and acetonitrile (60:40, v/v). Before use, the mobile phase was filtered and degassed through a 0.22 $\mu$m membrane filter. An Agilent Extend C18 (4.6 mm $\times$ 250 mm, 5.0 $\mu$m particle size) column was used and operated at 25 °C. Oseltamivir was detected with the UV detector at 215 nm. The run time under these conditions was 10 minutes. UV spectrophotometric method was carried out on a double beam spectrophotometer at 215 nm using 1.0 cm quartz cells for all absorbance measurements.

Method validation

Analytical methods have been validated in compliance with the recommendations of The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use [30, 31]. Validation parameters (Linearity, selectivity, accuracy, precision, limit of detection and quantification, and robustness) have been investigated. System suitability test was performed with respect to injection repeatability (relative standard deviation of retention time and peak area response), tailing factor, peak asymmetry, and theoretical plate number using a standard solution (oseltamivir, 30 $\mu$g mL$^{-1}$) [32–37]. Standard calibration curves in both methods were obtained by analyzing a series of standard solutions. These standard solutions have been prepared in triplicate and linearity was assessed using linear regression analysis.

Selectivity of both methods were assessed by comparison of the spectrums and chromatograms obtained from standard and sample preparations which take part in the pharmaceutical preparations. Limit of detection and quantification have been determined using the slope of calibration curve (m) and standard error (s) as displayed in following equations.

$$\text{LOD} = 3 \times s/m$$
$$\text{LOD} = 10 \times s/m$$

Precision of both methods were analyzed in terms of both repeatability (intraday precision) and intermediate precision (interday precision). The repeatability was determined from five replicate injections of a freshly prepared oseltamivir solution (assay concentration, 30 $\mu$g mL$^{-1}$) in the same equipment on the same day. In order to determine intermediate precision, the experiment was also replicated by analysing the newly prepared solutions at the same concentrations on three consecutive days. Precision was expressed as R.S.D. % of a series of measurement.

The percentage recovery was determined by using three preparations of three different levels of the reference drug of Fig. 2. The spectrum of oseltamivir standard solution (50 $\mu$g mL$^{-1}$)
oseltamivir for accuracy. The findings were expressed as the percentage of oseltamivir recovered in the sample and R.S.D. %.

The robustness of analytical methods was evaluated by making small changes in method conditions. For HPLC method, samples have been analyzed under different circumstances like changes in the flow rate of mobile phase (±0.1 mL min⁻¹) and in acetonitrile content (±2%) in the mobile phase and the effect of system suitability parameters have been observed. For the UV method, samples have been analyzed under different conditions such as using different brands of methanol (Sigma-Aldrich, 34,860/J.T. Baker 8402) as solvent and detection wavelengths (±2 nm).

**Application to pharmaceutical preparations**

Freshly prepared stock sample solution was diluted with methanol to obtain sample solution (30 µg mL⁻¹). This freshly prepared sample solution was filtered using a filter of 0.22 µm and then analyzed.

**Statistical comparison of methods**

From the validation results, it was determined that the above-mentioned methods were suitable for routine quality control analysis of oseltamivir in commercial formulations. The recovery percentages were statistically compared when both methods were applied to a commercial drug formulation. For this purpose, F-test and t-test were applied.

**RESULTS AND DISCUSSION**

**LC method**

A reversed-phase LC method for estimating oseltamivir in pharmaceutical forms has been proposed. In order to get a successful result, chromatographic conditions were adapted. The LC procedure has been optimized to develop an a reliable and repeatable method. Different conditions such as mobile phase compositions, different columns and configurations were tested to achieve a sharp peak. The mobile phase was chosen considering the peak parameters (tailing, symmetry), analysis time, easy preparation and cost. Figure 3 displays chromatogram of oseltamivir standard and sample solutions using the developed method. Oseltamivir was eluted to form symmetrical peak as seen in Fig. 3. The observed retention time (3.010 minutes) enables the rapid detection of oseltamivir, which is essential for routine research. The resulting symmetrical peak was observed at the retention time of oseltamivir in Fig. 3. Thus the method was proved selective. The selectivity of the UV method, the spectra of the standard, blank and tablet sample solutions were compared, and no interference was observed. Thus the method was selectively proved.

The equation of the calibration curve was produced from linear regression analysis of the peak area versus the concentration of oseltamivir (Fig. 4). Regression equations of the calibration curves for oseltamivir was calculated as $Y = 25.694 - 6.1333$ at the range of 10–60 µg mL⁻¹. Correlation coefficient ($r^2$: 0.9999) indicates a good linearity and high sensitivity; LOD and LOQ have been determined as 0.40 and 1.20 µg mL⁻¹, respectively (Table 2). The estimate of the standard deviation of the responses was based on the standard deviation of the calibration curve. The standard deviation of the y-intercept of the regression line was used as the standard deviation(s).

Precision of these methods have been determined by repeatability (intraday) and intermediate precision (interday). Precision was stated as RSD% of a sequence of measurement. Precision study data were presented in Table 3. The result obtained shows a good intra-day precision. Interday precision was also calculated from assays on 3 day.

The recovery of the analyte was determined by adding different levels of the standard analyte (80%, 100% and 120%) to the sample solution and analyzing it in the same way. The results of mean percentage recovery, R.S.D. % and standard error were given in Table 4. There was no significant change in system suitability parameters when the organic content and flow rate of the mobile phase were changed at small rates. Results were presented in Table 5. The low R. S. D. % values showed that these methods were sufficiently robust.

**UV method**

The spectrum of oseltamivir solution in methanol (60 µg mL⁻¹) against a blank has been shown in Fig. 5A and B. The most intense absorbance peak ($\lambda_{max}$) was observed at 215 nm. Several assays were carried out, and the best results have been achieved when using the amplitude from the valley at a wavelength of 215 nm to the zero base line. The overlay spectrum of oseltamivir standard solutions and spectrum of sample solution were given in Fig. 5C and D.

A good linearity was achieved in the concentration range of 10–60 µg mL⁻¹ of standard solutions of oseltamivir, this can be seen in Fig. 6. The exact data obtained for the evaluated methods are presented in Table 2. Less than 0.5 of R.S.D. % values have been determined. This shows that both methods provide good sensitivity, but the LC method is...
more sensitive compared to the UV method. Accuracy was studied by means of recovery experiments using the methods developed. Both spectrophotometric and

Table 1. System suitability test results (n = 6, 30 μg mL⁻¹)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(%) RSD of peak area</td>
<td>0.1847%</td>
<td>≤2%</td>
</tr>
<tr>
<td>(%) RSD of retention time</td>
<td>0.3882%</td>
<td>≤1%</td>
</tr>
<tr>
<td>Tailing factors</td>
<td>1.133 ± 0.0558</td>
<td>0.9–1.4</td>
</tr>
<tr>
<td>Theoretical plates number</td>
<td>4,310.67 ± 45.4078</td>
<td>N&gt;2000</td>
</tr>
<tr>
<td>Peak Asymmetry Ratio</td>
<td>0.6458 ± 0.0020</td>
<td>≤1.5</td>
</tr>
</tbody>
</table>

Fig. 3. Chromatogram produced of oseltamivir standard and sample solutions using the developed method. A. Chromatogram of standard oseltamivir solution (60 μg mL⁻¹). B. Chromatogram of blank solution. C. Overlap chromatogram of standard solutions (10–60 μg mL⁻¹). D. Chromatogram of sample solution (30 μg mL⁻¹)

Fig. 4. Calibration curve (LC method)
chromatographic methods displayed mean recoveries of close to 100 percent, showing adequate accuracy. This situation is shown in Table 4 (Fig. 6).

The method’s robustness was evaluated by testing the effect of minor variations on experimental variables like changes in different solvent and detection wavelengths on the analytical performance. The minor differences in each of the factors didn’t affect the findings dramatically. This indicates that the method developed for routine analysis is reliable; the situation is highlighted in Table 5.

### Application to pharmaceutical preparations

Chromatographic and spectropic methods have been applied in pharmaceutical formulations. Test results for tablet containing oseltamivir sold in pharmacies were presented in Table 6. These results are very close to the amounts indicated on the label of the tablets. The UV and LC methods recommended in this report can be applied appropriately for the analysis of oseltamivir in pharmaceutical preparations.

### Statistical comparison of methods

F-test and t-test and were applied for statistical comparison of both methods. Statistical tests revealed that there was no significant difference between the experimental values

### Table 2. Data of linearity tests

<table>
<thead>
<tr>
<th>Parameter</th>
<th>UV method</th>
<th>LC method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration range (n = 6) (µg mL⁻¹)</td>
<td>10–60</td>
<td>10–60</td>
</tr>
<tr>
<td>LOD/LOQ (µg mL⁻¹)</td>
<td>1.2/3.7</td>
<td>0.4/1.2</td>
</tr>
<tr>
<td>The regression equation’s slope</td>
<td>0.0171</td>
<td>25.694</td>
</tr>
<tr>
<td>Standard error (Slope)</td>
<td>0.00052</td>
<td>0.2700</td>
</tr>
<tr>
<td>The regression equation’s intercept</td>
<td>−0.0072</td>
<td>−6.1333</td>
</tr>
<tr>
<td>Standard error (Intercept)</td>
<td>0.01878</td>
<td>6.68515</td>
</tr>
<tr>
<td>The coefficient of determination</td>
<td>0.9998</td>
<td>0.9999</td>
</tr>
<tr>
<td>Standard deviation (Residuals)</td>
<td>1.00</td>
<td>0.29</td>
</tr>
</tbody>
</table>

### Table 3. Data of precision tests

<table>
<thead>
<tr>
<th>Standard solutions µg mL⁻¹</th>
<th>UV method</th>
<th>R.S.D.</th>
<th>LC method</th>
<th>R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absorbance</td>
<td>R.S.D.</td>
<td>Peak Area</td>
<td>R.S.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Intraday</td>
<td>Interday</td>
<td>Intraday</td>
<td>Interday</td>
<td>Intraday</td>
</tr>
<tr>
<td>30 0.502</td>
<td>0.246</td>
<td>0.349</td>
<td>766</td>
<td>0.145</td>
</tr>
</tbody>
</table>

### Table 4. Data of recovery tests

<table>
<thead>
<tr>
<th>Methods</th>
<th>Spiked level</th>
<th>Total amount added µg mL⁻¹</th>
<th>Mean recovery* %</th>
<th>S.D.</th>
<th>R.S.D.* %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC Method</td>
<td>80</td>
<td>32</td>
<td>99.83</td>
<td>0.331</td>
<td>0.332</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>40</td>
<td>99.77</td>
<td>0.341</td>
<td>0.342</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>48</td>
<td>99.88</td>
<td>0.308</td>
<td>0.309</td>
</tr>
<tr>
<td>UV Method</td>
<td>80</td>
<td>32</td>
<td>99.73</td>
<td>0.679</td>
<td>0.681</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>40</td>
<td>99.82</td>
<td>0.577</td>
<td>0.578</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>48</td>
<td>99.78</td>
<td>0.512</td>
<td>0.513</td>
</tr>
</tbody>
</table>

(n=3), R.S.D. (%) = Percentage Relative Standard Deviation,

### Table 5. Robustness study data

<table>
<thead>
<tr>
<th>Method</th>
<th>Parameter</th>
<th>Value</th>
<th>Tailing factor</th>
<th>Number of theoretical plates</th>
<th>Content %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC method</td>
<td>Acetonitrile composition (%)</td>
<td>38</td>
<td>0.775</td>
<td>4,324</td>
<td>100.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42</td>
<td>0.739</td>
<td>4,343</td>
<td>99.92</td>
</tr>
<tr>
<td></td>
<td>Flow rate (mL min⁻¹)</td>
<td>1.1</td>
<td>0.753</td>
<td>4,352</td>
<td>99.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.3</td>
<td>0.746</td>
<td>4,337</td>
<td>100.02</td>
</tr>
<tr>
<td>UV method</td>
<td>Solvent</td>
<td>Methanol Sigma-Aldrich Cat: 34860 Methanol JT Baker Cat: 8402.2500</td>
<td>100.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Detection wavelengths</td>
<td>213</td>
<td>99.89</td>
<td>100.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>218</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
obtained during the analysis with both methods. The calculated \( t \)-value and \( F \)-value were found to be lower than the table values of both methods in the 95% confidence interval. It is clear from this report that both of the recommended UV and LC methods are applicable to the determination of oseltamivir in drug formulations appropriately. Data for statistical comparison results of LC and UV methods has been shown in Table 7.

**DISCUSSION**

In this study, two different methods, Reverse Phase High Performance Liquid Chromatography, which are frequently used in drug analysis, and spectrophotometric method, were developed in order to determine the amount of oseltamivir active ingredient in pharmaceutical formulations. At the same time, the chromatographic and spectrophotometric conditions of these developed methods were optimized.

For quantification of oseltamivir in pharmaceutical form or biological fluids, a number of analytical procedures have been published. These include spectrophotometric methods, spectrofluorimetric method, high performance liquid chromatographic methods, high performance thin layer chromatographic method, capillary electrophoresis, and liquid
Table 6. Method application results

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Label claim mg</th>
<th>Found oseltamivir mg</th>
<th>Assay % ± S.D.</th>
<th>Found oseltamivir mg</th>
<th>Assay % ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enfluvir</td>
<td>30 mg</td>
<td>29.97 mg</td>
<td>99.90 ± 0.14</td>
<td>29.89 mg</td>
<td>99.63 ± 0.52</td>
</tr>
</tbody>
</table>

Table 7. Data for statistical comparison results of LC and UV methods ($\alpha=0.05$, 95% confidence interval, n=6)

<table>
<thead>
<tr>
<th>Statistical values</th>
<th>LC method</th>
<th>UV method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average value</td>
<td>102.21</td>
<td>103.33</td>
</tr>
<tr>
<td>Standard deviation (S.D.)</td>
<td>0.65</td>
<td>1.72</td>
</tr>
<tr>
<td>Relative standard deviation (R.S.D., %)</td>
<td>0.63</td>
<td>1.66</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.26</td>
<td>0.70</td>
</tr>
<tr>
<td>$F_{calculated}/F_{table}$</td>
<td>0.14/0.20</td>
<td></td>
</tr>
<tr>
<td>$t_{calculated}/t_{table}$</td>
<td>1.78/2.57</td>
<td></td>
</tr>
</tbody>
</table>

CONCLUSION

UV spectrophotometric methods generally do not require complex operations and procedures. It takes less time and is economical. These cases are advantages of UV method over LC method. Statistically compared, the LC method is more precise and accurate than the UV method. Statistical tests revealed that there was no significant difference between the experimental values obtained during the analysis with both methods. It is clear from this report that both of the recommended UV and LC methods are applicable to the determination of oseltamivir in drug formulations appropriately. Excipients in pharmaceutical preparations have not interfered with the and the mobile phase can be prepared very easily. Both suggested analytical methods are reproducible, precise and linear and can be used for routine analysis of oseltamivir in different pharmaceutical forms.

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