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# DEVELOPMENT AND VALIDATION FOR HIGH-PERFORMANCE MASS SPECTROMETRY METHOD FOR DETERMINATION OF BALOXAVIR MARBOXIL IN BIOLOGICAL MATRICES

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**Keywords:**

Baloxavir marboxil, Human plasma, HPLC-ESI-MS/MS, Bioanalysis

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**ABSTRACT:** The validated protein precipitation method was applied for the estimation of Baloxavir marboxil in human plasma with Oseltamivir as an internal standard (ISTD) by using HPLC-ESI-MS/MS. The chromatographic separation was achieved with 0.1% formic acid in combination with methanol (25:75 v/v) using the $C_{18}$ column Ascentis Express (50 mm × 4.6 mm, 2.7 µm). The total analysis time was 3 min and the flow rate was set to 0.6 ml/min. The mass transitions of Baloxavir marboxil and Oseltamivir obtained were m/z 572.6→250.3 and 313.2→269.1. The standard curve shows a correlation coefficient ($r^2$) greater than 0.9983 with a range of 5.00-10000.00 pg/ml using the linear regression model.

**INTRODUCTION:** Influenza virus can rapidly spread in populations and are responsible for seasonal influenza epidemics around the world every year [1]. Influenza virus infection can lead to serious and fatal outcomes, especially in elderly or immunocompromised patients [2]. Although, influenza vaccination represents the key option for preventing influenza virus infection and some strategies have been investigated to optimize immunogenicity by exploring new vaccines, vaccination doses, timing or adjuvants, its benefit in immunocompromised individuals is somewhat controversial [3, 4]. Additionally, vaccine mismatch has frequently occurred between the vaccine strain and the circulating strain [5].

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Therefore, anti-influenza drugs play an important role in the control of influenza virus infections especially for patients with or at risk of severe infection and complications.

Currently, neuraminidase (NA) inhibitors are the most widely used class of anti-influenza drugs [6]. However, the emergence of influenza viruses resistant to NA inhibitors is an issue of concern [7]. In addition, previous clinical studies have indicated that NA inhibitors must be administered within 48 hours of the onset of symptoms [8]. This is difficult to do because diagnosis is often delayed [9]. Thus, novel therapeutics that can extend the therapeutic window is needed if treatment is started from more than 48 h after the onset of symptoms.

Toward this aim, the recent availability of high-quality structural information of the influenza virus RNA polymerase complex [10] has led to the development of antiviral drugs that target the critical roles of the proteins involved in virus replication [11].

Baloxavir marboxil (BM) is an orally available small-molecule inhibitor of cap-dependent endonuclease (CEN), an enzyme residing on the PA subunit of the influenza virus polymerase that mediates the cap-snatching process during viral mRNA biosynthesis [13–15]. Baloxavir marboxil has been approved for clinical use in adults and adolescents in Japan and the United States. Baloxavir acid (BA), the active form of the prodrug Baloxavir marboxil [16], exhibited several times greater antiviral activity for type A virus than type B virus, but shows potent and broad-spectrum inhibitory activity against seasonal, avian, and swine influenza viruses *in-vitro* [17].

Baloxavir marboxil **Fig. 1** is an antiviral drug with activity against the influenza virus. It is supplied as white to light yellow film-coated tablets for oral administration and the molecular weight of 571.55. It is freely soluble in dimethyl sulfoxide, soluble in acetonitrile, slightly soluble in methanol and ethanol and practically insoluble in water. The chemical name of baloxavir marboxil is ({(12aR)-12-[(11S)-7, 8-Difluoro-6, 11-dihydrodibenzo[b, e] thiepin11-yl]-6, 8-dioxo-3,4,6,8,12,12a-hexahydro-1H-[1,4]oxazino[3,4-c]pyrido[2,1-f][1, 2, 4]triazin-7-yl}oxy) methylmethyl carbonate. The empirical formula of baloxavir marboxil is $C_{27}H_{23}F_2N_3O_7S$.

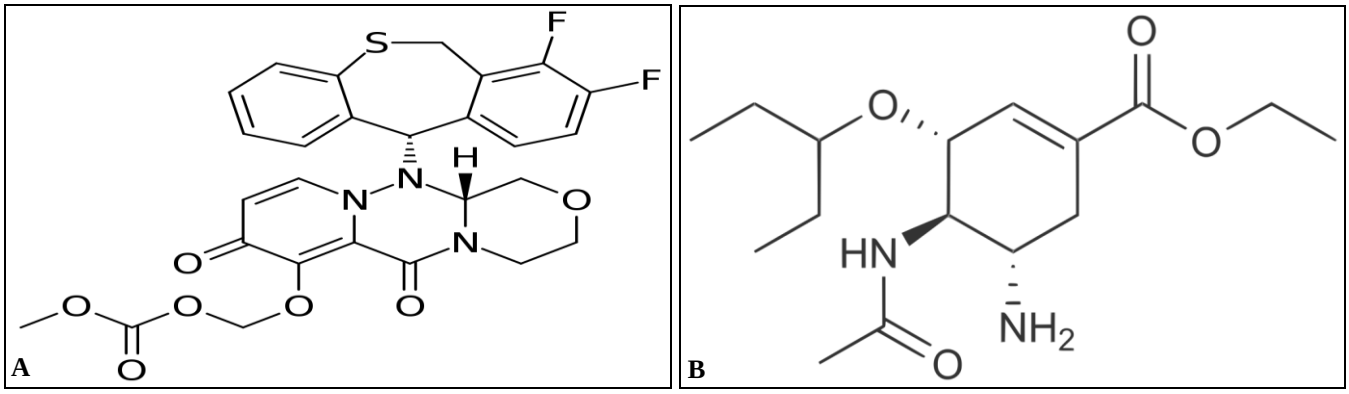


**FIG. 1: CHEMICAL STRUCTURES OF A) BALOXAVIR MARBOXIL (BM) B) OSELTAMIVIR (OMIS)**

As best our knowledge none of the methods were reported for estimation of Baloxavir marboxil (BM) in biological samples by LC-MS/MS using Oseltamivir (OMIS) as an internal standard.

The main goal of the present study is to develop and validate the novel simple, sensitive, selective, rapid, rugged and reproducible analytical method for the quantitative determination of Baloxavir marboxil (BM) in human plasma by HPLC-ESI-MS/MS with a small amount of sample volume [27-29].

## MATERIALS AND METHODS:

### Materials:

**Chemical Resources:** Baloxavir marboxil (MedKoo Biosciences, USA) and Oseltamivir (Clearsynth, Mumbai, India), methanol and acetonitrile (J.T Baker, USA), formic acid (Merck, Mumbai, India), Ultrapure water (Milli-Q system, Millipore, Bedford, MA, USA), human plasma (Doctors Pathological Labs, Hyderabad, India). The chemicals and solvents were used in this study analytical and HPLC grade.

**Instrument Resources:** An API 4000 HPLC-ESI-MS/MS system (Applied Biosystems), 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany), data acquisition and processing were accomplished using Analyst® Software 1.4.1.

### Methods:

**Chromatographic Conditions:** The chromatographic separation was achieved with 0.1% formic acid in combination with methanol (25:75 v/v), gave the best peak shape and low baseline noise was observed using the Ascentis Express $C_{18}$ (50 mm × 4.6 mm, 2.7 µm). The total analysis time was 3 min and the flow rate was set to 0.6 ml/min. The temperature was set to 40 °C for the column oven. The sample volume for the injection into mass spectrometry was adjusted to 10 µl for better ionization and chromatography.

**Detection:** The pure drug of Baloxavir marboxil and Oseltamivir were prepared in methanol (10.00 ng/mL) and injected with a flow rate of 5 µL/min into positive ion mode mass spectrometer for optimization of mass parameters like source

temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra-high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized. The analysis was performed using MRM positive ion mode with mass transitions of m/z (amu) 572.6→250.3 and 313.2→269.1 for Baloxavir marboxil and Oseltamivir. The mass fragmentation pattern of parent and product ions mass spectras were depicted in **Fig. 2** and **3**.

**Standard Calibration and Quality Control Samples Preparation:** Stock solutions of Baloxavir marboxil (1000.00 µg/ml) and Oseltamivir (1000.00 µg/ml) were prepared in methanol. The internal standard (Oseltamivir) spiking solution (500.00 ng/ml) was prepared in 75% methanol from the Oseltamivir stock solution. Stock solutions of Baloxavir marboxil, Oseltamivir and intermediate spiking solutions were stored in refrigerated conditions (2-8 °C) until analysis.

Calibration standards (5.00, 10.00, 50.00, 100.00, 500.00, 1000.00, 2000.00, 4000.00, 6000.00, 8000.00 and 10000.00 pg/ml), quality control samples of lower limit QC, low QC, mid-QC, high QC (5.00, 15.00, 3000.00, 7000.00 pg/ml) were used by spiking the appropriate amount of standard solution in the drug-free plasma and stored at -30 °C till analysis.

**Sample Extraction:** The protein precipitation method was applied to extract Baloxavir marboxil and Oseltamivir. To each labeled polypropylene tube 50 µl of Oseltamivir (500.00 ng/ml) was mixed with the 100 µl plasma sample, then 0.25 ml of acetonitrile were added, vortexed for 5 min and centrifuged at 4000 rpm for 10 min at 20 °C. The organic phase was transferred to autosampler vials containing 100 µl of 0.1% formic acid and injected into the HPLC-ESI-MS/MS for analysis.

**Method Validation:** The developed method was validated over a linear concentration range of 5.0–10000.0 ng/ml. The validation parameters include selectivity and specificity, LOQ, linearity, precision and accuracy, matrix effect, recovery, stability (freeze-thaw, autosampler, benchtop, long term) was evaluated under validation section.

**Selectivity and Specificity:** Ten lots of blank plasma samples were analyzed out of which six lots free from interference were selected for assessing the selectivity and specificity. The endogenous/ potential interfering peak areas for blank samples must be less than 20% of the LLOQ peak area of Baloxavir marboxil retention time and less than 5% for Oseltamivir retention time.

**Limit of Quantification (LOQ):** Six LLOQ standards were prepared in screened plasma lot along with IS (500.00 ng/ml) and signal to noise ratio (S/N) was calculated using analyst software.

**Linearity:** Calibration standards were prepared to obtain a linearity range of 5.00, 10.00, 50.00, 100.00, 500.00, 1000.00, 2000.00, 4000.00, 6000.00, 8000.00 and 10000.00 pg/ml and assayed in five replicates on five different days.

**Precision and Accuracy:** One set of calibration standards and one set contains four different concentrations of quality control standards of Lower limit QC (5.00 pg/ml), Low QC (15.00 pg/ml), Mid QC (3000.00 pg/ml) and High QC (7000.00 pg/ml) concentrations were prepared in screened plasma and analyzed each quality control (QC) standards in six replicates on the same day (Intraday) and five different days (Inter day).

**Matrix Effect:** Six extracted blank plasma samples in three replicates were spiked with the un-extracted concentration of mid-QC (3000.00 pg/ml) and compared with un-extracted standards of the same concentration.

**Recovery:** The recovery of samples was performed by protein precipitation method. The extraction recovery was determined in sextuplicate by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low (15.00 pg/ml), medium (300.00 pg/ml), high (7000.00 pg/ml).

**Stability Studies:**

**Bench Top Stability (Room Temperature Stability, 24 h):** Six replicates of spiked low and high concentrations (BT stability samples) were set aside at ambient temperature up to 24 h. Samples were processed and compared with newly prepared low and high concentrations (comparison samples).

**Freeze and Thaw Stability (After 3rd Cycle at -30°C):** Six replicates of low and high conc. (FT

stability samples) were frozen at -30 °C and subjected to three freeze-thaw cycles of 24, 36 and 48 h (-30 °C to room temperature) and compared with newly prepared low and high concentrations (comparison samples).

**Autosampler Stability (2-8 °C, 65 h):** Six replicates of low and high concentrations (AS stability samples) were stored in auto-sampler up to 65 h at 2-8 °C. Stability samples were compared with newly prepared low and high concentrations (comparison samples).

**Long-Term Stability (-30 °C, 45 Days):** After completion of the stability period stored at -30 °C (45 days) six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).

## RESULTS AND DISCUSSION:

**Method Development:** On the way to develop a simple and easily applicable method for the determination of Baloxavir marboxil in human plasma, HPLC-MS/MS was selected as the method of choice. During the method development process chromatographic (mobile phase composition, column, flow rate, injection volume, sample volume), mass spectrometric, sample extraction and internal standard parameters were optimized in logical and sequential manner to achieve the best results.

Separation of the Baloxavir marboxil was performed with different branded RP-HPLC $C_{18}$ columns. Initial separation was performed with isocratic elution of 10mM ammonium formate and acetonitrile was selected as a mobile phase in varying combinations that were tried, but a low response was observed. A mobile phase consisting of 0.1% acetic acid: acetonitrile (20:80 v/v) and 0.1% acetic acid: methanol (20:80 v/v) gave the best response, but poor peak shape was observed.

After a series of trials a mobile phase consisting of 0.1% formic acid in combination with methanol and acetonitrile in varying combinations was tried. Using a mobile phase containing 0.1% formic acid in combination with methanol (25:75 v/v), gave the best signal along with a marked improvement in the peak shape and low baseline noise was observed using the Ascentis Express $C_{18}$ (50 mm × 4.6 mm, 2.7 µm) analytical column with a flow rate of 0.6 ml/min and reduced runtime to 3 min. The column oven temperature was kept at a constant temperature of about 40 °C and the temperature of autosampler was maintained at 4 °C. An injection volume of 10 µl sample was adjusted for better ionization and chromatography. For the selection of internal standard, oseltamivir, zanamivir, rimantadine and amantadine were tried with optimized mobile phase and column conditions. Finally, Oseltamivir was selected as an internal standard in terms of better chromatography and extractability.

The retention times of analyte (Baloxavir marboxil) and internal standard (Oseltamivir) were eluted at 1.42 ± 0.2 min and 1.44 ± 0.2 min respectively with 3 min total runtime. Different procedures like PPT (Protein precipitation), SPE (solid phase extraction) and LLE (liquid-liquid extraction) methods were optimized. Out of all, it was observed that the PPT was suitable due to simple extraction, high recovery and the less ion suppression effect on drug and internal standard.

Electrospray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of the mobile phase to electrospray ion source operated at a flow rate of 20 µl/min. Baloxavir marboxil gave more response in positive ion mode as compared to the negative ion mode.

To get high intense production source dependent parameters were optimized like nebulizer gas flow 30 psi, CAD gas and curtain gas flow 25 psi, ion spray voltage 5500 V, and temperature 500 °C. The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized during tuning as 35, 25, 10, 20, 12 eV for Baloxavir marboxil and Oseltamivir, respectively. The collision activated dissociation (CAD) gas was set at 4 psi using nitrogen gas. Quadrupole-1 and quadrupole-3 were both maintained at a unit resolution and dwell time was set at 200 ms for Baloxavir marboxil and Oseltamivir.

The predominant peaks in the primary ESI spectra of Baloxavir marboxil and Oseltamivir correspond to the $MH^+$ ions at m/z 572.6 and 313.2 respectively. Productions of Baloxavir marboxil and Oseltamivir scanned in quadrupole-3 after a collision with nitrogen in quadrupole-2 had a m/z of 250.3 and 269.1 for both respectively. The parent and productions mass spectrums of Baloxavir marboxil and Oseltamivir were shown in **Fig. 2** and **3**.

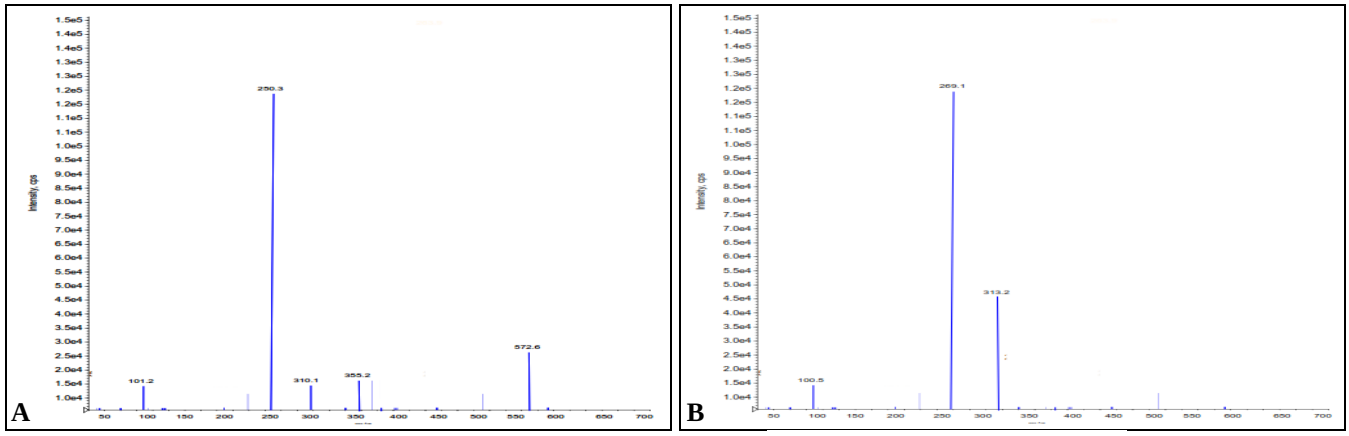


**FIG. 2: PARENT ION MASS SPECTRA (Q1) AND (Q3) OF A) BALOXAVIR MARBOXIL B) OSELTAMIVIR**

**Method Validation:**

**Selectivity and Specificity, Limit of Quantification (LOQ):** No significant response was observed at retention times of Baloxavir marboxil and Oseltamivir in blank plasma as compared to LLOQ and blank with IS samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 5.0 ng/ml. Represent chromatograms were shown in **Fig. 3**.

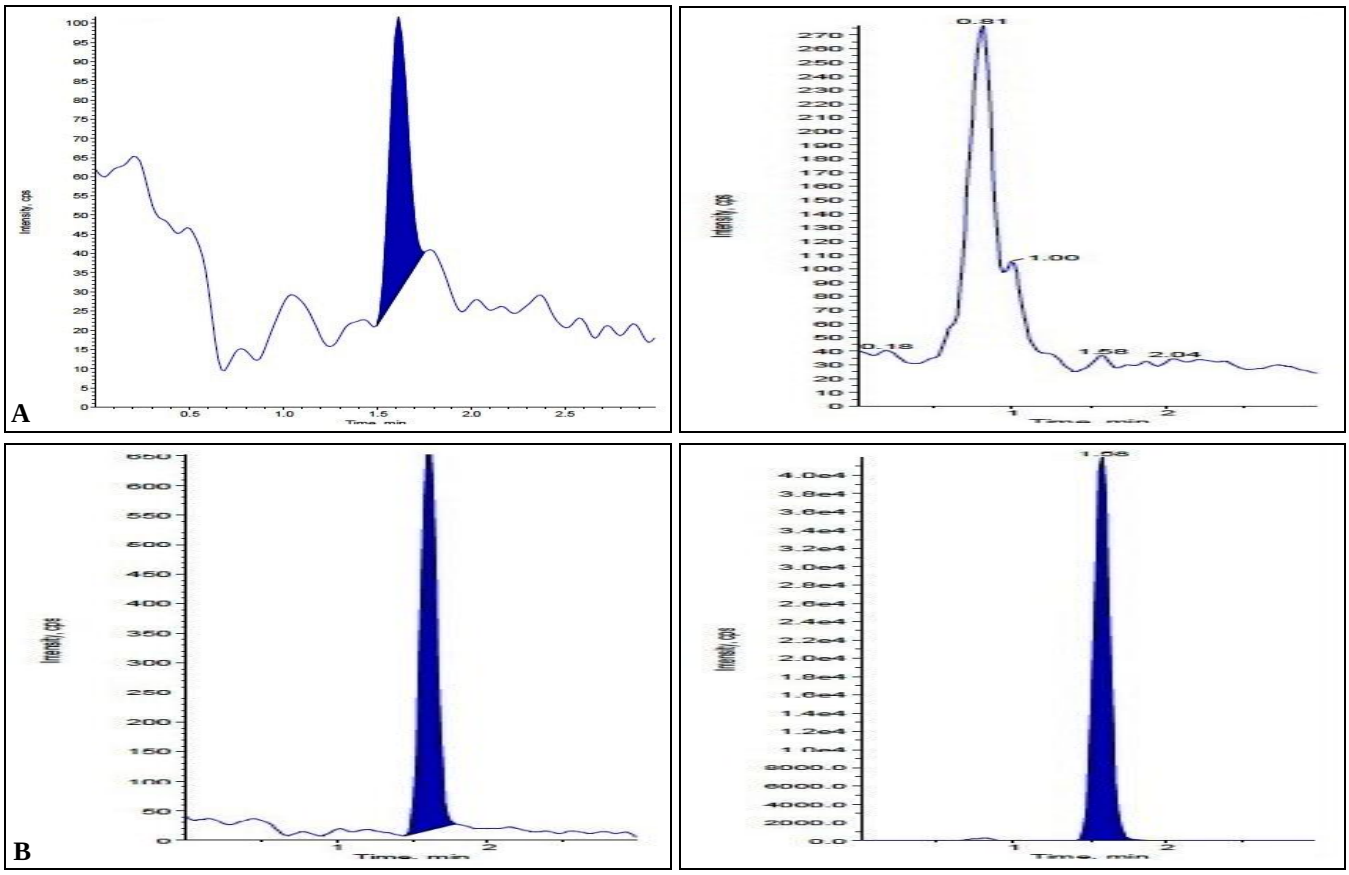


**FIG. 3: REPRESENTATIVE CHROMATOGRAMS OF BALOXAVIR MARBOXIL IN PLASMA SAMPLES A) BLANK PLASMA CHROMATOGRAM FOR INTERFERENCE FREE BALOXAVIR MARBOXIL AND OSELTAMIVIR B) CHROMATOGRAM OF LLOQ SAMPLE (BALOXAVIR MARBOXIL AND OSELTAMIVIR)**

**Linearity:** Linearity was plotted as a peak area ratio (Baloxavir marboxil peak area / Oseltamivir peak area) on the y-axis against Baloxavir marboxil concentration (pg/ml) on the x-axis. Calibration curves were found to be consistently accurate and precise for Baloxavir marboxil over a linearity range of 5 to 10000.00 pg/ml. The correlation coefficient was greater than 0.9980 for Baloxavir marboxil. The %CV was less than 15% and mean %accuracy was ranged between 99.40 - 102.67%. Results were presented in **Table 1**.

**TABLE 1: CALIBRATION CURVE DETAILS OF BALOXAVIR MARBOXIL**

| Spiked plasma Concentration (pg/ml) | Concentration measured (pg/ml) (Mean ± SD) | %CV (n=5) | %Accuracy |
|---|---|---|---|
| 5.00 | 5.00 ± 0.03 | 0.54 | 99.96 |
| 10.00 | 10.17 ± 0.14 | 1.37 | 101.7 |
| 50.00 | 50.69 ± 0.32 | 0.62 | 101.3 |
| 100.00 | 100.16 ± 0.70 | 0.70 | 100.1 |
| 500.00 | 500.70 ± 0.90 | 0.18 | 100.1 |
| 1000.00 | 10016.98 ± 27.55 | 2.71 | 101.7 |
| 2000.00 | 1988.00 ± 44.94 | 2.26 | 99.4 |
| 4000.00 | 4068.00 ± 72.59 | 1.78 | 101.7 |
| 6000.00 | 6160 ± 140.36 | 2.28 | 102.6 |
| 8000.00 | 8122.00 ± 137.19 | 1.69 | 101.5 |
| 10000.00 | 10057.43 ± 90.44 | 0.90 | 100.5 |

**Precision and Accuracy:** Intra and inter batch % accuracy for Baloxavir marboxil was ranged between 94.17-102.40 and 91.66 to 99.34. % CV is 2.16 to 5.64 and 1.64% to 3.24%. Results are presented in **Table 2**.

**TABLE 2: PRECISION AND ACCURACY (ANALYSIS WITH SPIKED SAMPLES AT THREE DIFFERENT CONCENTRATIONS) OF BALOXAVIR MARBOXIL**

| Spiked Plasma Concentration (pg/ml) | Within-run (Intra-day) | | | Between-run (Inter-Day) | | |
|---|---|---|---|---|---|---|
| | Concentration measured (n=6; pg/ml; mean ± SD) | % CV | % Accuracy | Concentration measured (n=6; pg/ml; mean ± SD) | % CV | % Accuracy |
| 15.00 | 14.22 ± 0.80 | 5.64 | 94.78 | 13.75 ± 0.44 | 3.24 | 91.66 |
| 3000.00 | 2880.07 ± 62.29 | 2.16 | 102.4 | 2980.07 ± 48.95 | 1.64 | 99.34 |
| 7000.00 | 6591.67 ± 245.83 | 3.73 | 94.17 | 6828.30 ± 163.88 | 2.40 | 97.55 |

**Recovery:** The mean % recovery for LQC, MQC, HQC samples of Baloxavir marboxil were 99.85%, 95.30%, and 93.54% respectively. The overall mean % recovery and % CV of Baloxavir marboxil across QC levels is 96.23% and 3.38%. For the Oseltamivir (internal standard) the mean % recovery and % CV is 91.68% and 7.09%.

**Matrix Effect:** No significant matrix effect found in different sources of rat plasma tested for Baloxavir marboxil, Oseltamivir. The % CV was found to be 3.71.

**Stability (Freeze-Thaw, AutoSampler, Bench Top, Long Term):** Quantification of the Baloxavir marboxil in plasma subjected to three freeze-thaw cycles (-30 °C to room temperature), autosampler (processed), room temperature (Benchtop), long-term stability details were shown in **Table 3**.

**TABLE 3: STABILITY STUDIES OF BALOXAVIR MARBOXIL IN SPIKED PLASMA SAMPLES**

| Spiked Plasma conc. (pg/ml) | Room temperature Stability | | Processed sample Stability | | Long term stability | | Freeze and thaw stability | |
|---|---|---|---|---|---|---|---|---|
| | 24h | | 65h | | 45 days | | Cycle (48h) | |
| | Concentration measured (n=6; pg/ml; Mean ± SD) | %CV (n=6) | Concentration measured (n=6; pg/ml; Mean ± SD) | %CV (n=6) | Concentration measured (n=6; pg/ml; Mean ± SD) | %CV (n=6) | Concentration measured (n=6; pg/ml; Mean ± SD) | %CV (n=6) |
| 15.00 | 12.89 ± 1.01 | 7.8 | 14.07 ± 0.74 | 5.3 | 14.30±1.26 | 8.8 | 14.27 ± 0.84 | 5.8 |
| 7000.00 | 6511.67 ±585.64 | 8.9 | 6066.67 ±575.21 | 9.5 | 6058.33 ±570.03 | 9.4 | 6665.0 ±182.51 | 2.7 |

**CONCLUSION:** The method described in this manuscript has been developed and validated over the concentration range of 5.0–10000.0 pg/ml in human plasma. The intra and inter-batch precision (%CV) was less than 6.0% and % accuracy ranged from 98.9%-102.4%.

The overall %recovery for Baloxavir marboxil, Oseltamivir was greater than 90%. The selectivity, sensitivity, precision, and accuracy obtained with this method make it suitable for the purpose of the present study. In conclusion, the method used in the present study is easy and fast to perform; it is also characterized by adequate accuracy, precision, selectivity and stability. The simplicity of the method, and using rapid protein precipitation extraction with less run time of 3.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of Baloxavir marboxil.

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**CONFLICTS OF INTEREST:** Authors declare that there is no conflict of interest.

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