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METHOD DEVELOPMENT AND VALIDATION OF BALOXAVIR MARBOXIL BY LCMS

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ABSTRACT:

Objective: The goal of this work was to create a validated LC-MS/MS method for measuring baloxavir Marboxil

Methods: Using a Phenomenex C18 HPLC column (50 mm 4.6 mm, 5 m, 100), the chromatographic separation was achieved with 0.1 percent formic acid in combination with methanol (30:70 v/v), which offered the best peak shape and minimum baseline noise. The flow rate was set at 0.3 ml/min and the entire analytical time was 3 minutes.

Results: The method presented in this paper was developed and verified in human plasma at concentrations ranging from 5.0 to10000.00 pg/ml. The intra-batch precision (% CV) was less than 6.0 percent, and the percent accuracy ranged from 94.08 percent to 98.4 percent. For Baloxavir marboxil, Oseltamivir, the total percent recovery was greater than 90%.

Conclusion: The method employed in this study is simple and quick to implement, as well as having appropriate accuracy, precision, selectivity, and stability. The method's simplicity, as well as the fact that it uses quick protein precipitation extraction with a shorter run time of 3.0 minutes per sample, make it appealing for high-throughput bioanalysis of Baloxavir marboxil.

KEYWORDS: Baloxavir, Oseltamivir, Lcms, Electrospray ionization, Influenza virus

INTRODUCTION:

Baloxavir, marketed under the brand name Xofluza, is an antiviral drug used to treat influenza A and B flu1 [1]. Baloxavir marboxil was created as a prodrug approach, with the active agent, baloxavir acid, released through its metabolism (BXA). The influenza virus's dependent endonuclease activity, one of the virus polymerase's activities, is then targeted by BXA, which acts as an inhibitor [2-4].

Baloxavir marboxil (BM) is an orally accessible small-molecule inhibitor of cap-dependent endonuclease (CEN), an enzyme that mediates the cap-snatching process during viral mRNA biosynthesis and is found on the PA subunit of influenza virus polymerase. In Japan and the United States, baloxavir marboxil has been licenced for clinical use in adults and adolescents. The active ingredient in baloxavir is baloxavir acid (BA)[5-9].

The antiviral medication baloxavir marboxil (Fig. 1) has activity against the influenza virus. It has a molecular weight of 571.55 and is delivered as white to light yellow film-coated tablets for oral administration [9-13]. It is soluble in dimethyl sulfoxide, acetonitrile, methanol, and ethanol to a lesser extent, but completely insoluble in water. ((12aR)- 12-[(11S)-7, 8-Difluoro-6, 11-dihydrodibenzo[b,e]thiepin11-yl] is the chemical name for baloxavirmarboxil.-6,8-dioxo-3,4,6,8,12,12a-hexahydro-1H[1,4]oxazino[3,4-c]methylcarbonate (pyrido[2,1-f][1, 2, 4]triazin-7-yloxy) C27H23F2N3O7S is the empirical formula for baloxavir marboxil[14-17].

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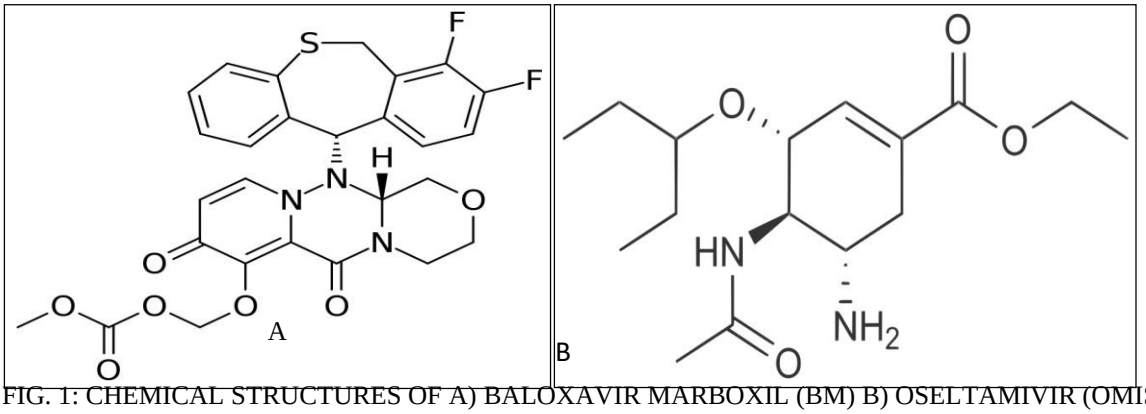

FIG. 1: CHEMICAL STRUCTURES OF A) BALOXAVIR MARBOXIL (BM) B) OSELTAMIVIR (OMIS)

The primary purpose of this work is to develop and validate a novel HPLC-ESI-MS/MS method for the quantitative detection of Baloxavir marboxil (BM) in human plasma using a little amount of sample volume [27-29].

MATERIALS AND METHODS:

Materials:

Chemical Resources: Methanol and acetonitrile, formic acid, Ultrapure water (Merck Mumbai India), human plasma (Doctors Pathological Labs, Hyderabad, India).Baloxavir marboxil gift sample is provided by spectrum pharma research solutions,Hyderabad. In this work, analytical and HPLC grade chemicals and solvents were used.

.**Instrument:** Shimadzu HPLC system (Kyoto, Japan) consisted of a pump (LC-20 AD), an automatic sampler (SIL-20 AC HT) and a unit for online degasser (DGU-20A3). An API 3200 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) with an electrospray ion source (ESI) operating in positive and negative mode was used for mass spectrometric analysis.

Methods:

Chromatographic Conditions: Using a Phenomenex C18 HPLC column (50 mm 4.6 mm, 5 m, 100), the chromatographic separation was achieved with 0.1 percent formic acid in combination with methanol (30:70 v/v), which offered the best peak shape and minimum baseline noise. The flow rate was set at 0.3 ml/min and the entire analytical time was 3 minutes. The column oven was preheated to 40 degrees Celsius.. For better ionisation and chromatography, the sample volume for injection into mass spectrometry was adjusted to 10µl.

Detection: The pure drugs Baloxavir marboxil and Oseltamivir were prepared in methanol (10.00 ng/mL) and injected into a positive ion mode mass spectrometer at a flow rate of 5 L/min for mass parameter optimization, including 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 optimised.. Baloxavir marboxil and Oseltamivir were analysed using the MRM positive ion mode, with mass transitions of m/z (amu) 572.8251.3 and 314.2268.8 for Baloxavir marboxil and Oseltamivir, respectively. Figures 2 and 3 show the mass fragmentation pattern of parent and product ions mass spectra.

Standard Calibration and Quality Control Samples Preparation: Stock solutions of Baloxavir marboxil (1000.00 g/ml) and Oseltamivir (1000.00 g/ml) were produced in methanol for standard calibration and quality control samples. The internal standard (Oseltamivir) spiking solution (500.00 ng/ml) was made from the Oseltamivir stock solution in 75 percent methanol. Until analysis, stock solutions of Baloxavir marboxil, Oseltamivir, and intermediate spiking solutions were kept refrigerated (2-8 °C).

Quality control samples of lower limit QC, low QC, mid-QC, and high QC (5.00, 15.00, 3000.00, 7000.00 pg/ml) and calibration standards (5.00, 10.00, 50.00, 100.00, 500.00, 1000.00, 2000.00, 4000.00, 6000.00, 8000.00, and

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10000.00 pg/ml) were used by spiking the appropriate amount of standard solution in the drug-free plasma and stored at -30°C until analysis.

Sample Extraction: Baloxavir marboxil and Oseltamivir were extracted using the protein precipitation technique. 50 µl of Oseltamivir (500.00 ng/ml) was combined with 100 µl plasma sample in each labelled polypropylene tube, then 0.25 ml of acetonitrile was added, vortexed for 5 min, then centrifuged at 4000 rpm for 10 min at 20 °C. The organic phase was injected into the HPLC-ESI-MS/MS for analysis after being transferred to autosampler vials containing 100µ l of 0.1 percent formic acid.

Method Validation: Over a linear concentration range of 5.0–10000.0 ng/ml, the described approach was validated. Under the validation section, selectivity and specificity, LOQ, linearity, precision and accuracy, matrix effect, recovery, and stability (freeze-thaw, autosampler, benchtop, long term) were assessed.

Selectivity and Specificity: Six lots of interference-free blank plasma samples were chosen for testing selectivity and specificity from ten lots of blank plasma samples. For blank samples, the endogenous/potential interfering peak areas must be less than 20% of the LLOQ peak area of Baloxavir marboxil retention time and less than 5% of the LLOQ peak area of 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 with 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 were created and evaluated in five duplicates on five distinct days..

Precision and Accuracy: One set of calibration standards and one set of quality control standards with four different concentrations 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) were prepared in screened plasma and analysed in six replicates on the same day (Intraday) and five different days (Inter day).

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

Recovery: The protein precipitation method was used to recover the samples. At three distinct concentrations of low (15.00 pg/ml), medium (300.00 pg/ml), and high (7000.00 pg/ml), the extraction recovery was measured in sextuplicate by comparing extracted QC standards with un- extracted QC standards.

Stability Studies:

Bench Top Stability (Room Temperature Stability, 24 h): Six duplicates of spiked low and high concentrations (BT stability samples) were stored for up to 24 hours at room temperature. Processed samples were compared to freshly created low and high concentrations (comparison samples).

Freeze and Thaw Stability (After 3rd Cycle at - 30°C): Six low and high concentration replicates (FT stability samples) were frozen at -30 °C and subjected to three freeze-thaw cycles of 24, 36, and 48 hours (-30 °C to room temperature) before being compared to newly generated low and high concentrations (comparison samples).

Autosampler Stability (2-8 °C, 60 h): Six replicates of low and high concentrations (AS stability samples) were kept at 2-8 °C for 60 hours in an auto-sampler. Newly manufactured low and high concentrations were compared to stability samples (comparison samples).

Long-Term Stability (-30 °C, 45 Days): Six duplicates of low and high concentrations (LT stability samples) were compared with newly generated low and high concentrations after the 45-day stability period at -30 °C (comparison samples).

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RESULTS AND DISCUSSION:

Method Development: HPLC-MS/MS was chosen as the method of choice for determining Baloxavir marboxil in human plasma on the road to developing a simple and simply applicable method.. Chromatographic (mobile phase composition, column, flow rate, injection volume, sample volume) and mass spectrometric (sample extraction and internal standard parameters) parameters were tuned in a logical and sequential manner during the technique development process to produce the best results.

Different brands RP-HPLC C18 columns were used to separate the Baloxavir marboxil.. The initial separation was done with isocratic elution of 10mM ammonium formate and acetonitrile as the mobile phase in a variety of combinations, however the response was modest. The best response was obtained with a mobile phase of 0.1 percent acetic acid: acetonitrile (30:70 v/v) and 0.1 percent acetic acid: methanol (30:70 v/v), however peak shape was unsatisfactory.

A mobile phase containing 0.1 percent formic acid, methanol, and acetonitrile in various combinations was explored after a series of trials.. The best signal was obtained using a mobile phase containing 0.1 percent formic acid in combination with methanol (30:70 v/v) utilising the Phenomenex C18 HPLC column (50 mm 4.6 mm, 5 m, 100) analytical column with a flow rate of 0.3 ml/min and a decreased runtime of 3 minutes. The temperature of the column oven was kept constant at around 40 °C, while the temperature of the autosampler was fixed at 4 °C. For improved ionisation and chromatography, a 10µ l sample injection volume was modified. Oseltamivir, zanamivir, rimantadine, and amantadine were tested as internal standards with optimised mobile phase and column conditions. Finally, because of its superior chromatography and extractability, Oseltamivir was chosen as an internal standard.

With a total runtime of 3 minutes, the retention periods of the analyte (Baloxavir marboxil) and internal standard (Oseltamivir) were eluted at 1.38± 0.2 min and 1.40± 0.2 min, respectively. Different processes were optimised, including PPT (Protein precipitation), SPE (solid phase extraction), and LLE (liquid-liquid extraction). First and foremost, the PPT was found to be useful because to its ease of extraction and high resolution.and the less ion suppression effect on drug and internal standard.

Electrospray ionisation (ESI) was used for this approach because it provided the best response over air pressure chemical ionisation (APCI). During infusion of the analyte in a continuous flow of the mobile phase to an electrospray ion source operating at a flow rate of 20µ l/min, the instrument was optimised for sensitivity and signal stability. Baloxavir marboxil elicited a stronger reaction.. gave more response in positive ion mode as compared to the negative ion mode.

Source dependent parameters such as nebulizer gas flow 30 psi, CAD gas and curtain gas flow 25 psi, ion spray voltage 5500 V, and temperature 500 °C were tuned to achieve high intensity production. During tuning, compound-dependent parameters such declustering potential (DP), focusing potential (FP), entry potential (EP), collision energy (CE), and cell exit potential (CXP) were tuned. as 35, 25, 10, 20, 12 eV for Baloxavir marboxil and Oseltamivir, respectively. Nitrogen gas was used to set the collision activated dissociation (CAD) gas at 4 psi. For Baloxavir marboxil and Oseltamivir, quadrupole-1 and quadrupole-3 were both kept at a unit resolution, and dwell time was set at 200 ms.

The MH+ ions at m/z 572.6 and 313.2 correspond to the prominent peaks in the primary ESI spectra of Baloxavir marboxil and Oseltamivir, respectively. After a collision with nitrogen in quadrupole-2, the m/z of Baloxavir marboxil and Oseltamivir produced in quadrupole-3 was 250.3 and 269.1, respectively. Baloxavir marboxil and Oseltamavir's parent and manufacturing mass spectrums were shown in **Fig. 2** and **3**.

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Fig. 2: parent ion mass spectra (q1) and (q3) of a) baloxavir marboxil b) oseltamivir

Method Validation:

Selectivity and Specificity, Limit of Quantification (LOQ): There was no significant difference in Baloxavir marboxil and Oseltamivir retention durations in blank plasma when compared to LLOQ and blank with IS samples. The lowest concentration of the calibration curve, which was verified to be 5.0 ng/ml, was established as the method's limit of quantification. Figure 3 depicts representative chromatograms..

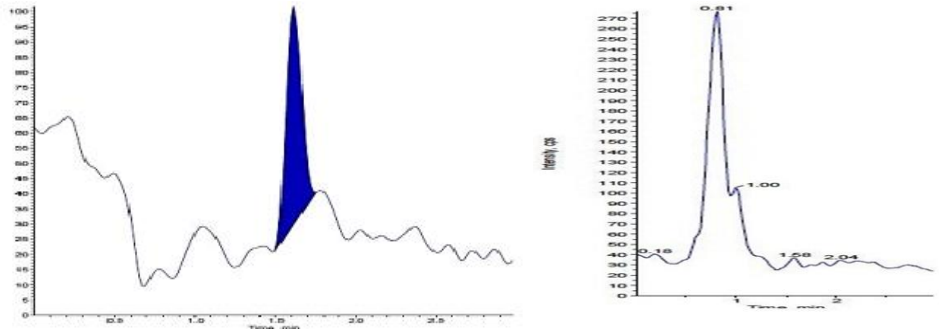

Fig. 3: representative chromatograms of baloxavir marboxil in plasma samples and oseltamivir**.**

Linearity : On the y-axis, linearity was represented as a peak area ratio (Baloxavir marboxil peak area / Oseltamivir peak area) versus Baloxavir marboxil concentration (pg/ml). Over a linearity range of 5 to 10000.00 pg/ml, calibration curves for Baloxavir marboxil were shown to be consistently accurate and exact. For Baloxavir marboxil, the correlation coefficient was greater than 0.9980. The mean percent accuracy ranged from 98.36 to 101.79 percent, and the percent CV was less than 15%. Table 1 summarises the findings.

TABLE 1: CALIBRATION CURVE DETAILS OF BALOXAVIR MARBOXIL

Spiked plasma Concentration (pg/ml)	Concentration measured(pg/ml) (Mean ± SD)	%CV (n=6)	%Accuracy
5.00	5.13 ± 0.03	0.58	99.42
10.00	10.18 ± 0.14	1.37	101.37
50.00	50.34 ± 0.32	0.63	99.37
100.00	100.09 ± 0.70	0.69	100.69
500.00	500.45 ± 0.90	0.17	99.83
1000.00	1001.98 ± 27.55	2.74	98.36
2000.00	2001.00 ± 44.94	1.11	101.11
4000.00	4035.00 ± 72.59	1.79	101.79
6000.00	6135 ± 140.36	2.28	97.82
8000.00	8022.00 ± 137.19	1.71	101.71
10000.00	10037.43 ± 90.44	0.90	99.10

Precision and Accuracy: The intra- and inter-batch percent accuracy for Baloxavir marboxil was 94.08-97.84 and 96.89-98.42, respectively. The percent CV was 2.16 to 5.92 and 1.58 to 3.11 percent. Table 2 summarises the findings.

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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	14.52 ± 0.86	5.92	94.08	14.75 ± 0.46	3.11	96.89
3000.00	2885.07 ± 62.59	2.16	97.84	2960.07 ± 46.95	1.58	98.42
7000.00	6811.67 ± 265.83	3.90	96.10	6878.30 ± 193.88	2.81	97.19

Recovery: The mean percent recovery for Baloxavir marboxil LQC, MQC, and HQC samples was 99.85 percent, 95.30 percent, and 93.54 percent, respectively. Baloxavir marboxil's overall mean percent recovery and percent CV throughout QC levels are 96.23 percent and 3.38 percent, respectively. The mean percent recovery and percent CV for Oseltamivir (internal standard) are 91.68 percent and 7.09 percent, respectively.

Stability (Freeze-Thaw, AutoSampler, Bench Top, Long Term): Table 3 shows the quantitative analysis of the Baloxavir marboxil in plasma after three freeze-thaw cycles (-30 °C to room temperature), autosampler (processed), room temperature (Benchtop), and long-term stability data.

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Table3: Stability Studies Of Baloxavir Marboxil In Spiked Plasma Samples

Spiked Plasma conc. (pg/ml	Room temperature Stability(24h)		Processed sample Stability(65h)		Long term stability(45days)		Freeze and thaw stability(cycle 48 h)	
	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	**12.91 ± 1.15**	**8.9**	**13.96 ± 0.72**	**5.1**	**14.40±1.28**	**8.8**	**14.17 ± 0.79**	**5.5**
7000.00	**6642.67±545.64**	**8.9**	**6166.67±579.21**	**9.5**	**6058.33±569.03**	**9.4**	**6685.0±181.51**	**2.7**

CONCLUSION: The method presented in this paper was developed and verified in human plasma at concentrations ranging from 5.0 to 10000.0 pg/ml. The intra-batch precision (% CV) was less than 6.0 percent, and the percent accuracy ranged from 94.08 percent to 98.4 percent. For Baloxavir marboxil, Oseltamivir, the total percent recovery was greater than 90%. This approach is appropriate for the current investigation because of its selectivity, sensitivity, precision, and accuracy. To summarise, the method employed in this study is simple and quick to implement, as well as having appropriate accuracy, precision, selectivity, and stability. The method's simplicity, as well as the fact that it uses quick protein precipitation extraction with a shorter run time of 3.0 minutes per sample, make it appealing for high-throughput bioanalysis of Baloxavir marboxil..

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

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