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Determination Of Entecavir In Human Plasma By LC-MS/MS And Method Validtion

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Abstract: An LC-MS/MS method for the determination of Entecavir in human plasma was developed and validated according to currently accepted FDA guidelines of bio analytical method validation. In the present method Shimadzu- LCMS/MS with analytical column X-bridge C18, 5µm, 4.68×50mm, an injection volume of 10µl was injected and eluted with mobile phase acetonitrile and methanol pumped at a flow rate of 0.6ml/min, with an internal standard Lamivudine with a less run time, Entecavir was quantified in human plasma.
Keywords: Entecavir, LCMS/MS.

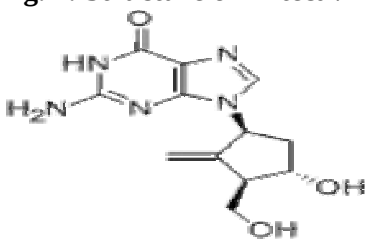
INTRODUCTION

Entecavir is a guanosine nucleoside analogue with selective activity against HBV. It is a white to off-white powder. It is slightly soluble in water (2.4 mg/mL), and the pH of the saturated solution in water is 7.9 at 25° ± 0.5° C.

Film-coated tablets are available for oral administration in strengths of 0.5 mg and 1 mg of Entecavir. Entecavir 0.5-mg and 1-mg film-coated tablets contain the following inactive ingredients: lactose monohydrate, microcrystalline cellulose, crospovidone, povidone, and magnesium stearate. The tablet coating contains titanium dioxide, hypromellose, polyethylene glycol 400, polysorbate 80 (0.5-mg tablet only), and iron oxide red (1-mg tablet only) [1].

An attempt has been made to develop a method for the quantification of Entecavir in human plasma by LCMS/ MS and liquid phase extraction. The literature survey revealed that there are several methods reported for the quantification of Entecavir drug in dosage forms as well as in human plasma. Also some methods have been reported for the determination of Entecavir by LCMS/MS in human plasma [2].

Fig. 1: Structure of Entecavir



MATERIALS AND METHODS

Chemicals and reagents
1.1 Mobile phase preparation
Solvent A: Acetonitrile
Solvent B: To 100 mL of water 0.500 mL of Formic acid was added, sonicated and filtered the solution using 0.22µ membrane filter.

Premixing:
The mobile phase was prepared by mixing Solvent A and Solvent B in the ratio of 90:10 v/v and ultrasonicated for 5 minutes.

1.2 Preparation of 50% Methanol in water solution:
To 50 mL of methanol, 50 mL of water was added, sonicate and filtered the solution using 0.22µ membrane filter.
1.3 Preparation of 50% Acetonitrile in water solution:
To 50 mL of Acetonitrile, 50 mL of water was added, sonicate and filtered the solution using 0.22µ membrane filter.
Needle wash: Acetonitrile: water (50:50v/v)
Seal wash: Water (MilliQ)

1.4. Preparation of Standards and Samples

1.4.1. Entecavir Standard Stock Solution
1mg/mL of Entecavir was prepared using Entecavir and methanol.

1.4.2. Spiking Solution for CC
Different concentrations of Entecavir i.e. 5, 10, 400, 1000, 2000, 2800, 3200 and 4000.00 ng/mL were prepared from standard stock solution using mixture of Methanol and water (50:50) as diluent.

1.4..3. Spiking Solution for QC
Different concentrations of Entecavir i.e. 15, 2400, and 3600 ng/mL were prepared from standard stock solution.

1.4..4. Calibration Curve Standards (CC)
The calibration standards of Entecavir 0.250, 0.500, 20, 50, 100, 140, 160 and 200 ng/mL were prepared from spiking solution using blank plasma as diluent.
The aliquots of the prepared solutions were transferred to different vials and stored at -70 ± 5^0C until processing.

1.4.5.Preparation of Quality Control (QC) Samples
The quality control samples of Entecavir 0.750 Low quality control (LQC), 120 Mid quality control (MQC), and 180 High quality control (HQC) ng/mL were prepared from spiking solution using blank plasma as diluent.
The aliquots of the prepared solutions were transferred to different vials and stored at –70 ± 5^0C until processing.

1.4.6. Lamivudine Stock Solution (Internal Standard) IS
1mg/mL of Lamivudine IS was prepared using Lamivudine and methanol.

1.4.7. Preparation of Plasma Samples
At the time of analysis, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw [3].
The resulting solution was processed as mentioned below-

- Transferred 250 µL of sample into a polypropylene micro centrifuge tube.
- Added 50 µL of 300 ng/mL internal standard solution.
- It was vortexed for about 30 sec.
- 3 µL of TBME was added
- It was shaken for 15 minutes in platform shaker.
- It was centrifuged about 5 min at ~ 4000 rpm and 10ºC.
- Collected the supernatant liquid in another glass tube.
- It was evaporated to dryness in low volume evaporator at 40ºC under nitrogen.
- The residue was reconstituted with 250 µL of Acetonitrile: water (50:50) and then used for analysis.

1.4.8. System Suitability Sample
Performed system suitability test using the chromatographic device during the following cases:

- At the start of each batch of method validation and subject sample analysis.
- After change of column in the middle of a project
- If, any components of the chromatographic device is replaced in the middle of a project
- Un-extracted standard equivalent to middle level of calibration curve concentration and internal standard were prepared and injected six times on the chromatographic device.

The retention times and responses of the analyte and internal standard were recorded. The system suitability is evaluated by inbuilt system suitability software calculating the mean, standard deviation and coefficient of variation for the retention time and area.
The % CV of area ratio of drug and internal standard is ≤3% for single analyte and for ≤5% for multiple analyte. The % CV of retention time of drug and internal standard is 2%.

Optimized chromatographic conditions are
Mass : API 3200
Ion source : Turbo ion spray
Polarity : Positive ion mode

Detection ions
Entecavir : 325.1amu (parent), 262 amu (product)
Lamivudine (IS): 322.1 amu (parent), 212 amu (product)
Column : Symmetry C18, 50x4.6, 5 µ
Column oven temperature : 30.0 ºC
Peltier temperature : 15.0ºC
Mobile phase : Acetonitrile: 0.5% Formic acid (90:10)
Flow rate : 0.6 mL/min.
Volume of injection : 10 µL
Retention time
Entecavir: 0.61 minutes
Lamivudine : 1.54 minutes
Run time : 3 minutes

MS Conditions

Curtain Gas (CUR)	: 15.0 PSI
Collision Gas (CAD)	: 5.0 PSI
Temperature (TEM)	:500.0 °C
Ion Source Gas 1 (GS1)	: 65 PSI
Ion Source Gas 2 (GS2)	: 40 PSI
Entrance potential (EP)	: 10.0V
Resolution Q1	: Unit
Q3	: Unit

Method Development:

Optimization of the LC-MS/MS system
MS/MS is a much more specific and selective method of detection than UV. Interference by co-eluting components is not considered as significant problem as with UV detection system, although the so-called "matrix effect" needs to be tested for. For this reason, the whole method development process was focused on mobile phase and extraction process optimization [4].
The LC- MS/MS instrument was calibrated with polypropylene glycol (PPG) standard in positive and negative ionization mode [5].
Infusion was done using 100ng/ml of Entecavir and Lamivudine IS separately in mobile phase. Using the spectra of the infused solutions, mass spectrometer parameters were optimized.

EXPERIMENTAL PROCEDURE:

10 µl of each sample was injected
The standard solutions, CC standard, QC samples were injected with the optimized chromatographic conditions and the chromatograms were recorded. The quantification of the chromatogram was performed using peak area ratios (response factor) of the drug to internal standard.

1. System suitability
System suitability was performed by injecting 6 sets of known concentrations of aqueous mixture for analyte and ISTD. CV% for retention time (RT) and area ratio (Analyte area/ISTD area) were calculated.
2. Selectivity
Selectivity was assessed by analysing blank plasma samples obtained from six different sources with six samples at LLOQ concentrations spiked using the biological matrix of any one source.
3. Sensitivity
Sensitivity was determined by limit of quantitation by analyzing six replicates of LLOQ that can be measured with acceptable accuracy and precision.
4. Recovery
Recovery of the developed method was evaluated by analysing six replicates for analyte along with internal standard by comparing the analytical results for extracted samples at three concentrations (equivalent to LQC, MQC and HQC) with unextracted samples that represent 100% recovery. The % recovery of analyte and ISTD were calculated using appropriate chromatographic conditions.
5. Precision
Precision of the method was determined by analyzing six replicates of LQC, MQC, and HQC samples.
6. Stability

- **Freeze and thaw stability**
  Sets of spiked plasma samples, six replicates of each LQC, MQC, and HQC stored at -70^{o}C were thawed completely unassisted at room temperature and refrozen immediately to -70^{o}C. This cycle was repeated three times with 12 hour intervals and the samples were extracted and analysed.
- **Bench top stability**
  The stability of samples on the bench i.e., when kept outside the freezer were studied to know the stability of samples at room temperature. Six replicate of LQC & HQC were kept at room temperature for 6 hrs these samples were processed and analyse with a freshly spiked calibration curve.

- **Stock solution stability (Short term stability)**
  The stability of stock solutions of analyte and IS at room temperature for 6 hours was evaluated by comparing with fresh solutions (zero time solutions) response.

- **Stock solution stability (Long term stability)**
  The stock solutions are refrigerated for the relevant period, and the stability of the solution were compared with the instrument response with that of freshly prepared solutions.
- **Long term stability**
  Long term stability of plasma sample at -70ºC were estimated by analyzing six replicates of stored low and high concentration quality controls with a freshly prepared calibration curve [6].

7. Dilution integrity

Dilution integrity test was done by diluting, 1.8 times the CC8 concentration in the ratio of 50:50 and 25:75 with matrix blank. This test was performed using 6 replicates. Concentration obtained was multiplied with dilution factor 2 (or) 4 to get the actual concentration [7].

8. Matrix effect

It has been noted that co eluting, undetected endogenous matrix components may reduce the ion intensity of the analyte and adversely affect the reproducibility and accuracy of the LCMS/MS assay [8].

In order to determine whether this effect (called the Matrix Effect) is present or not, 6 different plasma pools were extracted and then spiked with a known concentration of analyte. These samples were injected and peak areas compared. The reproducibility of the peak areas is an indication of the presence or absence of the matrix effect.

Figure 1: MASS SPECTRUM OF ENTECAVIR (Parent Ion)

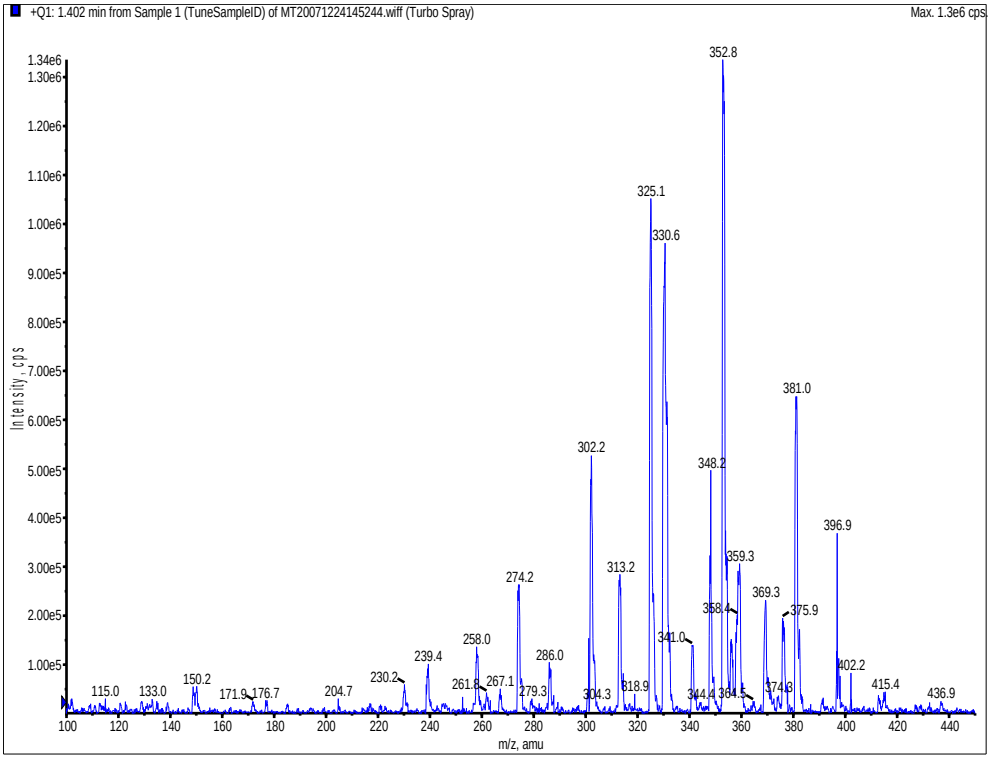


Figure 2:MASS SPECTRUM OF ENTECAVIR (Product ion scan)

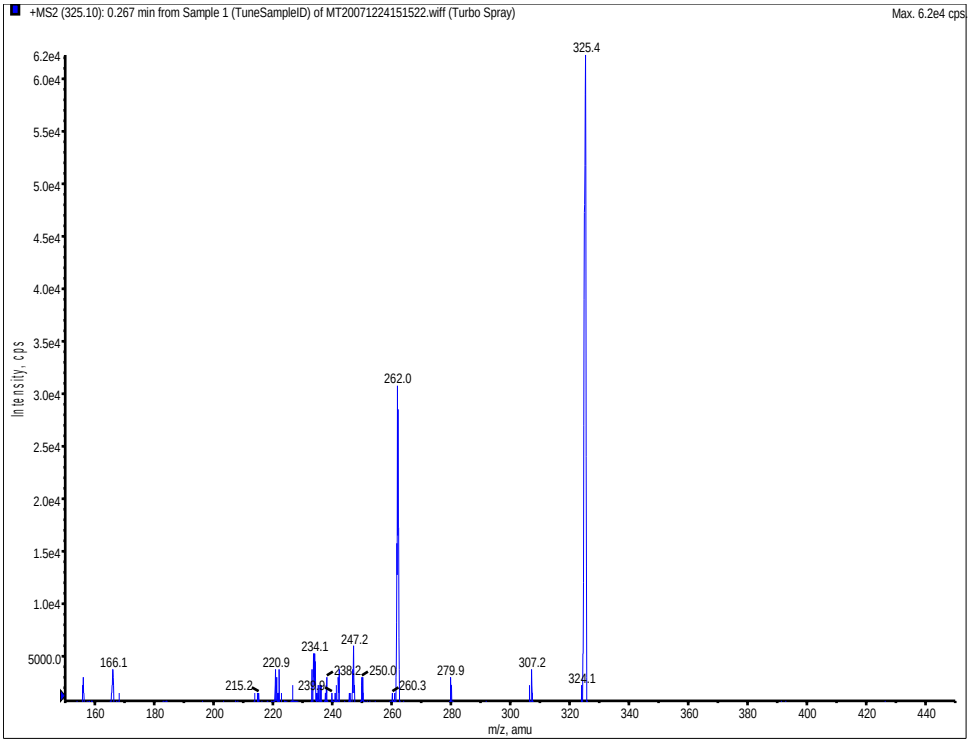


Figure 3: REPRESENTATIVE CHROMATOGRAM OF PROCESSED BLANK PLASMA

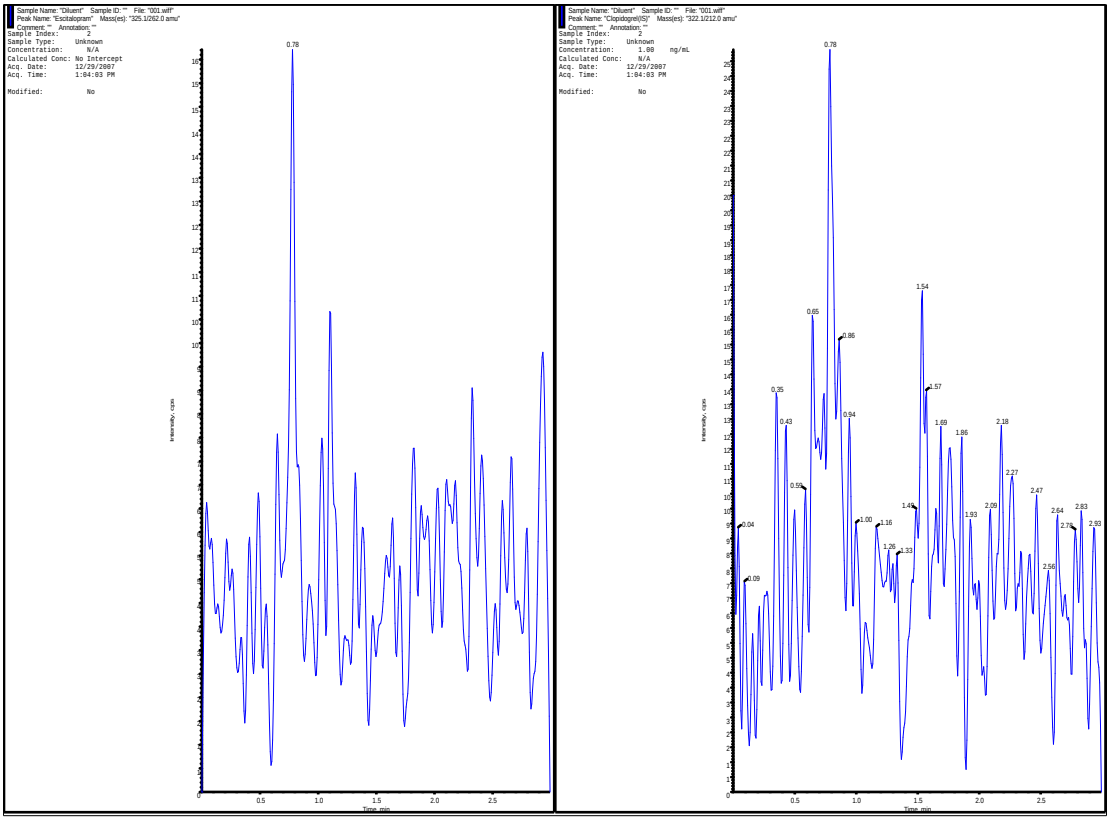


Figure 4:TYPICAL CHROMATOGRAMS OF ENTECAVIR & LAMIVUDINE

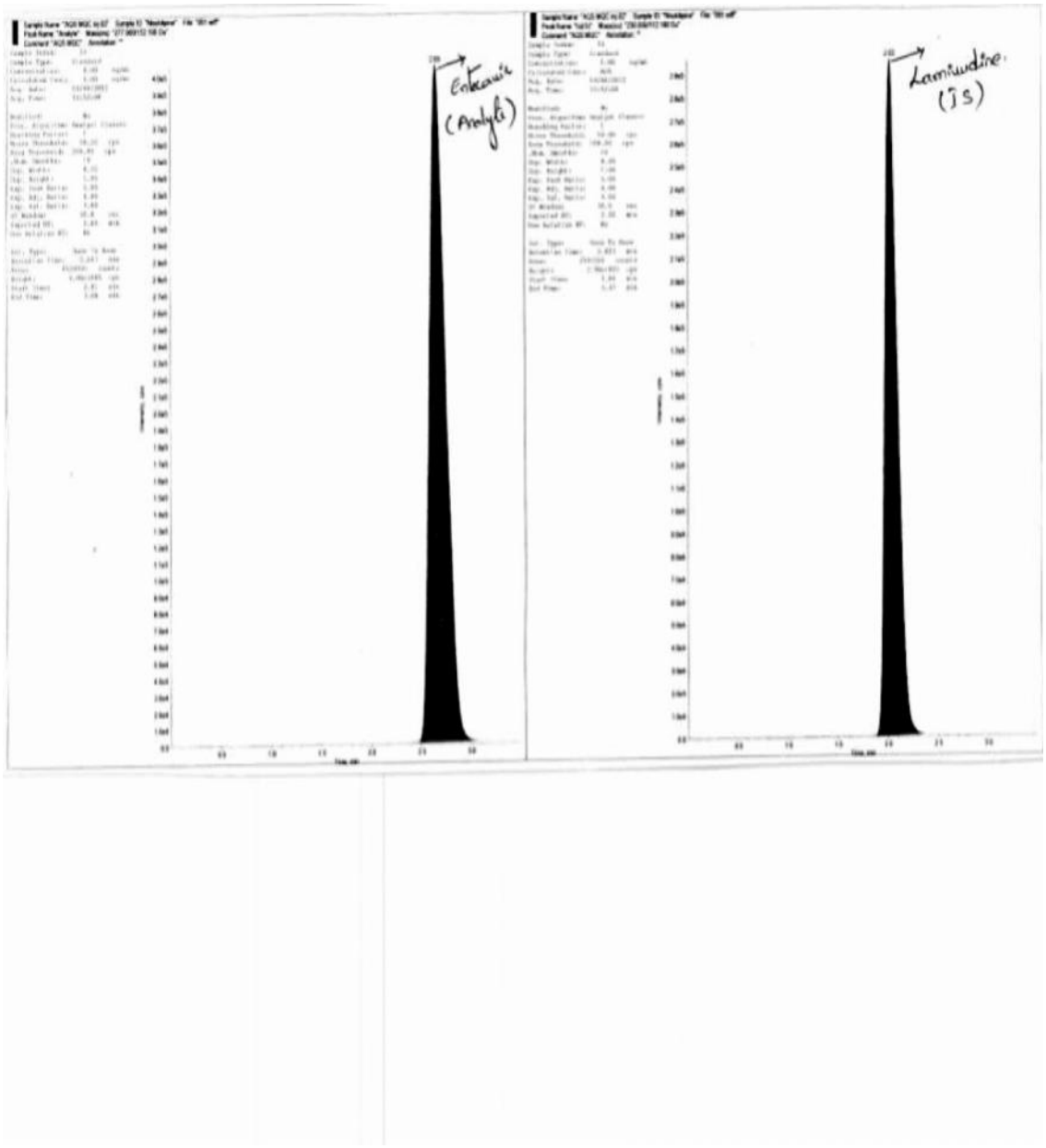
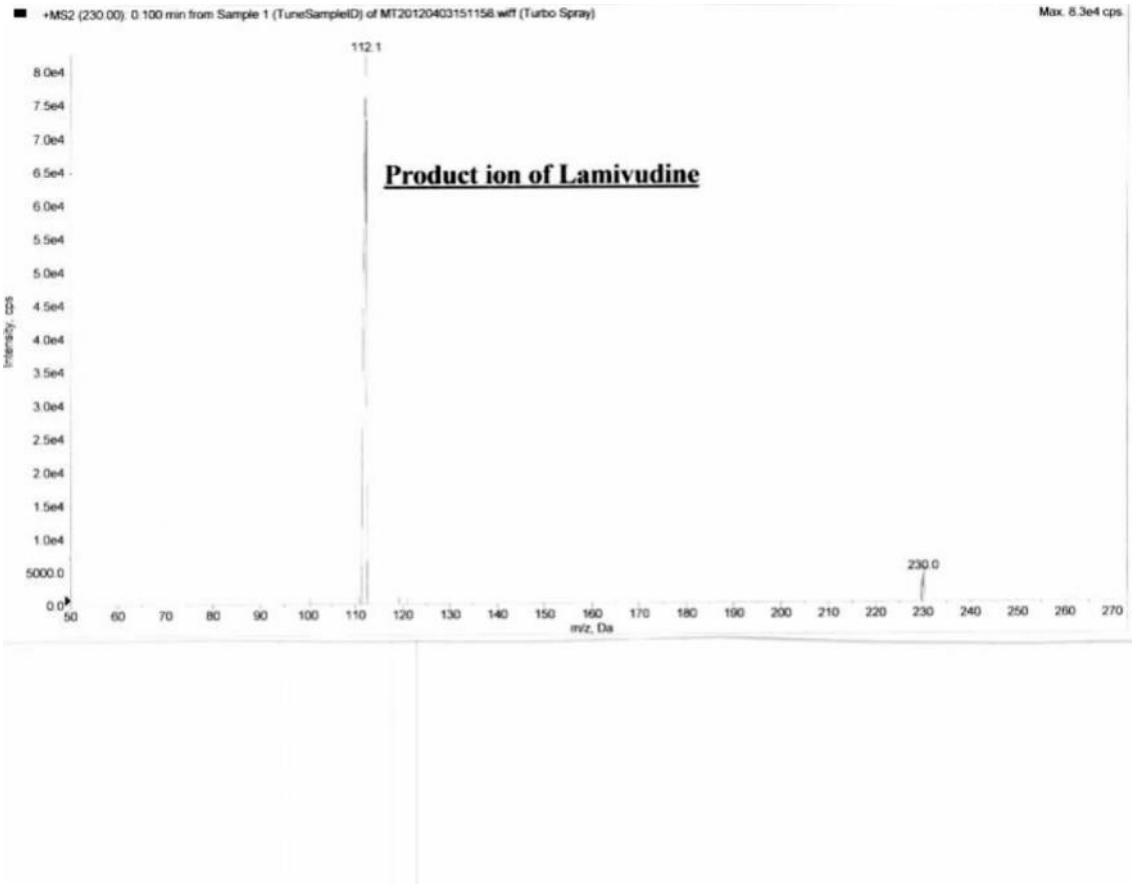


TABLE 1: ACURACY, PRECISION AND RECOVERY

Experiment	Concentration Taken	Concentration found (ng)	Mean	SD	%CV
Within run Accuracy & precision	LQC(0.750ng)	0.696	92.802%	3.58	3.86
	MQC(120ng)	117.535	97.947%	5.03	5.13
	HQC(180ng)	170.628	94.793%	4.13	4.36
Between run Accuracy & precision	LQC(0.750ng)	0.697	92.99%	3.20	3.44
	MQC(120ng)	117.98	98.32%	6.65	6.76
	HQC(180ng)	174.04	96.69%	4.79	4.95
Recovery of analyte	LQC(0.750ng)	0.594	79.25%	283.53	4.45
	MQC(120ng)	90.732	75.61%	19943.75	3.79
	HQC(180ng)	131.05	72.81%	25866.79	3.32

Figure 5: PRODUCT ION OF LAMIVUDINE



RESULTS & DISCUSSION:

An LC-MS/MS method for the determination of Entecavir in human plasma was developed and validated according to currently accepted FDA guidelines of bio analytical method validation.

In the present method Shimadzu-LCMS/MS with analytical column X-bridge C18, 5µm, 4.68×50mm, an injection volume of 10µl was injected and eluted with mobile phase acetonitrile and methanol pumped at a flow rate of 0.6ml/min, with an internal standard Lamivudine with a less run time, Entecavir was quantified in human plasma.

The developed chromatographic method for the determination of Entecavir in human plasma was simple, precise, accurate and economical. The internal standard used is easily available and economical. The mobile phase is simple to prepare and economical.

Based on the data presented in this report, it can be concluded that the present method is validated for the estimation of Entecavir in human plasma. Expected recoveries were observed in the present processing technique for LQC, MQC and HQC. The values obtained from system suitability studies demonstrated the suitability of the system for the analysis of the Entecavir in plasma.

The method can be applied for bioavailability studies and for analyzing patient samples in clinical trials.

Since the method has shown satisfactory results, it is deduced that the proposed method to be simple and short and most useful for the quantification of Entecavir in human plasma.

TABLE 2: LOWER LIMIT OF QUANTITATION (LLOQ)

	Mean	SD	%CV
Calculated Concentration ng/mL	0.243	0.01	5.71
Accuracy %	97.20	5.55	5.71

TABLE 3: DILUTION INTEGRITY

	50:50 Dilution (DQC 360ng)		25:75 Dilution (DQC 360ng)	
	Con. Found	%nominal	Con. Found	%nominal
Mean	348.73	96.87	347.55	96.54
SD	16.20	4.50	22.52	6.26
%CV	4.65	4.65	6.48	6.48

TABLE 4: MATRIX EFFECT

Matrix identification	Conc. found (LQC -0.750ng/ml)	% Nominal
MTP-005/07	0.758	99.33
MTP-009/07	0.744	97.29
MTP-013/07	0.774	96.22
MTP-012/07	0.722	100.75
MTP-014/07	0.746	100.75
MTP-015/07	0.739	98.71

TABLE 5: FREEZE-THAW STABILITY AT -70ºC

	LQC(0.750ng)		HQC(180ng)	
	FT		FT	
	Calculated con	Accuracy	Calculated con	Accuracy
Mean	0.737	98.222	179.443	99.691
SD	0.026	3.509	8.078	4.488
%CV	3.572	3.572	4.502	4.502

TABLE 6: STABILITY PARAMETERS

Stability parameter	Concentration	Type of sample	Mean	SD	%CV	%Change
Long-term stability of analyte in matrix at -70°c (60 days)	LQC	comparison sample	0.750	0.02	2.37	4.5
	HQC	stability sample	0.716	0.01	1.25	
	LQC	comparison sample	181.78	7.93	4.36	11.6
	HQC	stability sample	160.58	3.40	2.12	
Short term stability of analyte in matrix at room temperature (bench top)	LQC	comparison sample	0.742	0.027	3.670	-0.53
	HQC	stability sample	0.746	0.017	2.242	
	LQC	comparison sample	173.088	7.421	4.288	0.462
	HQC	stability sample	172.255	7.394	4.292	

TABLE 7: STABILITY PARAMETERS

Stability parameter	Type of sample	Mean Response	SD	%CV	%Change
Short-term stability of analyte in solution at room temperature (8 hrs)	comparison sample	652080.7	1447.767	0.222023	0.612
	stability sample	648088	3208.824	0.495122	
Short-term stability of internal standard in solution at room temperature (24 hrs)	comparison sample	122313.33	414.22	0.34	8.23
	stability sample	121714.67	313.46	0.26	
Long-term stability of analyte in solution at (2-10°c) - (30 days)	comparison sample	642330.17	2699.44	0.42	2.9
	stability sample	623546.00	8807.68	1.41	

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