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Development and Validation of a New HPLC Bio Analytical Internal Standard Method for the Analysis of Two Immunology Drugs (Lamivudine and Dolutegravir) in Human Plasma

Dr.K.R.S. Prasad*, Dr.V.D.N. Kumar Abbaraju,
Dr.K. Uma Maheswar, Chaitanya Darapureddy and
Dr.Phani RS CH

Abstract--- *Bioanalytical methods are useful for the quantitative analysis of drugs and their metabolites in biological fluids. Bio-analytical internal standards methods plays vital role in areas of human clinical pharmacology. The aim of this work is to development and validation of bio-analytical internal standard method for determination of two immunology drugs Lamivudine (LMVD) and Dolutegravir (DTGR) in plasma with Abacavir (ABVR) drug as internal standard (IS). Liquid-liquid extraction with Diethyl ether and methanol in the ratio of 50:50 (v/v) was used for the extraction of drugs from the biological matrix. The optimized chromatography conditions consist of Water, Acetonitrile and Methanol in the ratio of 40:25:35 (v/v) as a mobile phase with Ascentis, C18 Column (250 X 4.6 mm, 5μ) as stationery phase. Isocratic elution with 1.2 ml flow separates the LMVD at 3.5 min, DTGR at 9.8 min and ABVR at 6.8 min. The method was validated as per ICH guidelines. The relative standard deviation (%RSD) were found to be <5%for precision studies. Hence the method was found to be suitable fort the analysis of LMVD and DTGR in spiked human plasma.*

Keywords--- *RP-HPLC, Internal Standard, Bio-analytical, ICH Guidelines, Lamivudine, Dolutegravir and Abacavir.*

I. INTRODUCTION

Bio analytical method demonstrate that a particular method used for quantitative measurement of drugs or analytes in a given biological matrix, such as blood, plasma, serum, or urine is reliable and reproducible for the intended use. Determination of drugs in biological fluids plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic study data which supports regulatory filings. The quality of these studies is directly related to the quality of the underlying bio-analytical data. It is therefore validation of these bioanalytical methods are established and disseminated to the pharmaceutical community.

Lamivudine (LMVD is an antiretroviral medication used in the treatment of HIV/AIDS and chronic hepatitis B. It is effective against both HIV-1 and HIV-2[1-2].Dolutegravir (DTGR) is an antiretroviral medication in the treatment of HIV/AIDS. [3-4].

Dr.K.R.S. PRASAD, Department of Chemistry, K.L. University, Vaddeswaram, India.*
Dr.V.D.N. Kumar Abbaraju, Dept of Chemistry, GIS, GITAM University, Vishakapatnam, AP, India.
Dr.K. Uma Maheswar, A.P.J Abdul Kalam Campus, Rajiv Gandhi University of Knowledge Technologies, Ongole, AP, India.
Chaitanya Darapureddy, Department of Chemistry, K.L. University, Vaddeswaram, India.
Dr.Phani RS CH, Department of Chemistry, K.L. University, Vaddeswaram, India.

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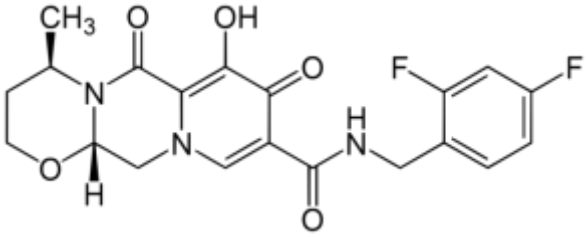

Figure 1: Structure of DTGR

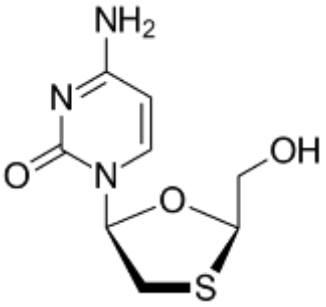

Figure 2: Structure of LMVD

II. MATERIALS AND METHODS

Instrumentation

The chromatographic system used is Agilent 1100 series HPLC with Quaternary G1311 A pump, COLCOM G1316A thermostat column temperature control, Thermostatic auto sampler G 1329A with sample volume of 0. 1 – 1500 µL and variable programmable UV detector (G 1314 A). The instrument was operated and integrated with Agilent chem station LC software. The separation of compounds was achieved by using KNAUER Eurospher II C18 Column (250 X 4.6 mm, 5µ).

Chemicals and Solvents

HPLC Grade Methanol, Acetonitrile and water were procured from Thermo Fisher Scientific India private limited, Mumbai. HPLC grade Acetonitrile purchased from Merck chemicals private limited, Mumbai.

Preparation of Mobile Phase

Water, Acetonitrile and Methanol in the ratio of 40:25:35 (v/v) sonicated the mobile phase for ten minutes to ensure the homogeneous mixing using ultrasonicater, and then it was filtered through 0.45 µ nylon membrane filter paper using vacuum filtration set. The solution was stored at room temperature and used within seven days from the date of preparation. Later the mixture was degassed.

Preparation of Diluent

An equal ratio of acetonitrile and methanol were used as diluent in the analysis. For the preparation of diluent, 50mL of methanol was transferred into a 100 mL reagent bottle and 50mL of acetonitrile was added, mixed and sonicated for ten minutes. The solution was stored at room temperature and used within one week from the date of preparation.

Preparation of Sample Solution

Different organic extraction solvents were evaluated in the experiment including methanol, acetonitrile, diethyl ether, chloroform and dichloromethane. Diethyl ether and methanol combination proved to be the most efficient extracting solvent. Blood samples from local diagnostic lab was collected in heparinized tubes and immediately placed on ice and taken to the lab. The tubes were centrifuged at 5000rpm for 5.0 min at room temperature for separation plasma. The plasma samples were stored at -30^{o}C. The drugs LMVD, DTGR and ABVR as internal

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standard was premixed with plasma. A liquid-liquid extraction method was employed by using Diethyl ether and methanol in the ratio of 50:50 (v/v) for the extraction of drugs from the biological matrix. 50 ml of Diethyl ether was added to 50 ml of methanol and vortex for 30sec, and then centrifuged at 4°C at 5000rpm. The blank plasma sample was prepared by adding 1ml of spiked plasma in to extraction solution and vortex for 30sec, and then centrifuged at 4°C at 5000rpm. Supernatant of these solutions were kept in a HPLC vial.

Preparation of Calibration Solutions[5-7]

Preparation of calibration curve (CC) standards and internal standard solutions by stock solution (100µg/mL) of LMVD, DTGR and ABVR drugs were appropriately diluted with diluents solution to get working standard solutions with concentrations of 50 – 5000 ng/mL of DTGR and LMVD. Aliquots of 0.9 mL of blank human plasma were spiked with 0.1 mL of the working standard solutions to get CC standards containing 50, 100, 250m 750, 100, 1500, 2000 and 3000 ng/mL of DTGR and LMVD. The internal standard ABVR samples were similarly prepared and maintained at constant concentration of 100µg/mL. The three concentrations [50 ng/mL low quality control (LQC), 1500 ng/mL middle quality control (MQC) and3000 ng/mL high quality control (HQC)] were selected for validation.

Method Development and Optimized Conditions[8-17]

Various chromatography parameters like columns, mobile phase, flow, and column temperatures were tested during development of the analytical method. The initial aim of the development is to develop a sensitive condition in order to detect the drug at very low concentration, where usual concentrations of the drug exist at biological samples. Different columns like C8 and C18 of the same length and diameter were tested and also mobile phase with solvents ratio were tested. UV detector wavelength was selected as iso-absorbtic point i.e 260nm. The mobile phase holdup time, resolution, asymmetry of drugs and quantity of fractions defined by the reading of area integrations from the chromatograms were assessed. The concentration of tested samples was 100 µg/mL throughout development. By keeping the same parameters and conditions other method parameters like mobile phase flow, injection volume, temperature of the column were optimized to get efficient chromatogram.

Table 1: Optimized chromatographic conditions:

S. No	Condition	Results
1	Mobile phase	Water, Acetonitrile and Methanol in the ratio of 40:25:35 (v/v)
2	Pump mode	Isocratic
3	pH	6.8
4	Diluents	Mobile phase
5	Column	Ascentis, C18 Column (250 X 4.6 mm, 5µ)
6	Column Temp	Ambient
7	Wavelength	260 nm
8	Injection Volume	20µl
9	Flow rate	1.2 ml/min
10	Run time	25 min

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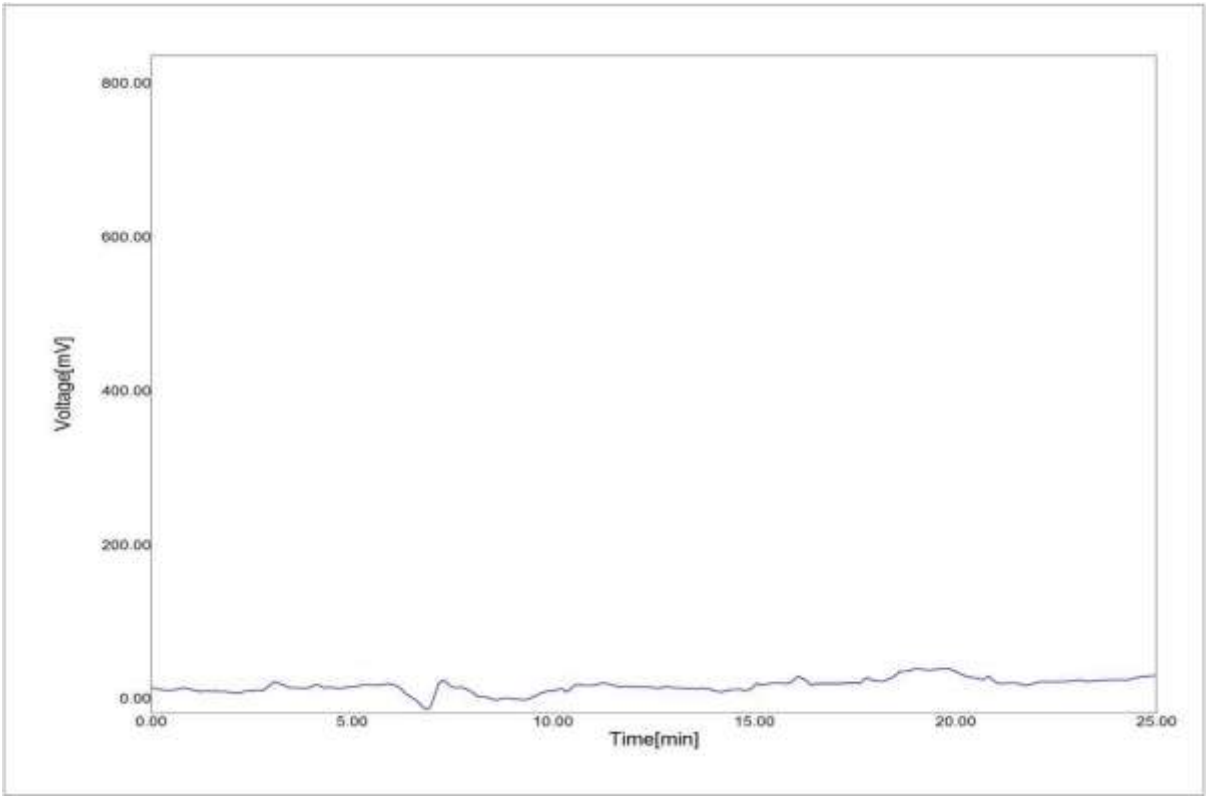

Figure 3: Blank Chromatogram of Plasma with Diluents and Further Subjected to Liquid-liquid Extraction

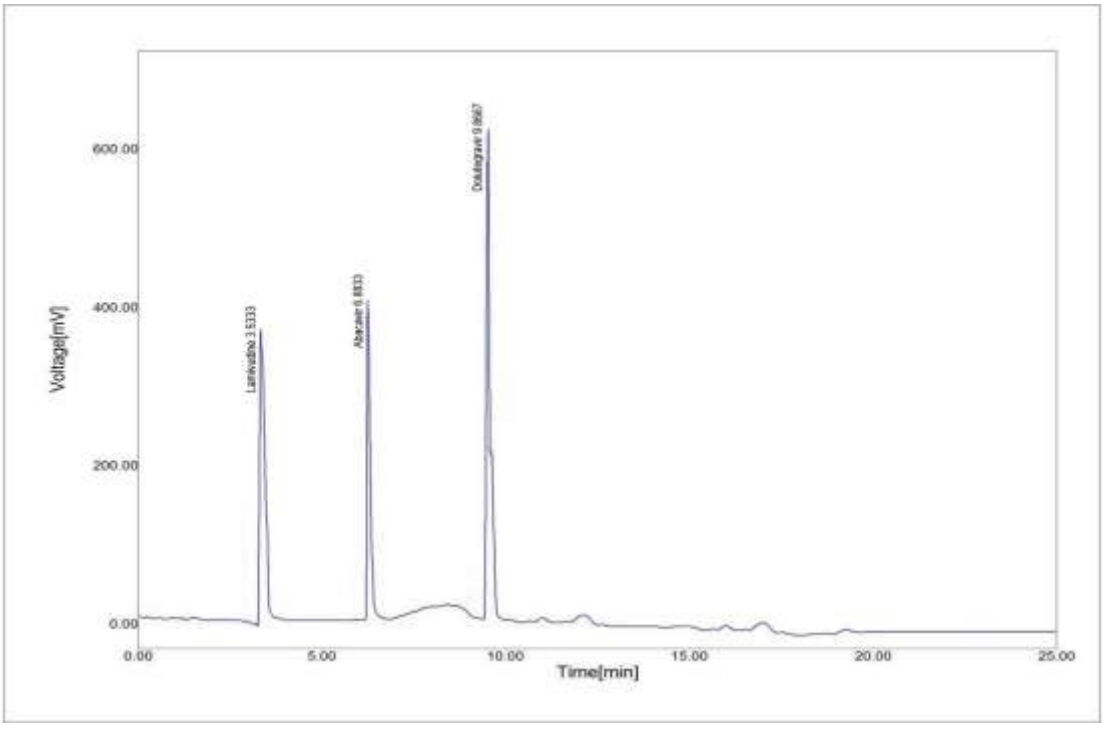

Figure 4: System Suitability Chromatogram of Plasma Spiked Drug of LMVD and DTGR with ABVR (IS) no Interfering Endogenous after Liquid-liquid Extraction

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Method validation [18-29]

The validation of the developed method was conducted as per the recommendations of US FDA guidelines. System suitability was studied at the middle of quantification (MQC) of 1500ng/mL by comparing blank responses of plasma. Accuracy was estimated as the mean RE while the precision was measured in terms of RSD. For each of the above validation tests, the analysis was performed at three QC concentrations (low, medium and high), with six determinations for each concentration. Stability of LMVD and DTGR in human plasma was evaluated under different conditions viz. three freeze–thaw cycles, stability of long term for 30 days and stability of short term at room temperature for 6 h. All the validation stability studies were performed at LQC, MQC and HQC concentration. The obtained results were compared with the nominal concentration of the analytes.

Table 2: Linearity test result of LMVD

S No	Concentration in ng/ml	Peak Area observed for		Ratio of Standard/IS	Sample Id
		LMVD - Standard	ABVR - IS		
1	50	12354	112613	0.109	LMVD-1
2	100	20536	110259	1.186	LMVD-2
3	500	52368	111314	0.470	LMVD-3
4	1000	90653	110652	0.819	LMVD-4
5	1500	144693	111985	1.292	LMVD-5
6	3000	263044	112369	2.340	LMVD-6
7	5000	426935	110688	3.857	LMVD-7
CC=0.9997 Slope:1327.701 IC= -128.554					

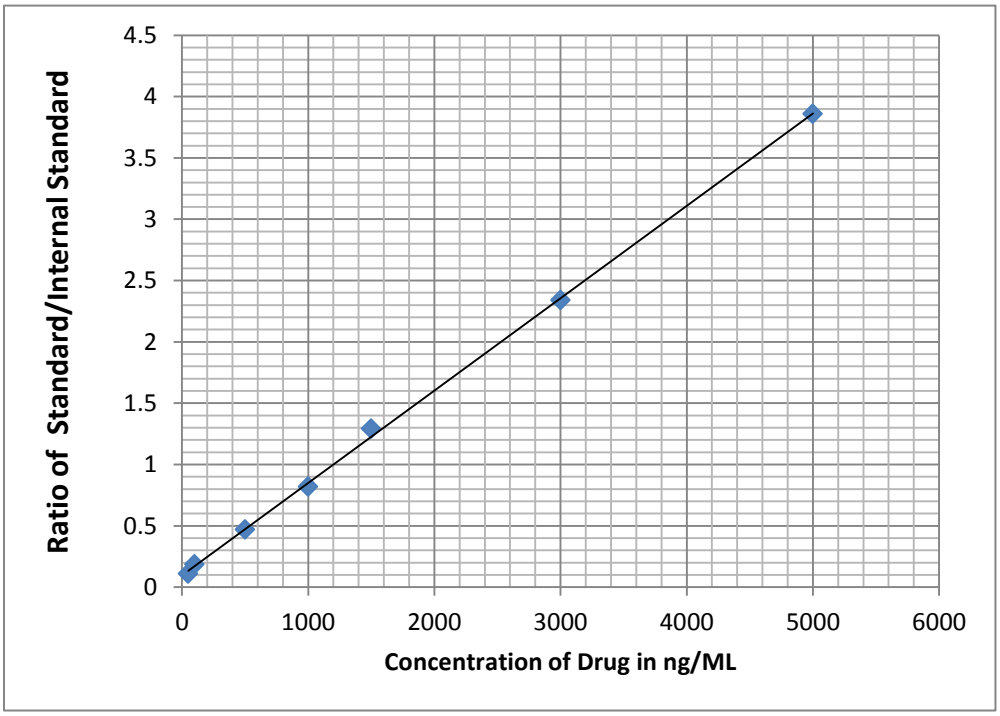

Figure 5: Calibration curve which ratio of LMVD to internal standard ABVR

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Table 3: Linearity test result of DTGR:

S No	Concentration in ng/ml	Peak Area observed for		Ratio of Standard/IS	Sample Id
		DTGR - Standard	ABVR - IS		
1	50	20639	112613	0.183	DTGR-1
2	100	28695	110259	0.260	DTGR-2
3	500	64302	111314	0.577	DTGR-3
4	1000	111069	110652	1.003	DTGR-4
5	1500	155037	111985	1.384	DTGR-5
6	3000	299876	112369	2.668	DTGR-6
7	5000	465311	110688	4.203	DTGR-7
CC=0.999 Slope:1229.975 IC= -213.78					

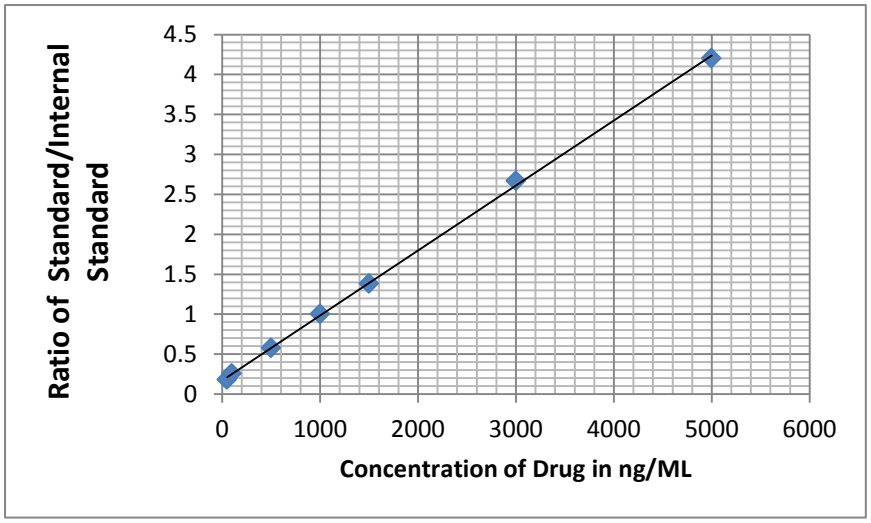

Figure 6: Calibration curve which ratio of DTGR to internal standard ABVR

Table 4: LMVD results of Precision and Recovery study with various QC concentrations:

S.NO	Parameter	Concentration	% of Drug Estimated	Standard deviation	Accuracy (%)
1	Intraday Precision	HQC	99.24-100.56	0.46	99.26
2		MQC	98.33-100.95	0.86	98.63
3		LQC	99.25-101.24	0.25	99.44
4	Interday Precision	HQC	99.64-100.08	0.90	98.26
5		MQC	98.25-99.85	0.75	98.38
6		LQC	99.06-100.25	0.47	99.48
7	Recovery	HQC	98.5-99.86	0.85	100.26
8		MQC	99.45-100.25	0.62	99.68
9		LQC	98.28-99.96	0.30	99.24

Table 5: DTGR results of Precision and Recovery study with various QC concentrations:

S.NO	Parameter	Concentration	% Of Drug Estimated	Standard deviation	Accuracy (%)
1	Intraday Precision	HQC	99.92-101.54	0.85	99.35
2		MQC	99.38-100.28	0.70	98.24
3		LQC	98.86-99.25	0.33	100.22
4	Interday Precision	HQC	98.63-99.25	0.92	100.44
5		MQC	99.67-100.34	0.80	99.46
6		LQC	98.05-99.88	0.50	99.02
7	Recovery	HQC	99.75-101.24	0.70	99.55
8		MQC	99.05-100.24	0.64	99.68
9		LQC	98.85-99.62	0.40	99.03

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Table 6: LMVD results of stability study with various QC concentrations:

S.NO	Parameter	Concentration	% Of Drug Estimated	Standard deviation	Accuracy (%)
1	Short term Stability	HQC	99.26-100.85	0.45	99.02
2		MQC	99.64-101.05	0.52	99.56
3		LQC	98.58-100.04	1.02	100.02
4	Long term Stability	HQC	98.25-100.33	0.88	99.85
5		MQC	98.62-99.98	0.63	100.24
6		LQC	99.02-100.25	1.24	99.63
7	Freeze Thaw Stability	HQC	99.55-101.82	0.88	99.37
8		MQC	99.26-100.24	0.45	100.56
9		LQC	98.93-100.43	1.06	100.25

Table 7: DTGR results of stability study with various QC concentrations:

S.NO	Parameter	Concentration	% Of Drug Estimated	Standard deviation	Accuracy (%)
1	Short term Stability	HQC	98.84-100.06	0.55	99.65
2		MQC	98.55-101.22	0.42	100.68
3		LQC	99.26-101.84	0.68	99.34
4	Long term Stability	HQC	98.35-100.44	1.02	100.38
5		MQC	99.65-101.32	0.46	100.49
6		LQC	98.35-100.22	0.83	99.63
7	Freeze Thaw Stability	HQC	98.24-99.86	0.36	100.62
8		MQC	100.32-101.2	1.36	99.54
9		LQC	99.45-100.88	0.55	99.29

III. RESULTS AND DISCUSSION

The chromatographic separation LMVD, DTGR using ABVR internal standard was optimized after several trials using the C8 and C18 columns mobile phase with different ratios of Ortho Phosphoric Acid buffer and at various pH. Optimized chromatography conditions includes Acetonitrile, water and Methanol, in the ratio of 40:25:35(v/v) (pH 6.8) as mobile phase with Ascentis, C18 Column (250 X 4.6 mm, 5µ) at 260 nm UV detection wavelength. The flow rate of the mobile phase 1.2 ml/min with isocratic elution at ambient temperature for 10 minutes was successfully achieved the separation of the LMVD at 3.5 min, DTGR at 9.8 min and ABVR at 6.8 min retention time with high resolution. The optimized chromatography conditions are presented on table-1 and the chromatograms of blank and system suitability are shown in figure-3 and figure 4.

All analytes eluted rapidly with good resolution within 10.0 min without any interfering of plasma matrix components with the analyte peaks. Peaks shape and retention time (Rt) were found to be same as that of pure standards.

Calibration curve is presented to confirm the relationship between the peak area ratios and the concentrations of LMVD, DTGR in the standard samples. The linearity of the method was evaluated at eight concentration range including the LQC. The calibration curves were found to be linear in the range 50–5000ng/ mL, with a correlation coefficients (r^2) of 0.999 for LMVD and DTGR. The data of calculated calibration standards are presented in Table-2 and Table-3 for LMVD and DTGR respectively and linear calibration graph is shown in Figure-5 and Figure-6. LMVD and DTGR concentrations in QC samples, recovery, and stability samples were calculated from the resulting

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area ratio and the regression equation of the calibration curve. The lower limits of quantification (LLOQs) under the optimized conditions were 50ng/mL for both drugs, which was determined from visual method of detection.

The precision evaluation were assessed by repeated analysis of plasma samples containing different concentrations of LMVD and DTGR with IS on separate occasions. Six replicates of LQC, MQC and HQC samples. Recovery of both drugs are evaluated by comparing mean analyte responses of six extracted samples of LQC, MQC and HQC samples. The Intraday Precision, Interday Precision, recovery of LMVD and DTGR showed in Table-4 and Table-5 Respectively.

Stability studies were performed to evaluate the stability of LMVD and DTGR in plasma after exposing to various stress conditions like long term, short term and freeze thaw. The results are showed in Table-6 and Table-7 for LMVD and DTGR respectively. The outcomes of other parameters like precision, accuracy, reproducibility, effect of potentially interfering drugs, dilution integrity, were found to be within the acceptance criteria as per ICH and USFDA guidelines.

IV. CONCLUSION

A simple, sensitive, accurate and precise RP-HPLC method was developed and validated for the estimation of LMVD and DTGR in plasma with ABVR as internal standard in plasma. The present method was employed with liquid – liquid extraction of the plasma spiked drug and successfully validated. The results of the all validation and stability studies were found in acceptable range of recovery. The developed RP-HPLC method is efficient and can be used in pharmacokinetics studies as well as in the monitoring of the investigated LMVD and DTGR in biological samples like body fluids.

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