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Development and validation of LC-MS/MS method for the simultaneous estimation of tenofovir disoproxil fumarate and pioglitazone HCL in human plasma

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ABSTRACT

For the simultaneous detection of tenofovir and pioglitazone in a human plasma LC-MS/MS technique was utilized. API-4000 mass spectrometer was used. It should be operated in positive ion mode and contains ion source of turbo spray. Inverted C18 column was used which produces good resolution and symmetrical peaks. Metoprolol is used as internal standard. It requires acidic PH for elution but Tenofovir disoproxil fumarate and Pioglitazone requires neutral PH after extraction which is equity diluted with H_2O for supernate. Finally, it provides good resolution and elution. By using Acetonitrile 100mg of Tenofovir Disoproxil fumarate and Pioglitazone HCL were dissolved and the final volume is made with H_2O and Acetonitrile in 1:1 ratio to give 1mg/ml. standard stock solution are stored at -2 to $\pm 2^0$C till analysis is completed. The guidelines were provided by the FDA to fulfill the essentials requires for the technique. To regulate the concentration the procedure starts with standard curve which is built form the quantitative analysis standard ranges form 1-1000ng/mL. To determine the stability freeze-thaw technique were used. To determine the stability the analyte is kept at room temperature for 24 hours. The correlation coefficients for all calibration curves were more than 0.99.

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INTRODUCTION

Tenofovir disoproxil fumarate is utilized in the treatment of Acquired Immuno Deficiency Syndrome (Yang *et al.*, 2019; Vergori *et al.*, 2020) and chronic serum hepatitis. From the age of 2 it is used for HIV-1 treatment by combining with ART (antiretroviral). From the age of 12, it is used to treat chronic serum hepatitis (De Nicolò *et al.*, 2015). Tenofovir disoproxil is a nucleotide analogue reverse-transcriptase inhibitor (NtRTI). It is so-called as pro-drug. Concentrations in blood plasma in abstaining people is 25% after an hour and in fatty food condition, it is 40% subsequently

two hours. Its hunks the efficiency of reverse transcriptase, which prevents from replicating. It results in a decrease in the content of virus in plasma. It is usually obtained in two forms as a tablet and as powder. Its melting point is 279°C. Tenofovir disoproxil fumarate is a white to off-white crystal-like powder, i.e., dissolvable in methyl alcohol, a little dissolve in H_2O and very little dissolvable in methylene chloride. Tenofovir eliminates waste through kidneys. It acts as a substrate analogue in cellular pigments. Tenofovir is detected in body fluids by liquid chromatography.

Pioglitazone is utilized for non-insulin-dependent diabetes type, which is Glucophage. Subsequent control of pioglitazone reduces insulin protest in the boundary and liver ensuing improvement in insulin subject to dextrose deed and reduces hepatic dextrose outcome. It is utilized together in monotherapy and in grouping with insulin in handling non-insulin-dependent diabetes type. Pioglitazone is also known as 5-[[4-[2-(5-ethyl pyridine-2-yl)ethoxy]phenyl]methyl]-1,3-thiazolidine-2,4-dione;hydrochloride. Its molecular formula is $C_{19}H_{21}ClN_2O_3S$.

It is utilized to regulate hyperglycaemia and is used to avoid kidney harm, sightless, nervous difficulties and amputate difficulties. Controlling diabetes reduces myocardial infarction. It is used alone or together with other diabetes mellitus pills. Pioglitazone is utilized with proper diet and physical activities and from time to time with extra medicines which are used to treat diabetes type 2 (Balaji, 2013; Hanefeld, 2007). Pioglitazone is a group of glitazones. It takes action to increase the ability to insulin, an organic element that serves to maintain the levels of sugar. It is not utilized for the treatment of diabetes type 1 and ketoacidosis.

Structure of Tenofovir Disoproxil Fumarate is shown in Figure 1 and Pioglitazone HCL is shown in Figure 2.

According to literature survey several techniques such as RP-HPLC (Panchagiri *et al.*, 2018; Reddy *et al.*, 2015), LC-MS (Yadav *et al.*, 2009; Takahashi *et al.*, 2007) were used for the assessment of Tenofovir disoproxil fumarate and to assess Pioglitazone HPLC (Barkil *et al.*, 2007; Souri *et al.*, 2008), HPLC-UV (Elrefay *et al.*, 2013; Tahmasebi *et al.*, 2009), RP-HPLC (Ramathilagam and Solairaj, 2014; Sultana *et al.*, 2011), C-MS/MS (Jagadeesh *et al.*, 2013) were used. Our present work determines the simultaneous estimation of Tenofovir disoproxil fumarate and Pioglitazone HCL by LC-MS using human plasma. Parameters such as linearity, LOQ, accuracy and precision, Recovery and sustainability/stability are validated.

EXPERIMENTAL

Chemicals and reagents

Working Standard of Tenofovir disoproxil fumarate and Pioglitazone was obtained from by Akshaya labs, Hyderabad, India. Tablets were acquired from the limited marketplace. Acetonitrile of HPLC grade by Merck, Ammonium Acetate AR grade obtained from Qualigens fine chemicals and Water HPLC grade from Milli-Q RO system was used. HPLC grade reagents were utilized.

LC-MS/MS Instrumentation and Chromatographic Methods

In positive ion mode, the LC-MS/MS instrument was functioned and equipped with ESI using nebulizer, curtain and collision gas which is embraced with AB Sciex API-4000 mass spectrometer. The HPLC setup contains online DGU-20A3 soluble degasser, segment boiler CTO20A, LC20AD drives-2, SIL-HTc sampler. C18-XBridge segment was split into 60:40 proportion with a stream rate of 1.0mL/min kept at 400C. Changeable phase contains 0.1% methanoic acid(A) and ethanenitrile (B). In the primary phase up to 0.8min it is arranged as 95% A/5%B then it gets changed to 5 % A/95% B for 2.2 min. Later it slowly stimulated to 95% A/5% B by 2.4 min and remained the same at 3.5 min.

Optimization of MS/MS detection conditions and parameters

Multi-reaction Monitoring settings were determined by using 500ng/mL of an analyte by distillation. Table 1 shows the optimum MS-parameters of Tenofovir Disoproxil fumarate and Pioglitazone HCL in positive ion mode.

Preparation of stock solution

By using Acetonitrile, 100mg of Tenofovir Disoproxil fumarate and Pioglitazone HCL were dissolved and the final volume is made with H_2O and Acetonitrile in 1:1 ratio to give 1mg/ml. The standard stock solution is stored at -2to $\pm$ 20 C till analysis is completed.

Selection of HPLC column

In HPLC column inverted C-18 columns were regularly used for the objective analyte. Primarily sio_2 based C-8 columns are verified and these results in poor separation. Several aspects were taken, such as the composition of the mobile phase and PH stages were examined, yet no enhancement in chromatographic condition. C18 results in fine resolution but presents the uneven peaks. Finally, Phenomenex Luna C18 showed substantially good

resolution, symmetrical peaks and less co-elution between target compounds due to its effectiveness in polarity and aromatic selectivity.

Internal standard selection

For positive mode, analysis metoprolol is used. It requires acidic PH for elution but Tenofovir disoproxil fumarate and Pioglitazone requires neutral PH after extraction, which is equity diluted with H_2O for supernate. Finally, it provides good resolution and elution.

RESULTS AND DISCUSSION

Validation

It is a secondary phase. It is a very hard and important phase in quantitation and produces absolute results. The guidelines were provided by the FDA to fulfil the essentials requires for the technique. If the guidelines were not obeyed, then it seems to the improper analysis and not used for assent. To regulate the concentration, the procedure starts with a standard curve, which is built form the quantitative analysis standard ranges form 1-1000ng/mL.

The standards were divided in the presence of Metoprolol as internal standard from Tenofovir disoproxil fumarate and Pioglitazone by LC-MS/MS using the MRM conversions. To determine the stability analyte is kept at room temperature for 24 hours.

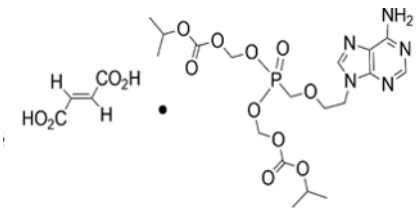

Figure 1: Tenofovir Disoproxil Fumarate

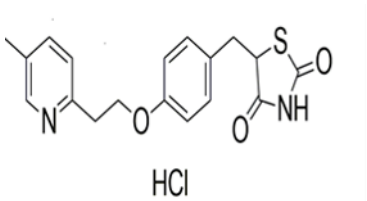

Figure 2: Pioglitazone HCL

Construction of calibration curve

To obtain the linear response, five samples were taken on an alternate day by using similar plasma and stock solution. Each one of these contains nine concentration levels which differed from one another. These are named as inter and intra-day data results in stability and reproducible on

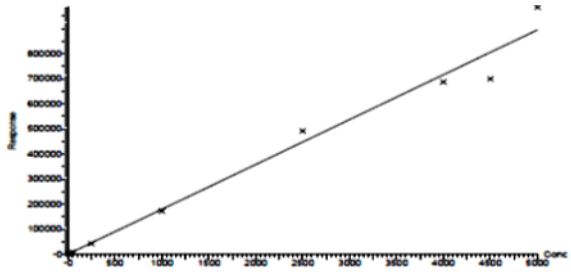

Figure 3: Calibration curve of Tenofovir

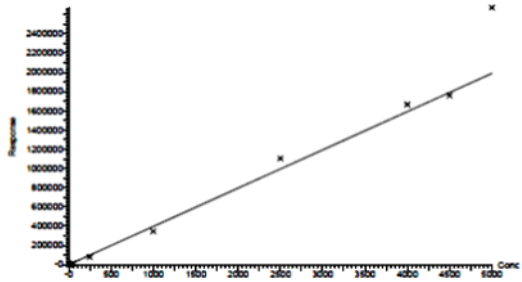

Figure 4: Calibration curve of Pioglitazone

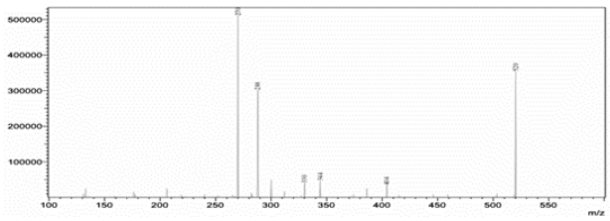

Figure 5: Product ion mass spectra of Tenofovir Disoproxil Fumarate in positive ionization mode

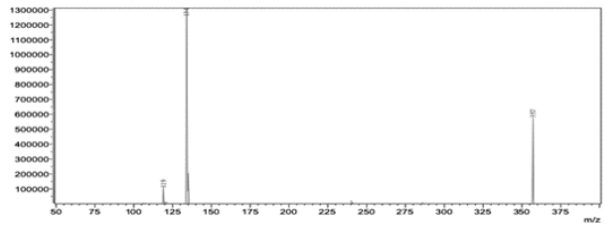

Figure 6: Product ion mass spectra of Pioglitazone HCL in positive ionization mode

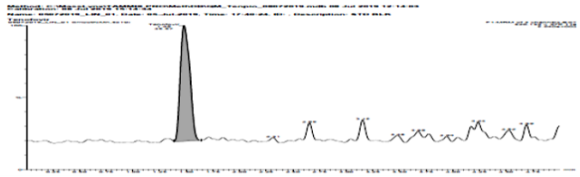

Figure 7: Representative example of the blank chromatogram for Tenofovir

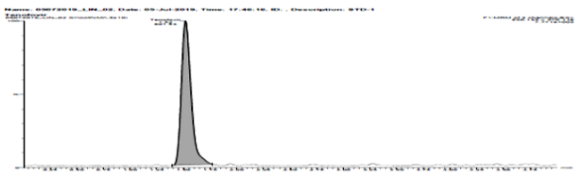

Figure 8: Representative example of LLOQ chromatogram of Tenofovir

Table 1: Positive ion mode (for Tenofovir Disoproxil fumarate and Pioglitazone HCL): Waters TQD MS conditions

Parameter	Value
Ionization type and polarity	ESI, positive ion mode
Ion source	Turbo spray
Scan type	MRM
Ion spray voltage	5500V
Source Temp	150
Desolvation temp	400
Desolvation gas	750
Cone gas	40

Table 2: Summaryof MRM transition

Compound	Mode of ionization	Q1 mass (m/z)	Q3 mass (m/z)	CE	Cone Voltage
Tenofovir Disoproxil Fumarate	Positive	520.13	270.12	30	38
Pioglitazone HCL	Positive	357.07	134.17	24	44

Table 3: Back-calculated standard curve data for Tenofovir in human plasma

	Concentration (ng/mL)						
Std conc.	Batch-1	Batch-2	Batch-3	Mean	SD	% CV	% Accuracy
5.000	0.94	1.00	1.00	0.98	0.035	3.53	98.0
10.000	1.96	2.01	2.03	2.00	0.036	1.80	100.0
50.000	9.78	9.76	9.77	9.77	0.010	0.10	97.7
250.000	51.05	50.67	50.86	50.86	0.190	0.37	101.7
1000.000	217.39	215.51	215.10	216.00	1.221	0.57	108.0
2500.000	491.02	486.71	485.74	487.82	2.811	0.58	97.6
4000.000	862.49	854.86	853.13	856.83	4.980	0.58	107.1
4500.000	851.03	888.39	841.80	860.41	24.670	2.87	95.6
5000.000	959.14	950.65	948.72	952.84	5.543	0.58	95.3

Table 4: Back-calculated standard curve data for Pioglitazone in human plasma

	Concentration (ng/mL)						
Std conc.	Batch-1	Batch-2	Batch-3	Mean	SD	% CV	% Accuracy
5.000	1.00	0.96	0.97	0.98	0.021	2.13	97.7
10.000	1.90	2.06	2.05	2.00	0.090	4.47	100.2
50.000	11.77	11.74	11.55	11.69	0.119	1.02	116.9
250.000	57.62	56.75	55.71	56.69	0.956	1.69	113.4
1000.000	225.88	221.88	217.77	221.84	4.055	1.83	110.9
2500.000	474.81	466.19	457.54	466.18	8.635	1.85	93.2
4000.000	794.61	793.17	778.33	788.70	9.012	1.14	98.6
4500.000	882.34	858.19	876.21	872.25	12.553	1.44	96.9
5000.000	936.18	970.12	960.75	955.68	17.528	1.83	95.6

Table 5: Calculated concentrations obtained for precision and accuracy batches of Tenofovir Disoproxil Fumarate and pioglitazone HCL

	Tenofovir Disoproxil Fumarate								
	Batch-1			Batch-2			Batch-3		
S. No.	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
1	3.70	441.93	818.23	4.39	515.75	908.50	4.42	511.24	1080.56
2	3.78	437.98	849.24	4.31	506.92	981.44	4.34	502.47	972.77
3	4.25	479.12	852.34	4.78	570.50	930.38	4.31	565.23	999.44
4	4.14	475.45	770.59	4.41	535.90	1008.37	4.81	570.43	941.42
5	4.52	414.43	832.56	4.45	559.64	949.82	4.44	531.19	969.64
6	3.97	448.59	860.21	4.59	575.50	965.34	4.48	554.71	978.56
Mean	4.06	449.58	830.53	4.49	544.04	957.31	4.47	539.21	990.40
SD	0.31	24.38	33.03	0.17	28.91	35.83	0.18	28.60	47.94
%CV	7.55	5.42	3.98	3.79	5.31	3.74	4.02	5.30	4.84
				Pioglitazone HCL					
1	4.45	510.19	1078.37	3.82	433.92	860.84	3.77	425.86	844.83
2	4.37	501.46	970.8	3.91	431.87	834.23	3.86	423.85	788.35
3	4.83	564.09	997.41	4.37	470.42	919.14	4.31	461.68	818.72
4	4.47	569.29	939.51	4.26	466.82	873.68	4.2	458.15	881.22
5	4.5	530.13	951.18	4.63	406.93	932.67	4.57	399.38	863.85
6	4.66	553.6	1020.21	4	440.46	908.86	3.95	432.28	858.21
Mean	4.55	538.13	992.91	4.17	441.74	888.24	4.11	433.53	842.53
SD	0.17	28.54	51.29	0.31	23.76	38.04	0.30	23.32	33.78
% CV	3.70	5.30	5.17	7.43	5.38	4.28	7.41	5.38	4.01

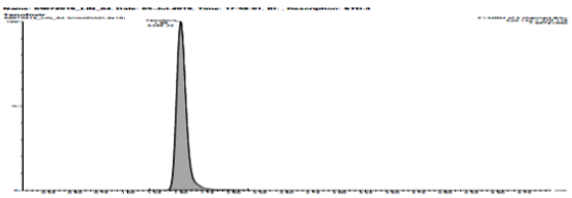

Figure 9: Representative example of LQC chromatogram of Tenofovir

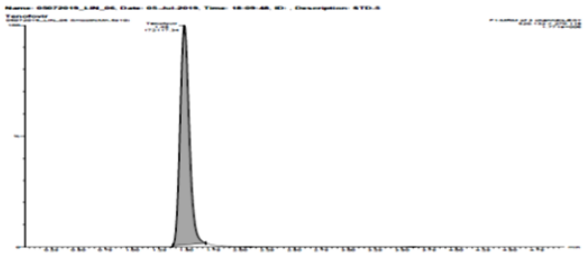

Figure 10: Representative example of MQC chromatogram of Tenofovir

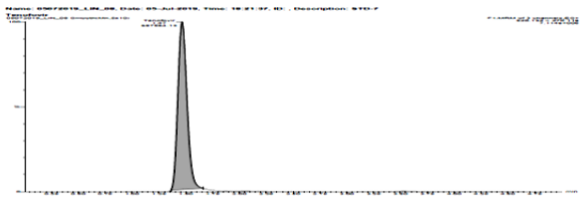

Figure 11: Representative example of HQC chromatogram of Tenofovir

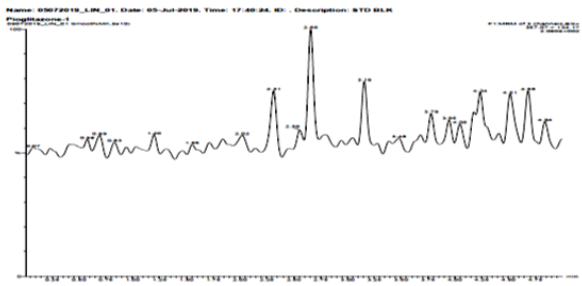

Figure 12: Representative example of the blank chromatogram of Pioglitazone

alternate days. The peak area is calculated from extracted ion to the internal standard. The main purpose of an experiment is to assess the least level. Form the nominal concentration of the analyte calibration curve was constructed. Calibration curve of Tenofovir Disoproxil Fumarate is shown in Figure 3 and pioglitazone HCL is shown in Figure 4.

MRM transition optimization

To provide the spectra, MRM tracks the precursor ion and their multiple product ions that are quan-

Table 6: Intra- and inter-run precision and accuracy of Tenofovir Disoproxil Fumarate and Pioglitazone HCL inhuman plasma

		Tenofovir			Pioglitazone HCL		
		LQC (4.2)	MOC (500)	HQC (900)	LQC (4.2)	MQC (500)	HQC (900)
BATCH-1 (N=6)	Intra-run mean	4.06	449.58	830.53	4.55	538.13	992.91
	Intra-run SD	0.31	24.38	33.03	0.17	28.54	51.29
	Intra-run % CV	7.55	5.42	3.98	3.70	5.30	5.17
	Intra-run % Accuracy	97	90	92	108	107	110
BATCH-2 (N=6)	Intra-run mean	4.49	544.04	957.31	4.17	441.74	888.24
	Intra-run SD	0.17	28.91	35.83	0.31	23.76	38.04
	Intra-run % CV	3.79	5.31	3.74	7.43	5.38	4.28
	Intra-run % Accuracy	107	109	106	99	88	89
BATCH-3 (N=6)	Intra-run mean	4.47	539.21	990.40	4.11	433.53	842.53
	Intra-run SD	0.18	28.60	47.94	0.30	23.32	33.78
	Intra-run % CV	4.02	5.30	4.84	7.41	5.38	4.01
	Intra-run % Accuracy	106	108	110	98	87	94
INTER-BATCH (N=18)	Inter-run mean	4.30	487.27	878.80	3.97	458.91	885.63
	Inter-run SD	0.12	22.6	53.45	0.26	28.93	42.67
	Inter-run % CV	2.79	4.63	6.0	6.5	6.3	4.81
	Inter-run % Accuracy	102	97	98	95	92	98

Table 7: Tenofovir Recovery Data

S.No.	LQC Area (Counts)		MQC Area (Counts)		HQC Area (Counts)	
	Aqueous	Extracted	Aqueous	Extracted	Aqueous	Extracted
1	2714	2687	47892	38421	98123	91588
2	2766	2680	48429	43568	96982	86088
3	2727	2549	46231	41098	94543	92702
4	2754	2628	49920	46532	93421	91676
5	2806	2782	47793	45874	92135	86165
6	2700	2547	45774	43217	90762	88362
Mean Area	2744.5	2645.5	47673.2	43118.3	94327.6	89430.2
% Recovery	96.4		90.4		94.8	

Avg. % Recovery= 93.9; SD= 2.5; % CV= 2.7

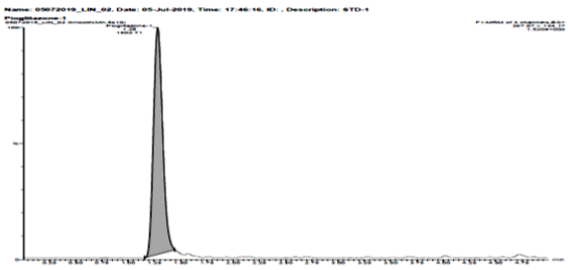

Figure 13: Representative example of LLOQ chromatogram of pioglitazone

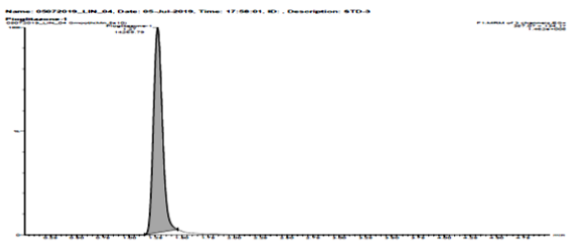

Figure 14: Representative example of LQC chromatogram for Pioglitazone

Table 8: Pioglitazone Recovery Data

S. No.	LQC Area (Counts)		MQC Area (Counts)		HQC Area (Counts)	
	Aqueous	Extracted	Aqueous	Extracted	Aqueous	Extracted
1	35298	37018	857071	845961	1428851	1518928
2	35440	36785	853245	847928	1456969	1463473
3	34354	36845	861064	888406	1476750	1509093
4	36789	36345	867852	864807	1483957	1469930
5	37296	37531	875386	884702	1466265	1361762
6	37807	37512	862120	881363	1452747	1445909
Mean Area	27330.67	27006.00	862789.7	868861.2	1460923	1461516
% Recovery	98.81		100.70		100.04	
Avg. % Recovery=99.85; SD= 0.96; % CV= 0.96						

Table 9: Stability data showing the % CV of analytes

Analyte name	Fresh samples	Freeze-thaw stability	Auto-sampler stability	Bench-top stability
Tenofovir				
LQC	9.12	12.09	8.98	4.19
MQC	5.06	9.12	5.42	9.13
HQC	8.63	6.34	3.09	8.01
Pioglitazone				
LQC	3.21	9.53	9.23	9.87
MQC	7.55	6.07	7.54	6.31
HQC	6.21	6.89	6.92	5.03

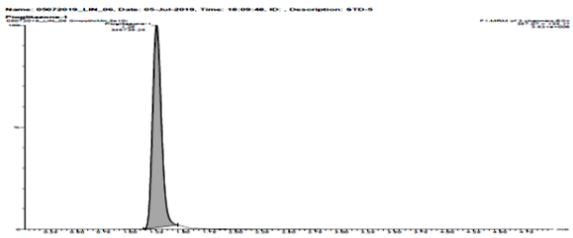

Figure 15: Representative example of MQC chromatogram for Pioglitazone

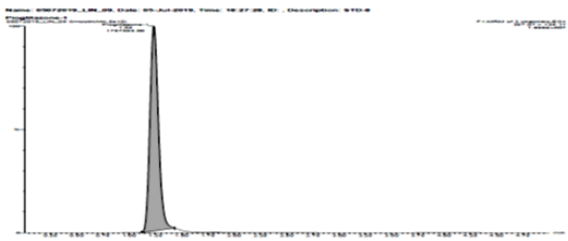

Figure 16: Representative example of HQC chromatogram for Pioglitazone

tifiable. They form different collision ions with each transition from parent ion to daughter ion. Each transition of collision energy is optimized by MRM transitions. Precursor ion is known as parent ion. Selective transmission of precursor ions (MS1) is passed through Q1 by m/z separation and are fragmented with collision energy to form product ions (MS2).

Product ions are known daughter ions. The m/z values of Tenofovir Disoproxil Fumarate are 520.13 →270.12 and Pioglitazone HCL is 357.07 →134.17. Table 2 shows the MRM transitions. Figure 5 & Figure 6 shows the product ion mass spectra of Tenofovir disoproxil fumarate and pioglitazone.

Linearity

It is defined as the relationship between analyte signals and analyte concentration in calibration samples. By using 1× linear regression calibration curves are generated by using analyst 1.5 software. The linearity ranges differ from one ion source to others. In ESI source, the linearity is lost in high concentrations and at lower concentrations, linearity is maintained. Table 3 and Table 4 show the back-calculated standards.

Accuracy and precision

Inter and intra batch precision and accuracy values are shown below. Precision is expressed as a standard deviation. In a multistep preparation

procedure, the results obtained show the acceptable accuracies. Accuracy refers to the closeness of true value and for measurement of uncertainty. The coefficients of variation are less than 15% for each analyte. The precision and accuracy analyzed by LQC, MQC and HQC are shown in Table 5. Table 6 shows the intra and inter run precision and accuracy. Figure 7, Figure 8, Figure 9, Figure 10 & Figure 11 shows the chromatogram of Tenofovir Disoproxil Fumarate and Figure 12, Figure 13, Figure 14, Figure 15 & Figure 16 shows the chromatogram of Pioglitazone HCL.

Recovery

The recovery is related to the separation approach inside variability stages**.** By comparing with the detector response, the recovery of an analyte is defined as the added amount of analyte to the extracted from the matrix. The average recovery rate of Tenofovir and pioglitazone is 93.9% and 99.85%. LQC, MQC and HQC values were also calculated as shown in Table 7 & Table 8.

Stability

Freezing and thawing quality control samples are validated by three cycles and the obtained values are shown in Table 9, which are within the range of inter-day precision and accuracy samples result in no instability. In autosampler, the quality control sample is kept for 24 hours at 4^0C and the values obtained are within the range of inter-day QC samples leads to the deduction that no instability is found during the runtime. In Bench-top stability quality control sample are kept for 4 hours at room temperature and the values obtained are within the range of inter-day QC samples leads to the deduction that no instability is found during the runtime.

CONCLUSION

This method describes a simple, rapid, sensitive, specific, LC-ESI-MS/MS assay of Tenofovir and Pioglitazone for simultaneous detection and accurate measurement in human plasma using Metoprolol as an internal standard and to extract the analytes the precipitation technique was used. Linearity is fixed at 5 to 5000ng/mL. The m/z values of tenofovir were 520.13→270.12 and for pioglitazone 357.07→134.17. They calculated the Intra-day and inter-day precision and accuracy values at n=6. The average recovery rate of tenofovir and pioglitazone is 93.9%and 99.85%. No instability was found during the runtime.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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