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DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL METHOD FOR THERAPEUTIC DRUG MONITORING OF DOLUTEGRAVIR AND RILPIVIRINE IN HUMAN PLASMA USING ULTRA FOW HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT:

An accurate, highly sensitive, and precise method for quantitative analysis of Dolutegravir (DOL) and Rilpivirine (RIL) by Ultra Flow High Performance Liquid Chromatography and Tandem Mass Spectroscopy in Human Plasma was proposed and validated successfully using Dolutegravir D4 as internal standard (ISTD). An aliquot of 200 μL of plasma was mixed with internal standard dilution and extraction was performed by using Liquid – Liquid extraction Technique. Peak resolution was achieved on symmetry C18 (4.6 X 150 mm, 5µm) column. The total analytical run time was 3 minutes. Both analytes were monitored using Multiple Reaction Monitoring scan (MRM) and the mass spectrometer was operated in positive polarity mode. The method was validated for specificity, sensitivity, precession, accuracy, and other analytical parameters. The results found were satisfactory over the linear calibration range of 2.000 ng/mL to 1001.734 ng/mL. The developed method can be ready to use by scientific community for quantification of analytes in plasma samples from various clinical studies of different dose strengths.

INTRODUCTION:

Dolutegravir(DOL)chemically is(4R,12aS)-N-(2,4-difluorobenzyl)-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido[1',2':4,5]pyrazino[2,1-b] [1,3]oxazine-9-carboxamide is an antiviral agent used for the treatment of HIV-1 infections in combination with other antiretroviral agents. HIV-1 integrase inhibitor that blocks that blocks the strand transfer step of the integration of the viral genome into the host cell. Unlike some other antiretroviral drugs, dolutegravir has no homology in human host cells, which contributes to its excellent tolerability and minimal toxicity1(1-2). Dolutegravir undergoes metabolism through three main pathways, one is by Glucuronidation by UGT1A, secondly metabolised by cytochrome P 450 and also by sequential oxidative defluorination and glutathione conjugation. Importantly, dolutegravir (DOL) does not form long-lived metabolites. 50 mg of Dolutegravir presents an apparent volume of distribution of 17.4L. The median dolutegravir concentration in CSF was 18ng/mL after 2 weeks of treatment. (3-4). As per literature surveyfew analytical methods like RP-HPLC, UPLC and LCMS/MS methods were reported for the estimation in combination forms in formulations and biological fluids.

Rilpivarin (RIL)4-{[4-({4-[(E)-2-cyanovinyl]-2,6-dimethylphenyl} amino) pyrimidin-2-yl] amino} benzonitrile. It is non-nucleoside reverse transcriptase inhibitor which is used to treat HIV – 1 infection (5-6). It is a diarylpyramidine derivative. RIL in combination with dolutegravir was approved as part of the first complete treatment regimen with only two drugs for the treatment regimen with only two drugs for the treatment of adults with HIV-1. Its binding results in the blockage of RNA and DNA-dependent DNA polymerase activities, like HIV-1 replicatipn. It does not present activity against human DNA polymerases α, β& γ.Rilpivirine's flexible structure around the aromatic rings allows the adaptation to changes in the non-nucleoside RT binding pocket, reducing the likelihood of viral mutations conferring resistance.It is predominantly metabolized by CYP3A4 and CYP3A5 to the hydroxylated metabolites M1, M2, M3, and M4. As per literature survey few analytical methods like RP-HPLC, UPLC and LCMS/MS methods were reported for the estimation in combination forms in formulations and biological fluids. (7-17).

EXPERIMENTATION

Reference standards and reagents:

The high purity reference standards of DOL, RIL and Dolutegravir D4 were procured from Honour labs Pvt Ltd. The LCMS grade Methanol, Acetonitrile & Mili Q water are purchased from Scharlau and AR grade TBME, Sodium Hydroxide and Ammonium Bicarbonate were procured from Merck specialities Pvt Ltd.

Analytical Instrumentation:

An ultra flow prominence high performance liquid chromatography (UF-HPLC) coupled with tandem mass spectrometer (MS/MS-3200 model, Sciex, Canada) was used for analysis. The mass spectrometer was assembled with electro spray ionization (ESI) interface. The HPCL was supplied with LC-20 binary pumps solvent degasser, column oven, and high-throughput SIL HTC auto sampler. After chromatographic separation, the positive polarity MS detection was performed in multiple reaction monitoring (MRM) mode. Analyst software 1.5.1 platform was used for data collection and hardware controlling.

Chromatographic Conditions:

Analytical peak resolution was achieved in Symmetry C18 (4.6 X 150 mm, 5µm) pumped with isocratic mobile phase Acetonitrile: 0.1 % Formic acid (55/45V /V). The flow rate was 0.8ml/min with 80% splitting Injection volume 15.000µL was injected into the LC-MS/MS system

MS/MS compound and source dependent conditions:

The Mass spectrometer was operated in positive mode to monitor parent product ion (m/z) transitions of analytes DOL&RIL and their internal standard Dolutegravir D4. The specific details of MRM transitions and their respective mass spectrophotometer voltage values like Declustering potential (DP), Entrance Potential (EP). Colllision Energy (CE), and Collision exit potential (CXP) used for quantification of respective and ISTD is summarized in Table 1.

Manual turning was performed to optimize the source dependent and compound dependent parameters to get highest credible intensities. The source dependent parameters like drying gas (GSI) and nebulizer gas (GS2) were set at 45 & 50 psi. The turbo ion spray temperature and ion spray voltage were set at 500^0C and -4,500 V respectively. The curtain gas (CUR) and collision associated dissociation gas (CAD) pressure were maintained at 30 psi and 5 psi. The ultrasound sound mode was employed in Q1 and Q3 (quadrupoles) with dwell time of 200 Milli seconds

Standard curve and control samples:

Stock solutions of DOL and RIL were prepared in methanol and respective working (spiking) dilutions were made using same solvent. Separate stock weighing was done for preparation of Calibration curve and Quality control stock solutions. Calibration curves in range 200-66000 ng/ml and 150-33000 ng/ml. Quality control samples were made at concentration of 100 ng/ml Lower limit of Quality Control (LLOQC), 335 ng/mL Lower quality Control (LQC), 3888ng/ml Middle Quality Control (MQC), 30000 ng/ml Highest Quality Control (HQC). The 2% of respective working dilution was spiked into total volume of plasma to get the above-mentioned concentrations for both analytes. The long term plasma stability samples at LQC and HQC level were prepared and stored at -70^0C.The spiked samples were freshly prepared based on the validation experimentation plan. All the stock solutions and working dilutions were stored in refregirator maintained at 2-8^{o}C

Bio-Analytical extraction procedure:

Required number of plasma samples were retrieved from deep freezer thawed them in water bath maintained at room temperature and vortexed to mix. 50 µL of internal standard solution (3000.000 ng/ml) was taken in to pre-labelled polypropylene tubes, except in standard blank samples where in 50 µL of solution was taken. 200 µL of plasma samples were aliquoted into above polypropylene tubes vortexed to mix then 50 µL of extraction buffer (0.2N Sodium Hydroxide Solution) solution was added and vortexed to mix. 2 mL of TBME was added to all polypropylene tubes and were vortexed for about 5 minutes at 2500 rpm. All the polypropylene tubes were centrifuged at 4000 rpm, at 5^0 C for about 5 minutes. 1.500 mL of supernatant was collected and transferred into pre-labelled ria vial and was evaporated under nitogen gas at 40^0C till dryness. The dried residue was reconstituted with 0.500 mL of reconstitution solvent and vortexed to mix then transferred to pre labelled auto sampler vials. The samples were injected to LC-MS/MS instrument for analysis.

METHOD VALIDATION:

System suitability:

System suitability is performed by System suitability experiment was performed by injecting six consecutive injections using Aqueous SYS prior to start of the day or in other occasions as per the SOP for “System Suitability, System performance and Autosampler Carryover”. The % C for area ratio (analyte /ISTD for both DOL&RIL) of high standard solution should be less tha 4.

Biological Matrix Screening and selectivity:

The percentage of interface due to exogenous and endogenous components at retention times of analytes and ISTD was evaluated by processing eight different lots of blank plasma along with each two lots of hemolytic and lipemic plasma. The interface due to concomitant medication at retention time was also investigated by spiking into drug free plasma at concentration equal to teir available literature Cmax values. The interface observed at the retention times of analytes and ISTDs in blank plasma lots was compared against mean response of extracted LLOQ (n=6) samples. The observed interface should be less than 20% and 5% at analyte and ISTD retention times, respectively, when compared to mean response of extracted LLOQ samples.

Reproducibility (precession) and Accuracy:

At four different quality control levels (LLOQQC, LQC, MQC, and HQC, n=12) within day (intrabatch) and between day (interbatch n = 24) precession and accuracy of DOL, RIL was evaluated by calculating the %CV and %accuracy. In together six reproducibility batches were performed on two different days by two different analysis.

Effect of Matrix:

The signal suppression or enhancement via ionization should be studied in Mass spectrometric detection methods. To prove that the method is free from matrix effect, post extraction response from 10 different lots (including each two lots of hemolytic and lipemic plasma) were compared with response of aqueous samples. The matrix effect was evaluated at LQC, HQC levels by calculating matrixfactor of analyte and ISTD. Later ISTD normalized matrix factor was calculated by using matrix factor of analyte and ISTD. If ISTD normalized matrix factor value is 1, that indicates there is no suppression or enhancement due to the presence of matrix. If the value is less than 1, that indicates ion suppression or more than 1, that indicates ion enhancement. The acceptable limits for ISTD normalized matrix factor are 0.85 – 1.15.

Linearity of Analytes:

The method Linearity was assessed by constructing three eight-point calibration curves. A linear least square regression analysis was applied for back calculated concentrations using weighing factors, none, 1/x, 1x2. The weighing factor with least regression value is 1/x2; therefore for 1/x2 was further used as weighing factor for constructing the calibration curves throughout the validation.

Extraction Recovery/Efficacy:

Good extraction recovery was needed for accurate and reproducible results. Stable and consistent recovery was the basic requirement to achieve method sensitivity at limit of quantification (LOQ) level. The analyte recovery might be low or medium or 100% but it should be steady at all levels (LQC, MQC, HQC). Care shouldbe taken while optimizing the procedure to achieve good extraction recovery. Relative recovery (RR) was evaluated at three different levels LQC, MQC, HQC (n=6) by comparing response in post spiked samples versus extracted samples. To evaluate true effect of matrix on recovery of analyte and ISTD (absolute recovery-AR), the response of extracted samples was also compared with aqueous samples. The recovery of analyte should not be more than115%.

Stability of Analytes/ISTD:

Stability of Analytes (DOL, RIL) was evaluated in different experimental conditions based on the requirement of real time unknown sample analysis conditions like freeze and thaw stability

(at– 70^0C), dry extract stability, spiked sample room temperature stability, auto sampler stability, long term stability and stability in whole human blood. For all stability experiments six replicates of LQC, HQC samples were processed and analysed against fresh calibration curve. The back calculated concentrations are compared to nominal concentration. Stability of aqueous samples were assessed by comparing the responses from high standard solutions prepared from stored aqueous stock solutions/ working dilutions (at 2-8^0C) with freshly prepared stock solutions/working dilutions.

RESULTS AND DISCUSSIONS:

For efficient quantification and reliable results, it is prerequisite to give equal importance to optimize the chromatographic conditions, extraction procedure and mass spectrometric conditions. All analytes dissolve in methanol, individually infused into MS (Mass Spectrometer) source for tuning and then selected positive mode because of better intensity. The Q1 scan was performed to select the parent ion. The declustering potential (DP), Entrance Potential (EP) voltage values were further optimized to get highest intensity for parent ion. After that, collision energy (CE), Collision Cell exit potential (CXP) values were optimized in MSMS scan to select product ion for DOL, RIL and DOL-D4. The observed $(M+H)^+$ peaks (parent ion) and respective consistent product ion was selected for Mass Spectrometric transitions and optimized voltage values were shown in table 1. The unit resolution mode with a dwell time 300 milliseconds was used for each MRM transition channel.

Several analytical bonded stationary phases of C_8 and C_{18} were checked and retention times of analytes are overlapped. Initially, aqueous solution of LLOQ level was injected into normal C_8 (50 × 4.6 mm, 5 μm) column, but theobserved peak resolution was not good and peak intensity is very low, the identical chromatogram of LLOQ solution in symmetry column was shown in Figure1.Then sample solution was injected into thermo high purityC_{18} (2.1 × 50 mm, 3.5 µm), column to improve the peak shape. The observed peak resolution was comparatively good with low intensity. The better peak shape and resolution with required sensitivity was achieved on Phenomenex, PFP (50×4.6mm, 2.6μm) column may be because of its combining C_{18} retention properties and unique aromatic PFP selectivity. A medium level buffer of Ammonium Bicarbonate gives high signaltonoiseratiowithnegligiblebaselinenoiseatLLOQlevel.

In sample extraction, Liquid- liquid and solid phase extraction techniques were investigated. In solid phase extraction high base line was observed because of possible matrix contaminants. Finally Liquid-Liquid extraction was selected due to its high consistent extraction recoveries with no matrix effect and cleaner extracts. Method was strictly optimized to get similar recoveries for analytes and ISTDs. The nearly same % recovery results for analytes and ISTD withacceptableISTDnormalizedfactorvaluesofthemethodassurereproduciblequantification.

Selectivity:

Eight plasma lots along with each two different lots of hemolytic and Lipemic plasma were processed and injected for LC-MS/MS analysis. Similar chromatography was observed with no significant interference at the retention times of analytes and ISTD in all analyzed blank lots, which indicates that the developed method was highly selective.

Linearity:

Three calibration curves were generated by plotting the area ratios (analyte response/ISTD response) on y-axis and concentration on x-axis. The plot was linear throughout the established calibration ranges, 2.000 ng/mL to 1001.734 ng/mL.The slope values are consistent and regression values were foundtobemorethan0.99.Thebackcalculatedconcentrationsfor individual calibrationstandards are meetingacceptancecriteriaforaccuracy(±15%)andprecision(≤15%).

Sensitivity:

Six replicates of LLOQ samples were processed and analysed against calibration curve. The accuracy, Precession values were 97.8% and 0.67% for both analytes. The observed signal to noise ratio is more that 5:1 for both analytes.

Precession and Accuracy:

Accuracy and reproducibility results of intra and inter batches of DOL and RIL were reported in tables 2 respectively. The intra and inter batches accuracy values were in the range of 91% to 98% and intra and inter day precession were found to be less than 6.3% for both analytes. The chromatogram at LOQ level was shown in figure 4.

Effect of Matrix:

In general considerations, effect of matrix does not influence peak resolution due to MS selectivity. However, in this method sufficient resolution between the analytes (DOL, RIL) was established chromatographically. Matric effect was evaluated in 10 different lots. The obtained ISTD normalized matrix factor at LQC and HQC level were 1.024 and 1.025. The resulted presented in table 4.

Recovery:

Absolute and relative recovery of analytes and ISTD was evaluated. The mean recovery results of DOL and RIL are represented in Table 5

Dilution Integrity:

Precession and Accuracy of diluted plasma samples were assessed at 1:4 dilution. The DQC was prepared by spiking at a concentration equal to two times of High-level calibration standard of proposed range for DOL and RIL respectively. Then ¼ th volume of plasma aliquot was diluted with drug free plasma and analysed against calibration curve. The accuracy values were 87.91% to 88.41%. The % CV was 0.66% and 0.96% for DOL and RILrespectively.The % C.V. for Dilution Integrity of 1/2 and 1/4 were found to be 0.66% and 0.96% respectively. The % Accuracy for Dilution Integrity of 1/2 and 1/4 were found to be 87.91% and 88.41% respectively.

Stability:

All the stock solutions and stock dilutions werestable for 21 days at refrigerated storage maintained at 2-80C. The processed stability samples in plasma at LQC and HQClevels were analyzed against freshly prepared calibrationcurve. The stability data results are given in Table 6. The DOL and RIL were stable in plasma at room temperature for about 4 hours. And for 7 freeze and thaw cycles. The established stability time for DOL and RIL were 49 h and 4 h 18 mins for auto sampler and dry extract stabilities. The analytes were found to be stable for 2 h in whole blood. The long-term stability was evaluated and analytes were stable at -700C.

CONCLUSION:

Full method validation was carried out using screened and pooled human plasma to ensure that developed procedure is accurate and precise for estimation of DOL and RIL simultaneously. The high though put LC ESI-MS/MS method is sensitive and specific.The recovery, precession and accuracy results were reproducible over the proposed calibration ranges for DOL and RIL. The

shorter run time allows the analysis of more samples per day. The method can be readily used by scientific community for application of sample analysis for therapeutic monitoring/ pharmacodynamics or bioequivalence studies.

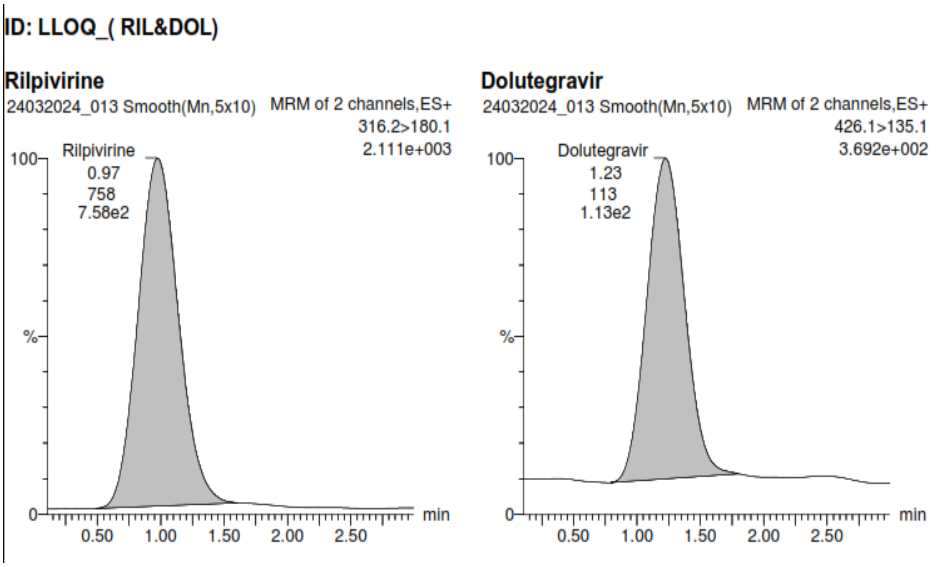

FIGURE 1

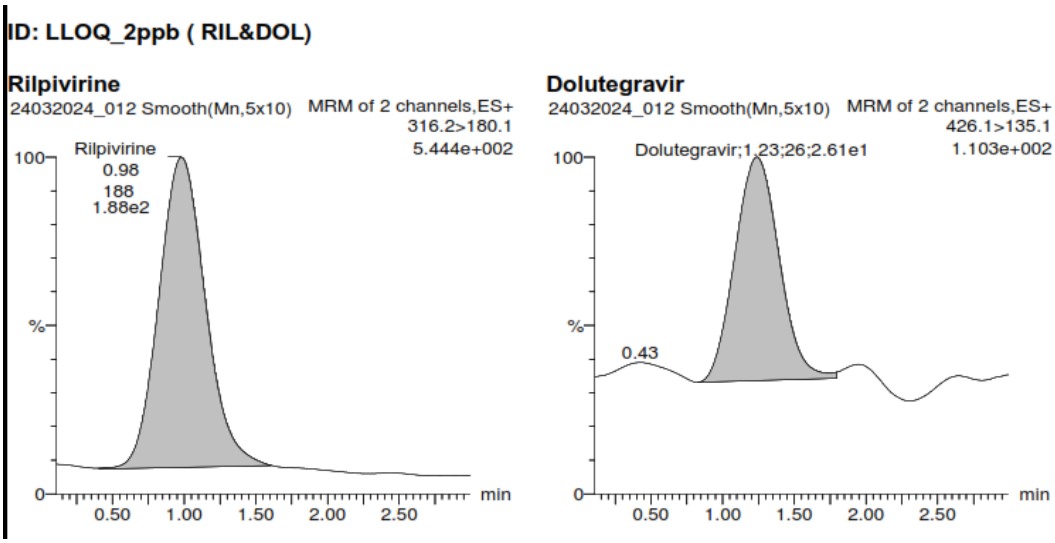

FIGURE 2

TABLE 1: MRM and Mass spectrometer Voltage details

NAME OF THE MOLECULE	DP	EP	CE	CXP
Doltegravir	-92	-10	-22	-14
Rilipivirinr	-92	-10	-22	-14
Doltegravir D4	-94	-10	-22	-14

TABLE 2: Precession and accuracy results

QC ID	LLOQ QC	LQC	MQC	HQC
Nominal conc. (ng/mL)	**2.000**	**6.71**	**77.76**	**600**
Nominal conc. Lower range (ng/mL)	**1.600**	**5.089**	**75.931**	**579.740**
Nominal conc. Upper range (ng/mL)	**2.400**	**6.885**	**79.965**	**612.236**
Batch ID & Date	**Back Calculated conc. (ng/mL)**			
AP1 and DI	1.708	5.524	76.702	534.660
	2.001	5.409	78.998	584.852
	2.075	5.475	76.184	598.878
	1.945	5.574	76.091	589.295
	2.000	5.474	78.793	580.738
	2.049	5.587	75.730	598.103
N (Number of samples)	**6**	**6**	**6**	**6**
Mean	**1.963**	**5.507**	**417.083**	**581.0877**
S.D	**0.133**	**0.068**	**1.439**	**23.84617**
%C.V	**6.78**	**1.23**	**0.35**	**4.103713**
%Accuracy	**98.15**	**91.98**	**94.05**	**94.25**
150424 AP2 and REC	1.917	5.468	79.849	579.278
	1.962	5.317	77.107	579.316
	1.983	5.339	76.778	579.431
	1.963	5.527	76.261	579.628
	1.964	5.557	78.628	758.215
	1.934	5.584	78.741	579.558
N (Number of samples)	**6**	**6**	**6**	**6**
Mean	**1.954**	**5.465**	**77.894**	**579.2377**
S.D	**0.024**	**0.113**	**1.386341**	**0.518837**
%C.V	**1.23**	**2.07**	**1.779779**	**0.089572**
%Accuracy	**97.70**	**91.28**	**94.31**	**94.05**
Intra-Day Accuracy & Precision				
N (Number of samples)	**12**	**12**	**12**	**12**
Mean	**1.958**	**5.486**	**417.655**	**729.663**
S.D	**0.091**	**0.092**	**1.656**	**2.702**
%C.V	**4.65**	**1.68**	**0.40**	**0.37**
%Accuracy	**97.90**	**91.63**	**94.18**	**94.15**
AP3 and RIR 150424	1.924	5.595	76.458	579.278
	2.063	5.596	78.433	579.316
	1.728	5.806	79.336	579.431
	2.021	5.772	78.193	579.628
	2.026	5.868	78.234	758.215
	1.967	5.741	78.672	579.558
N (Number of samples)	**6**	**6**	**6**	**6**
Mean	**1.955**	**5.730**	**78.221**	**579.2377**

S.D	**0.121**	**0.112**	**0.959238**	**0.518837**
%C.V	**6.19**	**1.95**	**1.226318**	**0.089572**
%Accuracy	**97.75**	**95.71**	**91.76**	**93.55**
Inter-Day Accuracy & Precision				
N (Number of samples)	**18**	**18**	**18**	**18**
Mean	**1.957**	**5.567**	**414.066**	**728.112**
S.D	**0.099**	**0.152**	**6.044**	**4.643**
%C.V	**5.06**	**2.73**	**1.46**	**0.64**
%Accuracy	**97.85**	**92.98**	**93.37**	**93.95**

TABLE 3: MATRIX EFFECT FOR EXTRACTED HQC SAMPLES

	Matrix I.Ds.	**Extracted HQC Samples**				
		Analyte Area Dolutegravir	**Matrix Factor for Analyte**	**Internal Standard Area**	**Matrix Factor for Internal Standard**	**IS Normalized Matrix Factor**
150424 **DR**	HQC-1-150424	568533246	1.028	4248	1.024	1.004
	HQC-2-**150424**	550863186	1.025	4227	1.019	1.006
	HQC-3-150424	498919137	1.077	4477	1.079	0.998
	HQC-4-150424	491948479	0.971	4068	0.981	0.990
	HQC-5-150424	507569136	1.057	4385	1.057	1.000
	HQC-6-150424	483665249	1.100	4576	1.103	0.997
	150424-LIPEMIC-1	558211649	0.992	4107	0.990	1.002
	150424-LIPEMIC-2	525378036	0.980	4052	0.977	1.003
	150424-HAEMOLYSED-1	53289818	1.030	4252	1.025	1.005
	150424-HAEMOLYSED-2	551665877	0.991	4130	0.996	0.995
Calculation of Matrix Factor		**Mean**	**1.025**		**1.025**	**1.000**
		S.D.	**0.043**		**0.043**	**0.005**
		C.V.	**4.20**		**4.2**	**0.50**

TABLE 4: RECOVERY RESULTS

	LQC		**MQC**		**HQC**	
	Extracted	**Unextracted**	**Extracted**	**Unextracted**	**Extracted**	**Unextracted**
	Analyte Peak Response	**Analyte Peak Response**	**Analyte Peak Response**	**Analyte Peak Response**	**Analyte Peak Response**	**Analyte Peak Response**
150424 AP2 and REC	48255.35	69632.38	530808.8	765956.2	4294726	6197282
	51636.67	66739.18	568003.4	734131	4595664	5939787
	53223.96	72943.79	585463.5	802381.7	4736932	6491997
	52688.19	64710.7	579570.1	711817.7	4689249	5759252
	48066.76	61519.71	528734.4	676716.8	4277942	5475254
	50368.7	66055.45	554055.7	726609.9	4482814	5878935
N(Number of samples)	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**50706.605**	**66933.54**	**557772.7**	**736268.9**	**4512888**	**5957085**
S.D. (±)	**2200.9266**	**3961.47**	**24210.18**	**43576.18**	**195882.4**	**352570.9**
% C.V.	**4.3405126**	**5.918513**	**4.340511**	**5.918515**	**4.34051**	**5.918514**
% Recovery	**74.79**		**77.55**		**75.76**	

Overall Recovery of Analyte	**76.03**
% C.V. of Mean Recoveries	**1.84**

TABLE 5: STABILTY RESULTS

STABILITY EXPERIMENT	STABILITY CONDITION	% MEAN STABILITY	
		LQC	HQC
Auto Sampler Stability	50 h	90.50	94.08
Free and Thaw stability	7 cycles at -70+/- 15^0C	90.75	94.80
Dry Extract stability	4h 18 mins	95.29	96.49
Room temperature stability	4 h 9 mins	95.84	96.72
Long Term stability	25 days 17 h	92.42	93.71
Stability in blood	2 h 57 mins	100.32	100.32

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