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DEVELOPMENT AND VALIDATION OF BIOANALYTICAL METHOD FOR THE ESTIMATION OF DARUNAVIR AND CEFALEXIN IN HUMAN PLASMA

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ABSTRACT: -

Rapid and sensitive HPLC method has been developed for the determination of Darunavir and Cephalexin in human plasma. The mobile phase employed is Acetonitrile: Water in the ratio of 50:50 v/v. Validation results proved that the developed method performs well with selectivity, precision, accuracy, stability and linearity for the concentration range of2- 12µg/ml of Darunavir and from 5µg/ml to 30µg/ml of Cephalexin to be found in human plasma. Ritonavir and Voriconazole is used as an internal standard and it suitable for the determination of drug in human plasma at different therapeutic dose levels. The Mean Peak plasma concentration reported is 6.7mg/lit. It utilizes liquid liquid extraction with Dichloromethane as the sample preparation technique. The mean recovery of Darunavir is found to be 94.26% and in

Fast analysis, Cephalexinutilizes protein precipitation as the sample preparation technique, which eliminates the drawbacks of less recovery due to liquid-liquid extraction or the use of solid phase extraction cartridges which is relatively costly. The mobile phase employed is Toluene: Methanol: Triethylamine in the ratio of 6:4:0.1 v/v/v. The % mean recovery of Cephalexin has been found to be 94.25%. The proposed method can be used for therapeutic drug monitoring in order to optimize drug dosage.

KEYWORDS

Darunavir, Cephalexin,Human plasma,Validation,protein precipitation

1.INTRODUCTION

Qualitative analysis gives an indication of the identity of the chemical species in the sample and quantitative analysis determines the amount of one or more of these components. Analytical chemistry is the study of the separation, identification, and quantification of the chemical components of natural and artificial materials. The use of analytical methods in the discovery, development and manufacture of pharmaceuticals. Various fields such as research, development and application of medicinal product

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require analytical input [1].Bio-analytical methods are developed in biological fluids such as plasma, serum, urine, cerebrospinal fluid, synovial fluid etc.to meet specific needs. For example, pharmacodynamic and pharmacokinetic assessment, bio- equivalence studies for the drugs require the ability to measure them in biological fluids. These methods should be developed under the 'Good Laboratory Practice' standards issued by the US Food and Drug Administration (FDA) and provide accurate and precise results [7].

The root of the word chromatography, chroma (Greek khrōma, color) and grafein is "to write", indicates that the separated components in some forms of the technique can be identified by their color alone. Chromatography consists of a diverse and important group of methods that allow the separation, identification, and determination of closely related components of complex mixtures.

Bioanalytical Method used to determine the concentration of drug or its metabolite or both in biological matrix such as plasma, serum, urine.Bioanalytical information used in human clinical pharmacology, bioavailability (BA) and bioequivalence (BE) studies requiring pharmacokinetic evaluation and Bioanalytical method is also used for non-human pharmacology/ toxicology studies. It is important for new drug discovery, new drug development, preformulation studies, formulation studies, validation of product, analysis of compound, method development studies and Bioanalytical research purpose.

1.1HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC utilizes a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result,HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures [12].

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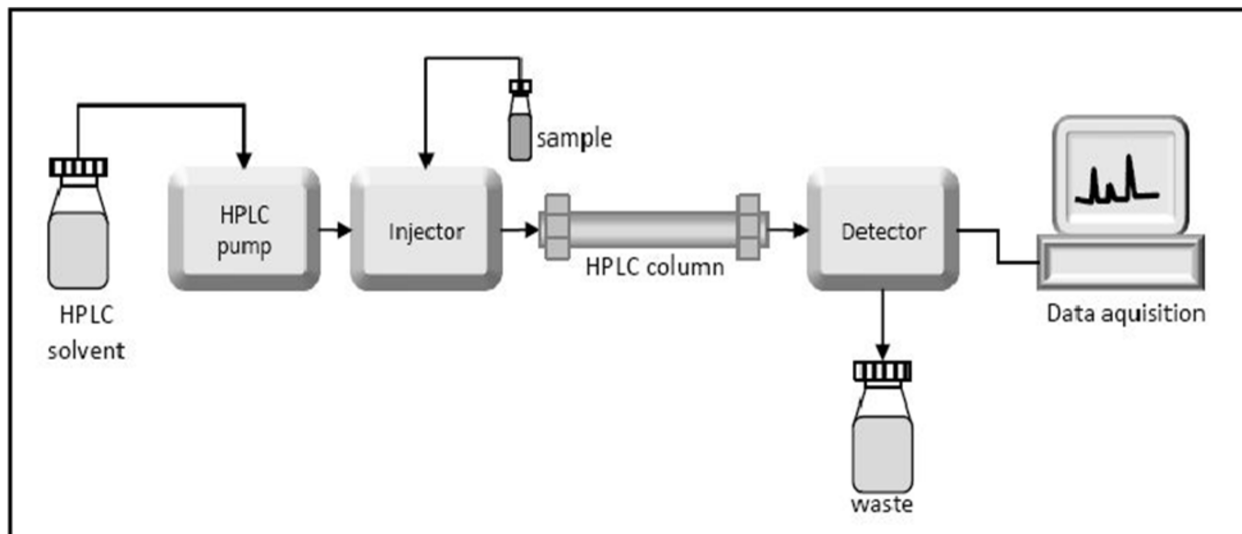

Figure: 1.1 Schematic diagram of HPLC

1.2 METHOD DEVELOPMENT IN HPLC [18]

In developing HPLC method for the quantitative analysis of pharmaceutical formulation the following general requirements should be fulfilled.

- The identity of the component to be analyzed should be established.
- Separation of specific components should be achieved.
- Sample preparation should be reproducible.
- Standard of known purity should be available, as accuracy will be directly related to the degree of purity of standards used in determination.
- A stationary phase that separates the component in reproducible manner.
- There must be a constant flow of mobile phase.
- Sample application or injection should be reproducible.

1.3 BIOANALYTICAL METHOD DEVELOPMENTS AND VALIDATION

The quantitative determination of drugs and their metabolites in biological matrices (bioanalysis) includes a number of steps from sample collection to the final report of the results. The intermediate steps typically include sample storage, sample preparation, separation, identification and quantification of

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analyte(s) [20]. Sample preparation prior to the chromatographic separation has three principal objectives:

- The dissolution of the analyte in a suitable solvent
- Removal of as many interfering compounds as possible
- Pre-concentration of the analyte

Measurement of drug concentration (levels) in blood, plasma or serum is the most direct approach to assessing the pharmacokinetics of the drug in the body. Assuming that drug in the plasma is in dynamic equilibrium within tissue and then changes in the drug concentration in plasma will reflect changes in tissue drug concentration.The intensity of the pharmacologic or toxic effect of drug is often related to the concentration of drug at receptor site. Because most of the tissue cells are richly perfuse with tissue fluids or plasma, measuring the plasma drug level is a responsive method of monitoring the course of therapy. Monitoring of plasma drug concentrations allows for the adjustment of the drug dosage in order to individualize and optimize therapeutic drug regimens. In case of alteration in physiologic functions due to disease, monitoring plasma drug concentration may guide to progress of the disease state and enable the investigator to modify the drug dosage accordingly. Mathematic analysis of plasma level vs. time curve permit, estimation of half-lives, absorption & excretion rates, extent of absorption (AUC) and other constants that are useful in describing fate of given drug in humans. Comparative bioavailability studies permit judgments as to the bioequivalence of drugs.

1.3.1 Sample collection

Human blood will be directly drawn from median cubical vein, which is generally easily found and accessed. Blood collect in polythene bags containing anticoagulants: buffered citrate, phosphate, dextrose, adenine solution. After collection of the blood specimen, the serum blood samples will be allowed to clot for 60 minutes followed by centrifugation (Cryo fuge 6000i centrifuge machine) at 3500 rpm for 12 min. at 2-6^{0}C. The plasma separated from cells by a mechanical separator (plasma expresser), removed from the blood collection bags, and will be transferred into separate bags, stored at -20ºC until next usage [23].

1.3.2 Sample preparation techniques

Protein Precipitation:

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In protein precipitation, acids or water-miscible organic solvents are used to remove the protein by denaturation and precipitation. Acids, such as trichloroacetic acid (TCA) and perchloric acid, are very efficient at precipitating proteins. The proteins, which are in their cationic form at low pH, form insoluble salts with the acids. A 5–20 per cent solution of these acids is generally sufficient and the best results can be achieved using cold reagents. Organic solvents, such as methanol, acetonitrile, acetone and ethanol, although having a relatively low efficiency in removing plasma proteins, have been widely used in bioanalysis because of their compatibility with high-performance liquid chromatography (HPLC) mobile phases. These organic solvents which lower the solubility of proteins and precipitate them from solutions have an effectiveness which is inversely related to their polarity [24].

Liquid–Liquid Extraction:

Liquid–liquid extraction (LLE) is the direct extraction of the biological material with a water-immiscible solvent. The analyte is isolated by partitioning between the organic phase and the aqueous phase [24].

The distribution ratio is affected by a number of factors:

- Choice of extracting solvent
- pH of aqueous phase
- Ratio of the volumes of the organic to aqueous phase.

1.4 BIONALYTICAL METHOD VALIDATION

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice [28].

Bioanalytical method validation (BMV) includes all of the procedures required to demonstrate that a particular bioanalytical method for the quantitative determination of the concentration of an analyte (or series of analytes) in a particular biological matrix is reliable for the intended application. The most widely employed bioanalytical techniques include, but are not limited to, conventional chromatographic based methods (such as GC-ECD and HPLC-UV), mass spectrometry-based methods (such as GC-MS and LC-MS), tandem mass spectrometry-based methods (such as LC-MS-MS) and ligand- based assays (such as RIA and ELISA) [29].

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2. MATERIALS AND METHODS

2.1 Materialsfor analysis of Darunavir

Procurement of Drug samples: Darunavir was obtained from Lupin Pharmaceuticals, Pune and Ritonavir was obtained fromMatrix laboratoires, Hyderabadas a gift sample.

Reagents and Chemicals used: Methanol AR grade, HPLC water, Acetonitrile HPLC grade and Dichloromethane AR Grade

2.2 Materials for analysis of Cephalexin

Procurement of Drug samples: Cephalexin was obtained from Maxim Pharmaceuticals, Pune and Voriconazole was obtained from Jubilant Organosys, Puru, Rajasthanas a gift sample.

Reagents and Chemicals used: Methanol AR grade, Toluene AR grade. and Triethylamine AR grade.

2.3 METHOD DEVELOPMENT FOR DARUNAVIR

Selection of mobile phase: Acetonitrile: Water (50:50 v/v) in which optimum system suitability parameters were obtained.

Selection of internal standard (IS): Ritonavir was selected as the internal standard, since the peaks of the drug and IS were well resolved**.**

Preparation of mobile phase: 200 ml of HPLC grade Acetonitrile was added to 200 ml of HPLC Grade Water i.e. in 50:50 v/v proportions. The solution was further filtered through 0.45μm membrane filter and sonicated in sonicator bath for 10 min.

Preparation of stock solutions of DRV: Stock solution was prepared by dissolving 10 mg DRV in methanol and then diluted to get the final concentrations of 2 to 12μg/ml.

Preparation of stock solution of IS:10 mg of Ritonavir (IS) was dissolved in methanol and it was diluted with methanol to get the final concentration of 300 μg/ml.

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Preparation of spiked plasma samples: The reported peak plasma concentration value for Darunavir is

Stock solution of DRV	Stock solution of IS (300 µg/ml)	Spiked Plasma	Dichlorometha-ne (Liquid Liquid Extraction)	Conc. of DRV (µg/ml)
-	-	1.0 ml	5.0 ml	-
0.1ml (20µg/ml)	0.1 ml	1.0 ml	5.0 ml	4 µg/ml
0.1ml (40µg/ml)	0.1 ml	1.0 ml	5.0 ml	8 µg/ml
0.1ml (60µg/ml)	0.1 ml	1.0 ml	5.0 ml	12 µg/ml
0.1ml (80µg/ml)	0.1 ml	1.0 ml	5.0 ml	16 µg/ml
0.1ml (100µg/ml)	0.1 ml	1.0 ml	5.0 ml	20 µg/ml
0.1ml(120µg/ml)	0.1ml	1.0ml	5.0ml	24 µg/ml

6.7mg/lit i.e. 6.7µg/ml. On this basis the linearity range was chosen as 2-12µg/ml. spiked plasma was prepared by taking 0.8ml plasma, to which 0.1ml solution of DRV and 0.1 ml stock solution of IS (300 µg/ml) were added.

Table 2.1: Preparation of spiked plasma samples

Selection of detection wavelength:

It was observed that drug and IS showed considerable absorbance at 266 nm.

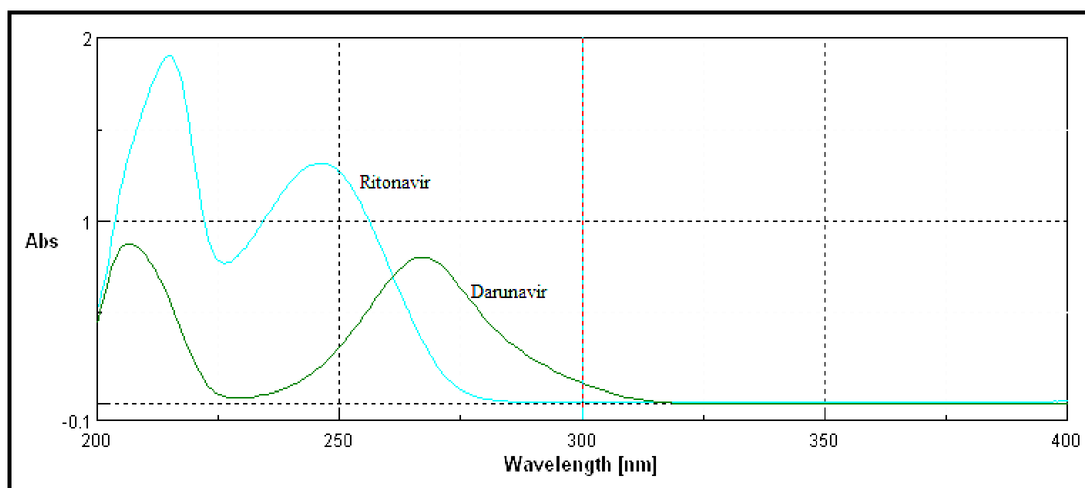

Figure 2.1: Overlain spectra of Darunavir and IS

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2.4 METHOD VALIDATION FOR DARUNAVIR

The analytical method should be able to differentiate the analyte(s) of interest and IS from endogenous components in the matrix or other components in the sample. The selectivity of the method was evaluated by analyzing pooled plasma samples obtained from two different sources spiked at LLOQ (Lower Limit of Quantification 1 µg/ml). No endogenous interferences are noted at the retention time of the drugs

Replicate No.	Nominal Conc. (LLOQ) (1µg/ml)				
	Area of DRV	Area of IS	Response Factor	Calculated Concentration	
				µg/ml	% Accuracy
1	147355.0	193988.1	0.759	0.91	91.0
2	144826.3	185769.5	0.779	0.96	96.0
3	148318.6	193222.3	0.767	0.93	93.0
4	136959.6	181257.2	0.755	0.90	90.0
5	147042.6	192563.1	0.763	0.92	92.0
6	144426.3	185256.0	0.779	0.96	96.0
Mean				0.93	**93.0**
SD				0.0252	**2.529**
%CV				**2.720**	

Acceptance Criteria:

At least 67 % (4 out of 6) sample should be within 80-120%

The % Mean accuracy should be within 80-120%

The % CV should be less than or equal to 20%

Table 2.2: Results for Selectivity of DRV

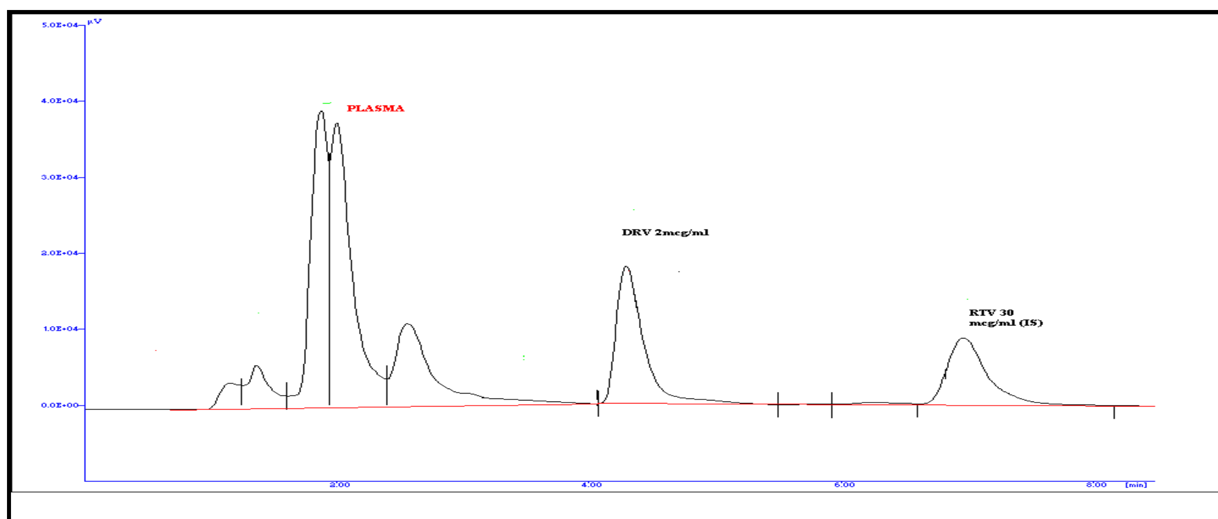

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Figure 2.2: Typical chromatogram of blank human plasma spiked with Darunavir 2µg/ml (Rt 4.29min) and IS 30 µg/ml (Rt 7.01min)

CALIBRATION CURVE

All the five calibration curves analyzed during the course of validation were found to be linear for the standards concentration ranging from 2-12µg/ml and best fitted by a linear equation y = mx + c, the correlation coefficient for standard DRV(r^2) is 0.999 and plasma spiked with DRV (r^2) is 0.997

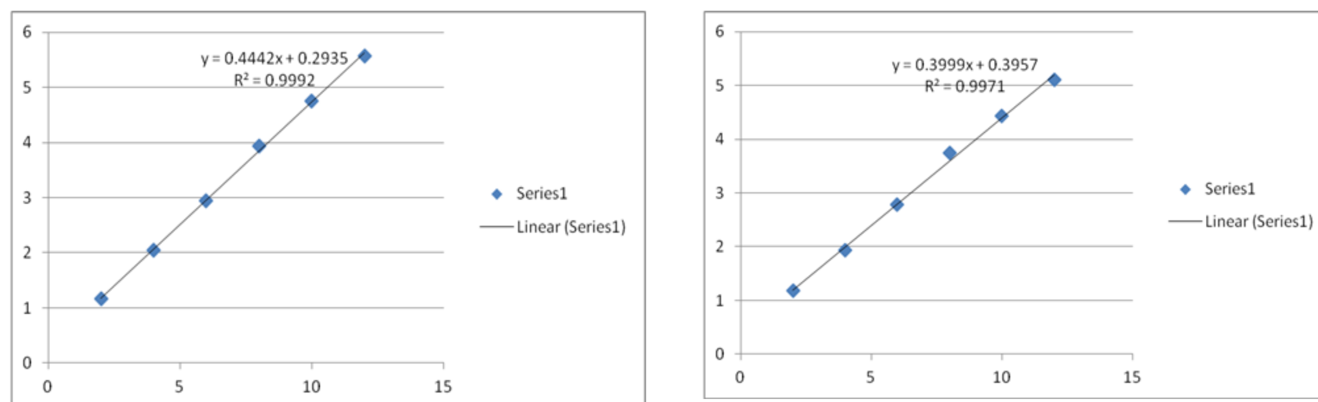

Figure 2.3:Calibration curve of DRV Standard solutions and plasma spiked

ACCURACY:

The % mean accuracy of calculated concentrations for all quality control samples at LQC, MQC and HQC concentration levels ranged from 86.12% to 92.90%, which is within acceptance limit 85- 115 %.

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A	Area of DRV	Area of IS	Response Factor	Calculated Conc. (µg/ml)
	Nominal concentration 2µg/ml (LQC)			
1	231649.2	211987.2	1.092	1.743
2	230589.1	210458.2	1.095	1.750
3	210016.3	190452.4	1.102	1.768
4	235968.6	214465.3	1.100	1.762
5	239748.7	214194.6	1.119	1.809
Mean				**1.767**
SD				**0.0258**
% CV				**1.46%**
% Mean Accuracy				**88.32%**
B	Nominal concentration 6 µg/ml (MQC)			
1	590150.0	229456.7	2.571	5.442
2	559105.4	216589.1	2.581	5.466
3	605055.7	227037.5	2.665	5.675
4	596935.4	229063.2	2.605	5.527
5	595561.6	223475.2	2.665	5.675
Mean				**5.556**
SD				**0.111**
% CV				**2.01%**
% Mean Accuracy				**92.61%**
C	Nominal concentration 10 µg/ml (HQC)			
1	896679.2	218965.2	4.095	9.251
2	924047.1	217963.4	4.239	9.612
3	913765.7	219789.5	4.157	9.407
4	906654.4	214385.4	4.229	9.586
5	900097.6	216589.9	4.155	9.403
Mean				**9.452**
SD				**0.148**
% CV				**1.57%**
% Mean Accuracy				**94.51%**
Acceptance Criteria: The % Mean Accuracy for all the samples should be within 85-115%.				

Table 2.5: Results for accuracy of DRV

PRECISION:

The precision of this method was evaluated by the % CV at different concentration levels corresponding to LQC, MQC and HQC during the course of validation.

Inter Day Precision (Reproducibility):

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	DAY 1	DAY 2	DAY 3
A	Nominal concentration 2µg/ml (LQC)		
	Calculated concentration (µg/ml)		
Mean	1.94	1.96	1.96
SD	0.075	0.0719	0.0717
% CV	3.90	3.65	3.66
B	Nominal concentration 6µg/ml (MQC)		
	Calculated concentrations (µg/ml)		
Mean	5.82	5.87	5.89
SD	0.134	0.064	0.037
% CV	2.312	1.090	0.630
C	Nominal concentration 10 µg/ml (HQC)		
	Calculated concentrations (µg/ml)		
Mean	9.85	9.90	9.88
SD	0.015	0.14	0.044
% CV	1.54	1.43	0.45
Acceptance Criteria: The % CV for LQC, MQC and HQC samples should be within 15%.			

Table 2.6: Results for Inter-day precision of DRV

INTRA DAY PRECISION (REPEATABILITY):

The repeatability (intra-assay precision) of the method was evaluated in five replicates on the same day for three different concentrations of DRV (2, 6, 10 µg/ml). The % CV of calculated concentrations for all quality control samples at LQC, MQC and HQC concentration levels are ranged from 1.30%- 1.71%, which is within acceptance limit 15%.

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Replicate No.	Nominal concentration		
	LQC (2.0µg/ml)	MQC (6.0 µg/ml)	HQC (10.0 µg/ml)
	Calculated concentrations (µg/ml)		
1	1.89	5.78	9.63
2	1.87	5.64	9.67
3	1.85	5.77	9.57
4	1.94	5.84	9.53
5	1.90	5.79	9.94
Mean	**1.89**	**5.76**	**9.67**
SD	**0.032**	**0.075**	**0.159**
% CV	**1.71**	**1.30**	**1.64**
Acceptance Criteria: The % CV for HQC, MQC, and LQC samples should be within15%.			

Table 2.7: Results for Intra-day precision of DRV

RECOVERY:

The % mean recovery for DRV at LQC, MQC and HQC levels are found to be 95.73%, 93.97% and 93.08% respectively.

Replicate No.	LQC (2.0 µg/ml)		MQC (6.0µg/ml)		HQC (10.0 µg/ml)	
	Standard	Spiked plasma	Standard	Spiked plasma	Standard	Spiked plasma
	Peak Area					
1	228975.2	218179.5	614598.2	575853.4	1011149.3	929452.0
2	230761.2	221868.4	637300.2	597563.5	1016629.3	945263.2
3	235565.4	225633.2	602278.6	568970.4	1024525.2	966598.6
Mean	**231767.3**	**221893.7**	**618059.0**	**580795.8**	**1017434.4**	**947104.6**
SD	**3408.3**	**3726.9**	**17765.4**	**14923.5**	**6724.5**	**18641.6**
% CV	**1.47**	**1.67**	**2.87**	**2.56**	**0.66**	**1.30**
% Mean Recovery	**95.73%**		**93.97%**		**93.08%**	
% Overall Mean Recovery	**94.26%**					
Overall SD	**7046.4**					
Overall % CV	**0.793%**					
Acceptance Criteria: The % CV of recovery at each QC levels should be within 15%.						

Table 2.8: Results for recovery of DRV

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Replicate No.	Nominal concentrations			
	LQC (2.0µg/ml)		HQC (10.0 µg/ml)	
	Comparison Sample	Stability Sample	Comparison sample	Stability sample
	Calculated concentrations (µg/ml)			
1	1.83	1.64	9.92	9.56
2	1.80	1.69	9.94	9.58
3	1.77	1.64	9.92	9.47
Mean	**1.80**	**1.66**	**9.92**	**9.54**
SD	**0.030**	**0.031**	**0.010**	**0.054**
% CV	**1.70**	**1.88**	**0.109**	**0.57**
% Mean stability	**92.22%**		**94.35%**	
Acceptance Criteria: The % CV for LQC and HQC should be sample should be within 15%. The % mean stability of LQC and HQC sample should be within 85-115%				

Table 2.9: Results for freeze and thaw stability of DRV

2.5 METHOD DEVELOPMENT FOR CEPHALEXIN IN HUMAN PLASMA BY HPTLC

Selection of mobile phase: Toluene: Methanol: Triethylamine (6:4:0.1 v/v)

Selection of internal standard (IS): Drugs which have considerable abs at 254 nm. Voriconazole was selected as the IS

Preparation of mobile phase: Mobile phase was, sonicated for 5 minutes in so-nicator bath.

Preparation of stock solutions of DRV: Stock solution was prepared by dissolving 10 mg 0floxacin in methanol and then diluted to get the concentration of 10 µg/ml.

Preparation of stock solution of IS:10 mg of Voriconazole (IS) was dissolved in methanol and then diluted to get the concentration of 40µg/ml.

Preparation of spiked plasma samples: Spiked plasma was prepared by taking 0.3ml plasma, 0.1ml solution of CFL and 0.1 ml stock solution of IS(20 µg/ml) were added. In the spiked plasma 0.5 ml of methanol was added. It was centrifuged for 10 minutes at 2000 rpm and thansupernatant was applied on TLC plate

Selection of detection wavelength: The developed plate was scanned over the range of 200-400 nm and the spectra were overlain.

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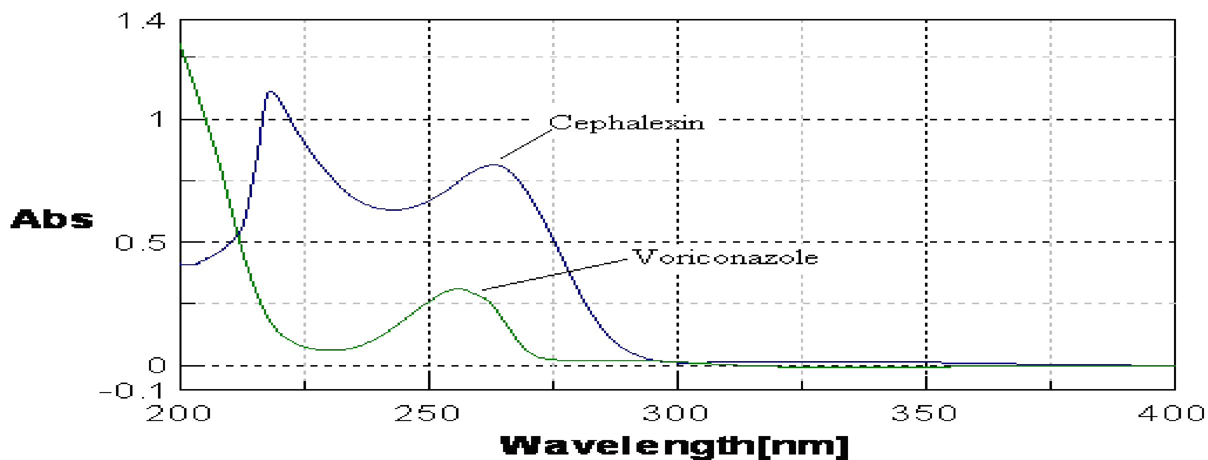

Figure 2.4: Overlain spectra of Cephalexin and Voriconazole (IS)

2.6 METHOD VALIDATION FOR CEPHALEXIN

SELECTIVITY:

The analytical method should be able to differentiate the analyte(s) of interest and IS from endogenous components in the matrix or other components in the sample

Replicate No.	Nominal Conc. (LLOQ) (300ng/band)				
	Area of CFL	Area of IS	Response Factor	Calculated Concentration	
				ng/band	% Accuracy
1	1167.7	4554.3	0.256	291.5	97.2
2	1127.2	4422.6	0.255	289.6	96.5
3	1163.0	4492.6	0.259	294.6	98.2
4	1099.5	4410.9	0.249	282.6	94.2
5	1048.6	4293.6	0.244	276.3	92.1
6	1109.6	4286.2	0.259	294.6	98.2
Mean				288.2	96.06%
SD				7.317	2.43
%CV				2.53%	
Acceptance Criteria: At least 67 % (4 out of 6) sample should be within 80-120%, The % Mean accuracy should be within 80-120% The % CV should be less than or equal to 20%					

Table 2.10: Results for Selectivity of CFL

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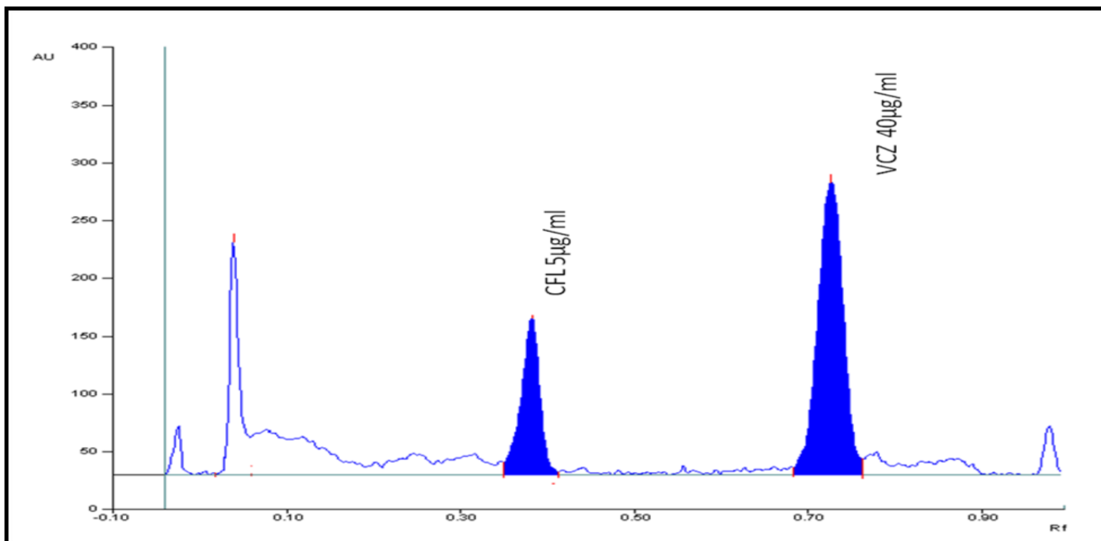

Figure 2.5: Densitogram of human plasma spiked with Cephalexin, 500 ng/band (Rf-0.41 ± 0.03) and IS 400ng/ band (0.70± 0.03) using LLE

CALIBRATION/STANDARD CURVE:

Linearity was tested for the range of concentrations 500- 3000ng/band. Each sample in 5 replicates was analyzed and peak areas were recorded. Response factor was calculated by taking the peak area ratio of CFL and IS

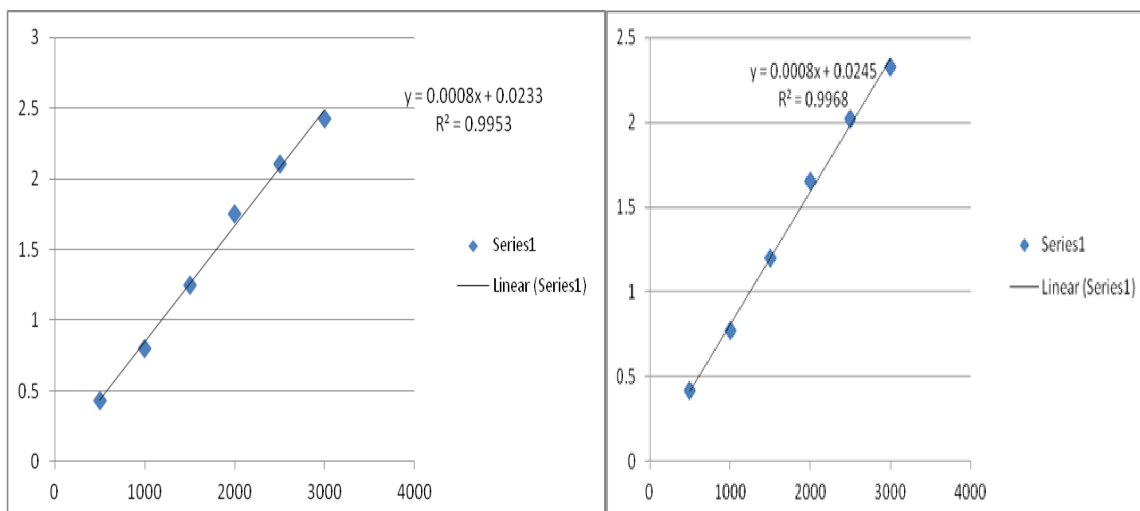

Figure 11: Calibration curve of CFL standard and spiked plasma

ACCURACY:

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The % mean accuracy of calculated concentrations for all quality control samples at LQC, MQC and HQC concentration levels ranged from **94.32%** to **97.96%** which is within acceptance limit 85-115%.

A	Area of CFL	Area of IS	Response Factor	Calculated Conc. (ng/band)
	Nominal concentration 500 ng/band (LQC)			
1	1672.2	4125.0	0.4053	477.7
2	1702.6	4095.6	0.4157	490.7
3	1689.6	4098.7	0.4122	486.3
4	1695.3	4172.2	0.4063	478.9
5	1706.5	4108.2	0.4153	490.2
Mean				**484.7**
SD				**6.15**
% CV				**1.27%**
% Mean Accuracy				**96.95%**
B	**Nominal concentration 1500 ng/band (MQC)**			
1	4804.2	4050.0	1.1862	1453.8
2	4782.2	4022.6	1.1890	1457.3
3	4901.8	4019.6	1.2196	1495.6
4	4871.9	4036.1	1.2071	1479.9
5	4879.8	4094.5	1.1919	1460.9
Mean				**1469.4**
SD				**17.73**
% CV				**1.20%**
% Mean Accuracy				**97.96%**
C	**Nominal concentration 3000 ng/band (HQC)**			
1	9196.77	4110.6	2.2373	2767.7
2	9500.16	4012.6	2.3675	2930.5
3	9297.54	4124.3	2.2543	2788.9
4	9443	4102.6	2.3017	2848.1
5	9382.14	4125.6	2.2741	2813.7
Mean				**2829.77**
SD				**63.76**
% CV				**2.25%**
% Mean Accuracy				**94.32%**
Acceptance Criteria: The % Mean Accuracy for all the samples should be within 85-115%.				

Table 2.11: Results for accuracy of CFL

INTER DAY PRECISION (REPRODUCIBILITY):

A	DAY 1	DAY 2	DAY 3
	Nominal concentration 500 ng/band (LQC)		
	Calculated concentration (ng/band)		
Mean	479.26	461.64	469.74
SD	7.48	9.01	11.97
% CV	1.56	1.95	2.55

B	Nominal concentration 1500 ng/band (MQC)		
	Calculated concentrations (ng/band)		
Mean	1462.02	1463.28	1469.26
SD	15.85	23.44	12.21
% CV	1.08	1.60	0.83

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C	Nominal concentration 3000 ng/band (HQC)		
	Calculated concentrations (ng/band)		
Mean	2852.7	2844.9	2850.5
SD	23.5	33.5	31.2
% CV	0.82	1.17	1.09
Acceptance Criteria: The % CV for LQC, MQC and HQC samples should be within 15 %.			

Table 2.12: Results for Inter-day precision of CFL

INTRA DAY PRECISION (REPEATABILITY):

Replicate No.	Nominal concentration		
	LQC (500 ng/band)	MQC (1500 ng/band)	HQC (3000 ng/band)
	Calculated concentrations (ng/band)		
Mean	479.26	1462.02	2830.12
SD	7.48	15.84	35.27
% CV	1.56	1.08	1.24

Table 2.13: Results for Intra-day precision of CFL

RECOVERY:

The % mean recoveries were determined by measuring the responses of the extracted plasma quality control samples against un-extracted quality control samples at HQC, MQC and LQC levels.

Replicate No.	LQC (500 ng/band)		MQC (1500 ng/band)		HQC (3000 ng/band)	
	Standard	Spiked plasma	Standard	Spiked plasma	Standard	Spiked plasma
	Peak Area					
Mean	1804.01	1682.51	5132.38	4882.94	9937.63	9373.97
SD	37.23	44.32	95.69	12.30	45.07	71.36
% CV	2.06	2.63	1.86	0.25	0.45	0.76
% Mean Recovery	93.28%		95.16%		94.33%	
% Overall Mean Recovery	94.25%					
Overall SD	50.99					
Overall % CV	1.33%					
Acceptance Criteria: The % CV of recovery at each QC levels should be within 15 %.						

Table 2.14: Results for recovery of CFL

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3. STABILITY:

Stability	Conc. (µg/ml)	Mean Stability (%)	% R.S.D.
Freeze thaw stability (three cycles)	2.0	92.22	2.64
	10.0	94.35	0.39
Short term stability (for 4h at RT)	2.0	94.95	2.80
	6.0	92.58	0.71
	10.0	91.85	1.95
Long term stability (for 15 days at 4^0 C)	2.0	91.26	1.54
	6.0	93.93	1.56
	10.0	95.43	2.97
Stock solution stability (for 11 days)	2.0	89.82	1.51
	6.0	91.96	2.35
	10.0	93.67	2.08
Post preparative stability (for 4hrs at RT)	6.0	92.40	0.693
	300 (IS)	98.29	1.31
Acceptance Criteria		**85-115%**	**≤ 15%**

Table 2.15: Stability studies for Darunavir

Stability	Conc. (µg/ml)	Mean Stability (%)	% R.S.D.
Freeze thaw stability (three cycles)	500	91.36	2.40
	3000	90.66	2.00
Short term stability (for 4h at RT)	500	96.43	1.06
	1500	97.18	0.54
	3000	97.70	0.56
Long term stability (for 12 days at 4^0 C)	500	92.01	1.60
	1500	92.54	0.66
	3000	92.61	1.75
Stock solution stability (for 7 days)	500	91.03	0.82
	1500	91.36	0.90
	3000	90.97	1.33
Post preparative stability (for 4hrs 30min at RT)	1500	98.91	1.01
	400	97.67	0.67
Acceptance Criteria		**85-115%**	**≤ 15%**

Table 2.16: Stability studies for CFL

4. SUMMARY & CONCLUSION

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Most published methods to quantify Darunavir in body fluids use tedious extraction, purification steps and sometimes solid phase extraction with multiple steps or some other tedious procedures have been applied to get rid of interfering proteins and other matter from the selected matrix. In this study, rapid and sensitive HPLC method has been developed for the determination of Darunavir in human plasma by liquid liquid extraction technique which is with simple and limited steps. Rapid and sensitive HPLC method has been developed for the determination of Darunavir in human plasma. The mobile phase employed is Acetonitrile: Water in the ratio of 50:50 v/v. Validation results proved that the developed method performs well with selectivity, precision, accuracy, stability and linearity for the concentration range of Darunavir to be found in human plasma. Ritonavir is used as an internal standard. The validated method covers the wide range of linearity over (2- 12µg/ml) and is therefore suitable for the determination of Darunavir in human plasma at different therapeutic dose levels. The Mean Peak plasma concentration reported is 6.7mg/lit. It utilizes liquid liquid extraction with Dichloromethane as the sample preparation technique. The mean recovery of Darunavir is found to be 94.26%.

Literature survey revealed that there are number of methods for quantitation of CFL in biological fluid viz. HPLC with UV detection, HPLC with DAD detection HPLC UV with using solid phase extraction and other tedious methods. Few methods have also been reported to determine the levels of CFL in biological fluids in combination with other cephalosporin antibiotics. HPTLC method has been reported for determination of CFL in bulk and pharmaceutical dosage form. To the best of our knowledge, no High-Performance Thin Layer Chromatographic (HPTLC) method has been reported for determination of CFL in human plasma. HPTLC technique, offers advantage of high throughput. As compared to HPLC methods, the present method is economical, simple and fast. The proposed method can be used for therapeutic drug monitoring in order to optimize drug dosage on an individual basis. The developed method is able to measure concentration of CFL to monitor drug concentration in body fluid, determination of drug level in plasma for dose regulation and bioavailability. The rapid and sensitive HPTLC method has been developed for the determination of Cephalexin in human plasma. Validation results proved that the developed method performs well with selectivity, precision, accuracy, stability and linearity for the plasma concentration range from 5µg/ml to 30µg/ml of Cephalexin. The Peak plasma concentration ranges between 9-32 mg/lit. Voriconazole is used as an internal standard. The present method involves minimal sample pretreatment, resulting in fast analysis, as it utilizes protein precipitation as the sample preparation technique, which eliminates the drawbacks of less recovery due to liquid-liquid extraction or the use of solid phase extraction cartridges which is relatively costly. The mobile phase employed is Toluene: Methanol: Triethylamine in the ratio of 6:4:0.1 v/v/v. The % mean recovery of Cephalexin has been found to be 94.25%. The proposed method can be used for therapeutic drug monitoring in order to optimize drug dosage. Hence it is concluded from the present study that the proposed HPLC methods for Darunavir and HPTLC method for Cephalexin can be used for therapeutic drug monitoring in order to optimize drug dosage on an individual basis. The developed methods are able to measure concentration of Darunavir and Cephalexin which can be used in plasma for dose regulation and bioavailability studies. These methods were also validated as per CDERguidelines on validation of bioanalytical methods.

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