

# Determination of Linezolid in Human Plasma Using Turbulent Flow Online Extraction and Tandem Mass Spectrometry

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## Research Article

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### ABSTRACT :

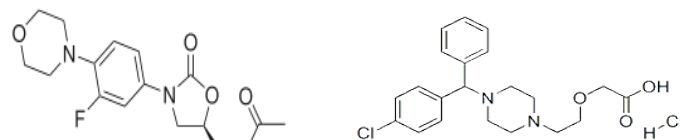
A rapid and sensitive HTLC-ESI-MS/MS method was developed for the determination of Linezolid in human plasma using an internal standard (cetirizine hydrochloride) with an advanced online sample preparation. This HTLC technique reduces the time required for sample cleanup since sample extraction and analysis are performed. A 10 $\mu$ l of prepared sample is directly injected into the HTLC-MS/MS system where analyte was retained on the extraction column (Cyclone P 50  $\times$  0.5mm, 50 $\mu$ m) and washed away the waste with the help of extraction solvent. Then the analyte was eluted from the extraction column and transferred to the analytical column (Zorbex XDB C18 50  $\times$  2.1mm, 5 $\mu$ m) using mobile phase of the mixture of 0.5% formic acid, 10mM ammonium formate and acetonitrile. The eluted analyte was then detected on mass spectrometer with ESI ion source and a positive selective reaction monitoring mode (SRM). The SRM transitions were m/z 383.20  $\rightarrow$  337.20 for Linezolid and m/z 389.10  $\rightarrow$  201.01 for internal standard. The developed method was validated as per USFDA guidelines. The method was linear over the concentration range of 0.409 – 20.310 ng/ml. The within batch and between batch accuracy for the three concentrations (LQC, MQC and HQC) were ranged from 98 -110.6% and 98 .6 – 108.3% respectively. The % RSD for all the QC samples was ranged from 3.0 – 8.1%. The percentage recovery of linezolid in HQC (16ng/ml), MQC (13ng/ml) and LQC (1,1ng/ml) was 60.3, 73 and 86.45% respectively. Stability studies were also performed and the results were within the acceptance range. This method was applied to the measurement of linezolid in human plasma and pharmacokinetic study.

**Keywords:** HTLC-ESI-MS/MS, selective reaction monitoring.

## INTRODUCTION:

Linezolid is a synthetic antibacterial agent of the oxazolidinone class. Chemically it is N-(((5S)-3-(3-fluoro-4-(morpholin-4-yl) phenyl)-2-oxo-1,3-oxazolidin-5-yl)methyl)acetamide. It has the molecular formula of C<sub>16</sub>H<sub>20</sub>FN<sub>3</sub>O<sub>4</sub> and molecular weight of 337.346g/mol. It has Invitro activity against aerobic gram positive bacteria, certain gram negative bacteria and anaerobic organisms. It selectively inhibits bacterial protein synthesis through binding to sites on the bacterial ribosome and prevents the formation of a functional 70S initiation complex, which is an essential component of the bacterial translation process.

Linezolid is rapidly and extensively absorbed after oral dosing. Maximum plasma concentrations (C<sub>max</sub>) are 15-27mg/l, reached approximately 1-2 hrs after dosing. The absolute bioavailability is approx. 100%. It is metabolized via morphine ring oxidation. In humans 30% of the linezolid dose is excreted in the urine as the parent drug. Plasma elimination half-life is 3.4-7.4hrs.



**Figure. 1 Structure of linezolid. Figure: 2 Structure of cetirizine hydrochloride**  
Literature survey reveals that very few high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods were published for the determination of linezolid in human plasma. This method requires effective sample cleanup procedure, such as solid phase extraction or liquid-liquid extraction. In the proposed method, High turbulence liquid chromatography (HTLC), sample cleanup is not necessary and offline time consuming. The challenge at the time was to design a chromatographic platform that would utilize turbulent flow properties to isolate small analytes from macromolecules present in complex matrices such as biological fluids. In this case the macromolecules are rapidly eliminated to waste with a high flow rate of aqueous mobile phase. While the small molecules are retained on extraction column and are subsequently eluted onto an analytical column with organic

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mobile phase for chromatographic separation.

In this paper we report the method for the determination of linezolid in human plasma by using HTLC-MS/MS. In addition we represent the validation parameters and the stability data for linezolid.

## 2. MATERIALS AND METHODS:

### 2.1 Materials:

Linezolid USP was obtained from Glenmark Pharmaceuticals, Mumbai, India. The internal standard cetirizine hydrochloride was procured from Praveen laboratories, Gujarat, India. HPLC grade acetonitrile, methanol and acetone were purchased from SD fine chemicals, Mumbai, India. Formic acid and propane-2-ol were purchased from Rankem Pharmaceuticals New Delhi, India. HPLC grade water is used for preparing all the solutions.

### 2.2 Instruments:

A Cohesive 2300 HTLC system (Cohesive Technologies, Franklin, MA, USA) with quaternary pump as a loading pump and a binary pump as a elution pump. Thermoelectron Finnigan TSQ Quantum Discovery Max (Thermoelectron corporation, USA) mass spectrometer equipped with electron spray ionization was used as detector. The

data was collected and analysed using Analyst software version 1.4.

### 2.3 Standard solutions and Quality control (QC) samples:

The standard stock solutions of linezolid and internal standard were prepared at about 649.5µg/ml and 1mg/ml respectively in methanol. From the stock solution the intermediate solution of linezolid (65µg/ml) and internal standard (10µg/ml) were prepared in methanol. Calibration curve (CC) spiking solutions in methanol were prepared in the concentration range of 1000ng/ml to 20ng/ml from the intermediate solutions as shown in the table1. Spiked plasma CC standards were prepared by spiking the respective CC standards in screened plasma in the range of 20ng/ml to 0.4ng/ml. (Table2). Similarly QC spiking solutions were also prepared from the intermediate solutions of linezolid and internal standard as shown in the table3. QC samples were prepared by spiking the respective QC spiking solutions in screened human plasma representing HQC (16ng/ml), MQC (13ng/ml) and LQC (1.1ng/ml) as described in the table 4.

Table: 1 Spiking solutions for calibration curve of linezolid

Stock Conc. (ng/mL)	Vol. Taken (mL)	Diluent's Vol. (mL)	Final Vol. (mL)	SS Conc. (ng/mL)	SS ID
650000	1.000	9.000	10.000	66500.000	INTM STK1
65000	1.600	8.400	10.000	10400.000	INTM STK2
10400.000	1.000	9.000	10.000	1040.000	SS STD1
1040.000	8.000	2.000	10.000	832.000	SS STD2
832.000	7.500	2.500	10.000	624.000	SS STD3
624.000	6.500	3.500	10.000	406.000	SS STD4
406.000	5.000	5.000	10.000	203.000	SS STD5
203.000	5.000	5.000	10.000	102.000	SS STD6
102.000	4.000	6.000	10.000	41.000	SS STD7
41.000	4.900	5.100	10.000	20.000	SS STD8

Table: 2 spiked plasma calibration curve standards of linezolid

SS ID	SS Conc. (ng/mL)	Spiking Vol. (mL)	Plasma Vol. (mL)	Final Vol. (mL)	Spiked Plasma Conc. (ng/mL)	STD ID
Diluent	0.000	0.200	9.800	10.000	0.000	STDBL
SS STD1	1040.000	0.200	9.800	10.000	21.000	STD8
SS STD2	832.000	0.200	9.800	10.000	17.000	STD7
SS STD3	624.000	0.200	9.800	10.000	13.000	STD6
SS STD4	406.000	0.200	9.800	10.000	8.000	STD5
SS STD5	203.000	0.200	9.800	10.000	4.000	STD4
SS STD6	102.000	0.200	9.800	10.000	2.000	STD3
SS STD7	41.000	0.200	9.800	10.000	0.800	STD2
SS STD8	20.000	0.200	9.800	10.000	0.400	STD1

Table:3 Spiking solutions for quality control samples of linezolid

Conc.(ng/mL)	Stock Vol.(mL)	Diluent's Vol. (mL)	Final Vol.(mL)	Conc. (ng/mL)	SS ID
10400.000	0.750	9.250	10.000	780.000	SS HQC
780.000	8.000	2.000	10.000	624.000	SS MQC
624.000	3.500	6.500	10.000	219.000	SS INT QC
219.000	2.500	7.500	10.000	55.000	SS LQC

Table:4 Spiked plasma quality control samples of linezolid

SS ID	SS Conc. (ng/mL)	Spiking Volume (mL)	Plasma Vol. (mL)	Final Vol. (mL)	Spiked Plasma Conc. (ng/mL)	QC ID
SS HQC	780	2	8	100	16	HQC
SS MQC	624	2	8	100	13	MQC
SS LQC	55	2	8	100	1.1	LQC

## 2.4 Sample preparation:

For sample preparation, the required number of spiked plasma samples (STD blank, CC, QC samples) were taken out from the deep freezer thaw them at room temperature and vortex the tubes to mix. Remove the caps from the polypropylene tubes, aliquot 0.5ml of CC, QC into prelabelled Eppendorf vials. Add 50µl of ISTD dilution (10µg/ml) to all the samples except STD blank and vortex for about 10 seconds. Centrifuge the Eppendorf vials at 14000rpm and 10degrees for 20min, transfer the 0.4ml of supernatant to prelabelled HPLC vials. Add 0.4ml of 10mM ammonium formate and vortex to mix and subsequently transfer the HPLC vials to the autosampler.

## 2.5 Chromatographic conditions:

A cyclone P HTLC column (50mm × 0.5mm, 50µm) from cohesive technologies Inc (Franklin,MA,USA) and a Zorbex XDB C<sub>18</sub> column (50 × 2.1mm, 5µm) from Agilent technologies Ltd, Bangalore were used as extraction and analytical columns respectively. Mobile phases used as follows

Mobile phase A : 10mM ammonium formate, 0.5% formic acid aqueous solution

B : 0.5% formic acid acetonitrile solution

C : acetonitrile solution

D : acetonitrile/IPA/acetone (60:25:15%vv/v)

Rinsing cycle conditions:

Rinsing volume: 100µl

Needle stroke: 38.2mm

Rinsing speed: 35µl/sec

Sampling speed : 15µl/sec

Purge time : 2min

Rinse dip time: 2sec

Rinse mode : before and after inspiration

The online extraction method consists of four general steps: loading, transfer, elution and equilibration. First the sample is applied during the loading step by the loading pump on to the turboflow column at a flow rate of 2ml/min. where the matrix components are rapidly washed away and the analytes are retained with mobile phase A. at the transfer step, the analytes are eluted from the extracted column and transferred on to the reverse phase analytical column, where the analytes are separated conventionally, at a flow rate of 0.2ml/min with a mobile phase that is stored in the transfer loop and filled before the loading step. At the same time, a mobile phase composed of 50%A and 50% B is delivered at a flow rate of 0.4ml/min from the eluting pump. At the elution step, the analytes are separated on to the analytical column and eluted to the mass spectrometer with the same mobile phase. Then the extraction column is washed to reduce the carry over. At the equilibration step, the loop is filled with the mobile phase of 100%B and then both extraction and analytical columns are equilibrated with 100%A for the next injection.

Table:5 Rinsing cycle by gradient programme

step			Turboflow column					Cut in loop		Analytical column				
Description	S t a r t (min)	sec	Flow rate (ml/min)	A%	B%	C%	D%	Tee	loop	Flow min	(ml/step)	A%	B%	
Loading	0	60	2	0	100	0	0	-	Out	0.6	Step	50	50	
Trasfering	1	60	0.2	50	0	50	0	T	In	0.4	Step	50	50	
Washing	2	60	2	0	0	0	100	-	In	0.6	Ramp	50	50	
washing	3	60	2	0	0	0	100	-	Out	0.6	Step	50	50	
Washing	3.5	60	2	0	0	0	100	-	In	0.6	Step	50	50	
Filling loop	4	60	2	100	0	0	0	-	in	0.6	Step	50	50	
equilibrating	4.5	60	2	0	100	0	0	-	out	0.6	step	50	50	

## 2.6 Mass spectrometry conditions:

Mass spectrometric analysis was carried out using Finnigan TSQ quantum discovery max from Thermo electron corporation, USA. Data acquisition for quantification and confirmation are performed in SRM mode (selective reaction monitoring) using analyst software version 1.4. Ionization mode: ESI (electron spray ionization)

Scan type: SRM

Polarity: positive ion mode

SRM transitions: Drug – Q<sub>1</sub> mass 383.20; Q<sub>3</sub> mass 337.20 (m/z)

ISTD - Q<sub>1</sub> mass 389.10; Q<sub>3</sub> mass 201.0 (m/z)

Spray voltage: 3000

Sheath gas pressure (arb): 40

Auxillary gas pressure (arb) : 10

Capillary temperature: 350°C

Collision energy: drug – 22; ISTD – 26

## 3. METHOD VALIDATION:

The present method was validated as per US FDA guidelines. The system suitability was performed by injecting 6 consecutive injections of AQ standard mixture of drug and ISTD. Autosampler carry over was determined by injecting one blank, STD along with AQ STD and RS in the sequence, to check any carry over in the blank sample. Specificity was proved by processing standard blanks and LLOQ in six different human plasma lots. A linear equation was established to provide the best fit for the concentration versus detector response using  $1/x^2$  weighed least square regression analysis of standard associated with eight point calibration curve. Accuracy was evaluated by measuring % mean accuracy at each concentration level of QC. Precision was measured by percent coefficient of variation over

the concentration range of QC samples of linezolid during the course of validation. Recovery was determined by comparing the areas of extracted QC samples against the areas of respective aqueous QC samples.

Stability studies like short term stock solution stability (after 6 hrs), long term stock solution stability (after 10 days), bench top stability (22±4°C), autosampler (wet extraction) stability (ambient temperature for 24hrs) and freeze thaw stability (-86°C) were also performed on the QC samples of linezolid.

## 4. RESULTS AND DISCUSSION:

### 4.1 Optimization of liquid chromatography and mass spectrometry conditions:

Complete resolution of linezolid and ISTD was achieved by using a cyclone P HTLC column (50mm × 0.5mm, 50µm) from cohesive technologies Inc (Franklin, MA, USA) as extraction column and a Zorbex XDB C<sub>18</sub> column (50 × 2.1mm, 5µm) from Agilent technologies Ltd, Bangalore, as analytical column with required mobile phase. Following detailed optimization of mass spectrometry conditions m/z 383.20 precursor ion to the m/z 337.20 for ISTD m/z 389.10 precursor ion to the m/z 201.01 was used for quantification purpose.

### 4.2 Method validation procedures:

#### 4.2.1 System suitability:

System suitability was performed by injecting six consecutive injections using aqueous standard mixture of drug and ISTD during the start of method validation. The % CV of system suitability was observed in the range of 3.7 – 4.2% for response of the drug and ISTD, which is not more than 5% as per the acceptance criteria. The results were shown in the following table 6.

Table:6 Results of system suitability

Injection	Drug Area	Drug Retention Time	ISTD Area	ISTD Retention Time	Area Ratio
1	4124427	2.2	126430	2.1	32.62222
2	4392474	2.2	112588	2.1	36.0137
3	4374485	2.2	122032	2.1	35.84703
4	4587745	2.2	125753	2.1	36.48219
5	4450798	2.2	122467	2.1	36.34284
6	4550489	2.2	125257	2.1	36.32922
Mean	4413403	2.2	122421.2	2.1	35.61
S.D	164952	0	5139.232	0	1.480
% CV	3.7	0	4.2	0	4.2

#### 4.2.2 Specificity:

To demonstrate that no interfering compounds elute at the retention times of linezolid or ISTD, the specificity was evaluated by testing six different lots of human plasma. No endogenous interferences were found at the retention times of linezolid or ISTD as seen in the table 7. Representative chromatogram of blank was shown in the figure 3.

Table: 7 Results showing specificity of linezolid

S.No	Area of interference at Rt of analyte	Area observed for extracted LOQ	% interference at Rt of Analyte	Area of interference at Rt of ISTD	Area observed for extracted LOQ	% interference at Rt of ISTD
1	0	20340	0	1689	751695	0.26
2	0	17898	0	2643	702759	0.41
3	0	21824	0	2399	608705	0.37
4	0	16635	0	3078	656531	0.48
5	0	17864	0	2229	551921	0.34
6	0	13073	0	3010	572453	0.47
Mean		17939	0		640677	0.39

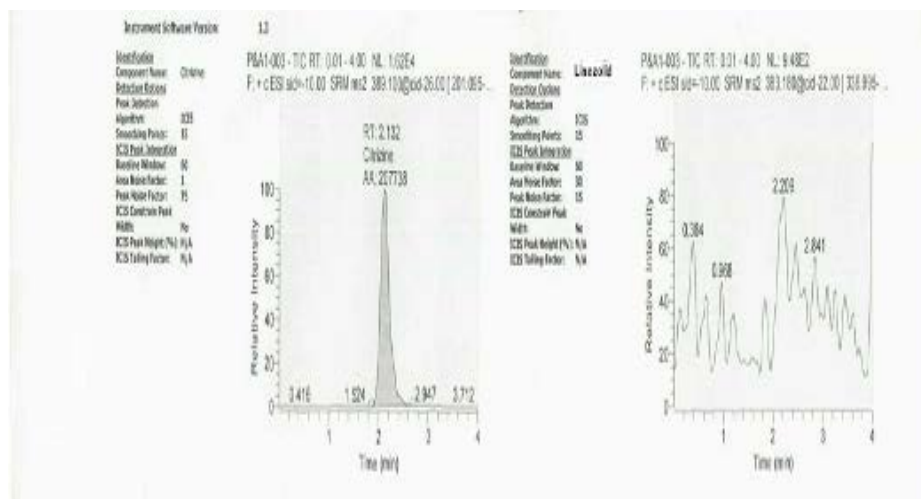


Figure: 3 A representative chromatogram of blank with ISTD

#### 4.2.3 Autosampler carryover:

Autosampler carryover was tested by injecting one blank and one extracted STD in the chromatographic system along with aqueous standard and reconstituted solution in the sequence. No significant carry over was found for linezolid while 0.006% of carryover was found for ISTD which was shown in the following table 8.

Table:8 Responses of linezolid with ISTD for autosampler carry over

Sample ID.	Response (Area Count)			
	Linezolid	Limits	ISTD	Limits
RS	0		1256	
AQ STD	912018		575203	
RS	0	18240	1534	11504
AQ STD	881911		566324	
RS	0	17638	0	11326
RS	0		833	
Extracted STD Blank	0		11865	
EXT STD 1	624394		552922	
Extracted STD Blank	0	12487	3711	11058
EXT STD 1	612607		555147	
Extracted STD Blank	0	12252	2127	11102

#### 4.2.4 Linearity and calibration curve:

A linear equation was established to provide the best fit for the concentration versus detector response using  $1/x^2$  as weighting factor. Calibration curve was drawn and it was found to be linear in the concentration range of 0.409 to 20.310ng/ml with the coefficient of 0.997 as seen in the figure 4.

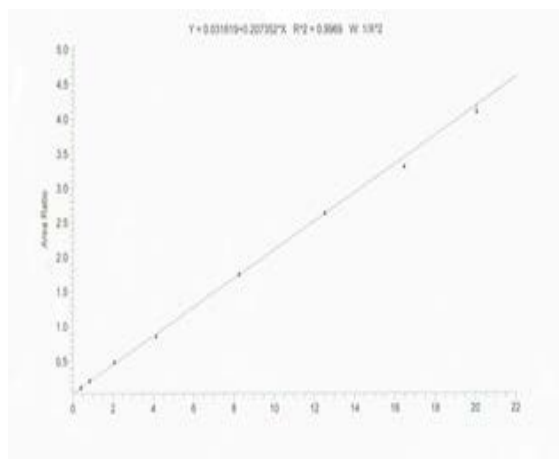


Figure: 4 Calibration curve of linezolid

**4.2.5 Precision:**

Precision is measured by percent coefficient of variation over the concentration range of QC samples of linezolid during the course of validation. The within batch precision for all low, middle, high and LOQ QC samples were ranged from 3.0% to 8.1% and 6.9 to 12.3% which is within the acceptance limits of 15% and ±20% for LOQ QC samples respectively. The between batch precision for all low, middle, high and LOQ QC samples were ranged from 4.4% to 6.9% and 9.6% which is within the acceptance limits of 15% and ±20% for LOQ QC samples respectively.

**4.2.6 Accuracy:**

The accuracy was defined as the absolute value of the ratio of back calculated mean values of QC samples to their respective nominal values expressed as percentage. The within batch accuracy for all low, middle, high and LOQ QC samples were ranged from 98% to 110.6% and 98.8 to 108.3% which is within the acceptance limits of 15% and ±20% for LOQ QC samples of nominal concentrations respectively. The between batch precision for all low, middle, high and LOQ QC samples were ranged from 98.6% to 108.3% and 99.2 % which is within the acceptance limits of 15% and ±20% for LOQ QC samples of nominal concentrations respectively. The representative chromatograms of HQC, MQC, LQC and LOQ QC samples were shown in the figures 5,6,7,8 respectively.

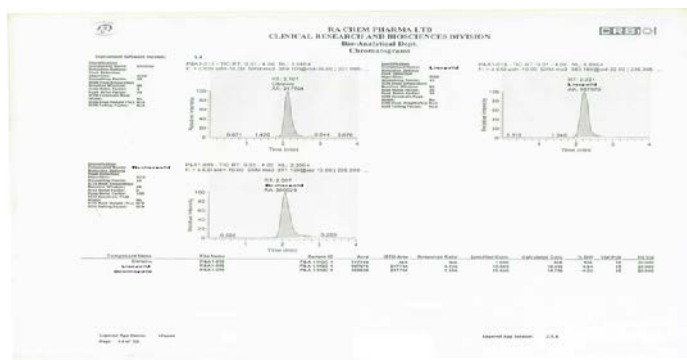


Figure:5 A representative chromatogram of HQC sample

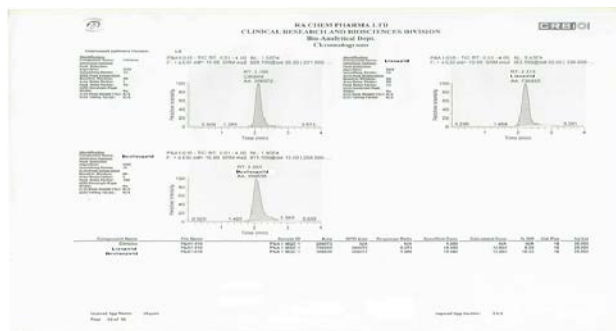


Figure:6 A representative chromatogram of MQC sample

4.2.7 Recovery:

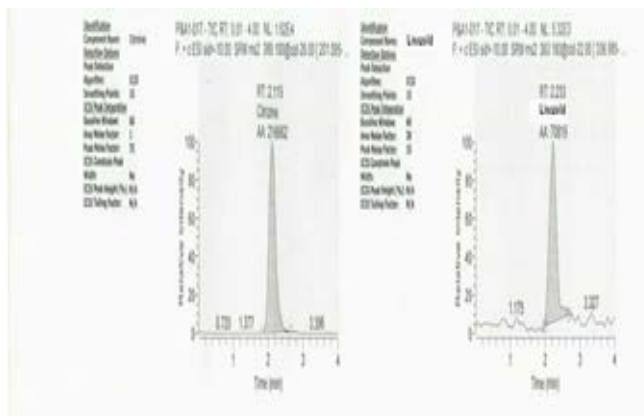


Figure:7 A representative chromatogram of LQC sample

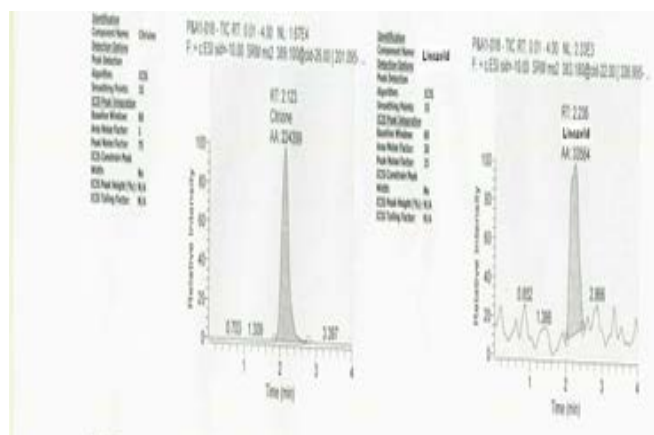


Figure:8 A representative chromatogram of LOQ QC sample

Recovery was determined by comparing the areas of extracted QC samples against the areas of respective aqueous QC samples. The % mean recovery of linezolid in HQC, MQC and LQC was 60.73, 73 and 86.45% respectively. Similarly the % mean recovery of ISTD in the same QC samples was 71.3, 58.5 and 54.3% respectively. The results of recovery of drug and ISTD were shown in the following tables 9,10.

Table: 9 Recovery of linezolid

Replicate No.	HQC		MQC		LQC	
	Aqueous response	Extract response	Aqueous response	Extract response	Aqueous response	Extract response
1	306132	360629	230072	306636	24089	25260
2	362551	426187	225158	323926	13966	29954
3	329471	363265	212962	342796	15171	26364
4	308896	368184	233893	289800	15790	28092
5	340985	457161	202959	308275	15153	23859
6	332468	410432	189034	318414	14641	37328
Mean	330083.8	397643	215679.7	314974.5	16468.3	28476.2
SD	20983.93	39850.79	17366.04	17967.55	3782.82	4833.42
% CV	6.4	10	8.1	5.7	23	17
% Mean Recovery	60.3		73		86.45	



**Table:10 Results of recovery of ISTD**

Replicate No.	HQC		MQC		LQC	
	Aqueous response	Extract response	Aqueous response	Extract response	Aqueous response	Extract response
1	299185	218147	353291	207004	379907	221375
2	311989	212549	340135	225420	413790	212676
3	297127	215132	342572	208676	370244	214687
4	302104	209983	365395	198558	410395	213150
5	295633	225750	383602	222087	379513	208193
6	300461	204017	391051	211995	383242	199703
Mean	301083.2	214263	362674.3	212290	389515.2	211630.7
SD	5819.36	7403.09	21216.86	9980.34	18046.19	7235.76
% CV	1.9	3.5	5.9	4.7	4.6	3.4
% Mean Recovery	71.3		58.5		54.3	

**4.2.8 Stability studies:**

Stability studies like short term, long term stock solution stability, bench top, autosampler and freeze thaw stability were determined by keeping the QC samples at different conditions. The results were within the acceptance criteria which were shown in the following table 11.

**Table:11 Results showing stability of linezolid at different conditions**

S.NO	PARAMETER	CONDITION	RESULTS	ACCEPTANCE CRITERIA
1	Short term stock solution stability	6hrs at ambient temperature	97.1%	90-110%
2	Long term stock solution stability	After storage of 10 days below 8°C	98.5%	90-110%
3	Bench top	6hrs at room temperature (22±4°C)	89-99.3%	85-115%
4	Autosampler	Stored in autosampler at ambient temperature for 24hrs	85.6-95.5%	85-115%
5	Freeze thaw	Stored at -86°C	92.1-92.3%	85-115%

**5. CONCLUSION:**

The use of turbulent flow chromatography combined with LC-MS/MS detection for analytical separation serves as a significant amount of time in sample preparation and increases sample throughput. The validation results reflected that this method is suitable for regulatory purposes. This method can be strongly recommended for use because it significantly speeds up sample analysis compared to traditional methods and is applicable for regulatory purposes.

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