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BIOANALYTICAL METHOD DEVELOPMENT, VALIDATION AND STABILITY FOR ESTIMATION OF CHLORTHALIDONE IN HUMAN PLASMA BY USING LC-ESI- MS/MS

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ABSTRACT

A simple, rapid and accurate bioanalytical method was developed and validated as per US-FDA guidelines for the estimation of Chlorthalidone in spiked human plasma using liquid-liquid extraction technique. **Results:** Chlorthalidone was well resolved from human plasma interference and internal standard (Chlorthalidone D4) using ZIC®HILIC (100X4.6mm,5µm) column with Methanol: 0.05 Formic acid in water (90:10,v/v) as mobile phase, at a flow rate of 0.900 ml/min. Detection was performed by electrospray ionization (ESI) operated in negative multiple reaction monitoring (MRM) mode. The lower limit of quantitation (LLOQ) of this method was 2071.057 pg/mL and the calibration curves were linear (r(2)≥0.995) over the concentration range of 2000-1200000 pg/mL for Chlorthalidone. The intra- and inter-day precision and accuracy were well within the acceptable limits. The mean extraction recoveries were found to be about 80% and no matrix effect was observed. Chlorthalidone wws found to be stable under all relevant storage conditions.

KEYWORDS: Chlorthalidone, Chlorthalidone D4, LC-MS/MS.

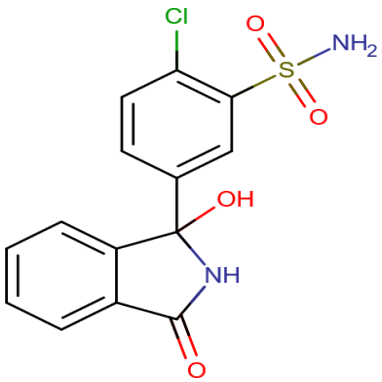
INTRODUCTION

Chlorthalidone is a thiazide-like diuretic used for the treatment of hypertension and for management of edema caused by conditions such as heart failure or renal impairment. Chlorthalidone improves blood pressure and swelling by preventing water absorption from

the kidneys through inhibition of the Na+/Cl− symporter in the distal convoluted tubule cells in the kidney. The exact mechanism of chlorthalidone's anti-hypertensive effect is under debate, however, it is thought that increased diuresis results in decreased plasma and extracellular fluid volume, decreased cardiac output and therefore overall reduction in blood pressure.[5]

Chlorthalidone is considered first-line therapy for management of uncomplicated hypertension as there is strong evidence from meta-analyses that thiazide diuretics such as chlorthalidone reduce the risk of stroke, myocardial infarction, heart failure, and cardiovascular all-cause mortality in patients with hypertension.[1] In particular, the ALLHAT trial confirmed the role of thiazide diuretics as first-line therapy and demonstrated that chlorthalidone had a statistically significant lower incidence of stroke and heart failure when compared to Lisinopril, Amlodipine, or Doxazosin. Further studies have indicated that low-dose thiazides are as good as, and in some secondary endpoints, better than β- blockers, ACE inhibitors, Calcium Channel Blockers or ARBs.

Chlorthalidone has been shown to have a number of pleiotropic effects that differentiate it from other diuretics such as Hydrochlorothiazide. In addition to its antihypertensive effects, chlorthalidone has also been shown to decrease platelet aggregation and vascular permeability, as well as promote angiogenesis in vitro, which is thought to be partly the result of reductions in carbonic anhydrase– dependent pathways. These pathways may play a role in chlorthalidone's cardiovascular risk reduction effects.[7]



MATERIALS AND METHODS

Reagents and Chemical

Chlorthalidone & Chlorthalidone D4 were kindly provided by the Research Laboratory of Clearsynth Private Limited). HPLC-grade methanol was purchased from Spectrochem.

Formic acid was purchased from Spectrochem. A Milli-Q® (Millipore Co., MA, USA) water purification system was used to obtain purified water used for the analysis. Blank human plasma was provided by Salvus Bio-Research Solutions.

Apparatus and chromatographic conditions

The quantification of Chlorthalidone in human plasma was carried out with LC–MS/MS system using an Shimadzu SIL-HTC (Shimadzu Corporation, Japan) coupled with an Applied Biosystems API 4000 triple quadrupole mass spectrometer equipped with an electrospray ionization source operated in negative ionization mode. All weighing were done on analytical balance & Microbalance. The analytical column used as a ZIC®HILIC (100X4.6mm,5µm). Methanol: 0.05 Formic acid in water (90:10,v/v) was used as mobile phase. The flow rate was maintained at 0.900 mL/min. The ion spray voltage was set at -4500 V and at a temperature of 500°C.

Preparation of Standard solutions

Preparation of Chlorthalidone Stock Solution For CC (A) (288759.319 ng/mL)

Weighed 3.063 mg of Chlorthalidone and transferred in to 10 mL of volumetric Flask and dissolved in 5 mL Methanol. The volume was made up to the mark with Methanol mixed well. The solution was then transferred into 15 mL tarson tube. It was stored in the refrigerator at 2-8 °C**.**

Preparation of Chlorthalidone Stock Solution For QC (B) ((287062.399ng/mL)

Weighed 3.045 mg of Chlorthalidone and transferred in to 10 mL of volumetric Flask and dissolved in 5 mL Methanol. The volume was made up to the mark with Methanol mixed well. The solution was then transferred into 15 mL tarson tube. It was stored in the refrigerator at 2-8 °C**.**

Preparation of internal standard

Preparation of Chlorthalidone D4 Internal Standard Stock Solution (99265.480 ng/mL)

Weighed 0.502 mg of Chlorthalidone and transferred in to 5 mL of volumetric Flask and dissolved in 2.5 mL Methanol. The volume was made up to the mark with Methanol mixed well. The solution was then transferred into 15 mL tarson tube. It was stored in the refrigerator at 2-8 °C**.**

Preparation of Chlorthalidone D4 Internal Standard Dilution (1488.9822 ng/mL)

Pipette out 0.750 mL of ISTD into 50 mL volumetric flask and make up the volume up to the mark with diluent (methanol: water, 50:50, v/v). Transfer the solution into 100 mL reagent bottle. Label and store the solution into Refrigerator at 2-8ºC.

Plasma sample preparation

Aliquot 200 µL from each sample into prelabeled polypropylene vials.
Add 50 µL of ISTD dilution solution [Chlorthalidone D4 in Diluent (i.e., Methanol: Water, 50:50 v/v)] to each sample except Blank, for "BLANK" sample add 50µL diluent instead of ISTD dilution solution. Vortex the samples for approx. 1 minute on vortexer
Add 50 µl of 2 %(v/v) Formic acid in water in each sample and vortex the samples for approx. 1 minutes on vortexer.
Add 2 ml of extraction solution (Methyl tert butyl ether: n-Hexane, 80:20, v/v) in each sample and vortex for 3 min. on vortexer.
Centrifuge the samples at 3000 RPM for 5 minutes at 5°C ±2°C and transfer the supernatant volume into prelabeled Ria vials.
Evaporate the supernatant volume under nitrogen gas stream at 45º±2º C up to dryness.
Reconstitute the dried samples with 400 µL of reconstitution solution (Methanol: 0.05 % (v/v) Formic acid in water) (90:10, v/v) and vortex for approx. 30 seconds and transferred the volumes into HPLC vials and injected it.

RESULT AND DISCUSSION

Method validation

System Suitability

To verify that the analytical system would work properly and would give accurate and precise results, the system suitability parameters were performed prior to any parameter and the results obtained were found to be well within the acceptance criteria.

Acceptance criteria: % CV should be within 5% and 2% for area and RT respectively for analyte and ISTD(s). % CV of Area Ratio should be within 5%.

Table 1: System suitability for LC-MS/MS method.

Sr. No.	Drug		ISTD		Area Ratio
	Area	RT	Area	RT	
System suitability	164492	2.403	53486	2.393	3.075
	165726	2.401	54897	2.394	3.019
	164049	2.407	54856	2.400	2.991
	163450	2.409	55364	2.401	2.952
	166622	2.406	54765	2.397	3.042
	167287	2.407	55296	2.403	3.025
Mean	**165271**	**2.406**	**54777**	**2.398**	**3.017**
S.D.	**1517.41**	**0.00**	**678.44**	**0.00**	**0.04**
% CV	**0.9**	**0.1**	**1.2**	**0.2**	**1.4**

Autosampler Carry Over

Carry over is the appearance of the analyte when a blank containing no analyte is injected. Carry over is observed when a blank is injected immediately after a high concentration of the analyte. Carry over should be checked at starting of the validation and during the validation if any part of the instrument is changed.

Design of the experiment

To check the carryover of the Autosampler one aqueous higher standard, one lower standard and mobile phase (MP) was processed according to extraction procedure. The processed sample was injected in the following sequence to check the carryover.

Acceptance criteria

Carryover of the analyte in MP should be less than or equal to 20.00% of the analyte area of AQ lower standard (LLOQ) STD 1.

Carryover of the analyte in MP should be less than or equal to 5% of the IS area of AQ lower standard (LLOQ) STD1.

Table 2: Auto Sampler carry over for LC-MS/MS method.

Sample name	Analyte Area	% of Interfering analyte Area	ISTD Area	% of Interfering ISTD Area
RS	0	0.0	0	0.0
AQ STD 8	3505368	NA	50431	NA
RS	0	0.0	0	0.0
RS	0	0.0	0	0.0
AQ STD 1	7980	NA	56976	NA

Selectivity

Screening and selectivity was done using six normal blank matrix lot and one lipemic, one Haemolyse lot of plasma and the result was found within the acceptance limits. No interferences from the plasma matrix were observed at the retention time of the analyte and ISTD. The results obtained were within acceptance criteria, not any interfering peak was found within ±10% of the retention times of analyte and ISTD.

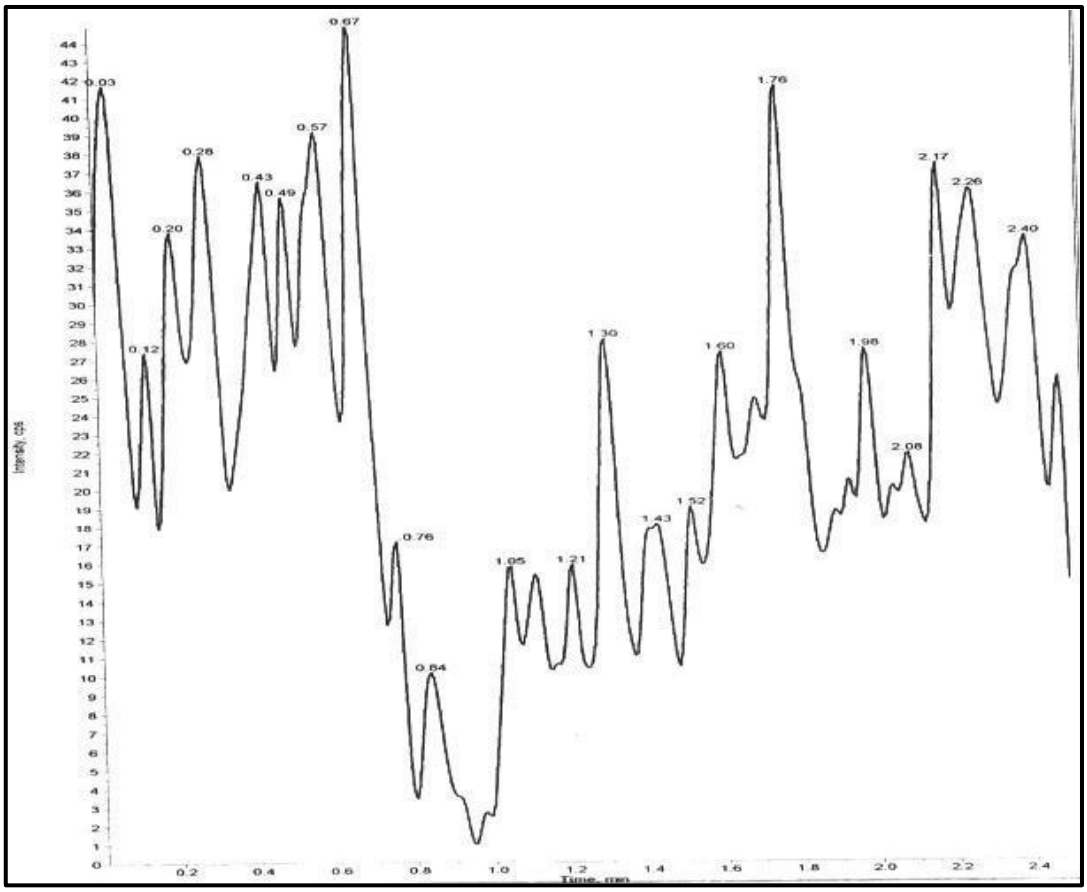


Figure 8.8.2: Chromatogram of blank plasma.

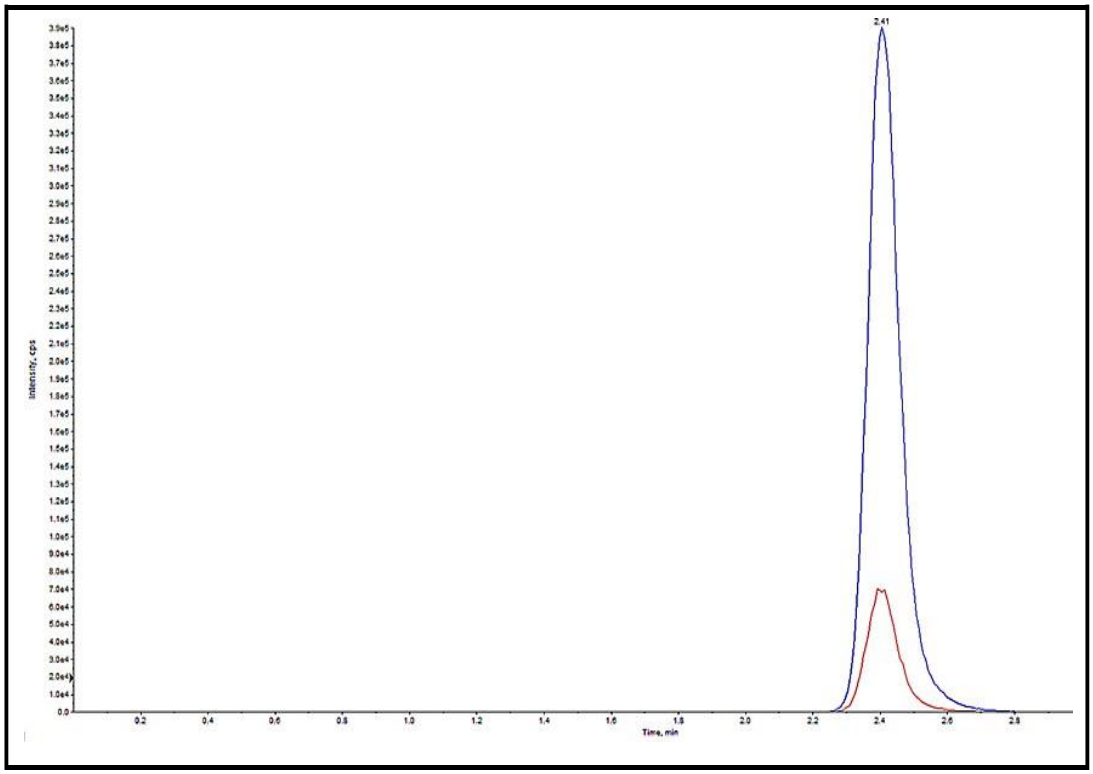


Figure 8.8.3: Chromatogram of LLOQ.

Table 3: Results of selectivity.

		Chlorthalidone			Chlorthalidone D4		
Sr. No	**Blank Plasma**	**Interference at RT of Analyte**	**LLOQ area**	**% LLOQ**	**Interference at RT of ISTD**	**IS area**	**% IS**
1	LOT-1	309	6385	4.8	0	49891	0.0
2	LOT-2	0	6160	0.0	0	55357	0.0
3	LOT-3	0	6705	0.0	0	52502	0.0
4	LOT-4	0	6920	0.0	0	54792	0.0
5	LOT-5	0	6358	0.0	0	50750	0.0
6	LOT-6	0	6666	0.0	0	51715	0.0
7	LOT-7(H)	0	6577	0.0	0	49091	0.0
8	LOT-8(L)	0	6812	0.0	0	53786	0.0
		Mean	6573		**Mean**	52236	
		S.D.	255.16		**S.D.**	2287.13	
		% CV	**3.9**		**% CV**	**4.4**	

DDR SELECTIVITY

(1% spiking of Mixed DDR solution in Blank plasma). The interference in BLANK was compared with the response of the respective extracted LLOQ samples. DDR SELECTIVITY (Drug metabolite)experiment performed in the presence of 1% spiking solution of DDR drug. DDR SELECTIVITY experiment performed in the presence of 1% spiking solution of mix solution of Paracetamol, Caffeine, Ibuprofen, Diclofenac, Ranitidine, Cetirizine, Domperidone, NicotineDDR drugs.

Acceptance criteria: Any significant interference peak corresponds to analyte and internal standard peak should not be present in Blank samples. If interfering peak observed at the retention time of analyte, area of interfering peak should be ≤ 20% of analyte area obtained at LLOQ samples. If interfering peak observed at the retention time of internal standard, area of interfering peak should be ≤ 5% of internal standard area obtained at LLOQ sample.

Table 4: Results of DDR selectivity.

		Chlorthalidone			Chlorthalidone D4		
Sr. No	**Blank Plasma**	**Interference at RT of Analyte**	**LLOQ area**	**% LLOQ**	**Interference at RT of ISTD**	**IS area**	**% IS**
1	LOT-1	0	8076	0.0	0	63058	0.0
2	LOT-2	0	7733	0.0	0	62730	0.0
3	LOT-3	143	7914	1.8	0	63547	0.0
4	LOT-4	0	7929	0.0	0	62032	0.0
5	LOT-5	0	8017	0.0	0	63387	0.0
6	LOT-6	0	6975	0.0	0	61919	0.0

	Mean	7774		**Mean**	62779	
	S.D.	408.40		**S.D.**	683.72	
	% CV	**5.3**		**% CV**	**1.1**	

Bench top stability

Six aliquots each of the low and high quality control samples were kept at room temperature (22±5°C) after spiking into Plasma. After completion of 06 Hrs. the samples were extracted and analyzed against freshly processed calibration curve and batch qualifying QC samples.

Acceptance criteria: The % Accuracy for all QC(s) should be within 85-115% of its nominal Concentration. The % CV for all QC(s) concentration at each concentration level should be less than or equal to 15%. At least 67% of all QC(s) should be within acceptance limits. At least 50% of all QC(s) at each level should be within acceptance limits.

Table 5: Bench top stability for LQC & HQC.

Sr. No.	LQC (6.072 ng/ml)	HQC (963.826 ng/ml)
1	5.766	943.917
2	5.929	940.015
3	5.877	956.948
4	5.866	938.483
5	5.877	986.615
6	5.802	929.278
Mean	5.853	949.209
S.D.	0.0588	20.4153
%CV	**1.0**	**2.2**
% Nominal	**96.4**	**98.5**
% Mean Change	**-3.6**	**-1.5**

Freeze and Thaw Stability

The freeze and thaw stability of analyte were determined after five freeze and thaw cycles. Six aliquots each of low and high quality control samples were stored at -30±10°C and -70±20°C and subjected to five freeze thaw cycles at a minimum interval of 12 Hrs. after completion of first cycle of minimum 24 Hrs. After the completion of five cycles the samples were analyzed against freshly processed calibration curve samples and batch qualifying QC samples.

Acceptance criteria: The % Accuracy for all QC(s) should be within 85-115% of its nominal Concentration. The % CV for all QC(s) concentration at each concentration level should be less than or equal to 15%. At least 67% of all QC(s) should be within acceptance limits. At

least 50% of all QC(s) at each level should be within acceptance limits.

Table 6: Freeze and Thaw Stability for LQC & HQC.

Sr. No.	For 5 Cycles at -30 ± 10 °C		For 5 Cycles at -70 ± 20 °C	
	LQC	HQC	LQC	HQC
	6.072	**963.826**	**6.072**	**963.826**
1	5.648	940.352	5.663	926.240
2	5.814	942.487	5.832	905.926
3	5.570	915.895	5.405	912.983
4	5.634	932.872	5.541	917.525
5	5.636	945.460	5.448	928.134
6	5.842	902.300	5.778	931.675
Mean	5.691	929.894	5.611	920.414
S.D.	0.1102	17.1844	0.1751	9.9380
%CV	**1.9**	**1.8**	**3.1**	**1.1**
% Accuracy	**93.7**	**96.5**	**92.4**	**95.5**

Whole blood stability

Sample of clinical study for analysis of Chlorthalidone will be collected from healthy human. The blood will be collected from the healthy human after administration of tablet containing Chlorthalidone. After collection of blood samples, sample will be centrifuged and plasma layer of the sample will be collected and stored in freezer at -20 ˚C. So from the collection time of samples to separation of the plasma layer Analyte will be present in the blood. Therefore it is necessary to establish the stability of the Analyte in blood for that period of time.

Design of the experiment

The fresh K3EDTA blood was collected. The spiking solution of HQC and LQC were spiked in fresh K3EDTA blood. The spiked blood samples were kept in to the ice cold water bath maintained at room temperature for 2 hrs. After 2 hrs, the spiking solution of HQC and LQC were spiked in blood and consider this sample as comparison or fresh sample and previously spiked sample as stability samples. Both comparison and stability HQC and LQC samples were centrifuged at 3000 rpm, for 15min and 5˚C. The plasma layer was separated from the centrifuged samples. Six aliquots from stability and comparison samples of HQC and LQC were processed as per the extraction procedure.

Acceptance criteria

The % CV at each level should be within 15%.

The % change at each level should be within ± 15%.

Table 7: Whole blood stability for LQC & HQC.

	Area Ratio			
	LQC		HQC	
	0 Hrs.	2 hours	0 Hrs.	2 hours
	0.54146	0.54833	80.32567	82.85563
	0.53636	0.57384	80.65157	81.74546
	0.54529	0.59371	82.00059	82.94083
	0.55264	0.58289	82.74003	82.21943
	0.55055	0.58187	84.67778	81.84267
	0.54358	0.56728	80.99486	81.78049
Mean	0.544980	0.574653	81.898417	82.230752
SD	0.005974	0.015691	1.630226	0.544732
% CV	**1.1**	**2.7**	**2.0**	**0.7**
% Accuracy	**105.4**		**100.4**	
% Change	**5.4**		**0.4**	

Auto sampler stability

During validation or study sample analysis of the sample, it might be possible that processed sample will not be analyzed immediately after completion of sample processing. In such a case processed samples are required to store in refrigerator at 2-8 ˚C. So the stability of the processed samples need to be evaluated at the above mentioned storage condition**.**

Design of the experiment

Auto sampler stability experiment processed HQC and LQC samples of the precision and accuracy were stored in refrigerator at 2-8 ˚C. After completion of desired storage period should be at least 24 hrs or longer duration, spiked HQC and LQC were retrieved for comparison samples. The comparison samples and freshly spiked calibration standard samples were processed as per the procedure. Compare the change in the nominal concentration of the HQC and LQC of stability samples with that of comparison samples.

Acceptance criteria

The % accuracy (nominal) for all QC should be within 85-115% of its nominal concentration.

The % CV for all QC concentration at each concentration level should be less than or equal to 15%. At least 67% of all QC should be within acceptance limits.

At least 50% of QC at each level should be within acceptance limits.

Table 8: Auto sampler stability for LQC & HQC.

	LQC.	HQC.
	6.072	**963.826**
	6.201	1010.061

	6.368	1012.321
	5.907	1020.377
	5.730	1014.979
	6.170	1010.740
	6.082	1010.602
Mean	6.076	1013.180
SD	0.2272	3.9522
% CV	**3.7**	**0.4**
% Accuracy	**100.1**	**105.1**
% Change	**0.1**	**5.1**

Stock solution stability and working solution stability Design of the experiment

Compare the analytical area results of stability solutions with freshly prepared solutions area results.

Acceptance criteria

The mean (%) change in area of the stability samples and comparison samples should be 90-110 % for both analyte and ISTD.

If above acceptance criteria are not meet then experiment should be repeated if on investigation any processing error is found or should be repeated for relevant shorter storage period or storage temperature.

Table 9: Stock & Working Solution Stability for analyte.

Factor	**W1 (OLD) (mg)**		3.08	0.9994				
	W2 (NEW) (mg)		3.08					
	COMPARISON		**LTSS**	**LTWS**		**STSS**	**STWS**	
	LLOQ	**ULOQ**	**18 DAYS 18 HOURS**	**18 DAYS 18 HOURS**		**06 HOURS**	**06 HOURS**	
				LLOQ	**ULOQ**		**LLOQ**	**ULOQ**
	7518	4083837	4018591	7747	3983369	4016624	7616	4058865
	7556	3986668	4007727	7767	3978907	4016332	7604	4014604
	7810	3969428	3998315	7638	4011344	3963671	7534	4014199
	7115	4018177	3981552	7521	3978882	4020485	7611	3945176
	7241	4000694	4027392	7621	3969658	3976279	7822	4001328
	7625	3904315	4012851	7859	4001406	3979019	7404	4009298
Mean	7478	3993853	4007738	7692	3987261	3995402	7599	4007245
SD	255.91	58992.94	16153.70	121.44	15780.25	25133.81	135.97	36493.63
% CV	**3.4**	**1.5**	**0.4**	**1.6**	**0.4**	**0.6**	**1.8**	**0.9**
% Stability			**100.4**	**102.9**	**99.9**	**100.1**	**101.7**	**100.4**
% Change			**0.4**	**2.9**	**-0.1**	**0.1**	**1.7**	**0.4**
Remark			**Stable in methanol at 2-8°C.**	**Stable in Methanol :Water (50:50) at 2-8°C**		**Stable for 06 hrs. in methanol at RT.**	**Stable for 06 hrs. in Methanol :Water (50:50) RT**	

Table 10: Stock & Working Solution Stability for ISTD.

Factor	W1 (OLD)	0.50	1.0000		
	W2 (NEW)	0.50			
	COMPARISO N	LTSS	LTWS	STSS	STWS
		18 DAYS 18 HOURS	18 DAYS 18 HOURS	06 HOURS	06 HOURS
	88868	88369	89299	88227	87914
	87824	88662	87049	87956	87470
	82881	83296	81808	81666	83027
	82383	82297	82393	81295	80847
	83162	83048	82902	83055	82294
	82063	82518	81219	82099	83066
Mean	84530	84698	84112	84050	84103
SD	2998.50	2979.76	3274.99	3186.66	2897.16
% CV	**3.5**	**3.5**	**3.9**	**3.8**	**3.4**
% Stability		**100.2**	**99.5**	**99.4**	**99.5**
% Change		**0.2**	**-0.5**	**-0.6**	**-0.5**
Remark		**Analyte was stable in methanol at 2-8°C for 18 days 18 hours.**	**Analyte was stable in Methanol: Water (50:50) at 2-8°C for 18 days 18 hours.**	**Analyte was stable for 06 hours in methanol at room temperature**	**Analyte was stable for 06 hrs. in Methanol: Water (50:50) at room temperature.**

Table 11: Within-batch or intra-batch precision and accuracy.

Result Table ID	QC-ID	LOQ QC	LQC.	MQC.	HQC.
		2.125	**6.072**	**481.913**	**963.826**
PA 01	1	2.142	5.648	469.479	940.352
	2	2.140	5.814	478.475	942.487
	3	2.080	5.570	465.855	915.895
	4	1.873	5.634	476.273	932.872
	5	1.926	5.636	436.759	945.460
	6	2.040	5.842	467.051	902.300
	Mean	2.034	5.691	465.649	929.894
	SD	0.11	0.11	15.02	17.18
	% CV	**5.5**	**1.9**	**3.2**	**1.8**
	% Accuracy	**95.7**	**93.7**	**96.6**	**96.5**
% Mean Change		**-4.3**	**-6.3**	**-3.4**	**-3.5**
PA 02	1	2.121	5.841	487.892	924.897
	2	2.186	5.898	489.241	945.681
	3	2.118	5.848	490.824	929.403
	4	2.206	5.995	480.381	918.522
	5	2.165	5.860	483.479	925.211
	6	2.052	5.998	493.737	947.092
	Mean	2.141	5.907	487.592	931.801
	SD	0.06	0.07	4.90	11.83
	% CV	**2.6**	**1.2**	**1.0**	**1.3**
	% Accuracy	**100.8**	**97.3**	**101.2**	**96.7**

% Mean Change		0.8	-2.7	1.2	-3.3
PA 03	1	2.043	5.815	467.005	920.815
	2	2.074	5.863	477.177	939.012
	3	1.928	5.702	494.230	925.784
	4	1.998	5.678	419.815	969.320
	5	2.090	5.713	495.926	963.342
	6	2.066	6.149	495.654	968.245
	Mean	2.033	5.820	474.968	947.753
	SD	0.06	0.18	29.49	21.97
	% CV	**3.0**	**3.0**	**6.2**	**2.3**
	% Accuracy	**95.7**	**95.8**	**98.6**	**98.3**
% Mean Change		**-4.3**	**-4.2**	**-1.4**	**-1.7**
PA 04	1	2.134	6.081	539.252	1016.191
	2	1.985	6.199	507.943	998.817
	3	2.168	6.116	506.098	945.657
	4	1.938	6.337	512.691	1011.403
	5	2.262	6.535	531.387	982.131
	6	2.002	6.335	497.336	1001.369
	Mean	2.082	6.267	515.785	992.595
	SD	0.13	0.17	16.12	25.84
	% CV	**6.0**	**2.7**	**3.1**	**2.6**
	% Accuracy	**98.0**	**103.2**	**107.0**	**103.0**
% Mean Change	**-2.0**	**3.2**	**7.0**	**3.0**	

Table 12: Between Batch Accuracy and Precision.

RESULT TABLE ID	QC-ID	LOQ QC	LQC	MQC	HQC
		2.125	**6.072**	**481.913**	**963.826**
PA 01	1	2.142	5.648	469.479	940.352
	2	2.140	5.814	478.475	942.487
	3	2.080	5.570	465.855	915.895
	4	1.873	5.634	476.273	932.872
	5	1.926	5.636	436.759	945.460
	6	2.040	5.842	467.051	902.300
PA 02	1	2.121	5.841	487.892	924.897
	2	2.186	5.898	489.241	945.681
	3	2.118	5.848	490.824	929.403
	4	2.206	5.995	480.381	918.522
	5	2.165	5.860	483.479	925.211
	6	2.052	5.998	493.737	947.092
PA 03	1	2.043	5.815	467.005	920.815
	2	2.074	5.863	477.177	939.012
	3	1.928	5.702	494.230	925.784
	4	1.998	5.678	419.815	969.320
	5	2.090	5.713	495.926	963.342
	6	2.066	6.149	495.654	968.245
PA 04	1	2.134	6.081	539.252	1016.191
	2	1.985	6.199	507.943	998.817
	3	2.168	6.116	506.098	945.657

	4	1.938	6.337	512.691	1011.403
	5	2.262	6.535	531.387	982.131
	6	2.002	6.335	497.336	1001.369
	Mean	2.072	5.921	485.998	950.511
	SD	0.10	0.25	25.92	31.79
	% CV	**4.8**	**4.3**	**5.3**	**3.3**
	% Accuracy	**97.5**	**97.5**	**100.8**	**98.6**
% Mean Change	**-2.5**	**-2.5**	**0.8**	**-1.4**	

Recovery

Recovery for analyte and internal standard is performed by comparing the area of extracted samples at three different concentrations (LQC, MQC and HQC) with un-extracted matrix that represents recovery.

$$\% \text{ Recovery} = \frac{\text{Mean peak area response of extracted samples X 100}}{\text{Mean peak area response of unextracted samples}}$$

Table 13: Recovery – ISTD.

RECOVERY - ISTD			
SR NO	**Extracted Sample**	**Extracted Sample (Abs. Recovery)**	**Aqueous Sample (Total Recovery)**
1	66625	71886	66994
2	64603	69692	66980
3	60022	68524	65577
4	61443	68429	60675
5	62608	68548	60446
6	62034	66925	59519
7	55777	68309	62232
8	55823	64541	63983
9	53857	64543	64310
10	58079	64163	57077
11	57829	63510	57136
12	56883	64051	56940
13	55748	63688	59692
14	51524	61262	59584
15	52052	61278	60456
16	55418	60643	60059
17	55292	60160	53714
18	56123	60185	54795
Mean	57874	65019	60565
SD	4195.21	3599.24	3884.34
% CV	**7.2**	**5.5**	**6.4**
% Recovery		**89.0**	**95.6**

	LQC RESPONSE		
	Extracted Sample	**Unextracted Sample (Absolute)**	**Aqueous Sample (Total)**
Recovery 01	22214	25411	23658
	21749	24258	24568
	20036	23921	24123
	21021	24288	22072
	20942	24509	22172
	21234	23711	22934
Mean	21199	24350	23255
SD	746.22	592.57	1031.04
% CV	**3.5**	**2.4**	**4.4**
%Recovery		**87.1**	**91.2**
	MQC RESPONSE		
	Extracted Sample	**Unextracted Sample (Absolute)**	**Aqueous Sample (Total)**
Recovery 01	1543128	1955934	1797781
	1548676	1851358	1826786
	1498964	1861432	1819264
	1582074	1819697	1651913
	1585433	1825622	1646616
	1592566	1828145	1639663
Mean	1558474	1857031	1730337
SD	35549.83	51084.46	92887.48
% CV	**2.3**	**2.8**	**5.4**
% Recovery		**83.9**	**90.1**
	HQC RESPONSE		
	Extracted Sample	**Unextracted Sample (Absolute)**	**Aqueous Sample (Total)**
Recovery 01	2923647	3612152	3408208
	2762855	3504704	3424304
	2743132	3498445	3456548
	2886330	3405014	3389390
	2900753	3393421	3119491
	3013950	3420013	3119400
Mean	2871778	3472292	3319557
SD	102341.19	83397.12	156565.22
% CV	**3.6**	**2.4**	**4.7**
%Recovery		**82.7**	**86.5**

Ruggedness

Ruggedness experiment was established by performing Precision and Accuracy batch having six replicates at four concentrations of quality control samples (LOQ QC, LQC, MQC and HQC) by different column Acceptance criteria

The % accuracy (nominal) for all QC should be within 85-115% of its nominal value except LOQ QC where it should be within 80-120% of its nominal concentration.

The % CV for QC samples at each concentration level should be less than or equal to 15% except LOQ QC where it should be less than or equal to 20%.

At least 67% of all QC should be within acceptance limits.

At least 50% of all QC at each level should be within acceptance limits.

QC-ID	LOQ QC.	LQC.	MQC.	HQC.
	2.125	**6.072**	**481.913**	**963.826**
1	2.123	5.748	471.364	912.676
2	2.152	5.795	474.953	956.742
3	2.170	5.690	494.540	933.300
4	2.170	5.677	418.276	986.329
5	2.129	5.784	497.427	955.083
6	2.114	5.939	490.942	989.305
Mean	2.143	5.772	474.584	955.573
SD	0.02	0.09	29.56	29.74
% CV	**1.1**	**1.6**	**6.2**	**3.1**
% Accuracy	**100.8**	**95.1**	**98.5**	**99.1**
% Mean Change	**0.8**	**-4.9**	**-1.5**	**-0.9**

Reinjection reproducibility

Reinjection reproducibility is performed to validate that the result produced can be reproduced by injecting the same samples again within the stability of the samples. When instrument failure occurs the samples might be required to reinject and because of this reason it is required to validate reinjection.

Reinjection reproducibility was performed by injecting the already acquired six set of HQC and LQC of precision and accuracy immediately after completion of Precision and Accuracy sequence. The % change will be evaluated by comparing with previously injected samples**.**

Acceptance criteria

The % Accuracy for all QC samples should be within 85-115% of its nominal concentration. The % CV for all QC concentration at each level should be less than or equal to 15%. At least 67% of all QC samples should be within the limits. The % change in accuracy at each level of re-injected QC sample should be within ± 15 % of comparison with earlier injected value.

	Original values		Reinjected values	
	LQC.	HQC.	LQC.	HQC.
	6.072	**963.826**	**6.072**	**963.826**
	5.648	940.352	5.663	926.240
	5.814	942.487	5.832	905.926
	5.570	915.895	5.405	912.983

	5.634	932.872	5.541	917.525
	5.636	945.460	5.448	928.134
	5.842	902.300	5.778	931.675
Mean	5.691	929.894	5.611	920.414
SD	0.1102	17.1844	0.1751	9.9380
% CV	**1.9**	**1.8**	**3.1**	**1.1**
% Accuracy	**93.7**	**96.5**	**92.4**	**95.5**
% Change			**-1.3**	**-1.0**

CONCLUSION

The method for estimation of Chlorthalidone from human plasma by LC-MS/MS was validated for performance characteristics related to accuracy, precision, linearity, selectivity, stability of the drug in biological matrix at ambient and at freezing conditions, stability of drug in extracted media. The method is linear over the concentration studied. The method is selective in presence of plasma interferences. Hence, % interference is Zero. Recovery is consistent at higher, middle and at lower level. % CV for recovery is well within acceptance criteria for all three QC levels studied. The results are precise for all three sets of QC and for LLOQ. Accuracy also holds well with all replicates of all QC levels and LLOQ lying within acceptance criteria for back-calculated concentration. Precision and accuracy also holds good on intra batch basis. % Difference(s) in the back calculated concentration obtained with fresh and stability samples for the following stability studies is within +15 %. Stability of Chlorthalidone and Chlorthalidone D4 (IS) in Mobile Phase when placed in auto sampler. Stability of Chlorthalidone in Biological matrix at ambient temperature. Stability of Chlorthalidone in Biological matrix subjected to freeze- thaw cycles. Method is also validated to re-inject any sample whenever re-injection required. The bioanalytical method validation report indicates the suitability of the method for analysis of subject samples.

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