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SIMULTANEOUS METHOD DEVELOPMENT AND VALIDATION OF AMLODIPINE BESYLATE AND HYDROCHLOROTHIAZIDE IN HUMAN PLASMA BY LC-MS/MS

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ABSTRACT

A rapid, simple, selective and sensitive LC-MS/MS method was developed for the determination of Amlodipine Besylate and Hydrochlorothiazide in human plasma using Amlodipine D4 and HCTZ 15N213C D2 as internal standard (IS). The method was developed with turbid ion spray (TIS) in the positive ion and multiple reaction monitoring (MRM) mode. The mobile phase was Water: Methanol (05:95) with 1 ml Ammonia Solution. Chromatographic separation was achieved on CHIRALCEL OZ-3R, 4.6 x 150 mm, column with a flow rate of 1.0ml/min. The MRM transitions monitored for Amlodipine Besylate and Hydrochlorothiazide and Amlodipine D4 and HCTZ 15N213C D2 were 296.00/205.00 (m/z), Mass 409.20/238.00 (m/z), Mass 301.00/207.00 (m/z), Mass 413.30/238.10(m/z) respectively. The developed method was validated as per FDA and ICH guidelines. Linearity was observed from of 51.910 pg/mL to 14464.765 pg/mL and0.509 ng/mL to 500789 ng/mL with correlation coefficient of 0.9969. The percent recovery for the drug and IS was found to be 45.5 and48.8 and,62.6 and 82.5% and respectively. Stability studies like freeze thaw, bench top, short term and long term were performed and the results were found to be within the acceptance limits according to FDA guidelines.

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INTRODUCTION

Amlodipine besylate is chemically described as 3-Ethyl-5-methyl (±)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-yridinedicarboxylate, monobenzene-sulphonate. Its empirical formula is C20H25CIN2O5• C6H6O3S.Amlodipine is a dihydropyridine calcium antagonist (calcium ion antagonist or slow-channel blocker) that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. Hydrochlorothiazide USP is a diuretic and antihypertensive. It is the 3,4-dihydro derivative of chlorothiazide. It is chemically designated as 6-chloro-3,4-dihydro-2 H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide It reduces the reabsorption of electrolytes from the renal tubules. This results in increased excretion of water and electrolytes, including sodium, potassium, chloride, and magnesium.

Literature review

Various LC-MS/MS and RP-HPLC methods are reported in the literature for the estimation of Amlodipine and HCTZ individually and in-combination with other drugs. According to literature survey there is no regulatory method reported for the simultaneous estimation of Amlodipine and HCTZ by LC-MS/MS in Human Plasma either in literature and pharmacopeia. Hence In this study, a Novel LC/MS/MS method was optimized and validated for simultaneous estimation and validation of Amlodipine and HCTZ in Human Plasma in accordance with the USFDA, EMEA and other relevant Regulatory guidelines

MATERIALS AND METHODS

Materials used

HPLC grade methanol, acetonitrile, HPLC grade water, Analytes free human plasma

Instrument used

The isocratic mobile phase consisted of water: Methanol at 5:95 ratios with Ammonia solution at a flow rate of 1.0 mL min^{-1}(with splitter out/in 50:50) and CHIRALCEL OZ-3R, 4.6 x 150 mm, 3 µm was used as stationary phase. The Mass parameters for Amlodipine is 409.20/238.00 (m/z), Amlodipine D4 is 413.30/238.10(m/z), HCTZ is 296.00/ 205.00 (m/z) and HCTZ 15N213C D2 is301.00/207.00 (m/z).

Drugs used: Amlodipine Besylate and Hydrochlorothiazide

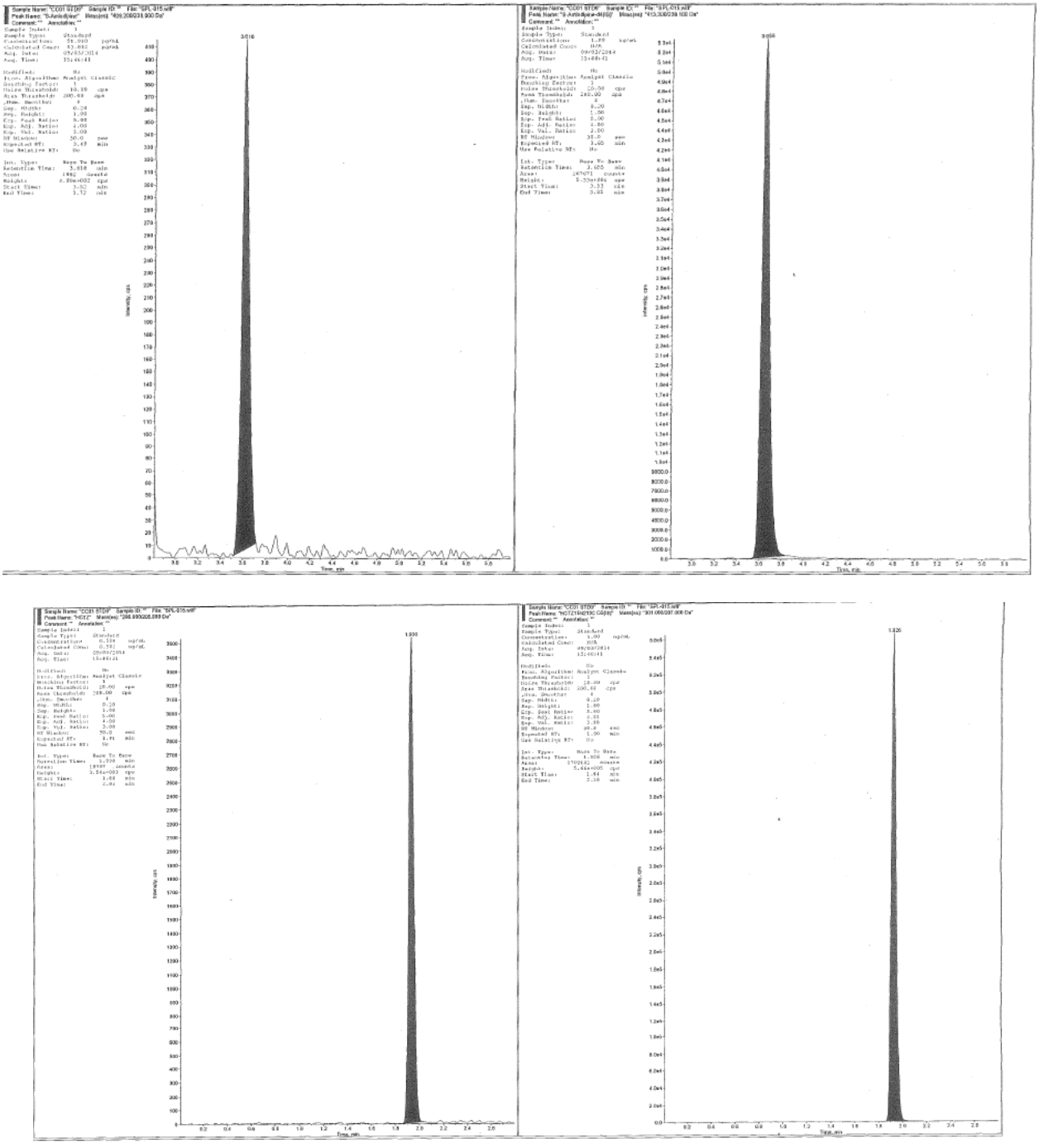
Standard & sample preparations

Hydrochlorothiazide

Weighed accurately 5.0000 mg Hydrochlorothiazide Reference standard, transfer into a 5 mL volumetric flask. 3 mL of methanol was added and sonicated to aid dissolution. Volume was made up to 5 mL with methanol to achieve 1000.000 µg/mL concentration.

Amlodipine

Weighed accurately Amlodipine BesilateHemipentahydrate Reference standard, equivalent to 5.0000 mg of Amlodipine transferred into a 10 mL volumetric flask. 5 mL of methanol was added and sonicated to aid dissolution. Volume made up to 10 mL with methanol to achieve the concentration 500.000 µg/mL.



A representative chromatogram of drug and internal standard

Preparationofworkingstandard solutions for AML&HCTZ

Spiking solutions were made and with serial dilution, the spiking solutions were spiked in screened pooled matrix to Standard concentrations.

RESULTS AND DISCUSSION

Optimization of liquid chromatography and mass spectrometry conditions

Amlodipine and HCTZ obtained for different water:Methanol (40:60, 35:65, 30:70, 25:75&10:90 v/v) indicated that the resolution between Amlodipine and HCTZ increased using higher Methanol (Figure 3.2). Thereafter, water: Methanol (05:95 v/v) with 1 ml of Ammonia solution ataflow rateof1.0mL min^{-1}(with splitter out/in 50:50). CHIRALCEL OZ-3R, 4.6 x 150 mm, 3 µmwasusedasthemobile and stationary phase respectively toimprove resolution, get short run timeand reducing tailingofbothpeakscloseto1. The Mass parameters for Amlodipine is 409.20/238.00 (m/z), Amlodipine D4 is 413.30/238.10(m/z), HCTZ is 296.00/ 205.00 (m/z) and HCTZ is HCTZ 15N213C D2. The retention time was found to be 3.500 ± 0.50 min & 1.800 ± 0.50 minfor Amlodipine and HCTZ, respectively and 3.500 ± 0.50 min & 1.800 ± 0.50 min for Amlodipine D1 and HCTZ 15N2 13C D2 respectively.

for Amlodipine and Results are shown in Table 2. Representative Chromatogram shown in Figure 2 for HCTZ.

Table 1. System Suitability results for Amlodipine

Injection No.	Retention time (min)		Area ratio
	Analyte-Amlodipine	ISTD-Amlodipine D4	
1	3.488	3.516	1.978
2	3.486	3.517	1.948
3	3.487	3.517	2.026
4	3.484	3.517	2.067
5	3.486	3.508	2.111
6	3.483	3.509	2.159
Mean	3.4857	3.5140	2.0482
SD	0.00186	0.00429	0.08004
%CV	0.1	0.1	3.9

Table 2. System Suitability results for HCTZ

Injection No.	Retention time (min)		Area ratio
	Analyte-HCTZ	ISTD-HCTZ 15N213CD2	
1	1.908	1.904	5.707
2	1.907	1.904	5.742
3	1.907	1.903	5.627
4	1.908	1.905	5.643
5	1.906	1.904	5.617
6	1.908	1.905	5.633
Mean	1.9073	1.9042	5.6615
SD	0.00082	0.00075	0.05075
%CV	0.0	0.0	0.9

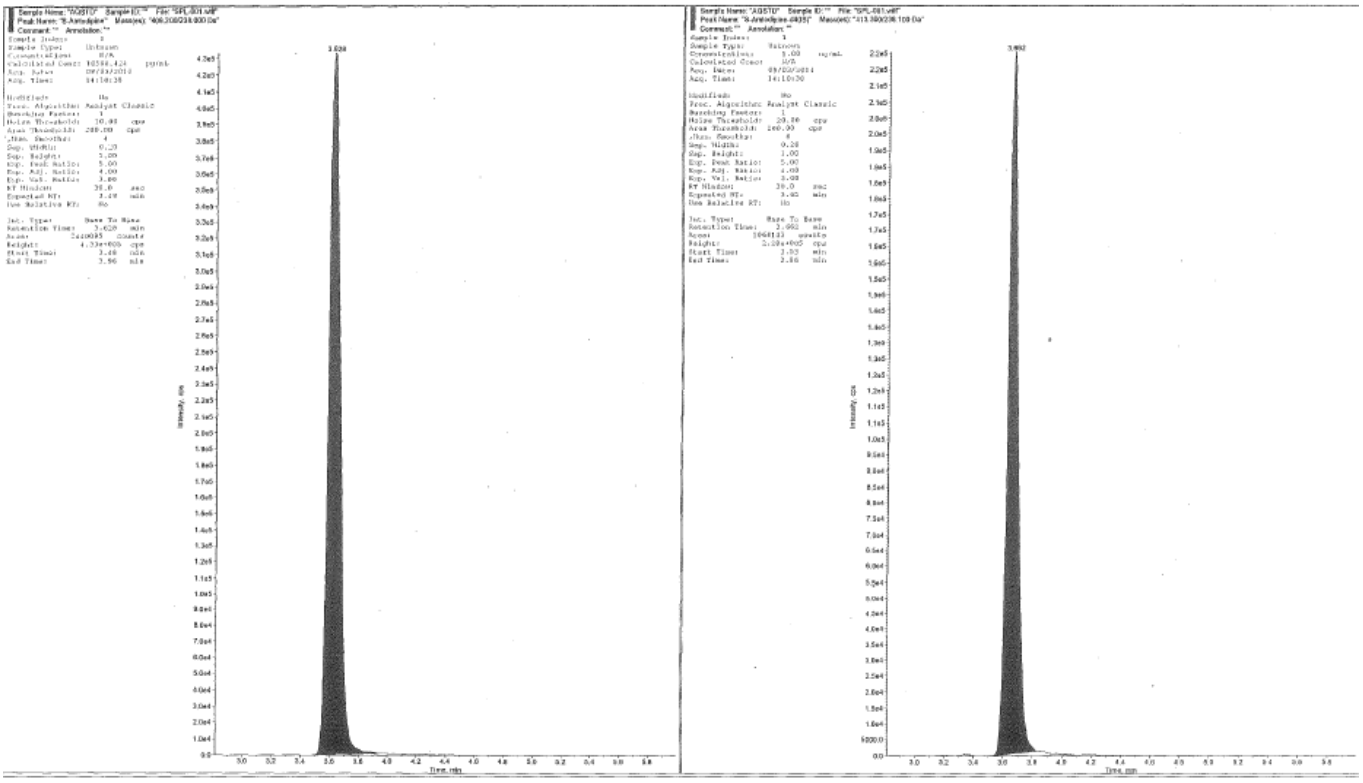


Figure 1. A Representative Chromatogram of Amlodipine for system suitability test

Method Validation

System Suitability Test

System suitability test was performed by acquiring six consecutive injections from a single sample of Highest Standard. The system was found to be sensitive, specific and reproducible for the current analytical run. Results are shown in Table 1. Representative Chromatogram shown in Figure 1

Auto Sampler Carry Over

Amlodipine

Carry Over was assessed by subsequently injecting reconstitution solution after an Aqueous Standard of Highest Concentration and Standard Blank (Extracted) after a calibration curve standard at the Upper limit of quantification. Aqueous and extracted LOQ samples were also injected. Carry

Over was not observed for Amlodipine, Amlodipine-D4 and Hydrochlorothiazide, and Hydrochlorothiazide 15N213C D2 with respect to aqueous LOQ and extracted LOQ samples. Results are shown in Table 3 for Aqueous Solution and Table 4 for extracted solution and Table 5 for Aqueous Solution and Table 6 for extracted solution

Selectivity & Specificity

Amlodipine

Thirteen lots (T-3213, T-3590, T-3540, K-1983, K-1992, K-2021, K-2079, K-2082, K-2084 including haemolytic (HK-2029, HK-2030) and lipemic (LT-1688, LT-1700) lots) of

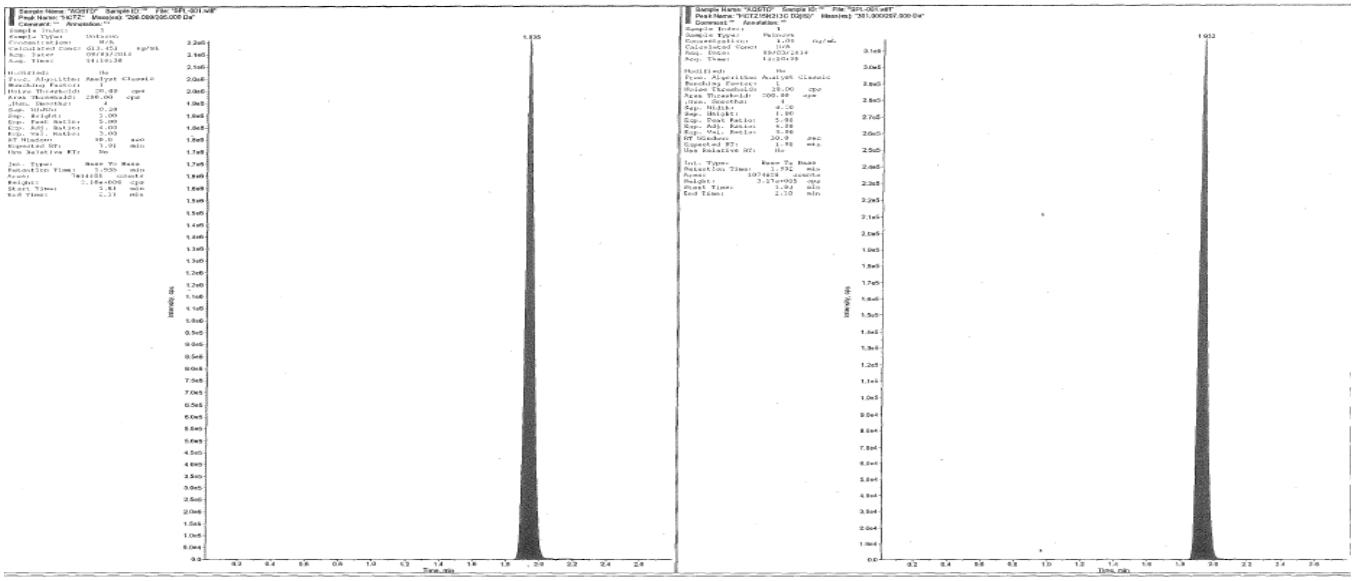


Figure 2. A Representative Chromatogram of HCTZ for system suitability test

Table 3. ASCOT results for Amlodipine – Aqueous solutions

Aqueous	Response Amlodipine	% Carry Over	Response Amlodipine D4	% Carry Over
RS	0	-	0	-
AQS STD 1	3569676	-	1697473	-
RS	0	0.0	0	0.0
AQS STD 1	3417600	-	1669078	-
RS	0	0.0	0	0.0
.AQ LOQ	13984	-	1612432	-

Table 4. ASCOT results for Amlodipine – Extracted solutions

Aqueous	Response Amlodipine	% Carry Over	Response Amlodipine D4	% Carry Over
Standard Blank	0	-	0	-
Extracted STD 1	1291447	-	681024	-
Standard Blank	0	0.0	0	0.0
Extracted STD 1	1295276	-	680739	-
Standard Blank	0	0.0	0	0.0
Extracted LOQ	6210	-	770669	-

Table 5. ASCOT results for HCTZ – Aqueous solutions

Aqueous	Response HCTZ	% Carry Over	Response HCTZ 15N213C D2	% Carry Over
RS	374	-	0	-
AQS STD 1	12504979	-	2185542	-
RS	583	2.3	0	0.0
AQS STD 1	12482144	-	2178568	-
RS	720	2.9	0	0.0
.AQ LOQ	25253	-	3289076	-

Table 6. ASCOT results for HCTZ – Extracted solutions

Aqueous	Response HCTZ	% Carry Over	Response HCTZ 15N213C D2	% Carry Over
Standard Blank	678	-	0	-
Extracted STD 1	11169017	-	1855087	-
Standard Blank	794	4.1	0	0.0
Extracted STD 1	11122930	-	1825557	-
Standard Blank	734	3.8	0	0.0
Extracted LOQ	19520	-	2404654	-

Table 7. Selectivity results for Amlodipine

S.No.	Plasma lot No.	Analyte - Amlodipine			ISTD - Amlodipine-d4		
		Area of interfering peak at RT of S-Amlodipine	Area observed for extracted LOQ	%. interference at RT of S-Amlodipine	Area of interfering peak at RT of ISTD	Area observed for extracted ISTD	Ok interference at RT of ISTD
1	T-3213	0	12222	0.0	0	1380828	0.0
2	T-3590	0	6147	0.0	0	558695	0.0
3	T-3540	0	4035	0.0	0	409875	0.0
4	K-1983	0	4268	0.0	0	547512	0.0
5	K-1992	0	4342	0.0	0	528609	0.0
6	K-2021	0	4367	0.0	0	539013	0.0
7	K-2079	0	4324	0.0	0	542484	0.0
8	K-2082	0	4252	0.0	0	542834	0.0
9	K-2084	0	4421	0.0	0	551389	0.0
10	HK-2029	0	4477	0.0	0	564488	0.0
11	HK-2030	0	4582	0.0	0	559955	0.0
12	LK-1688	0	4922	0.0	0	582185	0.0
13	LK-1700	0	4311	0.0	0	558582	0.0

H — Haemolyzed; L — Lipemic;
Degree of haemolysis: HK-2029: 1100 mg/dL HK-2030: 550 mg/dL

Table 8. Specificity results for Amlodipine

S.No.	Analyte- Amlodipine			ISTD - Amlodipine-d4		
	Area of interfering peak of HCTZ at RT of Amlodipine	Area observed for aqueous LOO	% interference of HCTZ at RT of Amlodipine	Area of interfering peak of HCTZ at RT of ISTD	Area observed for aqueous ISTD	% interference of HCTZ at RT of ISTD
1	0	12357	0.0	0	1497215	0.0
2	0	11399	0.0	0	1477098	0.0
3	0	11940	0.0	0	1386858	0.0
4	0	11131	0.0	0	1336551	0.0
5	0	10851	0.0	0	1341595	0.0
6	0	11321	0.0	0	1419250	0.0
	Mean	11499.8	11499.8	Mean	1409761.2	

Table 9. Selectivity results for HCTZ

S.No.	Plasma Lots	Hydrochlorothiazide			Hydrochlorothiazide 15N2 13CD2		
		Area of interfering peak at RT of HCTZ	Area observed for extracted LOQ	% interference at RT of HCTZ	Area of interfering peak at RT of ISTD	Area observed for extracted ISTD	% interference eat RT of ISTD
1	T-3213	888	31382	2.8	0	3705994	0.0
2	T-3590	0	37052	0.0	0	1210443	0.0
3	T-3540	490	31145	1.6	0	1041477	0.0
4	K-1983	0	8335	0.0	0	967683	0.0
5	K-1992	0	10117	0.0	0	1100185	0.0
6	K-2021	1010	10843	9.3	0	1182196	0.0
7	K-2079	0	10246	0.0	0	1186414	0.0
8	K-2082	0	10357	0.0	0	1177109	0.0
9	K-2084	0	10626	0.0	0	1210158	0.0
10	HK-2029	435	10755	4.0	0	1224808	0.0
11	HK-2030	0	11237	0.0	0	1263832	0.0
12	LK-1688	0	11132	0.0	0	1267089	0.0
13	LK-1700	0	10766	0.0	0	1262571	0.0

H — Haemolyzed; L — Lipemic;
Degree of hemolysis: HK-2029: 1100 mg/dL HK-2030: 550 mg/dL

Table 10. Specificity results for HCTZ

SNo.	Analyte- HCTZ			ISTD — HCTZ15N2130D2		
	Area of interfering peak of Amlodipine at RT HCTZ	Area observed for aqueous LOQ	% interference of Amlodipine at RTof HCTZ	Area of interfering peak of Amlodipine at RT of ISTD	Area observed for aqueous ISTD	% interference of Amlodipine at RT of ISTD
1	0	13123	0.0	0	1719401	0.0
2	0	13233	0.0	0	1720268	0.0
3	0	12328	0.0	0	1688743	0.0
4	0	12554	0.0	0	1683579	0.0
5	0	13097	0.0	0	1762313	0.0
6	0	12732	0.0	0	1764116	0.0
	Mean	12844.5		Mean	1723070.0	

plasma were evaluated for selectivity. No significant interference was observed at the retention time of S-Amlodipine and ISTD (S-Amlodipine-d4) and Hydrochiorothiazide and ISTD (Hydrochlorothiazide 15N2 13C D2) with respect to extracted LOQ (Table 7& 9). Specificity experiment was also evaluated by injecting six replicate injections of 50% of aqueous standard-1 of Hydrochlorothiazide at the method parameters of S-Amlodipine and ISTD (S-Amlodipine-d4) and compared the 96 interference with mean response of S-Amlodipine and (STD (S-Amlodipine-d4) and Hydrochiorothiazide and ISTD (Hydrochlorothiazide 15N2 13C D2) at aqueous LOQ concentration level. No significant interference of Hydrochlorothiazide was observed at the retention time of S-Amlodipine and ISTD (S-Amlodipine-d4) (Table 8 & 10).

Matrix effect

Amlodipine

Ten lots (K-1983, K-1992, K-2021, K-2079, K-2082, K-2084 including haemolytic (HK-2029, HK-2030) and lipemic (LT-1688. LT-1700) lots) of screened human plasma with K_2EDTA as anticoagulant were tested for the evaluation of matrix effect. Aqueous spiked samples considering 100% recovery for Amlodipine and [STD (Amlodipine-d4) and Hydrochiorothiazide and ISTD (Hydrochlorothiazide 15N2 13C D2) at LQC and HQC levels were prepared. Post extracted samples were prepared by processing two blank samples from each plasma lot as per method SOP and by reconstituting with LQC and HQC equivalent aqueous spiked

Table 11. Matrix effect results for Amlodipine

S.No.	Analyte - Amlodipine						
	Response of samples aqueous		Plasma Lot No.	Response of post extracted samples		Matrix factor	
	HOC	LQC		HQC	LQC	HOC	LQC
1	1334416	13686	HK-2029	1191819	13005	0.935	0.930
2	1216390	13519	HK-2030	1213685	13206	0.952	0.944
3	1202298	13802	K-1983	860640	11752	0.675	0.840
4	1210543	14308	K-1992	1146523	11597	0.899	0.829
5	1291840	13375	K-2021	1114720	13286	0.874	0.950
6	1226605	13291	K-2079	1258492	13875	0.987	0.992
7	1256020	14465	K-2082	1229778	13749	0.964	0.983
8	1285805	13972	K-2084	1371480	14212	1.076	1.016
9	1354650	14607	LK-1688	1242391	13252	0.974	0.948
10	1373390	14803	LK-1700	1229533	13868	0.964	0.992
Mean	1275195.7	13982.8					

Plasma Lot No.	Matrix factor- Analyte- HQC	Matrix factor- ISTD-HQC	ISTD Normalised Matrix factor-HQC	Matrix factor- Analyte- LQC	Matrix factor- ISTD-LQC	ISTD Normalised Matrix Factor- LQC
HK-2029	0.935	0.934	1.001	0.930	1.000	0.930
HK-2030	0.952	0.978	0.973	0.944	0.985	0.958
K-1983	0.675	0.689	0.980	0.840	0.894	0.940
K-1992	0.899	0.900	0.999	0.829	0.905	0.916
K-2021	0.874	0.935	0.935	0.950	0.950	1.000
K-2079	0.987	1.005	0.982	0.992	1.010	0.982
K-2082	0.964	1.009	0.955	0.983	1.033	0.952
K-2084	1.076	1.054	1.021	1.016	1.031	0.985
LK-1688	0.974	1.012	0.962	0.948	0.995	0.953
LK-1700	0.964	1.009	0.955	0.992	1.028	0.965
Mean			0.9763	Mean		0.9581
SD			0.02582	SD		0.02593

Table 12. Matrix effect results for HCTZ

S.No.	Analyte -Hydrochlorothiazide						
	Response of aqueous samples		Plasma Lot No.	Response of post extracted samples		Matrix factor	
	HQC	LQC		HQC	LQC	HQC	LQC
1	11485326	45124	HK-2029	9410317	40613	0.812	0.875
2	11311350	44989	HK-2030	9967172	41779	0.860	0.900
3	11307853	45832	K-1983	8356776	26275	0.721	0.566
4	11295022	46321	K-1992	8100526	27131	0.699	0.585
5	11765378	47137	K-2021	8059872	27069	0.696	0.583
6	11772964	47292	K-2079	8082664	29265	0.698	0.630
7	11638760	46081	K-2082	8197287	33462	0.708	0.721
8	11527314	46977	K-2084	8828083	38228	0.762	0.824
9	11774789	47468	LK-1688	10437692	41342	0.901	0.891
10	11972094	46947	LK-1700	10809399	42963	0.933	0.926
Mean	11585085.0	46416.8					

samples. Post extracted LQC and HQC samples were injected along with 10 replicate injections of aqueous spiked samples of LQC and HQC. Results were shown in Table 11 & 12.

Linearity and goodness of fit

Amlodipine & Hydrochlorothiazide

Calibration curve standards were prepared by spiking known concentration of analyte in human plasma containing K_2EDTA as anticoagulant. Nine-point calibration curve was found to be linear over a concentration range of 51.910 pg/mL to 14464.765 pg/mL and of 0.509 ng/mL to 500789 ng/mL. A linear equation was established to provide the best fit for the concentration vs. detector response using $1/x^2$ as weighting factor. The goodness of fit was consistently greater than 0.99 during the course of validation

Sensitivity

Amlodipine & Hydrochlorothiazide

Six replicate injections at LOQ level were injected to evaluate sensitivity of the method. The limit of quantification (LOQ) was 51.910 pg/mL & 0.509 ng/mL. Accuracy and precision at LOQ were 95.6%, 6.3% and 97.5% , 4.2% respectively, which were within the acceptance criteria of 80 - 120% of nominal concentration for accuracy and 20% for precision.

Precision and Accuracy

Amlodipine

The Intra day (P&A I) precision (%CV) for the low, dilution, middle and high quality control samples ranged from 2.3% to 5.9%. The inter day precision ranged from 3.2% to 6.2%, which were within the acceptance criteria of 15% (Table 19).

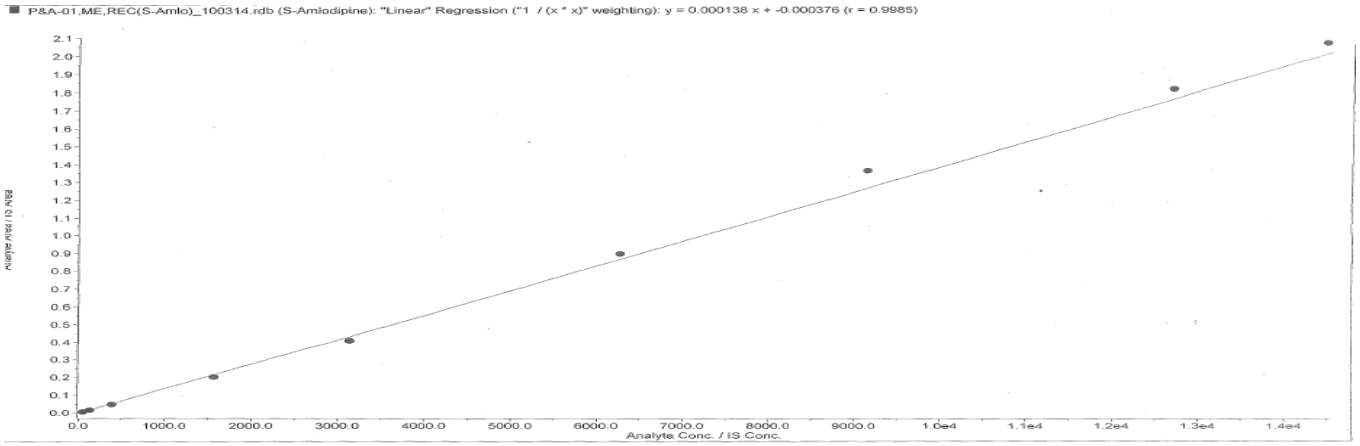


Figure 3. Calibration Curve for Amlodipine

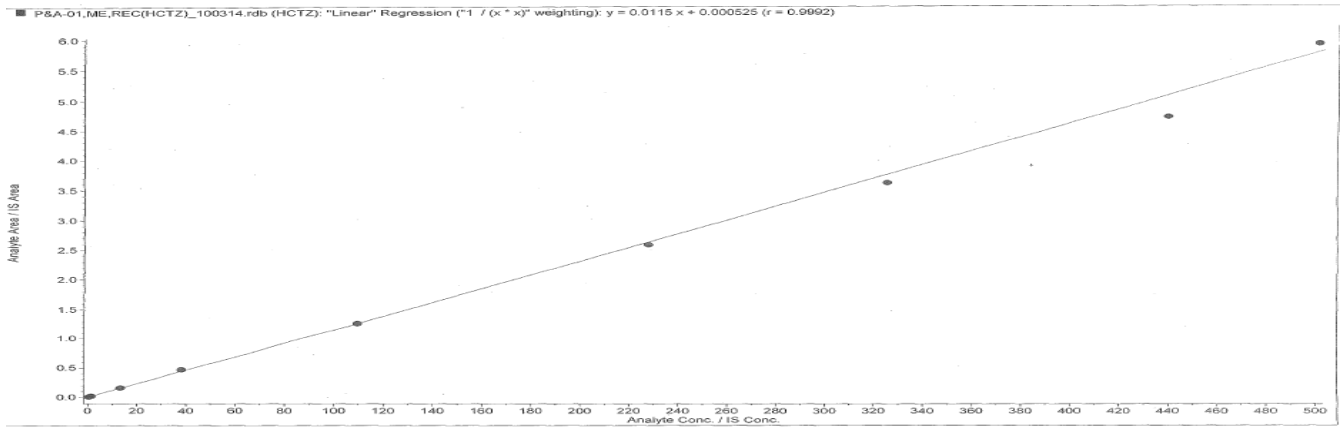


Figure 4. Calibration Curve for HCTZ

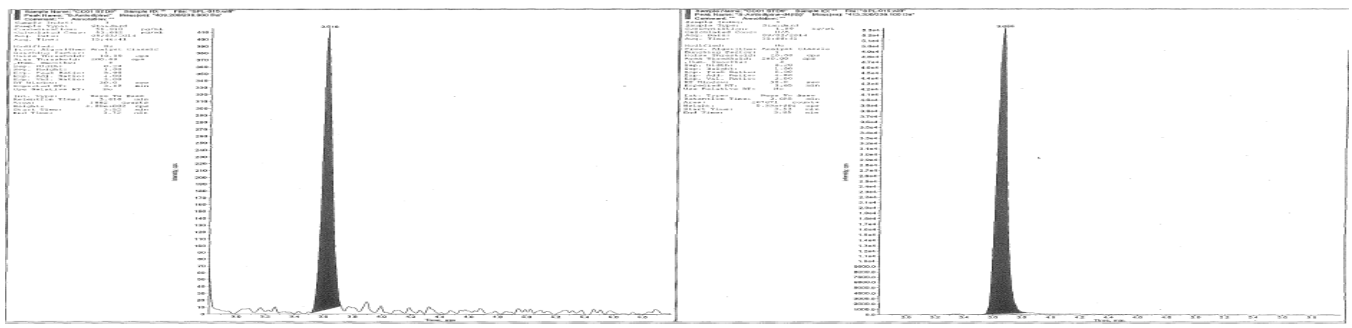


Figure 5. LOQ Chromatogram for Amlodipine

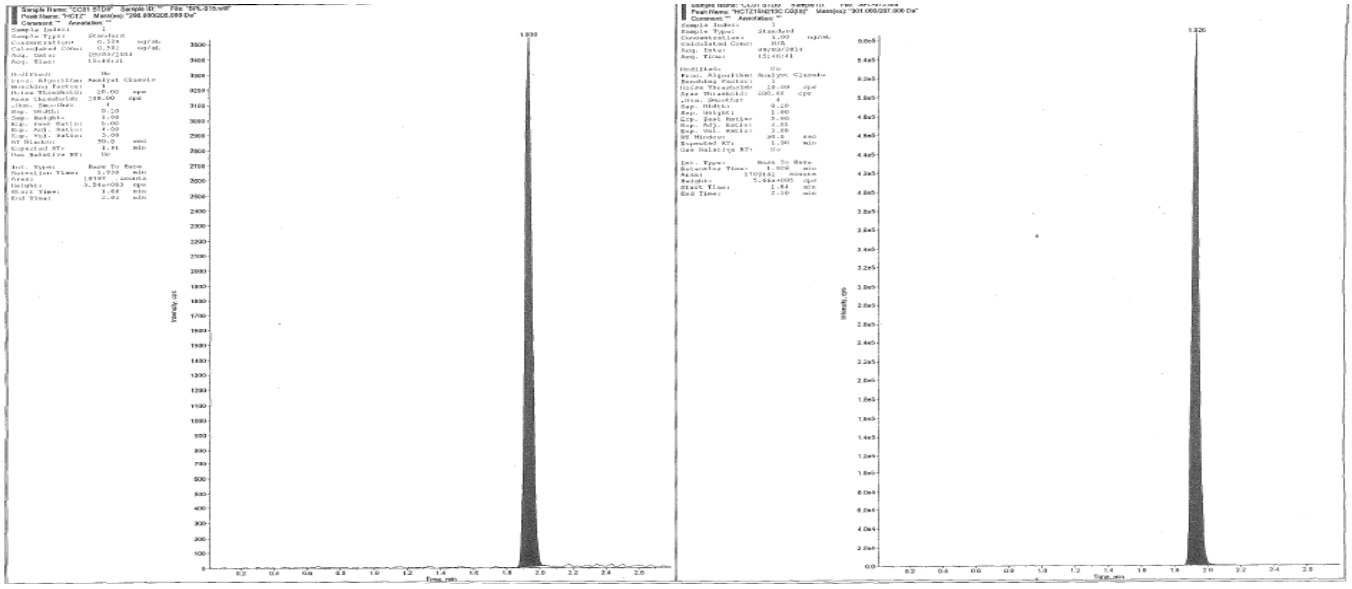


Figure 6. LOQ Chromatogram for HCTZ

Table 13. Precision and Accuracy Results of Amlodipine

Quality control samples		HQC	MQC	DQC	LQC	LOQ QC
Nominal concentration (ng/mL)		417.657	229.711	758.772	1.378	0.513
Maximum limit (ng/mL)		480.306	264.168	872.588	1.585	0.616
Minimum limit (ng/mL)		355.008	195.254	644.956	1.171	0.410
Batch ID	S.No.	Back calculated concentrations (ng/mL)				
P&A I (Intra day)	001	422.987	228.595	810.865	1.473	0.494
	002	424.082	229.678	784.041	1.444	0.514
	003	419.455	233.245	787.274	1.430	0.537
	004	429.964	230.073	790.097	1.441	0.509
	005	411.708	226.656	824.621	1.424	0.514
	006	423.154	230.132	790.281	1.450	0.482
	Mean	421.8917	229.7298	797.8632	1.4437	0.5083
	SD	6.03983	2.16217	16.16177	0.01721	0.01890
	% CV	1.4	0.9	2.0	1.2	3.7
	% Accuracy	101.0	100.0	105.2	104.8	99.1
	% Bias	1.0	0.0	5.2	4.8	-0.9
P&A II	007	435.890	231.446	788.334	1.471	0.521
	008	426.571	224.613	798.831	1.436	0.520
	009	393.735	225.850	787.037	1.425	0.480
	010	395.771	219.078	787.478	1.378	0.506
	011	412.340	222.141	805.768	1.399	0.519
	012	394.131	219.462	794.306	1.350	0.497
	Mean	40973A7	223.7650	793.6257	1.4098	0.5072
	SD	18.26909	4.63002	7.53924	0.04328	0.01636
	% CV	4.5	2.1	0.9	3.1	3.2
	% Accuracy	98.1	97.4	104.6	102.3	98.9
	°A Bias	-1.9	-2.6	4.6	2.3	-1.1
P&A III (Different column with different analyst)	013	401.179	224.677	818.203	1.367	0.458
	014	399.870	223.973	837.923	1.388	0.473
	015	395.443	228.143	780.702	1.414	0.462
	016	386.824	220.912	794.877	1.341	0.454
	017	395.978	232.463	771.270	1.369	0.492
	018	395.673	228.250	811.319	1.335	0.455
	Mean	395.8278	226.4030	802.3823	1.3690	0.4657
	SD	5.02469	4.05457	24.85011	0.02943	0.01462
	% CV	1.3	1.8	3.1	2.1	3.1
	% Accuracy	94.8	98.6	105.7	99.3	90.8
	% Bias	-5.2	-1.4	5.7	-0.7	-9.2
Global Statistics (Inter day)	Mean	409.1531	226.6326	797.9571	1.4075	0.4937
	SD	15.37473	4.33841	16.99130	0.04335	0.02576
	% CV	3.8	1.9	2.1	3.1	5.2
	% Accuracy	98.0	98.7	105.2	102.1	96.2
	% Bias	-2.0	-1.3	5.2	2.1	-3.8

Table 14. Precision and Accuracy Results of HCTZ

Quality control samples		HQC	MQC	DQC	LQC	LOQ QC
Nominal concentration (ng/mL)		417.657	229.711	758.772	1.378	0.513
Maximum limit (ng/mL)		480.306	264.168	872.588	1.585	0.616
Minimum limit (ng/mL)		355.008	195.254	644.956	1.171	0.410
Batch ID	S.No.	Back calculated concentrations (ng/mL)				
P&A I (Intra day)	001	422.987	228.595	810.865	1.473	0.494
	002	424.082	229.678	784.041	1.444	0.514
	003	419.455	233.245	787.274	1.430	0.537
	004	429.964	230.073	790.097	1.441	0.509
	005	411.708	226.656	824.621	1.424	0.514
	006	423.154	230.132	790.281	1.450	0.482
	Mean	421.8917	229.7298	797.8632	1.4437	0.5083
	SD	6.03983	2.16217	16.16177	0.01721	0.01890
	% CV	1.4	0.9	2.0	1.2	3.7
	% Accuracy	101.0	100.0	105.2	104.8	99.1
	% Bias	1.0	0.0	5.2	4.8	-0.9
P&A II	007	435.890	231.446	788.334	1.471	0.521
	008	426.571	224.613	798.831	1.436	0.520
	009	393.735	225.850	787.037	1.425	0.480
	010	395.771	219.078	787.478	1.378	0.506
	011	412.340	222.141	805.768	1.399	0.519
	012	394.131	219.462	794.306	1.350	0.497
	Mean	40973A7	223.7650	793.6257	1.4098	0.5072
	SD	18.26909	4.63002	7.53924	0.04328	0.01636
	% CV	4.5	2.1	0.9	3.1	3.2
	% Accuracy	98.1	97.4	104.6	102.3	98.9
	°A Bias	-1.9	-2.6	4.6	2.3	-1.1
P&A III (Different column with different analyst)	013	401.179	224.677	818.203	1.367	0.458
	014	399.870	223.973	837.923	1.388	0.473
	015	395.443	228.143	780.702	1.414	0.462
	016	386.824	220.912	794.877	1.341	0.454
	017	395.978	232.463	771.270	1.369	0.492
	018	395.673	228.250	811.319	1.335	0.455
	Mean	395.8278	226.4030	802.3823	1.3690	0.4657
	SD	5.02469	4.05457	24.85011	0.02943	0.01462
	% CV	1.3	1.8	3.1	2.1	3.1
	% Accuracy	94.8	98.6	105.7	99.3	90.8
	% Bias	-5.2	-1.4	5.7	-0.7	-9.2
Global Statistics (Inter day)	Mean	409.1531	226.6326	797.9571	1.4075	0.4937
	SD	15.37473	4.33841	16.99130	0.04335	0.02576
	% CV	3.8	1.9	2.1	3.1	5.2
	% Accuracy	98.0	98.7	105.2	102.1	96.2
	% Bias	-2.0	-1.3	5.2	2.1	-3.8

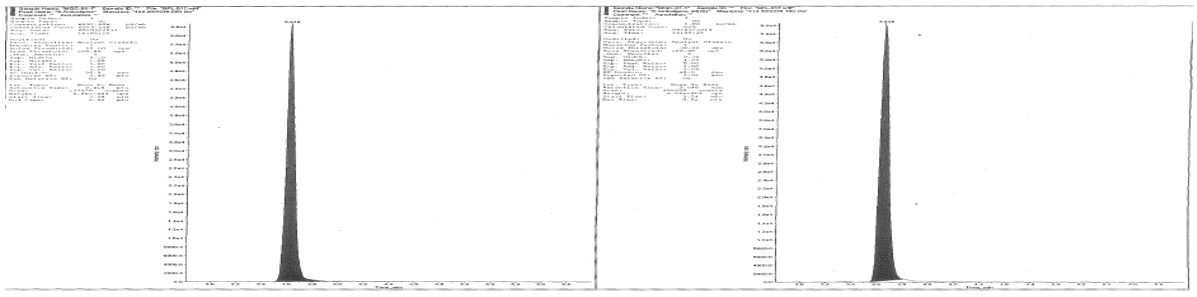


Figure 7. Recovery Representative Chromatogram for Amlodipine

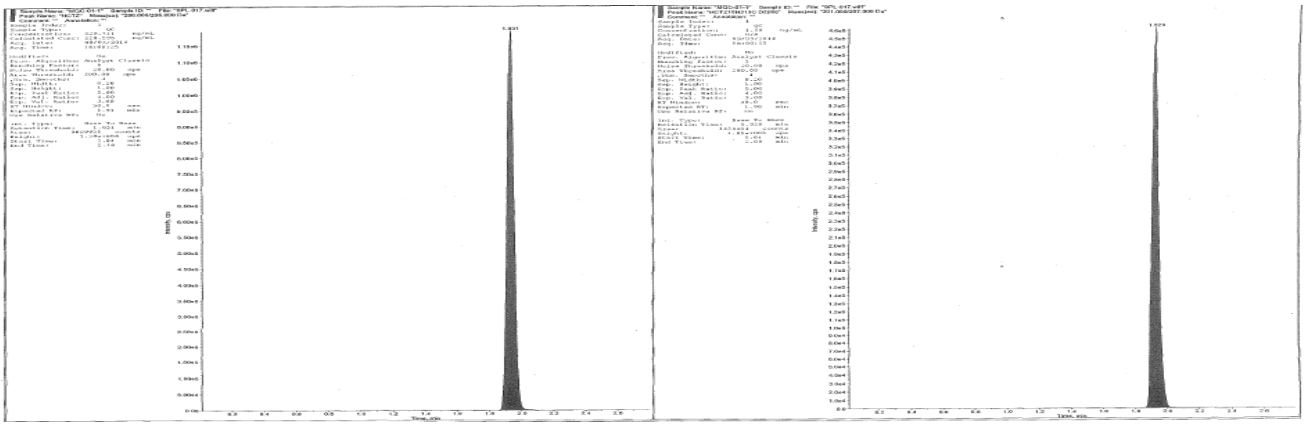


Figure 8. Recovery Representative Chromatogram for HCTZ

The Intra day (P&A I) precision (%CV) for the limit of quantification quality control samples was 7.6%. The inter day precision was 12.0%, which were within the acceptance criteria of 20% (Table 13). The Intra day (P&A I) accuracy for the low, dilution, middle and high quality control samples ranged from 98.9% to 110.7%. The Inter day accuracy ranged from 98.5% to 109.6%, which were within the acceptance criteria of 85 - 115% of nominal concentration (Table 13). The Intra day (P&A I) accuracy for the limit of quantification quality control samples was 108.9%. The Inter day accuracy was 97.4%, which were within the acceptance criteria of 80 - 120% of nominal concentration (Table 13).

Hydrochlorothiazide: The Intra day (P&A I) precision (%CV) for the low, dilution, middle and high quality control samples ranged from 0.9% to 2.0%. The inter day precision ranged from 1.9% to 3.8%, which were within the acceptance criteria of _15% (Table 14). The Intra day (P&A I) precision (%CV) for the limit of quantification quality control samples was 3.7%. The inter day precision was 5.2%, which were within the acceptance criteria of 20% (Table 14). The lntra day (P&A I) accuracy for the low, dilution, middle and high quality control samples ranged from 100.0% to 105.2%. The Inter day accuracy ranged from 98.0% to 105.2%, which were within the acceptance criteria of 85 - 115% of nominal concentration (Table 14).The Intra day (P&A I) accuracy for the limit of quantification quality control samples was 99.1%. The Inter day accuracy was 96.2%, which were within the acceptance criteria of 80 -120% of nominal concentration (Table 14).

Recovery

Amlodipine and Hydrochlorothiazide

The mean areas of extracted quality control plasma samples of S-Amlodipine were compared against the mean areas of post extracted quality control samples of HOC, MQC and LQC. The % recovery at HQC, MQC and LQC levels were 46.0%, 43.8% and 46.7% and 63.9%, 58.6% and 65.3% respectively. Global recovery for Amlodipine and Hydrochlorothiazide was 45.5% and 62.6%. The mean areas of extracted quality control plasma samples of ISTD (Amlodipine-c14) and (Hydrochlorothiazide 15N2 13C D2) were compared to the internal standard mean areas of the post extracted quality control samples. Recovery for ISTD (Amlodipine-d4) and (Hydrochlorothiazide 15N2 13C D2) is 48.8% and 82.5% which was within the acceptance criteria of 115%.

Conclusion

The validated regulaory bioanalytical method for simultaneous estimation of Amlodipine and HCTZ by LC-MS/MS in Human Plasma is useful for analysis of subject samples to support generic ANDA applications for different regulatory authorities for introducing new generic drugs in affordable rates for the patients. Also the validated regulatory method would be used as supporting literature for developing and validating better method in CROs and clinical research organisations to support new generic drugs.

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