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Development and validation of LC-MS/MS method for simultaneous determination of Azilsartan Medoxomil and Chlorthalidone from the human plasma.

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Abstract:

Background: Azilsartan Medoxomil (AZT) and Chlorthalidone (CTD) is potent combination used for the treatment of Hypertension. The combination has been proven to be beneficial than monotherapy.

Method: The present work provides with a single step protein precipitation method for simultaneous determination of AZT and CTD from human plasma by using an UFLC-MS/MS. The mobile phase chosen for the chromatographic separation was Methanol: Buffer (0.1 % Formic Acid) (65:35, %v/v). The column used was Shimapack C-8, 4.6 mm x 150 mm, 5µ. Electrospray Ionization was used as the interface for ionization, the quantitation was carried out using Multiple Reaction Monitoring (MRM) mode.

Result: The total run time was 12 minutes and Valsartan (IS) was used as an internal standard and retention time was 9.2 minutes that of CTD was 2.12 minutes and for AZT it was 9.2 minutes. Within batch, Intraday, Interday precision was found to be well below ±15% at each level. The method was found to be linear in the range of AZT (0.020 µg/mL to 4.000 µg/mL), and CTD (0.040 µg/mL to 8.000 µg/mL) with regression coefficient as 0.995 for Azilsartan and 0.993 for CTD when weighting factor of $1/x^2$ was employed. The recovery of AZT was found to be > 91% and that of CTD was found to be >93%, at all levels and for IS it was 104.91%.

Conclusion: The method was simple, reproducible and can be effectively used for determination of AZT and CTD in human plasma.

Keywords: Azilsartan Medoxomil, Chlorthalidone, protein precipitation, bioanalytical, validation, UFLC-MSMS

1. INTRODUCTION

Azilsartan Medoxomil (AM) is one of the latest entrant among the angiotensin II receptor blockers (ARBs) which was approved by the US Food and Drugs administration (FDA) in Feb 2011[1]. Further, AM in combination with Chlorthalidone (CTD) a diuretic was approved in Dec 2011[2]. AM and CTD are well tolerated by the patients and are having better clinical implication as compared to other ARB and diuretic combination[3–5].

AM is a hydrolysed to its active metabolite Azilsartan (AZT) in the gastrointestinal tract the inactive AM is rarely found in the plasma [6]. Thus it becomes essential to have a bioanalytical method which can effectively estimate the concentration of AZT in the plasma. Further, since the efficacy of the combination is better having a method for simultaneous estimation of AZT and CTD would be more beneficial.

Bioanalytical method for determination of AM on HPLC-PDA[7,8] are reported. However, the application of this method for analysis of AZT in clinical samples might be difficult. Time spent on the sample preparation is considered as a bottle neck in analysis. Manual liquid-liquid extraction (LLE) is more time consuming as compared to automated LLE or Solid Phase Extraction (SPE)[9]. Protein precipitation is a commonly used and faster technique for sample analysis in bioanalytical[10]. A simple protein precipitation UPLC-MS/MS method for determination of AZT in dog plasma is reported[11].

The literature study revealed only a single method for simultaneous estimation of AZT and CTD from rat plasma and human plasma, where the extraction method was liquid-liquid extraction [12].In the method development AZT was separately used for spiking, MS optimization and determination. However, in the Gong et. al. method AM was used for spiking in dog plasma[11]. Thus we understand that either AM or AZT can be used for spiking, MS optimization and successful determination of AZT from plasma.

Thus, in the current method we tried developing a single one step protein precipitation method for simultaneous determination of AZT and CTD from human plasma. AZT and CTD has substantial protein binding thus protein precipitation might be a better method for the sample preparation[1,2]. AM was used for spiking in the plasma and setting up the MS parameters.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

AM was obtained as a gift sample from Hetero Drugs Ltd, India and CTD was provided as a gift sample by IPCA Laboratories Ltd, Mumbai, India. Valsartan was used as an internal standard. LC-MS grade Acetonitrile and Methanol was purchased from SDFCL, Mumbai, India. Human plasma was procured from KEM blood Bank, Mumbai, India. Ultrapure water was obtained in-house using a Milli-Q PLUS PF water purifying system (Millipore, Bedford, MA). Further, other reagents and solvents were of analytical grade and purchased from standard chemical suppliers.

2.2 Liquid Chromatography and Mass spectrometry conditions

The bioanalytical method was developed on Shimadzu UFLC prominence (Shimadzu, Kyoto, Japan) equipped with Shimadzu LC-8040 mass spectrometer. The system was operated in an Electro Spray Ionization (ESI) mode. The LCMS−8040 are equipped with a LC−20 AD pump (two solvent delivery modules), a CTO−20 AC column oven, a DGU-20ASR degasser, a SIL−20 AC HT auto injector, and a CBM−20A system controller. LabSolutions® software (Shimadzu Corporation) was used for control and data processing. The injection volume was 1 µL for setting multiple reaction monitoring (MRM)

mode. The autosampler was operated at 4 °C and the autosampler needle was rinsed before and after aspiration of the sample using Methanol: Water (1:1, v/v).The MS analysis was performed by injecting 1 µg/mL directly in the MS using a mobile phase of Methanol: Buffer (0.1% Formic Acid) [4:1, v/v]. The separation was achieved on Shimadzu Shimpack C8 column, (150 mm x 4.6 mm,) maintained at 32℃. An isocratic flow consisting of Methanol: 0.1% Formic Acid in Water (3:2, v/v) as mobile phase was used at flow rate of 1 mL/min. The injection volume was 10 µL and the temperature of the autosampler was maintained at 4℃.

2.3 Preparation of stock and standard solutions

2.3.1 Preparation of AZT Stock Solution

25 mg of AZT standard was weighed and transferred to 25 mL volumetric flask. It was dissolved in HPLC grade methanol and volume was made up to produce of 1000 µg/mL concentration of AZT.

2.3.2 Preparation of CTD Stock Solution

25 mg of CTD standard was weighed and transferred to a 25-mL volumetric flask. It was dissolved in HPLC grade methanol and the volume was made up to produce 1000 µg/mL of CTD.

2.3.3 Preparation of Internal Standard (IS)

25 mg of VAL standard was weighed and transferred to a 25-mL volumetric flask. The stock dilution of VAL of concentration 5 µg/mL was prepared in methanol for preparation standard dilution.

2.4 Extraction of Plasma samples

In a 2 ml Eppendorf tube 100 µl of spiked plasma was transferred. IS 20µl was spiked and vortexed for uniform mixing of contents. To this 1 ml of Methanol was added and vortexed for 1 min. The solution obtained was centrifuged at 8000 rpm for 10 mins. 750 µl of the supernatant was collected and again centrifuged for 2 mins at 8000 rpm. 500 µl was transferred to the vial, 10 µl of which was injected for analysis.

2.5 Bioanalytical Method Validation

The method validation was performed as per the USFDA guidelines. The parameters which were analysed were sensitivity, selectivity, linearity, accuracy, precision, autosampler stability, stock solution stability, short-term stability and dilution integrity

2.5.1 Linearity:

The Linearity was established containing seven levels (non-zero standards) for AZT (0.020 µg/mL to 4.000 µg/mL), and CTD (0.040 µg/mL to 8.000 µg/mL) by plotting the response i.e. area ratio (Area of Analyte/ Area of IS) versus analyte concentration. The plot consisted of area ratio (Area of the analyte/ Area of IS) versus concentration of analyte using weighting factor $1/x^2$. Blank and Blank with IS was also analysed along with the calibration curve standards in order to confirm the absence of any interference.

2.5.2 Selectivity

Selectivity is the ability of the method to unambiguously identify the analyte from the other components. It was established by using plasma from 6 different individuals.

2.5.3 Matrix effect:

Matrix effect was determined by comparing the response of the aqueous samples with those spiked in the extracted blank.

2.5.4 Precision and Accuracy

Interbatch, Within Batch, Intraday and Inter-day precision and accuracy were determined by analyzing six replicates at four different Quality Control (QC) levels namely Low QC (LQC), Mid QC1(MQC1), Mid QC2 (MQC2) and High QC (HQC) for AZT LQC (0.100 µg/mL), MQC1 (0.270 µg/mL), MQC2 (1.500 µg/mL), and HQC (3.000 µg/mL) also for CTD LQC (0.200 µg/mL), MQC1 (0.540 µg/mL), MQC2 (3.000 µg/mL), and HQC (6.000 µg/mL).

2.5.5 Recovery

Recoveries of AZT, CTD and IS was determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. Recoveries of AZT and CTD was determined at concentration of 0.100 µg/mL, 0.270 µg/mL, 1.500 µg/mL, and 3.000 µg/mL. Whereas, that of CTD was determined at 0.200 µg/mL, 0.540 µg/mL, 3.000 µg/mL, and 6.000 µg/mL respectively, whereas for internal standards were determined at a concentration of 5 µg/mL.

2.5.6 Dilution Integrity

Was performed to extend the upper concentration limit with acceptable precision and accuracy. Six replicates each at a concentration of about 1.5 times of the uppermost calibration standard were diluted 2-and 4-fold with blank plasma. The diluted samples were processed and analysed.

2.5.7 Stability

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8 ˚C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Benchtop Stability (7h), processed samples stability (auto sampler stability for 12 hrs, freeze-thaw stability (3cycles), long-term stability (68days) were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (85–115%) and precision (15% RSD).

3. Results And Discussion

3.1 Optimization of Mass Spectrometric conditions:

All the three drugs were detected by triple- quadrupole tandem mass spectrometric detection with an ESI (Electrospray Ionization) running in positive mode. The MRM transition of m/z 457.95→279.15 was chosen for AZT, 383.00→145.95 for CLR and 436.10→235.15 for VAL with a dwell time of 100 milliseconds. AZM when used for optimization give similar transition as reported by Ramkrishna et. al [12] using AZT and that used by Gong et. al[13]. Nitrogen was employed as a desolvation gas at a flow rate of 3 L/min, the desolvation line temperature was 250 ℃, whereas the source temperature was 200 ℃, The Collision Gas (argon) flow was 0.1mL/min and capillary voltage was set at 4.0 kV. The compound specific

parameters like Q1 pre bias, collision energy and Q3 pre bias were set at -30V, -16eV and -30V for AZT, -30 V, -20 eV and -25 V for IS and -18V, -35 eV and -25 V for CLR.

3.2 Optimization of Chromatographic condition:

The mobile phase chosen for the chromatographic separation was Methanol: Buffer (0.1 % Formic Acid) (65:35, %v/v). CTD elutes at 2.2 min, IS at 9.2 min and AZT at 9.2 mins total run time was 12 mins (**Figure No. 1 and Figure No. 2**).

3.3 Sample Preparation technique and internal standard selection:

Methanol and Acetonitrile was considered for the protein precipitation. Methanol gave reproducible and consistent recovery at each QC level. Thus, methanol was chosen as an appropriate solvent for protein precipitation. Valsartan was used as an IS as it has reproducible recovery and had structural similarity to AZT.

3.4 Method Validation[14]:

3.4.1 Selectivity:

The method was found to be selective as no significant interfering peaks from any endogenous compound in blank matrix was detected at the retention time of the analytes AZT, CTD and IS was observed. No carry over effects was determined as randomly between the run blank methanol was injected.

3.4.2 Matrix effect:

The method showed no significant matrix effect at LQC or HQC for AZT or CTD. The overall mean response was within 80-120% of the nominal value. The precision at both the level was within 10%.

3.4.3 Linearity, Sensitivity:

The linearity was run in triplicate with new set of CC standards each time. The method for AZT in the range of 0.020 µg/mL to 4.000 µg/mL and for CTD in the range of 0.040 µg/mL to 8.000 µg/mL was found to be linear with the least R^2 value of 0.995 and 0.993 respectively.

3.4.3 Precision and Accuracy:

The Precision and Accuracy (PA) at Interbatch, Interday, and Intraday Within batch level is summarized in **TABLE I.** It can be concluded, the precision and accuracy was well within the ±15% at each level. (*Supplementary Table No 1-14.*)

3.4.4 Recovery:

Recovery of AZT was found to be 93.40% at LQC, 91.54 % at MQC1 and 98.23 % at MQC2 and 96.40 % at HQC. Similarly, the recovery of CTD was wound to be 95.95 % at LQC, 101.09 % at MQC1 and 93.31 % at MQC2 and 98.64 % at HQC. The recovery of IS was 104.90 %. The recovery was found to be reproducible at each level.

3.4.5 Dilution integrity:

Dilution integrity was determined by spiking 1.5 times the value of ULOQ in blank plasma. The spiked plasma was diluted two times (D2) and four times (D4). For AZT the mean was 1.656+-0.064 µg/mL with CV of 3.465% at D4 level and at D2 and mean was 3.267+-0.075 µg/mL with CV of 2.285%. .Similarly for CTD mean was found to be 2.818+-0.095 µg/mL with CV 3.376% at D2 and mean was 5.541+-0.265 µg/mL with CV 4.775% at D4. (**TABLE II**) (*Supplementary Table No 17-18.*)

Table No.1: Summary Precision and Accuracy for Azilsartan and Chlorthalidone

		Azilsartan				Chlorthalidone			
	Level	Mean	SD	%RSD	Accuracy	Mean	SD	%RSD	Accuracy
PA Batch I	LQC	0.096	0.011	11.865	95.691	0.208	0.02	9.85	103.939
	MQC1	0.264	0.026	9.89	97.933	0.56	0.036	6.36	103.69
	MQC2	1.475	0.097	6.589	98.315	3.302	0.16	4.848	110.066
	HQC	2.828	0.184	6.5	94.254	6.295	0.34	5.394	104.924
PA Batch II	LQC	0.094	0.009	10.074	93.968	0.213	0.023	10.984	106.574
	MQC1	0.295	0.014	4.893	109.406	0.518	0.013	2.52	95.883
	MQC2	1.543	0.039	2.544	102.887	3.121	0.06	1.932	104.036
	HQC	2.855	0.254	8.888	95.167	5.851	0.482	8.231	97.517
PA Batch III	LQC	0.111	0.004	3.693	110.687	0.208	0.009	4.306	104.11
	MQC1	0.295	0.019	6.446	109.385	0.563	0.041	7.244	104.241
	MQC2	1.478	0.132	8.913	98.509	3.079	0.138	4.492	102.638
	HQC	2.798	0.195	6.973	93.276	6.342	0.183	2.885	105.696
PA Batch Intraday	LQC	0.102	0.011	10.907	97.726	0.211	0.017	8.115	94.929
	MQC1	0.295	0.016	5.456	91.411	0.54	0.037	6.897	99.938
	MQC2	1.51	0.099	6.541	99.307	3.1	0.104	3.357	96.771
	HQC	2.827	0.218	7.706	106.133	6.096	0.432	7.081	98.419
PA Batch Interday	LQC	0.095	0.01	10.553	105.452	0.211	0.021	10.047	95.006
	MQC1	0.28	0.026	9.23	96.46	0.539	0.034	6.262	100.214
	MQC2	1.509	0.079	5.249	99.403	3.212	0.149	4.642	93.414
	HQC	2.841	0.212	7.452	105.585	6.073	0.46	7.576	98.794
PA Within Batch	LQC	0.1	0.011	11.339	99.885	0.21	0.018	8.453	95.352
	MQC1	0.285	0.024	8.554	94.72	0.547	0.037	6.748	98.745
	MQC2	1.499	0.097	6.468	100.096	3.167	0.155	4.904	94.715
	HQC	2.827	0.202	7.131	106.121	6.163	0.405	6.567	97.359

PA- Precision Accuracy, SD-Standard Deviation, RSD- Relative Standard Deviation

Table No.2: Dilution Integrity AZT and CTD

Azilsartan			Chlorthalidone		
Level	**D4**	**D2**	Level	**D4**	**D2**
Mean	1.656	3.267	Mean	2.818	5.541
SD	0.064	0.075	SD	0.095	0.265
%RSD	3.865	2.285	%RSD	3.376	4.775
Accuracy	110.417	108.898	Accuracy	93.923	92.347

Table No.3: Stability AZT and CTD

	Azilsartan			Chlorthalidone		
Sr. No	**Fresh**	**7 hours**	**7 days**	**Fresh**	**7 hours**	**7 days**
1	350,26,913	314,94,895	220,86,480	2661975	2610926	2710420
2	326,04,885	317,84,903	300,85,430	3029481	3607610	3557740
3	363,75,652	322,73,971	300,88,170	3385463	3476182	3693380
4	322,58,406	335,65,730	325,92,850	2914101	3571462	3919730
5	322347308	354,72,439	319,51,000	32145654	3488200	3851870
6	324913027	330,61,648	317,30,310	27854124	3652220	3901610
Mean	340,66,464	329,42,264	297,55,707	2997755	3401100	3605791.667
% Recovery		96.70	90.33		113.45	106.02
SD		149540052.21	1461897.74		14011661.85	393021.50

Table No.4: Freeze Thaw Stability AZT and CTD

Azilsartan					Chlorthalidone				
Freeze Thaw Stability					**Freeze Thaw Stability**				
Level	LQC	MQC1	MQC2	HQC	Level	LQC	MQC1	MQC2	HQC
Mean	0.094	0.269	1.574	2.765	Mean	0.195	0.537	2.947	5.413
SD	0.009	0.017	0.118	0.136	SD	0.017	0.031	0.187	0.303
%RSD	9.516	6.474	7.470	4.911	%RSD	8.825	5.836	6.348	5.599
Accuracy	93.904	99.565	104.936	92.154	Accuracy	97.529	99.515	98.231	90.213

3.4.6 Stability

3.4.6.1 Room temperature Stock Solution stability:

It was determined by comparing the response obtained for freshly prepared ULOQ with the one which was stored at room temperature for 7 hrs. The % recovery for AZT was 96.69% with CV of 5.79% and that for CTD was 113.45% with a CV of 10.02%. Thus both the drugs were found to be stable when stock solution was kept at room temperature for 7hrs. (**TABLE III**)

3.4.6.2 Refrigerated Stock solution Stability:

It was determined by comparing the response of freshly prepared ULOQ against the one which was kept in refrigerator for 7 days. The % recovery for area when compared with fresh standards was found to be 90.33 % with a CV of 4.43% for AZT. Similarly for CTD the recovery was found to be 106.02% with a CV of 11.55%.Thus, both the drugs were found to be stable when stored at 4 ˚C for 7 days. (**TABLE IV**)

3.4.6.3 Freeze thaw stability:

The spiked plasma samples containing AZT and CTD were evaluated for stability following three freeze thaw cycles. The freeze thaw samples stability was evaluated by back calculation of the concentrations of stability samples by comparing with the fresh plasma which was thawed just once. The decrease in concentration was well within the permitted ± 15% limit for Precision and Accuracy listed in **TABLE IV**.

3.4.6.4 Autosampler stability:

This was determined by processing the samples and keeping it in autosampler for 12 hrs at 5 ˚C and injecting it as per the normal procedure. The response was compared with the freshly prepared batch and injected. The variation at LQC and HQC was within 15 % and hence can be claimed to be stable for 12 hrs.

3.4.6.5 Short term and Long term storage stability:

The drugs were spiked in the blank plasma and stored at -20 ˚C and -80 ˚C degrees. The samples were processed after 14 days for short term storage and the precision and accuracy was within the permitted limit both at - 20 ˚C and - 80 ˚C degrees. Similarly, the samples of long term stability was processed on the 30th day and the precision and accuracy was within the allowed limit of ±15% for LQC, MQC1, MQC2, and HQC.

4. CONCLUSION

The one step protein precipitation was successfully developed and validated for simultaneous estimation of AZT and CTD. The method can be successfully used for simultaneous determination of AZT and CTD at clinical level. The method was found to fast, simple and robust.

Conflict Of Interest

The author declare no conflict of interest

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