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AN LC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TELMISARTAN AND HYDROCHLOROTHIAZIDE

GOPINADH VUYYALA[1*], D.RAMA SEKHARA REDDY[2]

1: Research Scholar, Dept. of Pharmaceutical Sciences, Krishna University, MachiliPatnam

2: Dept. of Chemistry Krishna University, MachiliPatnam, Andhra Pradesh

***Corresponding Author: Gopinadh Vuyyala: E Mail:gop626gnip@gmail.com**

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ABSTRACT

A new, simple, sensitive, selective, rapid, and high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for simultaneous quantification of Telmisartan and hydrochlorothiazide in human plasma. An HPLC system (Shimadzu, Kyoto, Japan) consisting of an advance C18 column, a binary LC-20AD prominence pump, an auto-sampler (SIL-HTc) and a solvent degasser (DGU-20A3) was used for the study. The separation of the analytes was carried out on an Aquasil-C18 (250×4.6mm×5µm) column. Temperature was set to 20^0C. The mobile phase composed of buffer solution, Methanol and Acetonitrile in the ratio of 60:20:20 (v/v) in isocratic condition at a flow rate of 0.5m L/min for 10min and the isocratic mobile phase comprised. The linearity ranges were found to be 40.064ng/ml - 801.272ng/ml for Telmisartan and 20.178ng/ml - 908.019ng/ml for Hydrochlorothiazide.

Keywords: Hypertension, Telmisartan, hydrochlorothiazide, LC-MS/MS, pharmacokinetics

INTRODUCTION

Telmisartan and hydrochlorothiazide are reported as the two most preferred drugs of choice for combination therapy of hypertension (Chrysant *et al*., 2004; Greathouse, 2006; Bramlage *et al*., 2013). Telmisartan medoxomil has the better antihypertensive effect when treatment is combined with diuretics (Zhang *et al*., 2017). A number of methods have been

reported for analysis of TLM and HCTZ simultaneously, separately or in combination with other drugs in pharmaceutical dosage forms which involves various techniques—namely, spectrophotometer (Wankhede *et al*., 2009; Rote and Bari, 2010), HPLC (Wankhede *et al*., 2009; Kamble *et al*., 2010; Rao *et al*., 2011; and Doshi *et al*., 2012), and HPTLC (Shah *et al*., 2007; Kamble *et al*., 2010), but these methods cannot be applied to the clinical pharmacokinetic studies. A few LC-MS/MS methods are reported for quantitation of TLM and HCTZ, separately or in combination with other drugs (Yu *et al*., 2006; Goswami *et al*., 2008; Vaidya *et al*., 2008; Rajasekhar *et al*., 2009; Shah *et al*., 2009; Tutunji *et al*., 2009; Gao *et al*., 2010; Sengupta *et al*., 2010; Tutunji *et al*., 2010; Bharathi *et al*., 2012; Gadepalli *et al*., 2014), but these are not suitable for the simultaneous quantitation of TLM and HCTZ. However, few methods are also reported for the simultaneous estimation of TLM and HCTZ in human plasma by LC-MS/MS (Liu *et al*., 2010; Kumar *et al*., 2012; Kumar *et al*., 2014; Sable *et al*., 2016), but all of them have applied the solid-phase extraction (SPE) method for drug extraction from plasma. The SPE method is quite tedious and costly due to the inevitable use of SPE cartridges. This results in a relatively long extraction time and increases the burden on the laboratory budget. Therefore, this method has been rendered tedious and time-consuming especially for those clinical studies with a considerable sample size and could not turn into the method of choice for pharmacokinetic analysis in clinical studies.

MATERIALS AND METHODS

Instrumentation

An HPLC system (Shimadzu, Kyoto, Japan) consisting of an advance C18 column, a binary LC-20AD prominence pump, an auto-sampler (SIL-HTc) and a solvent degasser (DGU-20A3) was used for the study. Aliquots of the processed samples (20 ml) were injected into the column, which was kept at 30 1 C. The isocratic mobile phase was delivered into the electro-spray ionization chamber of the mass spectrometer. Quantitation was achieved with MS–MS detection in positive ion mode for both the analytes using a MDS Sciex API-4000 mass spectrometer equipped with a Turbo ion spray TMinterface at 500 1 C. The ion spray voltage was set at 5500 V. The source parameters, viz.the nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 45, 20, 45 and 10 psi, respectively. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM)

Chemicals and standard drugs:

The working standard drug Telmisartan having a purity of 99.36% and Hydrochlorothiazide with 98.91% pure were kindly provided by Medley Pharmaceuticals Ltd, Mumbai; Maharashtra, India. All the chemicals used were of laboratory reagent grade and were purchased from Merck chemicals private limited, Mumbai; Maharashtra, India.

Preparation of aqueous calibration curve dilutions for Hydrochlorothiazide:

From the standard stock solution of Hydrochlorothiazide, pre-calculated dilutions were made accurately and a working standard stock solution concentration of 504.455ng/ml was prepared.

Plasma spiked calibration curve for Telmisartan:

The prepared aqueous dilutions were used to spike the screened blank human plasma matrix to prepare plasma calibration curve standards. The plasma spiked calibration curve was prepared with in the concentration range of 801.272ng/ml-40.064ng/ml.

Plasma spiked calibration curve for Hydrochlorothiazide:

The prepared aqueous dilutions were used to spike the screened blank human plasma matrix to prepare plasma calibration curve standards. The plasma spiked calibration curve was prepared with in the concentration range of 908.019ng/ml-20.178ng/ml.

Extraction of drugs from plasma:

Prior to sample analysis, 100µL of each solution was extracted using 300µL of diethyl ether: dichloromethane (60:40% v/v) for protein precipitation. Further, each of the mixtures was vortex for a period of 5 min in a vortex mixer with subsequent centrifugation at 10000 rpm, for a period of 10 min at 4°C using a centrifuge. For each sample, an aliquot of a supernatant was isolated and subjected to dryness. The residue was reconstituted in 100µL of mobile phase and subsequently centrifuged at 10000 rpm for10 min at 4°C in a centrifuge. The supernatant was finally collected and directly injected for analysis. This procedure was followed for all samples of calibration curve plasma spiked dilutions and plasma spiked samples.

Method Development:

The separation of the analytes was carried out on an Aquasil-C18 (250×4.6mm×5µm) column. Temperature was set to 20^0C. The mobile phase composed of buffer solution, Methanol and Acetonitrile in the ratio of 60:20:20 (v/v) in isocratic condition at a flow rate of 0.5m L/min for 10min and the isocratic mobile phase comprised. The full scan MS and M S/M S spectra of each analyte were obtained by direct infusion of the respective sample solution at a

concentration of 801.272ng/ml of Telmisartan and 908.019ng/ml of hydrochlorothiazide solution prepared in the mobile phase. The flow rates of sheath gas and auxiliary gas were optimized and set to 30 psi and 5 psi, respectively. The needle spray voltage was set to 4.5 k V. Protonated form of each analyte ion was the parent ion in the Q 1 spectrum and was used as the precursor ion to obtain Q 3 product ion spectra. The most sensitive mass transition was monitored from m/z 298.12→204.36 (Q1/Q3) for hydrochlorothiazide and m/z of 513.56→469.19(Q1/Q3) for Telmisartan.

At the optimized conditions, the standard drug Telmisartan elute at a retention time of 4.39min and Hydrochlorothiazide elutes at 5.73min. The separation was found to be accurate and symmetric peaks with high resolution was observed.

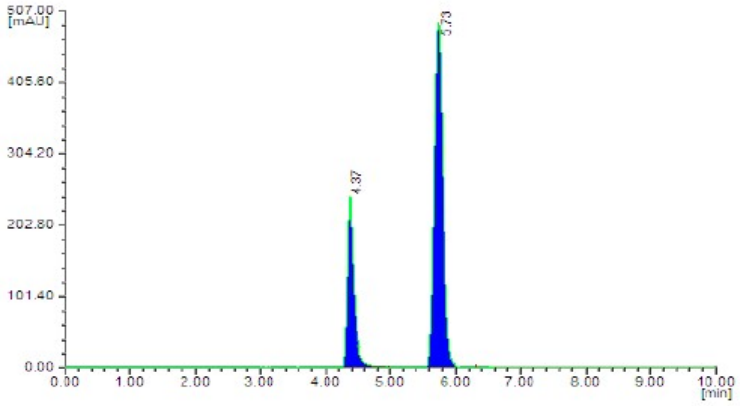

Figure 1: Standard LC chromatogram of Telmisartan and Hydrochlorothiazide

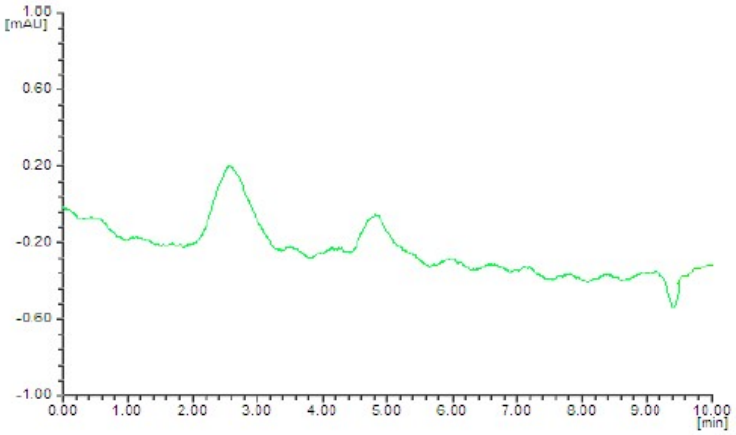

Figure 2: Blank chromatogram of Telmisartan and Hydrochlorothiazide

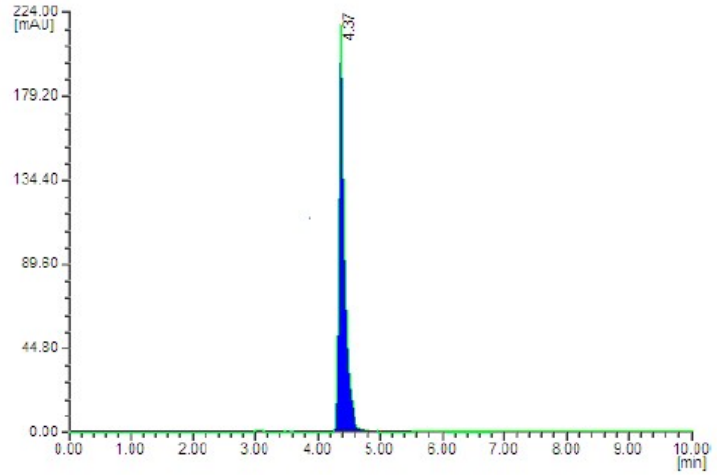

Figure 3: Standard LC chromatogram of Telmisartan

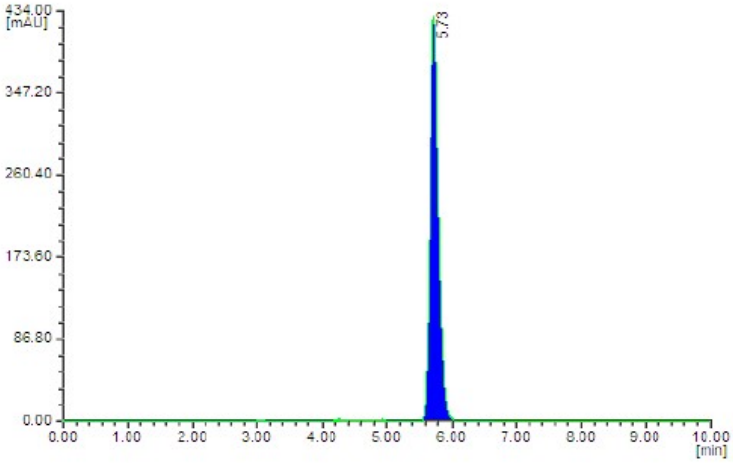

Figure 4: Standard LC chromatogram of Telmisartan

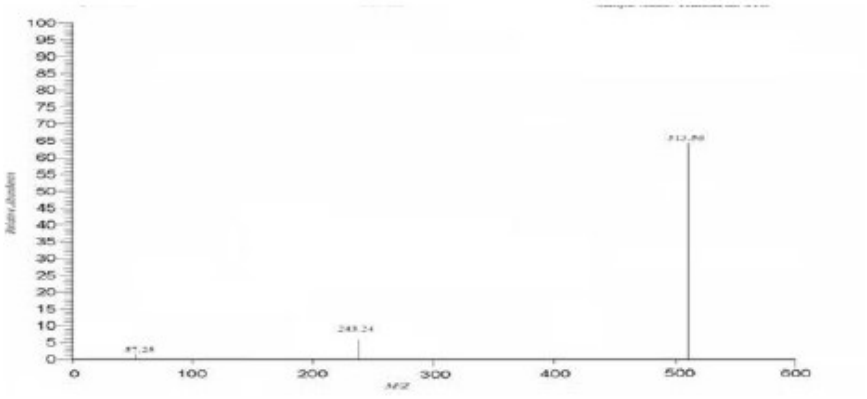

Figure 5: Mass spectrum of Telmisartan

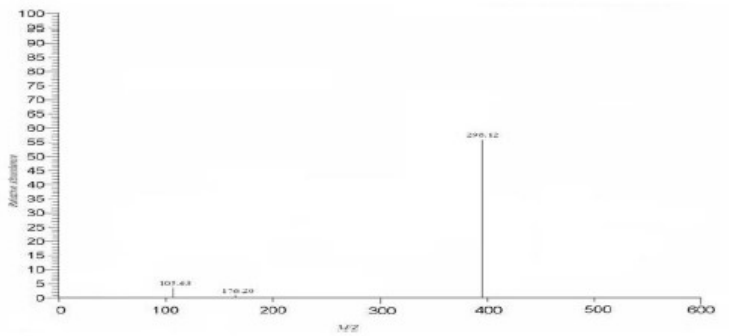

Figure 6: Mass spectrum of Hydrochlorothiazide

Method Validation

A thorough validation of the method was carried out as per the US FDA guidelines. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability.

Selectivity:

Selectivity of the method was assessed by analyzing six blank human plasma matrix samples. The responses of the interfering substances or background noises at the retention time of the Telmisartan and hydrochlorothiazide acceptable if they are less than 20% of the response of the lowest standard curve point or LLOQ. There is no remarkable noise was observed at the retention time of Telmisartan and hydrochlorothiazide and hence the proposed method was selective for the standard drugs Telmisartan and hydrochlorothiazide only and hence the method selective.

Linearity:

The plasma spiked calibration curve dilution for both the drugs were prepared. The prepared plasma spiked dilutions were used for the determination of the linearity of the method. Linearity was tested for Telmisartan and Hydrochlorothiazide in the concentration range of 40.064ng/ml-801.272ng/ml for Telmisartan and 20.178ng/ml- 908.019ng/ml for Hydrochlorothiazide in the method.The results were given in **Table 1**.

Precision and accuracy

Intra-day assay precision and accuracy were determined by analyzing six replicates at four different QC levels in the same day on two runs. Precision was carried out at HQC, MQC, LQC and LLOQC for both the drugs in calibration curve range. Detector response at the retention time of both the drugs in each level was determined and the %CV of the response was calculated. The acceptance criteria included accuracy within ±15% deviation (SD) from the nominal values, except LLOQ QC, where it should be ±20% and a precision of $\leq$15% relative standard deviation (RSD), except for LLOQ QC, where it should be ±20%. Whereas batch acceptance criteria included

67% for overall quality control samples and 50% at each level respectively. The results confirmed that the method was found to be precise and accurate. Results were given in **Table 2, 3**.

Recovery:

Recovery of the analytes from the extraction procedure was determined by comparing the peak areas of the analytes inspiked plasma samples (six each of HQC, MQC, and HQC samples) with those of the analytes in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of the analytes at the step immediately prior to chromatography **(Table 4)**.

The results of the recovery conforms that the % recovery was found to be 3.27 for Telmisartan and 6.011 for Hydrochlorothiazide in three levels. The results of the recovery were given in **Table 4 and 5** for Telmisartan and Hydrochlorothiazide respectively.

Table 1: Plasma spiked calibration curve

S.NO	Telmisartan		Hydrochlorothiazide		Sample vial code
	Concentration	Area at the retention	Concentration	Area at the retention time	
1	40.064ng/ml	88251	20.178ng/ml	42814	PSCC 001
2	70.111ng/ml	97123	50.445ng/ml	63817	PSCC 002
3	90.143ng/ml	141935	70.624ng/ml	93871	PSCC 003
4	170.27ng/ml	187936	100.891ng/ml	115827	PSCC 004
5	350.557ng/ml	360369	403.564ng/ml	298710	PSCC 005
6	500.795ng/ml	478251	605.346ng/ml	436107	PSCC 006
7	801.272ng/ml	712258	908.019ng/ml	652814	PSCC 007
	Slope Intercept r^2	831.27 54897 0.9976	Slope Intercept r^2	671.61 36274 0.9984	

Table 2: Precision at HQC and LQC levels

Precision at HQC					
S.NO	Sample ID	Telmisartan		Hydrochlorothiazide	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at HQC	PA001	651758	906.55	651758	906.55
	PA002	651963	906.835	651963	906.835
	PA003	653911	909.545	653911	909.545
	PA004	652459	907.525	652459	907.525
	PA005	652988	908.261	652988	908.261
	PA006	651790	906.595	651790	906.595
Nominal Conc.		801.272ng/ml		908.019ng/ml	
N		6		6	
Average		712193	1.46381	845.094	1.17547
SD		1203.06	800.956	652478	907.552
%CV		0.169	0.18276	0.12952	0.12952
Accuracy (%)		99.961		99.949	
Precision at MQC					

S.NO	Sample ID	Telmisartan		Hydrochlorothiazide	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at MQC	PA007	184722	98.2898	115028	99.3102
	PA008	185936	98.9358	114693	99.021
	PA009	182299	97.0006	114239	98.629
	PA010	185258	98.575	114069	98.4822
	PA011	185563	98.7373	116025	100.171
	PA012	186936	99.4679	115025	99.3076
Nominal Conc.		170.27ng/ml		100.891ng/ml	
N		6		6	
Average		1568.15	0.83441	700.113	0.60445
SD		185119	98.5011	114847	99.1535
%CV		0.8471	0.8471	0.60961	0.60961
Accuracy (%)		98.5011		99.1535	

Table 3: Precision at LQC and LLOQC levels

Precision at LQC					
S.NO	Sample ID	Telmisartan		Telmisartan	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at LQC	PA013	97021	99.895	63021	98.7527
	PA014	96578	99.439	63229	99.0786
	PA015	97367	100.251	63182	99.005
	PA016	97442	100.328	63589	99.6427
	PA017	96987	99.860	62950	98.6414
	PA018	96875	99.745	62597	98.0883
Nominal Conc.		70.111ng/ml		50.445ng/ml	
N		6		6	
Average		320.069	0.32931	329.868	0.5169
SD		97045	99.9197	63094.7	98.8681
%CV		0.330	0.330	0.52281	0.52281
Accuracy (%)		99.9197		98.8681	
Precision at LQC					
S.NO	Sample ID	Telmisartan		Telmisartan	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at LLOQC	PA019	88012	99.729	42142	98.4304
	PA020	87896	99.598	42969	100.362
	PA021	87936	99.643	42551	99.3857
	PA022	88693	100.501	42367	98.9559
	PA023	88581	100.374	42914	100.234
	PA024	87158	98.761	42636	99.5842
Nominal Conc.		40.064ng/ml		20.178ng/ml	
N		6		6	
Average		553.016	0.6268	316.996	0.7404
SD		88046	99.768	42596.5	99.492
%CV		0.628	0.628	0.744	0.744
Accuracy (%)		99.768		99.492	

Table 4: Recovery of Telmisartan

S. No.	Recovery at HQC level				Recovery at MQC level				Recovery at LQC level			
	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery
1	736925	908211	650.154	81.1403	382863	441893	303.728	86.642	90176	105863	59.722	85.182
2	729963	909917	642.805	80.223	381190	459631	290.731	82.934	91589	106397	60.353	86.082
3	731580	905583	647.312	80.7855	380326	432816	308.043	87.872	93691	118580	55.395	79.011
4	739281	914792	647.541	80.8141	385601	445021	303.750	86.648	93902	107951	60.987	86.986
5	728284	909882	641.351	80.0416	376920	446179	296.141	84.477	93698	105821	62.079	88.544
6	731417	904861	647.684	80.832	382047	446377	300.036	85.588	90581	107486	59.084	84.272
SD	4261	3591	3.3435	0.417	2878.38	8658	6.204	1.770	1698.42	4925	2.306	3.290
Mean	732908	908874	646.141	80.64	381491	445319	300.405	85.693	92272.8	108683	59.603	85.013
CV	0.581	0.395	0.517	0.517	0.75451	1.9443	2.065	2.065	1.841	4.533	3.870	3.869
Standard Deviation					2.74313							
Average recovery of three levels					83.782							
% Recovery					3.27413							

Table 5: Recovery of Hydrochlorothiazide

S. NO	Recovery at HQC level				Recovery at MQC level				Recovery at LQC level			
	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery
1	682239	849631	729.124	80.2983	275819	315896	352.365	87.3132	64281	85861	37.7663	74.8664
2	679228	845822	729.175	80.3039	286932	319671	362.233	89.7585	61820	82661	37.7265	74.7874
3	671852	841028	725.368	79.8846	265102	328476	325.703	80.7067	60528	86394	35.342	70.0604
4	642811	842836	692.525	76.2676	295017	339178	351.02	86.98	65936	84105	39.5475	78.3972
5	637492	849928	681.063	75.0054	259670	329541	317.998	78.7975	62820	80281	39.4733	78.2501
6	637028	842014	686.964	75.6553	279954	330208	342.146	84.7811	64559	84028	38.7571	76.8303
SD	21540.5	3886	22.81	2.512	13230.5	8295	16.970	4.205	1979.76	2225.1	1.567	3.105
Mean	658442	845210	707.37	77.902	277082	327162	341.911	84.723	63324	83888	38.102	75.532
C V	3.271	0.460	3.224	3.224	4.77493	2.536	4.963	4.963	3.126	2.652	4.111	4.111
Standard Deviation					4.771							
Average recovery of three levels					79.386							
% Recovery					6.011							

DISCUSSION

The isocratic mobile phase, a mixture of Acetate buffer (pH 4.4), Methanol and Acetonitrile in the ratio of 60:20:20 (v/v) acetonitrile and 5 mM ammonium acetate (pH- 4.0) (50:50, v/v) was delivered at 0.5mL/min into the electrospray ionization chamber of the mass spectrometer. Quantitation was achieved with MS–MS detection in positive ion mode for both the analytes using a MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray™ interface at 500 °C. The ion spray voltage was set at 5500 V. The source parameters, viz. the nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 45, 20, 45 and 10 psi, respectively. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM). At the optimized conditions, the standard drug Telmisartan elute at a retention time of 4.39min and Hydrochlorothiazide elutes at 5.73min. The separation was found to be accurate and symmetric peaks with high resolution was observed. Recovery comparison sample at LQC, MQC and HQC concentration level along are prepared and screened. The results confirmed that no significant matrix effect was observed for the method developed in the study.

The calibration curves were plotted between response factor and concentration of the standard solutions against concentration of the analyte. The linearity ranges were found to be 40.064ng/ml - 801.272ng/ml for Telmisartan and 20.178ng/ml - 908.019ng/ml for Hydrochlorothiazide. The calibration curves were constructed on 11 different days over a period of four weeks to determine the variability of the slopes and intercepts. The results indicated no significant interday variability of slopes and intercepts over the optimized concentration range. A high correlation of 0.997 (y = 831.2x + 54897) and 0.998 (y = 671.6x + 36274) for was observed for Telmisartan and Hydrochlorothiazide respectively. Figure 1 and 2 shows the plasma spiked calibration curves obtained in the developed method. Table 1 shows the results the plasma spiked calibration curve results for Telmisartan and Hydrochlorothiazide. High % stabilities were obtained for different stability studies like short term, long term, bench top, freeze thaw and auto- injector stabilities. This confirms that the method was stable.

CONCLUSION

A method using LC-MS/MS for the determination of Telmisartan and Hydrochlorothiazide in plasma employing simple liquid-liquid extraction was developed. The method is rapid, simple, specific and sensitive, and additionally demonstrates good accuracy and precision.

Compared with the available methods, the present method features high selectivity and sensitivity. We believe that this high through put method could provide a useful tool for the determination of Telmisartan and Hydrochlorothiazide in plasma. The established method was successfully applied to a pharmacokinetic study and to assess the plasma concentration.

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