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Research Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF PRAZOSIN AND POLYTHIAZIDE DRUGS IN SPIKED HUMAN PLASMA BY RP-HPLC

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ABSTRACT

A simple, novel, sensitive, rapid, precise and accurate high performance liquid chromatography method has been developed and validated for simultaneous determination of Prazosin and Polythiazide in human plasma using Hydrochlorothiazide as internal standard (ISTD).The analytes were extracted from 500 µl aliquots of human plasma sample by direct protein precipitation technique using acetonitrile. Evaluation of content of the drugs were done by employing a mixture of acetonitrile and potassium dihydrogen orthophosphate buffer in ratio of 35: 65 v/v as the mobile phase with a flow rate of 1 ml/min and injection volume of 10µl. Chromatographic separation was accomplished using Zorbax C18, (150×4.6 mm; 5µm) analytical column and the effluents were monitored at 265 nm with PDA detector. The total run time was 8 min with retention time of Prazosin, Polythiazide and Hydrochlorothiazide was 6.598 min, 5.214 min and 3.579 min respectively. Linearity was established at a concentration range of 5.0-500 ng/ml for Prazosin and 2.5-250 ng/ml for Polythiazide. The method was validated as per the US-FDA guidelines and the results were within the acceptance criteria and proposed method was successfully applied for the simultaneous determination of Prazosin and Polythiazide in human plasma.

Keywords: Prazosin, Polythiazide, Protein precipitation, Human plasma, RP-HPLC.

INTRODUCTION

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites are critical for the successful evaluation of preclinical, biopharmaceutical and clinical pharmacological studies. Bioanalytical method validation includes all of the procedures which demonstrate that a particular method used for the quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine. These methods are reliable and reproducible[1].

Prazosin is a quinazoline derivative, is the first of that chemical class of antihypertensive. Chemically it is designated as 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furoyl) piperazine and its structural formula is shown in Fig. 1. Prazosin is a sympatholytic alpha-adrenergic blocker used in the treatment of anxiety, hypertension, refractory pulmonary oedema and panic disorders. It reduces peripheral resistance and blood pressure by vasodilatation of peripheral vessel in arterioles and veins without increasing the heart rate or significantly impairing sympathetic function[2-5]. It is official in Indian pharmacopoeia[6], British pharmacopoeia[7], United States Pharmacopoeia[8].

Polythiazide is an orally effective benzothiadiazine sulfonamide derivative belonging to the class of the thaiazide diuretics. Chemically it is designated as 2*H*-1,2,4-Benzothiadiazine-7-sulfonamide,6-chloro-3,4-dihydro-2-methyl-3-[[(2,2,2-trifluoroethyl) thio]methyl]-1,1dioxide and its structural

formula is shown in Fig. 2. Its mechanism of action results in an interference with the renal tubular mechanism of electrolyte reabsorption[9-11].

Hydrochlorothiazide is a diuretic and antihypertensive. Chemically it is designated as 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiazine-7-sulfonamide1,1-dioxide. It structural formula is shown in Fig. 3. It acts by inhibiting the kidney's ability to retain water. This reduces the volume of the blood, decreasing blood return to the heart and thus the cardiac output, is believed to lower peripheral vascular resistance [12-13].

Prazosin and Polythiazide combination is used in the treatment of high blood pressure (hypertension). Prazosin works by relaxing blood vessels so that blood passes through them more easily. The Polythiazide in this combination is a thaiazide diuretic (water pill) that helps to reduce the amount of water in the body by increasing the flow of urine. Both of these actions help to lower blood pressure [14].

Literature survey revealed that few analytical methods have been reported for estimation of Prazosin and Polythiazide individually or in combination with other drugs. The reported methods include Spectrophotometric[15-19], HPLC[20-26], Bioanalytical HPLC[27-30], simultaneous estimation of Prazosin and Polythiazide in combined pharmaceutical formulations by RP-HPLC[31-34]. There are no reports as per our knowledge that methods developed for the analysis of these two drugs in combination in blood plasma. The present study was aimed to develop a simple, sensitive, rapid, precise, accurate and validated the bioanalytical method for the simultaneous estimation of Prazosin and Polythiazide in human plasma. The developed method was validated according to US-FDA guidelines by using high performance liquid chromatography[35].

MATERIAL AND METHODS

Chemicals and reagents

Blank human plasma and pure drug samples including Prazosin, Polythiazide and Hydrochlorothiazide were obtained from Spectrum Pharma Research Solutions, Hyderabad, India. HPLC grade Acetonitrile was obtained from Merck Chemical Division, Mumbai, India. Analytical grade of Potassium dihydrogen orthophosphate and orthophosporic acid was obtained from SD Fine Chemicals Ltd., Mumbai, India. The double distillation and purification with Milli-Q water purification system of purified water helped to prepare HPLC grade water.

Instrumentation

The analysis was performed by using Waters 2695 series HPLC comprised of vacuum degas, auto injector, and dual gradient pump with photo diode array detector and Empower 2 software.

Chromatographic Conditions

The drug samples were analysed with Zorbax C18, (150 x 4.6 mm, 5 µm) column as stationary phase and was maintained at 30^0 C. The mobile phase used was a mixture of potassium dihydrogen orthophosphate buffer and acetonitrile in the ratio 65:35 v/v. The flow rate of the mobile was 1.0 ml/min and sample injection volume 10 µl. The detection of the effluents was carried out at 265 nm with PDA detector. Samples of Prazosin and Polythiazide were prepared using water and acetonitrile diluents in 50:50 ratios.

Preparation of mobile phase

Preparation of buffer was accomplished by transferring 1.36 g of potassium dihydrogen orthophosphate in about 1000 ml reagent bottle and made up the volume to 1 litre with HPLC water and pH was adjusted to 3.0 (± 0.5) with dilute orthophosporic acid. The buffer was filtered through 0.2 µ filter and sonicated for 5 min. Mobile phase was prepared from 650 ml of 0.01 M potassium dihydrogen orthophosphate buffer (pH 3 ± 0.5) and 350 ml of HPLC grade acetonitrile mixed well and sonicated for 5 min.

Preparation of stock and working solutions of analytes and internal standard

Primary stock solutions of Prazosin, Polythiazide and Internal standard (Hydrochlorothiazide) were prepared individually by dissolving accurately weighed compounds in acetonitrile-water (50:50, v/v) to give a concentration of 0.1 mg/ml, 0.2 mg/ml and 1.0 mg/ml, respectively. Working solutions of analytes over the desired concentration range were prepared by further dilution of these stock solutions with acetonitrile-water (50:50, v/v). A combined internal standard working solution was prepared in same diluent for analysis at a concentration 450 ng/ml. All stock and working solutions were stored at –20°C. Polypropylene vials were used to store the solutions at - 20°C and brought to room temperature before use.

Preparation of calibration standards and quality control samples

The calibration standards and quality control samples were prepared by spiking bank human plasma with suitable volume of respective working solutions of analytes to achieve

concentrations of 5-500 ng/ml for Prazosin, 2.5-250 ng/ml for Polythiazide. In the same way quality control (QC) samples were prepared at five levels, 500.0/250.0 ng/ml (ULOQ, upper lower limit of quantification quality control) 400.0/200.0 ng/ml (HQC, high quality control), 300.0/150.0 ng/ml (MQC-2, medium quality control), 250.0/125 ng/ml (MQC-1, medium quality control), 100.0/50.0 ng/ml (LQC-2, low quality control), 15.0/7.5 ng/ml (LQC-1, low quality control), 5.0/2.5 ng/ml (LLOQ-QC, lower limit of quantification quality control), for Prazosin and Polythiazide, respectively.

Sample preparation

An aliquot of 500 µl of human plasma sample was spiked with 450 µl of internal standard, 0.05 µl of Prazosin and 0.05 µl of Polythiazide working standard solutions into 10 ml centrifuge tube then,1000 µl of acetonitrile was added to precipitate the protein by vortex mixing for 2 min. The plasma sample was subjected to centrifugation at 3200 rpm for 5 min. After centrifugation, 10 µl of the supernatant layer was collected and injected into the chromatographic system for the analysis.

Method validation

The analytical method was validated for the fundamental validation parameters following the US-FDA guidelines for the bioanalytical method validation[35].

System suitability test

The system suitability test was performed before analysis of every batch of sample to ensure the reproducibility of the chromatographic system. The HPLC system suitability test was performed by running six injections of diluted drugs and ISTD in the linear region of the calibration curve and measuring the percentage coefficient of variance (% CV).

Specificity and Selectivity

The specificity was verified by analysing six different batches of blank human plasma so as to ensure that no endogenous substances interfere with analytes and internal standard existed in plasma. The selectivity of the method was demonstrated by comparing chromatograms obtained from blank plasma and spiked plasma.

Matrix effect

Matrix effect was assessed with six different lots of chromatographically screened plasma sample. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (total thirty six quality control samples).

Calibration and linearity

The linearity of the method was established from the standard calibration curve constructed at eight concentrations (non-zero standards) ranges from 5-500 ng/ml and 2.5-250 ng/ml for Prazosin and Polythiazide respectively. The peak area of each concentration was recorded and then plotted against the corresponding concentration to obtain the calibration graph. In addition, a blank and zero samples were prepared to conform the absence of direct interferences. The working standards were prepared by adding different concentrations of Prazosin and Polythiazide fixed concentration of internal standard (450 ng/ml) solutions spiked in plasma to obtain the required concentration range. Samples were extracted and injected into the HPLC system. The drug/IS peak area ratio was plotted against the concentration of the drug and expressed in terms of coefficient of variance (CV).

Sensitivity (LLOQ)

The sensitivity of the method was determined by analysing six replicates of plasma samples spiked with the lowest level of the calibration curve concentrations (LLOQ-QC, lower limit of quantification quality control) of Prazosin and Polythiazide, respectively.

Precision and accuracy

Intra-day, inter-day precision, and accuracy were determined by analysing six replicates at four different quality control levels (LLOQ, LQC, MQC and HQC) on three different days. Concentrations covering the low, medium and higher range of the calibrations curve. Intra-day variation of the assay was done by injecting six samples for each concentration on the same day. Inter-day variation was assessed by injecting six sample of each concentration over a period of three days. The precision of the method is expressed in terms of percentage coefficient of variance (% CV) and accuracy was expressed as a percentage of the theoretical concentration (observed concentration / theoretical concentration × 100)[36].

Recovery

The recovery of each analyte and internal standard was determined by analysing six replicates of quality control samples at three different concentration levels (LQC, MQC and HQC) and was calculated as the ratio of peak areas obtained from extracted spiked samples to that of non-extracted standard at corresponding concentrations.

Stability

Stability studies were performed as zero hours, long term at -28ºC and long term at -80ºC. Day zero having two samples with six replicates of HQC and LQC levels. LT at -28ºC and LT at -80ºC have HQC and LQC level with % Stability finding by comparison sample and stability sample.

RESULTS AND DISCUSSION

In the present study, Acetonitrile was the solvent of choice, in order to obtain satisfactory values for recovery of Prazosin and Polythiazide which showed good resolutions with no interferences peak. Hence, extraction with Acetonitrile was optimized as the sample treatment procedure[37]. The mobile phase was optimized to provide sufficient selectivity towards the drugs. Potassium dihydrogen orthophosphate buffer contribute high sensitivity and selectivity when compared with other buffers. The optimized mobile phase consisted acetonitrile and potassium dihydrogen orthophosphate buffer in ratio of 35:65 v/v. Injection volume was optimized to 10 µl. The column temperature was maintained at 30°C (ambient). Retention times were 6.589 ± 0.05 min for Prazosin, 5.214 ± 0.05 min for Polythiazide and 3.579 ± 0.05 min for Hydrochlorothiazide (ISTD).

System Suitability Test

System suitability test was done by MQC level sample as six homogenous injections and calculated the % CV for retention time and peak areas and number of peak area ratios of analytes and internal standard. The values obtained are listed in Table 1. The % CV calculated for the method was found to be less than 2%, which revealed the suitability of the developed method and the optimized chromatographic conditions. These values met the requirements of USP24/ NF19[38] and were therefore found to be satisfactory.

Specificity and Selectivity

Representative chromatogram of blank plasma confirmed there is no significant interference from the endogenous component as shown in Fig. 4. Chromatogram of spiked plasma samples of Prazosin and Polythiazide at a concentration of 250.00 ng/ml and 125.00 ng/ml, respectively with the internal standard at a concentration 450.00 ng/ml, conformed that the Prazosin and Polythiazide and internal standard were well resolved and completely separated at retention time of 6.589 ± 0.05 min, 5.214 ± 0.05 min and 3.579 ± 0.05 min, respectively as shown in Fig. 5.

Matrix effect

Matrix effect was assessed with six different lots of chromatographically screened plasma samples of two quality control levels (LQC and HQC). The results obtained were well within the acceptable limits, as the % CV of the area ratios of post spiked recovery samples at LQC were 2.57 and at HQC were 0.43 for Prazosin and at LQC were 5.42 and at HQC were 5.52 for Polythiazide which were within 10%. Hence minor suppression or enhancement of analyte signal due to endogenous matrix interferences did not affect the quantification of analytes and internal standard peak.

Calibration and linearity

Calibration curves of Prazosin and Polythiazide in human plasma demonstrated linearity in the concentration ranges from 5-500 ng/ml and 2.5-250 ng/ml. The method was exhibited excellent linearity for this range. A typical calibration curve of spiked plasma samples with the regression equation and their respective correlation coefficient (R^2) for Prazosin and Polythiazide were shown in Fig. 6 and Fig. 7. The average correlation coefficient was found to be 0.999 for both analytes with goodness of fit; the results were shown in Table 3.

Sensitivity (LLOQ)

The lower limit of quantification was experimentally determined by diluting known concentrations of Prazosin and Polythiazide in human plasma for six replicate determinations. The present assay method offered an LLOQ of 5.0 ng/ml for Prazosin and 2.5 ng/ml for Polythiazide in human plasma. Intra-day precision of the method was found to be 4.53 and 2.34 % CV and accuracy of the method was found to be 100.75 % and 98.85 % for Prazosin and Polythiazide, respectively. The results of sensitivity were listed in Table 3.

Precision and Accuracy

The precision of the method was measured by the percentage coefficient of variance (% CV) over the concentration range of HQC, MQC and LQC samples, respectively of drugs during course of validation. Intra-day precision of the method ranged from 0.077 to 3.78 % CV where as Inter-days precision was found to be 0.08 to 3.28 % CV. Nominal values (%) for recovery of Prazosin and Polythiazide from QC samples were tested of intra-day and inter-days. Intra-day accuracy ranged from 99.72 to 101.82 % whereas Inter-days accuracy from 99.72 to 101.83 %. The result from determination of intra-day and inter-day, accuracy and precision were given in Table 4. Percentage coefficient of

variance (% CV) was found to be less than 15% for both intra and inter day samples over the concentration range assayed.

Recovery

The recovery for the Prazosin, Polythiazide and internal standard were determined by spiking known quantitative of analytes and ISTD into drug free human plasma to obtain three different concentrations covering the low, medium and higher ranges of the calibration curve. The samples were then extracted and analyzed as described earlier. The recovery was calculated by comparing the peak areas of the drugs with those obtained from pure standards in mobile phase and ISTD in mobile phase at the same concentration[39]. The recovery of Prazosin and Polythiazide ranges from 88.41 ± 142.90 % to 93.41 ± 2080.95 %, while the absolute recovery for ISTD was 95.08 ± 3622.21%, the results of the study were shown in Table 5.

Stability

Low value percentage difference (<15) between area ratio for stability test samples and fresh QC samples confirm the stability of drug on Zero hours, LT at -28ºC and LT at -80ºC results of LQC, MQC, and HQC were more than 85% which are within acceptance limits. The results of stability study were given in Table 6.

CONCLUSION

The developed RP-HPLC Bioanalytical method is an accurate, specific and simple method for simultaneous determination of Prazosin and Polythiazide. The method involves simple extraction procedure, separation on a reversed phase column with an internal standard and PDA detector. The validation data demonstrated good precision and accuracy, which proves the reliability of proposed method. Thus the method suits for routine therapeutic drug monitoring (TDM), specializes in the measurement of medication concentrations in blood for Prazosin and Polythiazide. It is also helpful in pharmacogenetic, demographic and clinical information, and/or on the posterior measurement of blood concentrations of drugs (pharmacokinetic monitoring) of Prazosin and Polythiazide in human plasma. The present developed method could be adapted for the determination of bioavailability and bioequivalence required for filing NDA and ANDA.

ACKNOWLEDGEMENTS

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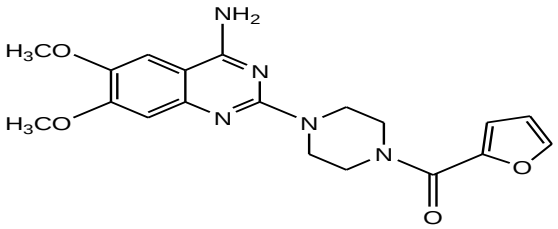

Fig. 1: Chemical Structure of Prazosin

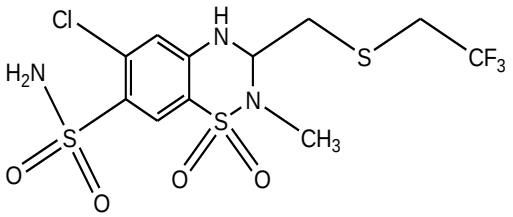

Fig. 2: Chemical Structure of Polythiazide

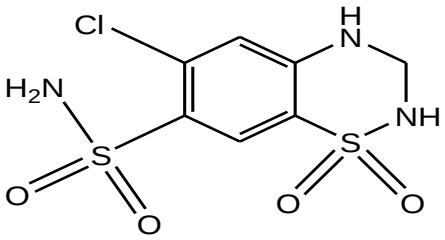

Fig. 3: Chemical Structure of Hydrochlorothiazide

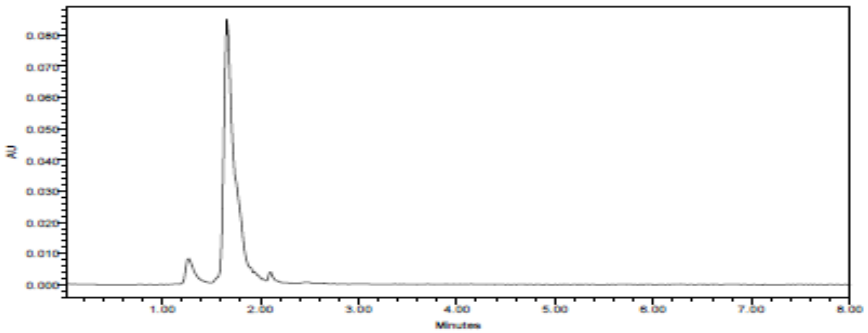

Fig. 4: A representative chromatogram of human blank plasma

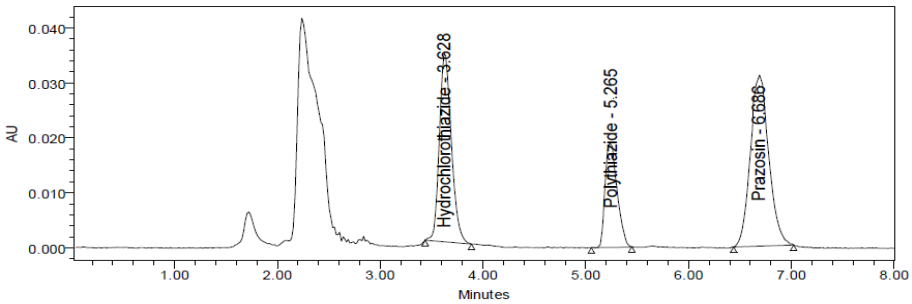

Fig. 5: A representative chromatogram of spiked plasma sample (HQC) of analytes

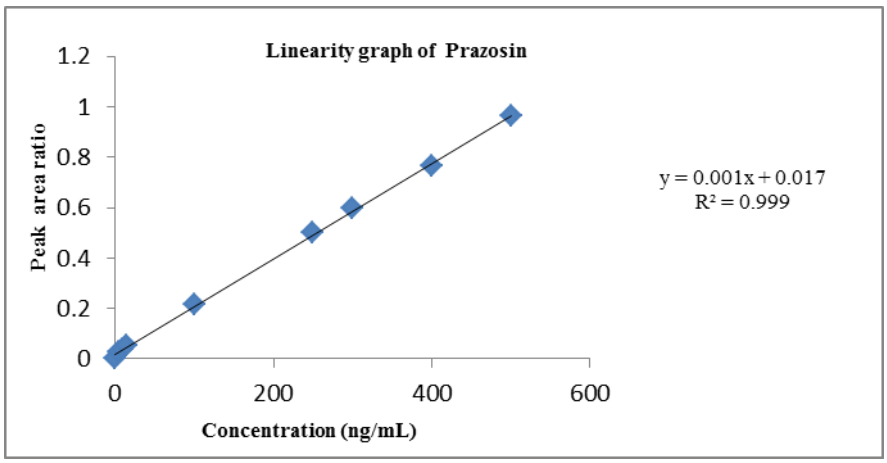

Fig. 6: Calibration curve of Prazosin

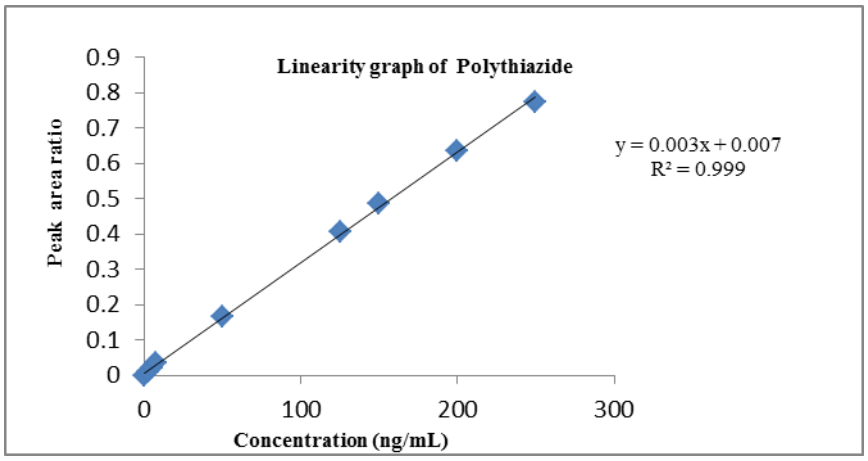

Fig. 7: Calibration curve of Polythiazide

Table 1: System Suitability data of Prazosin and Polythiazide

S. No.	Prazosin		Internal standard		Peak area ratio	Polythiazide		Internal standard		Peak area ratio
Sample Name	RT (min)	Peak Area	RT (min)	Peak Area		RT (min)	Peak Area	RT (min)	Peak Area	
AQMQC	6.70	213691	3.63	427110	0.5003	5.21	178111	3.55	433590	0.0410
	6.62	222502	3.60	432353	0.5146	5.21	177671	3.57	434678	0.4087
	6.60	223146	3.63	444696	0.5018	5.23	179992	3.59	433246	0.4154
	6.64	219877	3.61	432711	0.5081	5.23	177695	3.60	433832	0.4096
	6.61	218796	3.64	451135	0.4850	5.25	177712	3.62	433791	0.4097
	6.62	219062	3.64	423901	0.5168	5.27	177522	3.65	431852	0.4111
Mean	6.631		3.625		0.50444	5.234		3.595		0.4108
SD	0.034		0.0143		0.01159	0.0221		0.034		0.0023
% CV	0.52		0.39		2.30	0.42		0.96		0.58

Acceptance Criteria: The % CV of the retention time (RT) should be ≤2.00 %. The % CV of the area ratio should be ≤ 5.00%.

Table 2: Sensitivity results of lower limit of quantitation (LLOQ)

Sample / Parameter	Prazosin		Polythiazide	
	Nominal concentration (5.000 ng/ml)		Nominal concentration (2.500 ng/ml)	
	Cal. conc. (ng/mL)	% Nominal conc.	Cal. conc. (ng/mL)	% Nominal conc.
LLOQ-1	4.835	96.7	2.507	100.28
LLOQ-2	4.235	84.7	2.541	101.64
LLOQ-3	4.737	94.74	2.410	96.41
LLOQ-4	5.238	84.76	2.402	96.08
LLOQ-5	5.239	104.78	2.454	98.16
LLOQ-6	4.940	98.8	2.513	100.52
Mean cal. conc.	5.0373 ng/ml		2.4712 ng/ml	
SD	0.22831		0.05785	
% CV	4.53		2.34	
% Mean accuracy	94.08		98.85	

Acceptance Criteria: At least 67% (4 out of 6) of samples should be within 80.00-120.00%. %Mean accuracy should be within 80.00-120.00%. % CV accuracy should be ≤ 20.00%.

Table 3: Calibration curve (Linearity) data of Prazosin and Polythiazide

Nominal concentrations (ng/ml)	Prazosin			Nominal concentrations (ng/ml)	Polythiazide		
	*Mean	% CV	% Mean accuracy		*Mean	% CV	% Mean accuracy
5.000	5.103	4.82	102.07	2.500	2.580	3.85	103.23
10.000	10.160	2.31	101.60	5.000	5.034	3.18	100.69
15.000	14.306	2.27	95.38	7.500	7.462	1.49	99.50
100.000	100.243	0.53	100.24	50.000	50.709	3.96	101.42
250.000	250.610	0.26	100.24	125.000	120.883	2.14	96.71
300.000	300.386	0.13	100.13	150.000	151.644	2.43	101.10
400.000	399.923	0.16	99.98	200.000	198.331	2.32	99.17
500.000	500.016	0.09	100.00	250.000	256.677	2.18	102.67
*Mean values represent three different samples for each concentration							

Acceptance Criteria: The regression coefficient should be $R^2 = 0.999$.

Table 4: Precision and Accuracy data of Prazosin and Polythiazide

Drugs	Concentration added (ng/ml)	Recovery (% Mean ±S.D)	Intra Day # % CV	Accuracy (%)	Recovery (%Mean ±S.D)	Inter Days $ % CV	Accuracy (%)
Prazosin	5.000	4.986 ± 0.139	2.80	99.73	4.986 ± 0.150	3.02	99.73
	15.000	15.088± 0.418	2.78	100.19	15.029± 0.430	2.87	100.20
	300.000	300.08 ± 0.600	0.2	100.03	300.087 ± 0.609	0.20	100.03
	400.000	400.275 ±0.317	0.077	100.07	400.275±0.336	0.08	100.07
Polythiazide	2.500	2.545± 0.093	3.68	101.82	2.5457 ±0.090	3.54	101.83
	7.500	7.558 ± 0.173	2.32	100.77	7.5582±0.24014	3.18	100.78
	125.000	124.65 ± 2.082	0.79	99.72	124.65 ± 2.758	2.21	99.72
	200.000	202.92 ±3.612	1.78	101.46	202.92 ± 3.698	1.82	101.46

Mean values represent six different plasma samples for each concentration. $Interday was determined from nine different runs over two-week period. The concentration of each run was determined from a single calibration curve run on the first day of the study.

Acceptance Criteria: The within and between batch precision for LQC, MQC and HQC samples should be ≤15.00% and for the LLOQ QC, It should be ≤20.00%.

Table 5: Recovery study data of Prazosin, Polythiazide and internal standard drugs from human plasma

Drugs	Concentration added (ng/ml)	Recovery (% Mean ± S.D)	% CV	Overall % CV
Prazosin	15.000	92.29 ± 22474	1.23	1.37
	250.000	90.12 ± 25.35	0.01	
	400.000	92.2 ± 3890.24	1.16	
Polythiazide	7.500	88.41 ± 142.90	0.95	2.98
	125.000	89.17 ± 2405.93	1.37	
	150.000	93.41 ± 2080.95	0.74	
Internal Standard	450.000	95.08 ± 3622.21	0.81	-

Acceptance Criteria: The % CV of recovery at each QC level and for ISTD should be ≤ 15.00%. The overall mean recovery %CV for all QC levels should be ≤ 20.00%.

Table 6: Stability study data of Prazosin and Polythiazide

Sample	Prazosin				Polythiazide			
	Nominal concentration (ng/ml)	Mean calculated conc. ± S.D (ng/ml)(n=6)	%CV	% Mean accuracy	Nominal concentration (ng/ml)	Mean calculated conc. ± S.D (ng/ml) (n=6)	% CV	% Mean accuracy
Stability at day Zero								
HQC	400.00	400.525±1.2718	0.32	100.13	200.00	199.980±0.027	2.39	99.99
LQC	15.00	0.1518±0.00403	2.66	101.17	7.500	7.4257±0.265	3.58	99.01
Long term at -28ºC								
HQC	400.00	3.99.856 ±1.315	0.33	99.96	200.00	200.120 ±3.152	1.58	100.06
LQC	15.00	14.8717±0.380	2.56	99.14	7.500	7.446 ± 0.256	3.44	99.28
Long term at -80ºC								
HQC	400.00	400.355 ±1.155	0.29	100.09	200.00	100.25 ± 4.305	2.13	101.25
LQC	15.00	15.136 ±0.256	1.69	100.91	7.500	7.443± 0.265	3.57	99.24

Acceptance Criteria: At least 67% (8 out of 12) of total QC samples and 50% (3 out of 6) at each level should be within 85.00 - 115.00%. The % Mean accuracy of LQC and HQC should be within 85.00-115.00%. The % CV of LQC and HQC samples should be ≤ 15.00%.

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