New Validated Stability-Indicating Reverse-phase High-performance Liquid Chromatography Method for the Simultaneous Estimation of Prazosin and Polythiazide in their Formulations in Human Plasma

G. Dharmamoorthy¹, K. S. Nataraj², A. Krishna Manjari Pawar³

¹Research Scholar, Andhra University, Visakhapatnam, Andhra Pradesh, India, ²Department of Pharmaceutical Analysis, Shri Vishnu College of Pharmacy, Bhimavaram, Andhra Pradesh, India, ³Department of Pharmaceutical Analysis, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India

Abstract

Objective: A new simple and precise stability-indicating bioanalytical reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of prazosin and polythiazide in their formulation and in human plasma. Materials and Methods: The developed method was successfully used for assaying drug contents in plasma. Isocratic elution mode was carried at Agilent C18 column (150 mm × 4.6 mm, 5 µm particle size) using 0.01 N disodium hydrogen phosphate pH 5.0:acetonitrile (55:45) as mobile phase at flow rate 1.0 ml min at detection wavelength 265 nm. Celecoxib was taken as an internal standard. The method was validated as per ICH guidelines. Results and Discussion: The present validated method can be successfully applied for the estimation of prazosin and polythiazide in human plasma over the concentration range of 12.5–500 ng/ml of prazosin and 6.25–250 ng/ml of polythiazide. The method for the determination of prazosin and polythiazide in human plasma using HPLC detection met the acceptance criteria with respect to selectivity, precision, accuracy, linearity, and recovery. Conclusion: The proposed method is simple, rapid, accurate, precise, and appropriate for pharmacokinetic and therapeutic drug monitoring in the clinical laboratories.

Key words: Celecoxib internal standard, ICH guidelines, prazosin and polythiazide K₂EDTA plasma, reverse-phase high-performance liquid chromatography, validation

INTRODUCTION

Prazosin is an antihypertensive agent which lowers arterial blood pressure by effecting blockade of vascular postjunctional α-adrenoceptors¹⁻³ and is the first of such agents to show long-term efficacy in the treatment of hypertension. Alpha-adrenergic receptors are essential for the regulation of blood pressure in humans. Two types of alpha receptors, alpha 1 and alpha 2, both play a role in regulating blood pressure. Alpha-1 receptors are postsynaptic (located after the nerve junction or space between a nerve fiber and target tissue).

Polythiazide is a diuretic with actions and uses similar to those of hydrochlorothiazide⁴. As a thiazide diuretic, polythiazide inhibits the sodium-chloride symporter which decreases solute reabsorption leading to a retention of water in the urine, as water normally follows solutes. The short-term antihypertensive action is based on the fact that thiazides decrease preload, decreasing blood pressure, polythiazide also inhibits sodium ion transport across the renal tubular epithelium through binding to the thiazide sensitive sodium-chloride transporter. This results in an increase in potassium excretion through the sodium-potassium exchange mechanism.

Address for correspondence:
G. Dharmamoorthy, Research Scholar, Andhra University, Visakhapatnam - 530 003, Andhra Pradesh, India. Phone: +91-9603774847. E-mail: dharmamoorthy111@gmail.com

Received: 28-03-2020
Revised: 14-04-2020
Accepted: 19-04-2020
Extensive survey of literature few methods has been reported for the simultaneous estimation of prazosin and polythiazide using spectroscopic and chromatographic methods.[5-8] However, there is no reported for the bioanalytical methods. The main aim of the present study is to develop a precise, sensitive, accurate, selective, reproducible, and rapid bioanalytical technique for the estimation of prazosin and polythiazide in human plasma[9-13] and validated as per ICH guidelines.[14,15]

**MATERIALS AND METHODS**

**Materials**

List of chemicals and solvents [Table 1] and solvents [Table 2] given below.

**API**

Prazosin and polythiazide, API, were gifted by BMR Chemicals, Hyderabad.

**Human plasma**

K<sub>2</sub>EDTA control plasma procured by Deccan Pathological Labs, Hyderabad.

**Method development**

**Diluent**

Based on the solubility of the drugs, diluent was selected, 0.01 N disodium hydrogen orthophosphate and acetonitrile taken in the ratio of 50:50.

**Preparation of prazosin stock solution (25 µg/ml)**

Take 2.5 mg of prazosin in 100 ml volumetric flask and make the volume with diluent to produce 25 µg/ml.

---

**Table 1: List of chemicals and solvents**

| Chemical name | Grade | Manufacturing company |
|---------------|-------|-----------------------|
| Distilled water | HPLC | Rankem, Avantor Performance Materials India Limited |
| HPLC water | Analytical reagent | Rankem, Avantor Performance Materials India Limited |
| HPLC water | Analytical reagent | Rankem, Avantor Performance Materials India Limited |
| Acetonitrile | Analytical reagent | Rankem, Avantor Performance Materials India Limited |
| Phosphate buffer | Analytical reagent | Rankem, Avantor Performance Materials India Limited |
| Methanol | Analytical reagent | Rankem, Avantor Performance Materials India Limited |
| Sodium dihydrogen phosphate | Analytical reagent | Rankem, Avantor Performance Materials India Limited |
| Orthophosphoric acid | Analytical reagent | Rankem, Avantor Performance Materials India Limited |

HPLC: High-performance liquid chromatography

**Table 2: List of instruments**

| Instrument | Company name | Brand name |
|------------|--------------|------------|
| Electronic balance | Sartorius | Denver |
| pH meter | Metsar | BKV enterprises |
| Sonicator | Lab man | BKV enterprises |
| Centrifuge | Thermo Fisher | - |
| Vertex | Remi CM101 | - |
| HPLC water | Alliance | Water HPLC 2695 SYSTEM |

HPLC: High-performance liquid chromatography

**Table 3: System suitability of prazosin**

| Sample name | Prazosin | ISTD | Celecoxib |
|-------------|---------|------|-----------|
|             | Analyte area | Analyte RT (min) | ISTD Area | ISTD RT (min) | Area ratio |
| AQ MQC      | 29,558 | 3.98 | 69,634 | 2.683 | 0.4245 |
| AQ MQC      | 29,056 | 4.01 | 69,452 | 2.684 | 0.4184 |
| AQ MQC      | 29,993 | 4.02 | 69,667 | 2.688 | 0.4305 |
| AQ MQC      | 29,390 | 4.03 | 69,151 | 2.697 | 0.4250 |
| AQ MQC      | 29,884 | 4.06 | 69,724 | 2.723 | 0.4286 |
| AQ MQC      | 29,717 | 4.08 | 69,956 | 2.724 | 0.4248 |
| Mean±SD     | 4.029±0.0357 | 2.700±0.0190 | 0.42529±0.004179 |
| %CV         | 0.89 | 0.70 | 0.98 |

RT: Retention time, MQC: Middle quality control
Preparation of prazosin spiking solutions
From the above prazosin stock solution, 0.05 ml, 0.1 ml, 0.15 ml, 0.6 ml, 1.0 ml, 1.2 ml, 1.6 ml, and 2.0 ml were pipette and transferred to eight individual 10 ml volumetric flask and make up the volume up to the mark with diluent to produce 0.125 µg/ml, 0.25 µg/ml, 0.375 µg/ml, 1.0 µg/ml, 2.5 µg/ml, 3.0 µg/ml, 4.0 µg/ml, and 5.0 µg/ml. Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.0125 µg/ml, 0.025 µg/ml, 0.0375 µg/ml, 0.10 µg/ml, 0.25 µg/ml, 0.3 µg/ml, 0.4 µg/ml, and 0.5 µg/ml.

Preparation of polythiazide stock solution (12.5 µg/ml)
Take 1.25 mg of polythiazide in 100 ml volumetric flask and make the volume with diluent to produce 12.5 µg/ml.

Preparation of polythiazide spiking solutions
From the above polythiazide stock solution, 0.05 ml, 0.1 ml, 0.15 ml, 0.6 ml, 1.0 ml, 1.2 ml, 1.6 ml, and 2.0 ml were pipette and transferred to eight individual 10 ml volumetric flask and make up the volume up to the mark with diluent to produce 0.0625 µg/ml, 0.125 µg/ml, 0.1875 µg/ml, 0.25 µg/ml, 1.25 µg/ml, 1.5 µg/ml, 2.0 µg/ml, and 2.5 µg/ml. Calibration standards and QC samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.00625 µg/ml, 0.0125 µg/ml, 0.01875 µg/ml, 0.05 µg/ml, 0.125 µg/ml, 0.15 µg/ml, 0.20 µg/ml, and 0.25 µg/ml.

Preparation of internal standard solution (celecoxib)
- Stock-1: Take 5 mg of celecoxib in 100 ml volumetric flask and make up the volume with diluent to produce 50 µg/ml.

### Table 4: System suitability of polythiazide

| Validation No. | SOP No. | Column ID. | Analyte | Polythiazide | ISTD | Celecoxib |
|---------------|---------|------------|---------|--------------|------|-----------|
| Acquisition batch ID | Date | | Analyte area | Analyte RT (min) | ISTD area | ISTD RT (min) | Area ratio |
| Sample name | | | | | | | |
| AQ MQC | | | 16,193 | 4.89 | 69,634 | 2.683 | 0.2325 |
| AQ MQC | | | 16,231 | 4.93 | 69,452 | 2.684 | 0.2337 |
| AQ MQC | | | 16,028 | 4.95 | 69,667 | 2.688 | 0.2301 |
| AQ MQC | | | 16,141 | 4.96 | 69,151 | 2.697 | 0.2334 |
| AQ MQC | | | 16,041 | 5.01 | 69,724 | 2.723 | 0.2301 |
| AQ MQC | | | 16,118 | 5.05 | 69,956 | 2.724 | 0.2304 |
| Mean±SD | | | 16,062 | 5.00 | 69,451 | 2.693 | 0.2317±0.001714 |
| %CV | | | 1.15 | | | 0.70 | 0.74 |

RT: Retention time, MQC: Middle quality control

### Table 5: Auto-sampler carryover of prazosin

| Acquisition batch ID | Date |
|----------------------|------|
| Sample ID | Peak area | % carryover |
| | Drug | ISTD | ISTD | Drug | ISTD |
| Unextracted samples | | | | | | |
| RS | 0 | 0 | N/A | N/A |
| AQ ULOQ | 61,732 | 70,558 | 0.00 | 0.00 |
| RS | 0 | 0 | N/A | N/A |
| AQ LLOQ | 1515 | 71,349 | N/A | N/A |
| Extracted samples | | | | | | |
| STD Blk | 0 | 0 | N/A | N/A |
| ULOQ | 59,694 | 69,246 | 0.00 | 0.00 |
| STD Blk | 0 | 0 | N/A | N/A |
| LLOQ | 1489 | 69,231 | N/A | N/A |

LLOQ: Lower limit of quantitation

### Table 6: Auto-sampler carryover of polythiazide

| Acquisition batch ID | Date |
|----------------------|------|
| Sample ID | Peak area | % carryover |
| | Drug | ISTD | Drug | ISTD |
| Unextracted samples | | | | | | |
| RS | 0 | 0 | N/A | N/A |
| AQ ULOQ | 33,275 | 70,558 | 0.00 | 0.00 |
| RS | 0 | 0 | N/A | N/A |
| AQ LLOQ | 844 | 71,349 | N/A | N/A |
| Extracted samples | | | | | | |
| STD Blk | 0 | 0 | N/A | N/A |
| ULOQ | 32,502 | 69,246 | 0.00 | 0.00 |
| STD Blk | 0 | 0 | N/A | N/A |
| LLOQ | 818 | 69,231 | N/A | N/A |

LLOQ: Lower limit of quantitation
Table 7: Matrix factor evaluation of prazosin (absence of matrix factor)

| Acquisition batch ID | Plasma Lot No. | Date | HQC | LQC |
|----------------------|----------------|------|-----|-----|
| S. No.               |                |      | Nominal concentration (ng/mL) | Nominal concentration range (ng/mL) |
|                      |                |      | 400.000 | 37.500 |
|                      |                |      | (340.000–460.000) | (31.875–43.125) |
|                      |                |      | Calculated concentration (ng/mL) |     |
| 1                    | LOT1           |      | 404.00 | 37.55 |
|                      |                |      | 401.00 | 37.86 |
|                      |                |      | 398.00 | 37.87 |
| 2                    | LOT2           |      | 402.00 | 37.86 |
|                      |                |      | 396.00 | 37.90 |
|                      |                |      | 398.00 | 37.14 |
| 3                    | LOT3           |      | 403.00 | 36.91 |
|                      |                |      | 405.00 | 37.88 |
|                      |                |      | 400.00 | 37.78 |
| 4                    | LOT4           |      | 399.00 | 37.91 |
|                      |                |      | 398.00 | 36.84 |
|                      |                |      | 405.00 | 37.98 |
| 5                    | LOT5           |      | 399.00 | 37.86 |
|                      |                |      | 398.00 | 37.79 |
|                      |                |      | 402.00 | 37.80 |
| 6                    | LOT6           |      | 404.00 | 37.94 |
|                      |                |      | 401.00 | 37.00 |
|                      |                |      | 406.00 | 37.99 |
| n                    | 18             |      | Mean±SD | 401.0556±2.97978 | 37.6589±0.39279 |
|                      | 18             |      | % CV | 0.74 | 1.04 |
|                      |                |      | % mean accuracy | 100.26 | 100.42 |
|                      |                |      | Number of QC failed | 0 | 0 |

LQC: Low-quality control, HQC: High-quality control

• Stock-2: From the above solution, take 1 ml of solution into 10 ml volumetric flask and make up the volume with diluent to produce 5 µg/ml solutions.

Final concentration

From the above solution, take 0.5 ml of solution and spiking blank plasma with working stock dilutions of analyte to produce 1 µg/ml Internal standard solution (ISD) concentration.

Optimized chromatographic conditions

Mobile phase : 0.01 N disodium hydrogen orthophosphate pH (5.0): acetonitrile (55:45)
Flow rate : 1.0 ml/min
Column : Agilent C18 (150 mm × 4.6 mm, 5 µ)
Detector wavelength : 265 nm
Column temperature : 30°C
Injection volume : 50 µL

Extraction procedure

Take 750 µl of plasma and 500 µl of internal standard, 250 µl of prazosin from the spiking solutions of both into a centrifuging tube and add 1 ml of acetonitrile go for cyclomixer for 15 s. Then vortex for 2 min and finally centrifuge for 5 min at 3200 rpm speed. After the centrifugation, collect the sample and filter it directly inject 50 µL into high-performance liquid chromatography (HPLC).
Method validation

System suitability
All the system suitability parameters were within the range and satisfactory as per ICH guidelines. The % CV for system suitability test was in the range of 0.89 for retention time (RT) of prazosin, 1.15 for RT of polythiazide, and 0.74% for the area ratio (analyte area/IS area) of celecoxib.

Auto-sampler carryover test
The carry over effect due to the auto sampler was investigated by injecting the sequence of un extracted and extracted samples. Results demonstrated that no significant carry over was observed during this experiment

Matrix factor evaluation
Matrix effect is played a key role in the assessment of pharmacokinetic studies. It was expressed as internal standard normalized matrix factor and it was varied from 0.90 to 0.99 which was close to 1 which indicates that there is no ionization suppression or enhancement in plasma samples.

Table 8: Matrix factor evaluation of polythiazide (absence of matrix factor)

| Acquisition batch ID | Date | HQC | LQC |
|----------------------|------|-----|-----|
| S. No.   | Plasma Lot No. | Nominal concentration (ng/mL) | Calculated concentration (ng/mL) |
|          |                  | 200.000  | 18.750 |
|          |                  | (170.000–230.000) | (15.938–21.563) |
| 1        | LOT1             | 196.463  | 18.180 |
|          |                  | 199.621  | 18.201 |
|          |                  | 192.325  | 18.155 |
| 2        | LOT2             | 201.925  | 18.766 |
|          |                  | 201.899  | 17.891 |
|          |                  | 200.946  | 18.155 |
| 3        | LOT3             | 199.942  | 17.974 |
|          |                  | 200.908  | 18.762 |
|          |                  | 199.889  | 18.690 |
| 4        | LOT4             | 201.906  | 19.083 |
|          |                  | 200.896  | 18.858 |
|          |                  | 202.119  | 18.016 |
| 5        | LOT5             | 200.601  | 19.002 |
|          |                  | 196.902  | 19.095 |
|          |                  | 198.914  | 19.092 |
| 6        | LOT6             | 201.884  | 18.760 |
|          |                  | 200.954  | 18.682 |
|          |                  | 200.921  | 18.488 |
| n        |                  | 18       | 18   |
| Mean±SD  |                  | 199.9453±2.49617 | 18.5472±0.41815 |
| % CV     |                  | 1.25     | 2.25 |
| % mean accuracy |         | 99.97 | 98.92 |
| Number of QC failed |     | 0  | 0  |

LQC: Low-quality control, HQC: High-quality control

Table 9: Linearity of prazosin

| Final conc. in µg/mL | ISD (area) | Drug (area) | Area ratio |
|----------------------|------------|-------------|------------|
| 0.0125               | 69,638     | 1496        | 0.021      |
| 0.025                | 69,756     | 2981        | 0.043      |
| 0.0375               | 69,793     | 4478        | 0.064      |
| 0.1                  | 69,586     | 11,936      | 0.172      |
| 0.25                 | 69,547     | 29,828      | 0.429      |
| 0.3                  | 69,684     | 35,765      | 0.513      |
| 0.4                  | 69,726     | 47,735      | 0.685      |
| 0.5                  | 69,675     | 58,672      | 0.842      |
**QC samples**

The chromatography observed during the course of prazosin and polythiazide which was acceptable and representative chromatograms of standard blank, standard zero (standard blank with internal standard) QC-lower limit of quantitation (LLOQ), QC-L, QC-M1, QC-M2, and QC-H samples.

**Table 10: Linearity of polythiazide**

| Final conc. in µg/ml | ISD (area) | Drug (area) | Area ratio |
|----------------------|------------|-------------|------------|
| 0.00625              | 69,638     | 811         | 0.0116     |
| 0.0125               | 69,756     | 1236        | 0.0177     |
| 0.01875              | 69,793     | 2495        | 0.0357     |
| 0.05                 | 69,586     | 6586        | 0.0946     |
| 0.125                | 69,547     | 16,438      | 0.2364     |
| 0.15                 | 69,684     | 19,478      | 0.2795     |
| 0.2                  | 69,726     | 25,234      | 0.3619     |
| 0.25                 | 69,675     | 31,679      | 0.4547     |

**Selectivity/specificity**

To establish the selectivity of the method, possible interference at the RT of prazosin, polythiazide, and internal standard due to endogenous plasma components was checked during the validation. Selectivity was performed by testing six batches of K$_2$EDTA blank plasma and the mass detection of extracted blank plasma gave good selectivity of both drug and internal standard. No interferences were found at the RTs of analytes and internal standard.

**Linearity**

Calibration was found to be linear over the concentration range of 0.0125–0.5 µg/ml for prazosin and 0.00625–0.25 µg/ml polythiazide. The coefficient correlation ($r^2$) value was found consistently greater than 0.999 in all the cases. This indicates linearity of results and an excellent correlation between peak area ratios for each concentration of analytes.

**Precision and accuracy**

The intraday and interday accuracy and precision were assessed by analyzing six replicates at five different QC levels such as LLOQ, low QC (LQC), middle QC (MQC), and high QC (HQC). Accuracy and precision method performance were evaluated by determined by six replicate analyses for prazosin at four concentration levels, i.e., 0.0125 µg/ml (LLOQ), 0.0375 µg/ml (LQC), 0.25 µg/ml (MQC), and 0.40 µg/ml (HQC), polythiazide at 0.00625 µg/ml (LLOQ), 0.01875 µg/ml (LQC), 0.125 µg/ml (MQC), and 0.2 µg/ml (HQC), the intraday and interday accuracy of plasma samples were assessed and excellent mean % accuracy was obtained with range varied from 98.16 to 100.42% and 98.75 to 100.41% for intraday and 98.83 to 100.03 and 99.70 to 99.86 for interday, respectively. The precision (%CV) of the analytes and plasma samples were calculated and found to be 0.70–4.34% and 0.39–2.73% for intraday and 0.21–2.83% and 0.22–0.74% for interday, respectively.

**Recovery**

Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those.
extracted blank plasma spiked with standards containing the same area with known amount of prazosin and polythiazide. The overall % mean recovery for prazosin and polythiazide was found to be 98.16% and 98.23%. The overall % mean recovery for celecoxib was found to be 98.11%.

**Long-term stock solution stability for polythiazide**

In bench-top stability, six replicates of LQC and HQC samples (0.01875 and 0.2 µg/ml) were analyzed for 9 h at room temperature on the laboratory bench. The % mean stability was calculated and found to 99.95% for LQC and 99.87% for HQC, respectively.
Figure 7: Chromatogram of quality control-high-quality control sample prazosin and polythiazide

Figure 8: Chromatogram of ULOQ sample prazosin and polythiazide

Figure 9: Chromatogram of prazosin and polythiazide

Figure 10: Calibration curve of prazosin

Figure 11: Calibration curve of polythiazide
Table 11: Precision and accuracy results for prazosin

| Acquisition batch ID | Date | HQC | MQC1 | LQC | LLOQ QC |
|----------------------|------|-----|------|-----|---------|
| 400.000              |      |     |      |     |         |
| 250.000              |      |     |      |     |         |
| 37.500               |      |     |      |     |         |
| 12.500               |      |     |      |     |         |
| Nominal concentration (ng/mL) |      |      |      |      |         |
| 400.000–460.000      |      |      |      |      |         |
| (340.000–460.000)    |      |      |      |      |         |
| Nominal concentration range (ng/mL) |      |      |      |      |         |
| 250.000              |      |     |      |     |         |
| 212.500–287.500      |      |      |      |      |         |
| (31.875–43.125)      |      |      |      |      |         |
| (10.000–15.000)      |      |      |      |      |         |
| Back calculated concentration (ng/mL) |      |      |      |      |         |
| 400                  |      |     |      |     |         |
| 250.900              |      |     |      |     |         |
| 246.300              |      |     |      |     |         |
| 251.500              |      |     |      |     |         |
| 252.100              |      |     |      |     |         |
| 248.800              |      |     |      |     |         |
| 400                  |      |     |      |     |         |
| 250.900              |      |     |      |     |         |
| 251.500              |      |     |      |     |         |
| 252.100              |      |     |      |     |         |
| 248.800              |      |     |      |     |         |
| 402                  |      |     |      |     |         |
| 250.900              |      |     |      |     |         |
| 251.500              |      |     |      |     |         |
| 252.100              |      |     |      |     |         |
| 248.800              |      |     |      |     |         |
| 6                   |      |     |      |     |         |
| Mean±SD              |      |      |      |      |         |
| 400.0167±0.88412     |      | 250.0833±2.16187 | 37.8860±0.64906 | 12.1353±0.42526 |
| % CV                 |      |      |      |      |         |
| 0.22                 |      | 0.86 | 1.71 | 3.50  |
| % mean accuracy      |      |      |      |      |         |
| 100.00               |      | 100.03 | 101.03 | 97.08 |
| 400                  |      | 250.800 | 37.900 | 12.330 |
| 401                  |      | 250.100 | 37.910 | 12.280 |
| 400                  |      | 244.010 | 37.920 | 11.322 |
| 389                  |      | 252.300 | 38.860 | 12.295 |
| 400                  |      | 252.700 | 36.880 | 12.310 |
| 399                  |      | 253.600 | 36.630 | 12.268 |
| 6                   |      |     |      |     |         |
| Mean±SD              |      |      |      |      |         |
| 397.9167±4.65507     |      | 250.5850±3.46459 | 37.6833±0.81163 | 11.9675±0.52146 |
| % CV                 |      |      |      |      |         |
| 1.17                 |      | 1.38 | 2.15 | 4.36  |
| % mean accuracy      |      |      |      |      |         |
| 99.48                |      | 100.23 | 100.49 | 95.74 |
| 400                  |      | 256.100 | 37.880 | 12.908 |
| 401                  |      | 250.500 | 36.940 | 12.920 |
| 400                  |      | 250.300 | 37.860 | 12.990 |
| 399                  |      | 250.800 | 36.880 | 11.980 |
| 399                  |      | 250.600 | 37.910 | 12.810 |
| 399                  |      | 250.900 | 36.930 | 12.699 |
| 6                   |      |     |      |     |         |
| Mean±SD              |      |      |      |      |         |
| 399.7867±1.02033     |      | 251.5333±2.24737 | 37.4000±0.53009 | 12.7178±0.37533 |
| % CV                 |      |      |      |      |         |
| 0.26                 |      | 0.89 | 1.42 | 2.95  |
| % mean accuracy      |      |      |      |      |         |
| 99.95                |      | 100.61 | 99.73 | 101.74 |
| Between batch precision and accuracy |      |      |      |      |         |
| 18                  |      |     |      |     |         |
| Mean±SD              |      |      |      |      |         |
| 399.2400±2.80107     |      | 250.7339±2.60255 | 37.6564±0.66511 | 12.2736±0.53298 |
| % CV                 |      |      |      |      |         |
| 0.70                 |      | 1.04 | 1.77 | 4.34  |
| % mean accuracy      |      |      |      |      |         |
| 99.81                |      | 100.29 | 100.42 | 98.19 |

LLOQ: Lower limit of quantitation, LQC: Low-quality control, MQC: Middle quality control, HQC: High-quality control

Matrix samples stability at −28 ± 5°C for 37 days and −80 ± 5°C

Long-term stock solution stability for the prazosin was determined at a concentration of LQC-HQC level after a storage period of 37 days at −28°C and −80°C in refrigerator. The % mean stability of the prazosin was found to be 100.87% and 99.96% at 28 ± 5°C and 100.72% and 99.81% at 80 ± 5°C, respectively.
Long-term stock solution stability for the polythiazide was determined at a concentration of LQC-HQC level after a storage period of 37 days at −28°C and −80°C in refrigerator. The % mean stability of the polythiazide was found to be 100.41% and 100.01% at 28 ± 5°C and 100.11% and 99.98 at 80 ± 5°C, respectively.

### Table 12: Precision and accuracy results for polythiazide

| Acquisition batch ID | Date   | HQC  | MQC1 | LQC  | LLOQ QC |
|----------------------|--------|------|------|------|---------|
|                      |        | Nominal concentration (ng/mL) |        | Nominal concentration range (ng/mL) | Back calculated concentration (ng/mL) |
|                      |        | 200.000 | 125.000 | 18.750 | 6.250 |
|                      |        | (170.000–230.000) | (106.250–143.750) | (15.938–21.563) | (5.000–7.500) |
|                      |        | 199.89 | 125.18 | 18.678 | 6.156 |
|                      |        | 200.15 | 125.00 | 18.462 | 6.258 |
|                      |        | 199.91 | 126.02 | 18.592 | 6.359 |
|                      |        | 198.93 | 125.09 | 18.188 | 6.626 |
|                      |        | 200.91 | 126.03 | 18.276 | 6.260 |
|                      |        | 201.82 | 125.02 | 18.568 | 6.161 |
| n                    |        | 6      | 6     | 6     | 6     |
| Mean±SD              |        | 200.2677±0.99104 | 125.3880±0.49407 | 18.4607±0.19207 | 6.2093±0.10414 |
| % CV                 |        | 0.49   | 0.39  | 1.04  | 1.68  |
| % mean accuracy      |        | 100.13 | 100.31 | 98.46 | 99.35 |
|                      |        | 201.89 | 125.20 | 18.501 | 6.260 |
|                      |        | 200.88 | 126.19 | 18.663 | 6.259 |
|                      |        | 200.92 | 125.18 | 17.920 | 6.574 |
|                      |        | 198.88 | 125.22 | 18.884 | 6.294 |
|                      |        | 196.90 | 126.16 | 18.669 | 6.262 |
|                      |        | 200.94 | 125.21 | 18.774 | 6.161 |
| n                    |        | 6      | 6     | 6     | 6     |
| Mean±SD              |        | 200.0697±1.83772 | 125.5250±0.49984 | 18.5865±0.34232 | 6.3016±0.14092 |
| % CV                 |        | 0.92   | 0.40  | 1.84  | 2.24  |
| % mean accuracy      |        | 100.03 | 100.42 | 99.03 | 100.83 |
|                      |        | 198.96 | 125.09 | 18.466 | 6.589 |
|                      |        | 200.95 | 125.17 | 18.592 | 6.157 |
|                      |        | 201.62 | 125.24 | 18.688 | 6.055 |
|                      |        | 201.24 | 126.20 | 18.476 | 6.205 |
|                      |        | 201.32 | 125.22 | 18.494 | 6.659 |
|                      |        | 200.59 | 126.24 | 18.389 | 6.225 |
| n                    |        | 6      | 6     | 6     | 6     |
| Mean±SD              |        | 200.7770±0.95631 | 125.5282±0.53988 | 18.5175±0.10589 | 6.3151±0.24732 |
| % CV                 |        | 0.48   | 0.43  | 0.57  | 3.92  |
| % mean accuracy      |        | 100.39 | 100.42 | 98.76 | 101.04 |

Between batch precision and accuracy

| n                       | 18     | 18    | 18    | 18    |
| Mean±SD                 | 200.3714±1.28263 | 125.4804±0.48531 | 18.5156±0.22509 | 6.2753±0.17135 |
| % CV                    | 0.64   | 0.39  | 1.22  | 2.73  |
| % mean accuracy         | 100.19 | 100.38 | 98.75 | 100.41 |

LLOQ: Lower limit of quantitation, LQC: Low-quality control, MQC: Middle quality control, HQC: High-quality control
RESULTS AND DISCUSSION

The method has been developed after performing several trails. In each trail, different columns, mobile phase, and flow rates were selected. The suitable wavelength for quantization was determined in K2 EDTA human plasma and fixed chromatographic conditions, and the developed method is validated as per ICH guidelines; validated results are listed in Tables 3-15 and representative chromatograms present in Figures 1-11.

All the system suitability parameters were within the range and satisfactory as per ICH guidelines.

| Table 13: Recovery of prazosin |
|--------------------------------|
| Acquisition batch ID | HQC | MQC1 | LQC |
| Replicate No. | Unextracted response | Extracted response | Unextracted response | Extracted response | Unextracted response | Extracted response |
| 1 | 60,570 | 59,132 | 30,526 | 29,564 | 4530 | 4462 |
| 2 | 60,294 | 59,512 | 30,243 | 29,498 | 4486 | 4478 |
| 3 | 60,552 | 59,607 | 30,105 | 29,543 | 4529 | 4488 |
| 4 | 60,203 | 59,226 | 30,714 | 29,627 | 4523 | 4446 |
| 5 | 60,156 | 59,707 | 30,369 | 29,528 | 4509 | 4464 |
| 6 | 60,437 | 59,276 | 30,450 | 29,454 | 4509 | 4454 |
| n | 6 | 6 | 6 | 6 | 6 | 6 |
| Mean±SD | 60,369±177.24 | 59,410±230.88 | 30,401±214.29 | 29,536±58.90 | 4514±16.71 | 4465±15.42 |
| % CV | 0.29 | 0.39 | 0.70 | 0.20 | 0.37 | 0.35 |
| % mean recovery | 98.41 | 97.15 | 98.91 |
| Overall % mean recovery | 98.160 |
| Overall SD | 0.9074 |
| Overall % CV | 0.92 |

LQC: Low-quality control, MQC: Middle quality control, HQC: High-quality control

| Table 14: Recovery of polythiazide |
|-----------------------------------|
| Acquisition batch ID | HQC | MQC1 | LQC |
| Replicate No. | Unextracted response | Extracted response | Unextracted response | Extracted response | Unextracted response | Extracted response |
| 1 | 33,087 | 32,484 | 16,831 | 16,254 | 2518 | 2429 |
| 2 | 33,032 | 32,411 | 16,845 | 16,370 | 2520 | 2473 |
| 3 | 33,008 | 32,511 | 16,774 | 16,228 | 2527 | 2433 |
| 4 | 33,731 | 32,335 | 16,966 | 16,235 | 2536 | 2453 |
| 5 | 33,053 | 32,314 | 16,871 | 16,324 | 2542 | 2464 |
| 6 | 33,182 | 32,407 | 16,813 | 16,290 | 2520 | 2424 |
| n | 6 | 6 | 6 | 6 | 6 | 6 |
| Mean±SD | 33,182±275.62 | 32,410±78.14 | 16,850±65.46 | 16,284±55.62 | 2527±9.85 | 2446±20.22 |
| % CV | 0.83 | 0.24 | 0.39 | 0.34 | 0.39 | 0.83 |
| % mean recovery | 97.67 | 96.64 | 96.79 |
| Overall % mean recovery | 97.033 |
| Overall SD | 0.5598 |
| Overall % CV | 0.58 |

LQC: Low-quality control, MQC: Middle quality control, HQC: High-quality control
The % CV for system suitability test was in the range of 0.89 for RT of prazosin, 1.15 for RT of polythiazide, and 0.74% for the area ratio (analyte area/IS area) of celecoxib.

Due to the auto-sampler was investigated by injecting a sequence of un-extracted and extracted samples. Results demonstrated that no significant carryover was observed during this experiment.

Matrix effect is played a key role in the assessment of pharmacokinetic studies. It was expressed as internal standard normalized matrix factor and it was varied from 0.90 to 0.99 which was close to 1 which indicates that there is no ionization suppression or enhancement in plasma samples.

QC samples

The chromatography observed during the course of prazosin and polythiazide which was acceptable and representative chromatograms of standard blank, standard zero (standard blank with internal standard) QC-LLOQ, QC-L, QC-M1, QC-M2, and QC-H samples sample are shown in Figures 3-9, respectively.

This indicates linearity of results and an excellent correlation between peak area ratios for each concentration of analytes.

The intraday and interday accuracy and precision of plasma samples were assessed and excellent mean % accuracy was obtained with range varied from 98.16 to 100.42% and 98.75 to 100.41% for intraday and 98.83 to 100.03 and 99.70 to 99.86 for interday, respectively. The precision (%CV) of the analytes and plasma samples was calculated and found to be 0.70–4.34% and 0.39–2.73% for intraday and 0.21–2.83% and 0.22–0.74% for interday, respectively. The results are summarized in Tables 11 and 12.

The overall % mean recovery for prazosin and polythiazide was found to be 98.16% and 98.23%.

The overall % mean recovery for celecoxib was found to be 98.11%.

CONCLUSION

Based on the results obtained in this study, it is concluded that the present validated method can be successfully applied for the estimation of prazosin and polythiazide in human plasma over the concentration range of 12.5–500 ng/ml of prazosin and 6.25–250 ng/ml of polythiazide. The method for the determination of prazosin and polythiazide in human plasma using HPLC detection met the acceptance criteria with respect to selectivity, precision, accuracy, linearity, and recovery.

REFERENCES

1. Piascik MT, Perez DM. Alpha1-adrenergic receptors: New insights and directions. J Pharmacol Exp Ther 2001;298:403-10.
2. Madden CJ, Tupone D, Cano G, Morrison SF. α2 adrenergic receptor-mediated inhibition of thermogenesis. J Neurosci 2013;33:2017-28.
3. Giovannitti JA, Thomas SM, Crawford JJ. Alpha-2 adrenergic receptor agonists: A review of current clinical applications. Anesth Prog 2015;62:31-9.
4. Roush GC, Abdelfattah R, Song S, Ernst ME, Sica DA, Kostis JB. Hydrochlorothiazide vs chlorothalidone, indapamide, and potassium-sparing/hydrochlorothiazide diuretics for reducing left ventricular hypertrophy: A systematic review and meta-analysis. J Clin Hypertens (Greenwich) 2018;20:1507-15.
5. Eswarudu MM, Rao AL, Vijay E. Bioanalytical method development and validation for simultaneous determination of prazosin and polythiazide drugs in spiked human plasma by RP-HPLC. Int J Pharm Chem Biol Sci 2019;9:61-70.
6. Dokladalova J, Coco SJ, Lemke PR, Quercia GT, Korst JJ. Determination of polythiazide and prazosin in human plasma by high-performance liquid chromatography. J Chromatogr Biomed Sci Appl 1981;224:33-41.
7. Sultana N, Arayne MS, Shah SN. Liquid chromatographic analysis of prazosin in API, dosage form and serum: Application to drug-metal interaction studies. J Chromatogr Sep Tech 2013;4:197.
8. Pawar VT, Pawar SV, More HN, Kulkarni AS, Gaikwad DT. RP-HPLC method for simultaneous estimation of cilnidipine and chlorothalidone. Res J Pharm Technol 2017;10:3990-6.
9. Sonawane LV, Poul BN, Usmale SV, Waigmare PV, Surwase LH. Bioanalytical method validation and its pharmaceutical application-a review. Pharm Anal Acta

Table 15: Recovery – internal standard (celecoxib)

| Acquisition batch ID | Date | S. No. | Unextracted area ratio | Extracted area ratio |
|----------------------|------|--------|------------------------|----------------------|
|                      |      |        |                        |                      |
| 1                    |      |        | 70,491                 | 69,335               |
| 2                    |      |        | 70,130                 | 69,594               |
| 3                    |      |        | 70,493                 | 69,555               |
| 4                    |      |        | 70,561                 | 69,218               |
| 5                    |      |        | 70,498                 | 69,002               |
| 6                    |      |        | 70,558                 | 69,113               |
| n                    |      |        |                        |                      |
| Mean±SD              |      |        | 70,455.2±162.52        | 69,269.5±280.14      |
| % CV                 |      |        | 0.23                   | 0.40                 |
| % mean recovery      |      |        |                        | 98.32                |

Table 15: Recovery – internal standard (celecoxib)
Dharmamoorthy, et al.: RP-HPLC method for the estimation of prazosin and polythiazide

10. Darkunde SL, Borhade RN. Bioanalytical method validation: A quality assurance auditor view point. Asian J Pharm Technol Innov 2017;5:59-60.
11. Tijare LK, Rangari NT, Mahajan UN. A review on bioanalytical method development and validation. Asian J Pharm Clin Res 2016;9:6-10.
12. Kumar A, Kishore L, Kaur N, Nair A. Method development and validation: Skills and tricks. Chron Young Sci 2012;3:3-11.
13. Latha EP, Sailaja B. Bioanalytical method development and validation by HPLC: A review. J Med Pharm Innov 2014;1:1-9.
14. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2 (R1). Current Step 4 Version, Complementary Guideline on Methodology. Geneva: ICH Expert Working Group; 1996.
15. ICH Expert Working Group. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Validation of Analytical Procedures: Text and Methodology. Geneva: ICH Expert Working Group; 1996.

Source of Support: Nil. Conflicts of Interest: None declared.