**IJPSR (2018), Volume 9, Issue 1** **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 27 April, 2017; received in revised form, 12 July, 2017; accepted, 25 July, 2017; published 01 January, 2018

# SIMULTANEOUS ESTIMATION OF HYDROCHLORTHIAZIDE AND CANDESARTAN CILEXTIL IN BULK AND PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC PDA METHOD

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**Keywords:**

Candesartan cilextil, Hydrochlorothiazide, Phenomenex C18RP column, Method Validation, Photo diode array (PDA detection)

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**ABSTRACT:** Hydrochlorothiazide and Candesartan cilextil are used in combination for the treatment of hypertension. The aim of the present investigation was to develop and validate a simple, efficient, economical RP-HPLC method for the simultaneous estimation of Hydrochlorothiazide and Candesartan cilextilin bulk and dosage forms. Chromatographic analysis was performed on Phenomenex C18RP column (250 x 4.6mm; 5µm) with mobile phase containing 10mM ammonium acetate: acetonitrile (65:35% v/v) at a flow rate of 1.2mL/min and eluents were monitored at 262nm. The retention times of Hydrochlorothiazide and Candesartan cilextil were 3.6 min and 6.8 min respectively and showed a good linearity in the concentration range of 7.8-18.8µg/mL for Hydrochlorothiazide and 10-26µg/mL for Candesartan cilextil with a Regression coefficient ($R^2$) of 0.996 and 0.998 respectively. The validation parameters performed were specificity, linearity, limit of detection, limit of quantification, precision, robustness and stability. Validation parameters fulfilled regulatory requirements in all cases. The percentage recoveries ranged in between 98 - 102 (RSD < 2). The RP-HPLC method developed can be successfully used for the simultaneous estimation of Hydrochlorothiazide and Candesartan cilextilin bulk and pharmaceutical dosage forms.

**INTRODUCTION:** CST is an orally active non-peptide tetrazole derivative and is chemically 2-ethoxy- 3- [21- (1H- tetrazol- 5- yl) biphenyl-4yl methyl] -3H-benzoimiadazole-4-carboxylic acid 1-cyclohexyloxycarbonyloxy ethyl ester.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(1).150-57

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(1).150-57

CST selectively blocks the binding of angiotensin II to AT1 in many tissues including vascular smooth muscle and the adrenal glands. HCTZ chemically is 6-chloro-3,4-dihydro-2H-1,2,4-benzo thiadiazine-7-sulfonamide 1,1-dioxide [1 - 3]. HCTZ is frequently used for the treatment of hypertension, HCTZ is believed to lower peripheral vascular resistance [2, 3]. Fixed-dose combination of anti-hypertensive drugs can simplify dosing regimens, improve compliance, improve hypertension control, decrease dose-dependent side effects and reduce cost as the first-line treatment of hypertension [1 - 3].

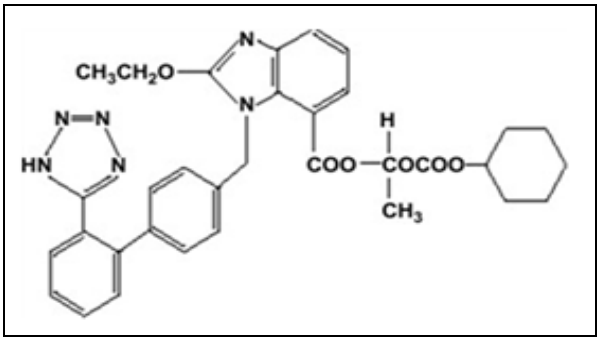


**FIG. 1: CANDESARTAN CILEXITIL**

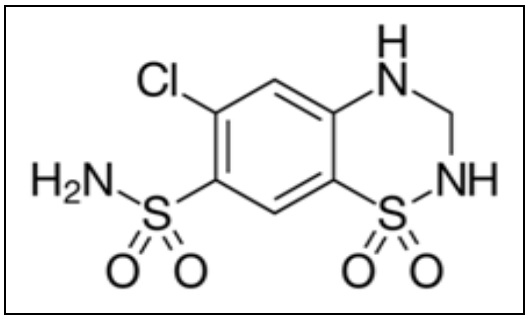


**FIG. 2: HYDROCHLOROTHIAZIDE**

Literature review reports for the quantitative determination of HCTZ and CST in combination with other drugs like Losartan [4], Amlodipine besylate [5, 17],Lisinopril [6], Irbesartan [7], Valsartan [8], Olmesartan [9, 17], Telmisartan [10] by HPLC, stability indicating method [11] Spectrophotometry [12] and Fluorimetry [13] were reported. Literature survey reveals that there were only few validated RP-HPLC-PDA methods reported for the simultaneous estimation of HCTZ and CST in bulk and tablet dosage forms [14 - 16]. However, the reported HPLC methods for the estimation of HCTZ and CST in combination with other anti-hypertensive and diuretics used non-volatile buffers like phosphate buffers in mobile phase which are not LC-MS compatible. Hence, the present investigation was aimed at developing a validated RP-HPLC-PDA method for the simultaneous estimation of HCTZ and CST in bulk and dosage forms.

**MATERIAL AND METHODS:** CST and HCTZ were procured as a gift samples from Hetero drugs Ltd, India. Ammonium acetate, water and methanol were purchased from E. Merck, Mumbai, India. All the solvents and reagents used were of HPLC grade. Tablet formulation contains HCTZ (25mg) and CST (32mg) was punched by direct compression technique, due to the unavailability of tablet formulation in Indian market.

**Equipment:** A Shimadzu Prominence HPLC system provided with DGU-20A3 degasser, LC-20AD binary pumps, SIL-20AHT auto sampler, and SPD-M20A PDA detector was used. Data acquisition was carried out using LC solutions software. The chromatographic analysis was performed using Phenomenex C18 RP column (250 × 4.6mm; 5µm) as a stationary phase.

**Chromatographic Conditions:** Mobile phase was pumped at a flow rate of 1.2mL/min using a binary mixture of 10mM ammonium acetate: acetonitrile (65:35% v/v) in isocratic mode. The mobile phase was filtered through nylon disc filter of 0.45µm (Millipore) and sonicated for 3 min before use for degassing. The injection volume of 10µL was given and the detection wavelength for HCTZ and CST was set at 262nm and the separation was achieved at ambient temperature.

**Preparation of Stock and Standard Solutions:** The stock solutions of concentration 1000ppm were prepared for HCTZ and CST by dissolving 10mg of the reference standards separately using methanol as diluents in 10ml volumetric flasks. Further sub stocks were prepared by consuming the appropriate volume of the stock solution and diluted with ammonium acetate to get the required concentrations of the working standard solutions at a concentration range of 7.8-18.8µg/mL HCTZ and 10-26µg/mL of CST.

**Validation of the HPLC Method:** The proposed method was validated as per ICH guidelines.

**Linearity:** Calibration graphs were constructed across the range of the analytical procedure with a minimum of five concentrations. A series of standard dilutions of HCTZ and CST were prepared over a concentration range of 7.8-13.8µg/mL (7.8, 10.8, 13.8, 15.8, 18.8 µg/mL) and 10-26 µg/mL (10, 14, 18, 22, 26 µg/mL) respectively from stock solution and injected in triplicate (n = 3). Linearity was evaluated by a plot of peak areas as a function of analyte concentration, and the test results were evaluated by appropriate statistical methods where by slope, intercept, and regression ($R^2$) and correlation coefficients (R) were calculated and the data was given in **Table 1**.

**Precision:** Precision is the measure of closeness of the data values to each other for a number of measurements under the same analytical conditions. Repeatability was assessed by using a minimum of six determinations at 100% of the test concentration. The standard deviation and the relative standard deviation were reported for precision. Less than 2% RSD for peak areas indicates the precision of the developed method.

**Specificity:** Specificity is a measure of the degree of interference in the analysis of the complex sample mixtures such as analyte mixed with the formulation excipients or the known impurities. Specificity of the method was carried out by comparing chromatogram of the placebo with that of the sample for checking any interference peaks. Absence of interference peaks of excipients in the tablet indicates the specificity of the proposed method.

**Accuracy:** Accuracy was established across the specified range of the analytical procedure. Accuracy of the method was tested by spiking 80, 100, and 120% of standards to 7.8µg/mL of HCTZ and 10µg/mL of CST test solution. These solutions were analyzed by developed method in triplicate. The % recovery and % RSD were calculated at each level of addition.

**Limit of Detection (LOD) and Limit of Quantification (LOQ):** LOD and LOQ were calculated based on calibration curves and were expressed as LOD = (3.3 ×σ)/m; LOQ= (10.0×σ)/m (Where, σ is the standard deviation of the y-intercepts of the three regression lines and m is mean of the slopes of the three calibration curves).

**Robustness:** To determine the robustness of the method developed, the experimental conditions were deliberately altered and the chromatographic parameters *viz.*, capacity factor, tailing factor, theoretical plate number and % assay were recorded. The flow rate of the mobile phase was 1.2mL/min. To study the effect of flow rate, the flow rate was changed by 20%. The effect of wavelength was studied by changing wavelength by ±1nm and the effect of mobile phase was studied by changing the mobile phase by ±2% v/v of organic phase.

**System Suitability:** System suitability was carried out by injecting a standard concentration by increasing the injection volumes in the range of 10-50µL. The system suitability test parameters were noted and % RSD was calculated.

**Assay:** Twenty tablets were weighed and finely powdered and the powder equivalent to 10mg was accurately weighed and transferred into a 10mL volumetric flask. Dissolve in 5mL methanol and vortexed for 5min and made up to the mark with methanol, centrifuged and filtered using Nylon disposable syringe filter (13mm, 0.45µm). An aliquot of filtrate was diluted with ammonium acetate and analyzed in triplicate. The amount present in the each tablet was quantified by comparing the area of HCTZ and CST standards with that of the sample.

**Preparation of HCTZ and CST Tablets by Direct Compression Technique:** Due to the unavailability of marketed tablet formulation for HCTZ and CST combination in Indian market, tablets were prepared in house by direct compression method using carboxy methyl cellulose calcium, hydroxyl propyl cellulose, lactose monohydrate, corn starch and polyethylene glycol 8000 as excipients. All the ingredients were passed through sieve # 80 before mixing. Initially drugs and filler were mixed thoroughly, followed by the addition of required amount of colloidal silicon dioxide and mixed thoroughly for 5min in a poly bag. Required amounts of SSG was added and mixed thoroughly for 5 min. Finally, the resultant powder blend (for 20 tablets) of the required formulation was compressed on single punch tablet press (Cadmach, India) using 10mm punches (round shape) maintaining a hardness range of compressed tablets between 4 - 5 kg/cm$^2$.

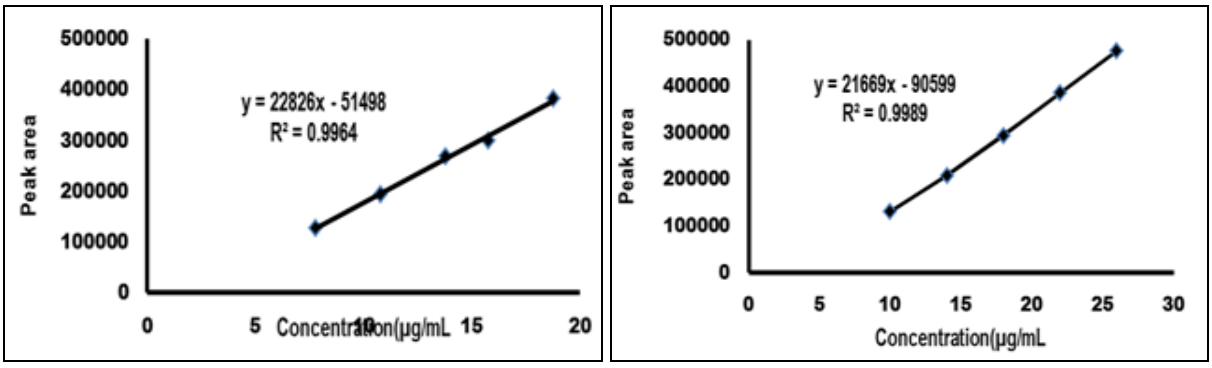
**RESULTS AND DISCUSSION:** Various HPLC, UV methods were published for the estimation of HCTZ and CST in combination with other drugs like Losartan, Amlodipine besylate, Lisinopril, Irbesartan, telmisartan and olemesartan but only few methods were reported on the simultaneous quantification of HCTZ and CST in bulk and dosage forms using phosphate buffers. Hence, the present investigation was aimed to develop a simple, economical RP-HPLC-PDA method for the

determination of HCTZ and CST in bulk and dosage forms.

**Method Development:** Mobile phase optimization was initially carried with Phenomenex C18RP column (250 x 4.6 mm) using 10mM ammonium acetate and methanol as mobile phase at different ratios at 1mL/min flow rate, the HCTZ and CST were eluted at 3.17 and 14.90 min respectively but asymmetrical peaks were seen. In the final trail, mobile phase ratio was changed to 10mM ammonium acetate and acetonitrile (65:35%v/v) using Phenomenex C18RP column at a flow rate of 1.2mL/min and peaks were monitored at 262nm. Under these conditions, HCTZ and CST peaks were eluted with in desired run time and the peak symmetry was satisfactory. The retention times were 3.65 min and 6.49 min respectively for HCTZ and CST. For quantitative analytical purpose wavelength was set at 262 nm, which provided better reproducibility with no interference. The method was validated as per ICH guidelines. The peak purity index was found to be greater than 0.9999 and peak purity profiles were shown in **Fig. 7** along with UV spectra.

**Method Validation:** The method has been validated as per ICH - Guidelines for following parameters.

**Linearity:** The range for the reliable quantification was set at the concentrations of 7.8-18.8µg/mL and 10-26µg/mL of HCTZ and CST respectively. This range was selected based on 80-120% of the standard concentration used for accuracy and were analyzed in triplicate. Peak areas and concentrations were subjected to least square regression analysis to calculate regression equation. The regression coefficient ($R^2$) was found to be 0.996 and 0.998 indicating a linear response over the range used. The data from the calibration curve was given in **Table 1** and shown in **Fig. 3**.




**CALIBRATION CURVE OF HCTZ** **CALIBRATION CURVE OF CST**

**FIG. 3: CALIBRATION CURVE OF HCTZ AND CST**

**Precision:** Precision was carried out in terms of repeatability. Repeatability of standard application was assessed by using six replicates at a concentration of 10.8 µg/mL of HCTZ and 14 µg/mL of CST. The data was given in **Table 1** shown in **Fig. 4**. The % RSD was found to be NMT 2, indicating the repeatability of the method.

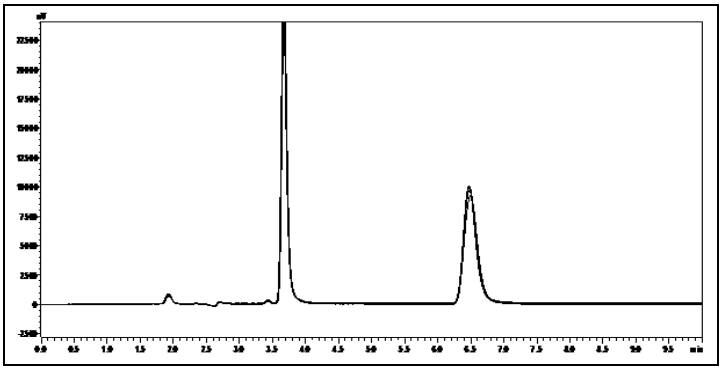


**FIG. 4: OVERLAY OF SYSTEM PRECISION CHROMATOGRAMS OF STANDARD MIXTURES (7.8µg/ml OF HCTZ AND 10µg/ml OF CST) WITHOUT BASE SHIFT**

**Specificity:** The specificity of the method was established by injecting the solutions of diluent, placebo, standard, test sample (formulation) individually to examine for any interference, from the overlay of chromatograms as shown in **Fig. 5** and **6** and the 3D plots of placebo and test samples it can be inferred that there were no co-eluting peaks at the retention time of HCTZ and CST, this shows that peak of analyte was pure and the excipients in the formulation did not interfere with the analysis and the peak purity indices for sample and standard was found to be greater than 0.999 and this confirms specificity of the method.

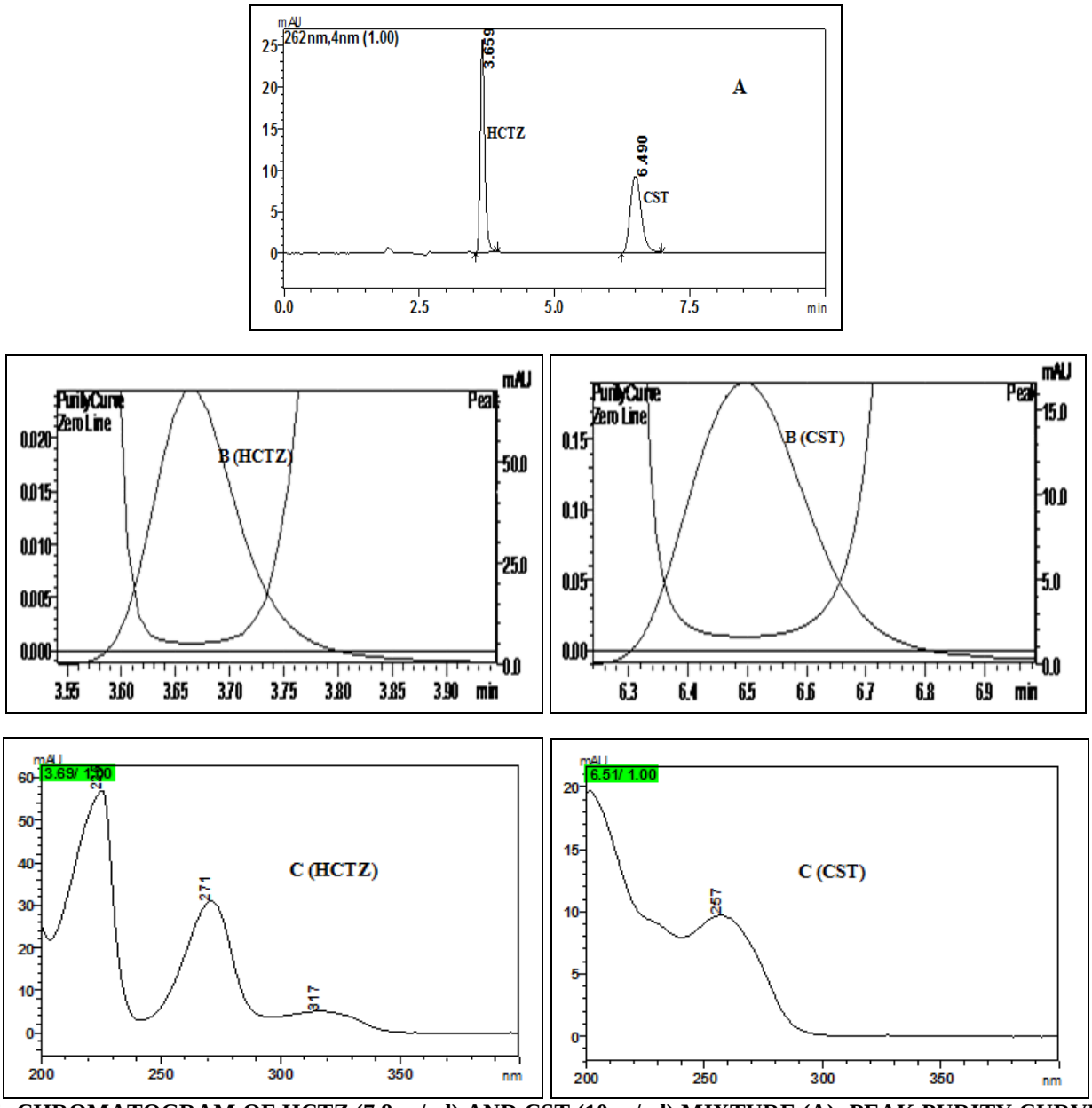



**FIG. 5: CHROMATOGRAM OF HCTZ (7.8µg/ml) AND CST (10µg/ml) MIXTURE (A); PEAK PURITY CURVES OF HCTZ AND CST (B) AND (C) - UV SPECTRA**

**Accuracy:** Accuracy of the proposed method was ascertained by performing recovery studies using external standard addition method by spiking the known quantities of standard at 80%, 100% and 120% to the test solution of 10ug/mL. These solutions were analyzed in triplicate in each level of addition. The % RSD and the % recovery were within the acceptable limit in all cases. It is evident from the results of accuracy study given in **Table 1**, that the proposed method enables very accurate quantitative estimation of HCTZ and CST.

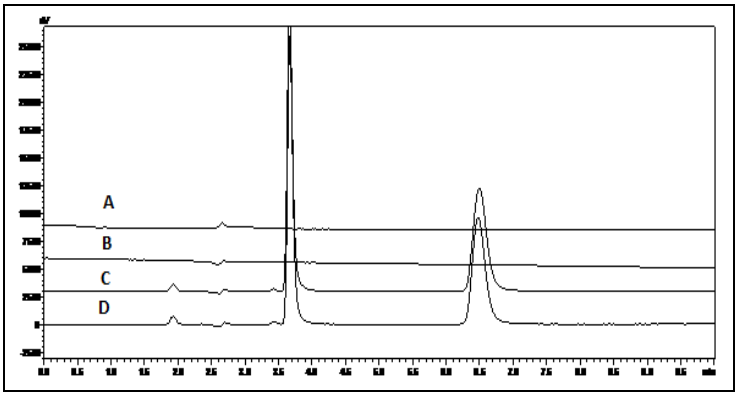



**FIG. 6: OVERLAY OF THE DILUENT (A) PLACEBO (B) STANDARD (C) AND SAMPLE (D) CHROMATOGRAMS**

**Assay:** Assay of tablets containing 25mg of HCTZ and 32mg of CST was performed by the proposed method and the % assay of the formulation was calculated as an average of 3 determinations, which was about 100.29 ± 0.45 and 100.52 ± 0.44 for HCTZ and CST respectively. These results indicate that the present HPLC method can be successfully used for the assay of HCTZ and CST in bulk and combination dosage forms.

**TABLE 1: LINEARITY, PRECISION, ACCURACY AND ASSAY DATA OF HCTZ AND CST**

| Validation data of HCTZ and CST | | | |
|---|---|---|---|
| | **Parameters** | **HCTZ** | **CST** |
| | Range | 7.8-18.8µg/mL | 10-26µg/mL |
| | Regression equation | Y=22826x-51498 | Y=21669x-90599 |
| Linearity (n = 3) | Regression coefficient ($R^2$) | 0.996 | 0.998 |
| | Correlation coefficient (r) | 0.998 | 0.999 |
| Accuracy (n = 3) | % Level of Addition | Mean % Recovery (RSD) | Mean % Recovery (RSD) |
| | 80 | 94.55(0.45) | 100.59 (0.15) |
| | 100 | 101.40(0.33) | 100.67 (0.51) |
| | 120 | 103.86(1.08) | 100.86 (1.78) |
| Precision (n = 6) | | HCTZ | CST |
| System Precision | Average Peak area of the standard sample (RSD) | 147786 (0.82) | 131045 (0.66) |
| Method Precision | Average peak area of the Assay sample (RSD) | 112120(0.63) | 143443 (1.06) |
| Assay in mg (n = 3) | Mean ± SD | 25.08 (0.41) | 32.26 (0.99) |

**Limit of Detection (LOD) and Limit of Quantification (LOQ):** LOD and LOQ were determined based on statistical calculation from the calibration curves, where LOD = (3.3 ×σ)/m; LOQ= (10.0×σ)/m (σ is the standard deviation of the y-intercepts of the three regression lines and m is mean of the slopes of the three calibration curves). The limit of detection for HCTZ and CST was found to be 0.803µg/mL and 2.435µg/mL respectively; the drug peak can be detected without any base line noise at this concentration. The limit of quantification for HCTZ and CST was found to be 0.377µg/mL and 1.143µg/mL respectively.

**Robustness:** As part of the robustness, deliberate changes in the flow rate, mobile phase and wavelength were made to evaluate the impact on the method. Retention times were significantly changed with flow rate, mobile phase and there was no alteration of the retention time was observed with the change in wavelength. % assay was also estimated under these changed conditions and the results were given in **Table 2**. The symmetry parameters like capacity factor, theoretical plate number and assay were significant and were within the limits. These results indicate the method is robust in terms of change in flow rate, mobile phase and wavelength.

**System Suitability:** System suitability testing is an integral part of the analytical procedure. System suitability studies were carried out by injecting the standard concentration of 7.8µg/mL and 10µg/mL for HCTZ and CST respectively at injection volumes ranging from 10µL to 50µL respectively.

The RSD values for system suitability test parameters like retention time [$R_t$ = 3.7 (0.5)], tailing factor [$T_f$ = 1.4 (1.5) of HCTZ], [$R_t$ = 6.56 (0.17)], tailing factor [$T_f$ =1.33 (1.18) of CST] and theoretical plate number [8603 (0.43) for HCTZ and 5755 (0.34) for CST] were less than 2% indicating the present conditions were suitable for the analysis of HCTZ and CST in tablets.

**TABLE 2: ROBUSTNESS DATA OF HCTZ AND CST**

| Drug | Composition | Retention time (min) | Theoretical plates (N) | Tailing factor | % Assay |
|---|---|---|---|---|---|
| **% Mobile phase composition** | | | | | |
| HCTZ | 63:37 | 3.50 | 8136 | 1.42 | 98.7 |
| | 65:35 | 3.64 | 8191 | 1.45 | 99.7 |
| | 67:33 | 3.85 | 8463 | 1.42 | 98.5 |
| CST | 63:37 | 5.26 | 6926 | 1.33 | 101.1 |
| | 65:35 | 6.68 | 6119 | 1.27 | 101.9 |
| | 67:33 | 8.90 | 5355 | 1.16 | 101.5 |
| **Wavelength (nm)** | | | | | |
| HCTZ | 260 | 3.64 | 8191 | 1.45 | 99.4 |
| | 262 | 3.64 | 8183 | 1.45 | 99.7 |
| | 264 | 3.64 | 8190 | 1.45 | 99.8 |
| CST | 260 | 6.68 | 6119 | 1.27 | 101.6 |
| | 262 | 6.68 | 6119 | 1.27 | 101.9 |
| | 264 | 6.68 | 6123 | 1.27 | 101.7 |
| **Flow rate (mL/min)** | | | | | |
| HCTZ | 1.1 | 3.98 | 8346 | 1.45 | 99.4 |
| | 1.2 | 3.64 | 8191 | 1.45 | 99.7 |
| | 1.3 | 3.38 | 7252 | 1.43 | 100.4 |
| CST | 1.1 | 7.17 | 5961 | 1.27 | 100.6 |
| | 1.2 | 6.68 | 6119 | 1.27 | 101.9 |
| | 1.3 | 6.23 | 6110 | 1.26 | 101.4 |

**Stability of the Stock Solution:** The stability of the stock solution was determined by analyzing the samples under refrigeration (8 ± 1 °C) at different time intervals up to 48hrs. The % variation in assay values at different time intervals were found to be less than 2 of the initial zero time interval solution, thus indicating that the solutions were stable for a period of 48hrs when stored at 8 ± 1 °C and the results were given under **Table 3**.

**TABLE 3: STABILITY DATA FOR HCTZ AND CST**

| Time interval | % Variation in peak area | |
|---|---|---|
| | HCTZ | CST |
| 0 days | 0 | 0 |
| 48 hrs | 0.880 | 1.030 |

**CONCLUSION:** The proposed method has the advantage of simplicity and convenience for the simultaneous estimation of HCTZ and CST by RP-HPLC-PDA method in bulk and pharmaceutical dosage forms. The method was validated as per International Conference on Harmonisation (ICH) Guidelines, and found to be applicable for routine quality control analysis for the simultaneous estimation of HCTZ and CST in using isocratic mode of elution. The results of linearity, precision, accuracy and specificity, proved to be within the limits. The method provides selective quantification of HCTZ and CST without interference from diluents and placebo. Therefore, this method can be employed in quality control to estimate the amount of HCTZ and CST in bulk and pharmaceutical dosage forms.

**ACKNOWLEDGEMENT:** The authors are thankful to Hetero Drugs Ltd, India for providing gift samples and also to the Siddhartha Academy of General and Technical Education, Vijayawada, for providing necessary facilities to carry out the research work**.**

**CONFLICT OF INTEREST:** The authors declare no conflict of interest.

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**How to cite this article:**
Srikalyani V, Madhuri T, Sareesh K and Kumar RM: Simultaneous estimation of Hydrochlorthiazide and Candesartan cilextil in bulk and pharmaceutical dosage forms by RP-HPLC PDA method. Int J Pharm Sci Res 2018; 9(1): 150-57.doi: 10.13040/IJPSR.0975-8232.9(1). 150-57.