## 3.1. Introduction

At various stages of formulation development, analytical methods serve as an important tool for the quantification of the drug. They are helpful in establishing critical formulation characteristics like entrapment efficiency, loading efficiency, stability, in vitro drug release behavior and in vivo bioavailability etc [1]. The most commonly used analytical methods for quantitative estimation of drugs and metabolites in different solvents, media and biological samples such as blood, serum, plasma, or urine includes UV-Spectrophotometry, High-performance liquid chromatography (HPLC), Thin layer chromatography (TLC), Gas chromatography (GC), combined GC, LC and mass spectrometric (MS) procedures such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS etc [2].

The key requirement in the practice of an analytical method development is validation [3]. The purpose of validation of an analytical method is to ensure that every impending measurement/analysis will be close enough to the unknown true value for the content of the analyte in the sample [1]. The various parameters of method validation include Linearity, Accuracy, Precision, Range, Robustness, Ruggedness, Limit of Quantification (LOQ) and Limit of Detection (LOD) [4].

## 3.2. Materials and Instruments

### 3.2.1. Materials

Paclitaxel (PTX) and Carboplatin (CBP) were obtained as a gift sample from Sun Pharmaceutical Industries Ltd, Vadodara. Acetonitrile and Methanol of HPLC grade were purchased from S.D. Fine Chemicals, Mumbai. Triple distilled water was prepared in laboratory.

### 3.2.2. Instruments

The chromatographic system was LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector with a 20µl fixed loop and SPD-20A Prominence UV-Visible detector (Shimadzu, Kyoto, Japan). The separation was performed on a C-18 column (250 mm [L] x 4.6 mm [ID] x 5 µm [particle size]; Phenomenex, Torrance, USA).

Chromatographic data were recorded and processed using LC Solution software. Other instruments used are Electronic weighing balance (Shimadzu, Japan), Ultrasonic bath 120W (Vibronics Co. Pvt. Ltd., Mumbai, India).

## 3.3. Analytical Method for Estimation of PTX

Estimation of PTX by HPLC has been reported in USP29-NF 24. The same method was adapted here with a few modifications for estimation of PTX in formulation.

### 3.3.1. Chromatographic setup

Method: RPHPLC method

Column specifications: Supelco® C18 [25cm (L) × 4.6mm (ID) with 5µm (PS)]

Mobile Phase: Acetonitrile: Water (60:40)

Flow Rate: 1ml/min

Wavelength for Detection: 227nm

Sample injection volume (loop size): 20µl

Diluent: Methanol

Run time: 10 min

### 3.3.2. Mobile Phase Preparation

Triple distilled water and Acetonitrile were mixed according to proportions described in the chromatographic setup. The solvent mix was filtered through 0.22 µm membrane filter (Ultipor® Nylon 66 membrane filter, Pall Life Sciences, USA), transferred to reagent bottle and degassed using bath sonication for 10 minutes.

### 3.3.3. Preparation of Stock solution

Accurately weighed quantity (10 mg) of PTX was transferred to 10 mL calibrated volumetric flasks and dissolved in Methanol. The final volume was made up to 10 mL with methanol. The resulting solution (1000 µg/ml) was further diluted 10 times to obtain stock solution (100 µg/ml).

### 3.3.4. Preparation of standard solutions

40 to 200 µl of the stock solution was transferred to 2.0 ml microcentrifuge tubes using micropipette. The final volume was made upto 2 ml with methanol to obtain standard solutions of concentrations of 2, 4, 6, 8 and 10 µg/ml.

### 3.3.5. Preparation of calibration plot

The mobile phase was set to a flow rate of 1.0 ml/min at room temperature. 20 µl of each standard solution was injected into the HPLC system through rheodyne injector using Hamilton syringe. The absorption wavelength was set to 227 nm and the chromatogram was run for 10 minutes. The area of the peak was obtained from the software. Calibration graph was drawn by plotting peak area versus drug concentration.

### 3.3.6. Validation of HPLC method

Following method development, method validation is required. Recommendations for pharmaceutical analysis may be found in the ICH guideline Q2 "Validation of Analytical Procedures: Text and Methodology"[4]. Adapted from the terms in FDA Guidance for Industry on Process Validation [5] "method validation" is also called "method performance qualification". This guideline was revised in 2011 to better align with the US Food and Drug Administration's "Pharmaceutical Current Good Manufacturing Practices (cGMPs) for the 21st Century – a Risk Based Approach" initiative and the ICH Q8, 9 and 10, and comprised a product life cycle concept. Consequently, Ermer and Ploss [6] define method validation as "the collection and evaluation of data and knowledge from the method design stage throughout its life cycle of use which established scientific evidence that a method is capable of consistently delivering quality data".

#### *3.3.6.1. Linearity*

Linearity of an analytical method is the capability to produce the test results that are directly or by well-defined transformation, proportional to the concentration of the analyte in the samples within the given range. Different concentrations in the range of

2 to 10 µg/mL were prepared from the stock solution of 100 µg/mL by suitable dilutions using methanol. Linear regression was performed using MS office excel software and the correlation coefficient ($R^2$) was generated to demonstrate linearity.

### 3.3.6.2. Precision

It refers to the extent of variability of a group of measurements observed under similar conditions. Precision provides an indication of random errors and is generally subdivided into two cases: repeatability and reproducibility, which were determined by calculating RSD (Relative standard deviation) of inter-day and intra-day determinations. The inter-day and intra-day precisions were measured in terms of % Relative Standard Deviation (% RSD). The 3 concentrations (2 µg/ml, 6 µg/ml and 10 µg/ml) were selected and experiment was repeated 3 times in a day for intra-day and on 3 different days for inter-day precision. Criteria: % RSD ≤ 2 % as per the requirements of ICH guidelines.

### 3.3.6.3. Accuracy

Accuracy refers to the closeness of an individual observation or mean of the observations to true value. Accuracy was performed by standard addition method in which known amounts of standard drug (80%, 100% and 120% of 5 µg/mL) were added to the pre-analyzed samples and the measurements done in triplicate. Accuracy was assessed by calculating the percent recovery of active ingredient.

### 3.3.6.4. Sensitivity

Calibration plot was repeated for 3 times and the standard deviation (SD) of the intercepts was calculated. The average of the slope of calibration plot was calculated. Then Limit of detection (LOD) and limit of quantification (LOQ) were measured as follows

$\text{LOD} = 3.3 * (\sigma/s)$

$\text{LOQ} = 10.0 * (\sigma/s)$

Where, $\sigma$ = Standard deviation of intercepts

S = Slope of calibration plot

## 3.4. Results and Discussion

### 3.4.1. Preparation of calibration plot

**Table 3-1: Mean Peak area values for calibration plot for PTX**

| Concentration of PTX (μg/ml) | Peak Area |
|---|---|
| **0** | 0 |
| **2** | 87630.67 ± 897.7 |
| **4** | 167551.7 ± 975.9 |
| **6** | 251423.3 ± 1013.9 |
| **8** | 327721 ± 948.3 |
| **10** | 420412.3 ± 1273.5 |

The experiment was performed in triplicate and the values of area corresponding to the peaks are represented as mean ± SD.

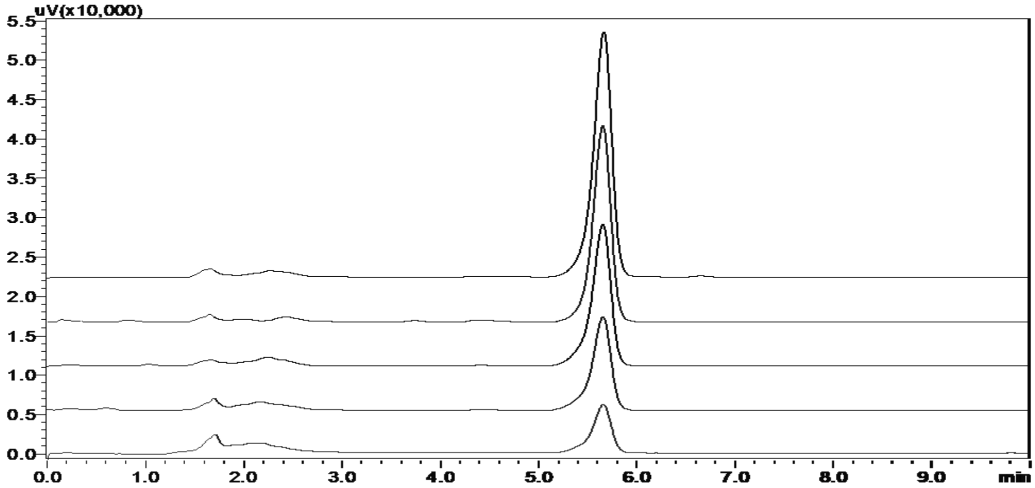

**Fig. 3-1: Overlay chromatogram for PTX**

The chromatograms obtained against standard concentrations were depicted in **Fig. 3-1**. Sharp, symmetric peaks were observed with retention time of 5.6 min at 227 nm detection wavelength and 1 ml/min flow rate.

### 3.4.2. Validation of HPLC Method

#### *3.4.2.1. Linearity*

The mean peak area values with the standard deviation are represented in **Table 3-1** and **Fig. 3-2**. The value of correlation coefficient $R^2$ = 0.9995 indicated that area and concentration of the drug was in significant linear correlation which said to obey beer's law in the range of 2 to 10 µg/ml. The results indicated the linearity of the method for selected concentration range for calibration.

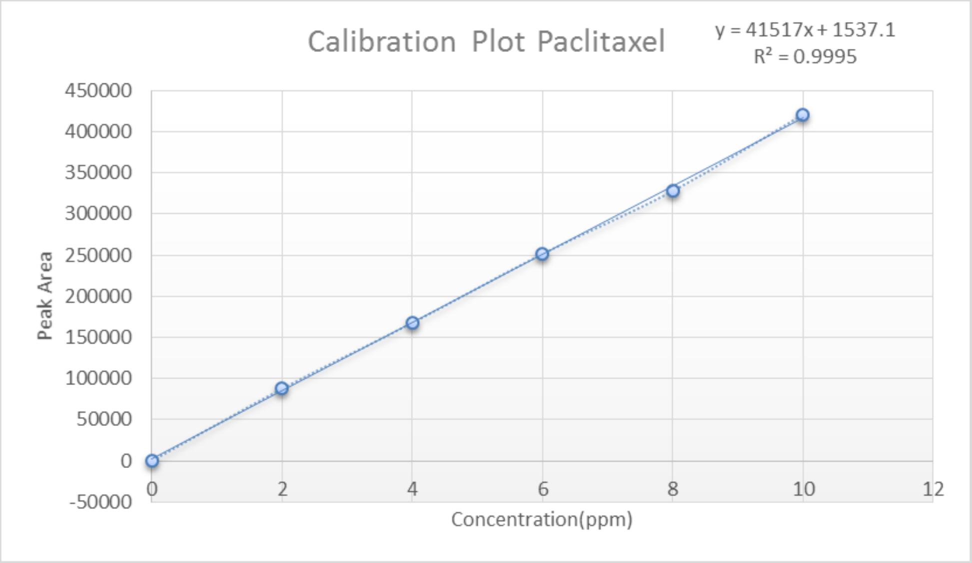

**Fig. 3-2: Calibration Plot for PTX (n=3)**

#### *3.4.2.2. Precision*

The results for intraday and interday precision are summarized in **Table 3-2**. The results were found to be precise under the same operating conditions over the interval of time. Additionally, the RSD values for the analytical methods were within the acceptable range of < 2% indicating that the method is precise.

**Table 3-2: Intraday and Interday precision values for PTX**

| Conc. (µg/ml) | Intraday Precision | | | | Interday Precision | | | |
|---|---|---|---|---|---|---|---|---|
| | Peak Area | | | %RSD | Peak Area | | | %RSD |
| | Set 1 | Set 2 | Set 3 | | Set 1 | Set 2 | Set 3 | |
| 2 | 87698 | 88261 | 87051 | 0.69 | 88261 | 86952 | 86666 | 0.97 |
| 6 | 251665 | 252476 | 250753 | 0.34 | 252554 | 250617 | 252169 | 0.41 |
| 10 | 420271 | 422617 | 420886 | 0.29 | 419402 | 419799 | 421340 | 0.24 |

### *3.4.2.3. Accuracy*

Accuracy of the method was confirmed by recovery study from prepared laboratory sample at three level of standard addition (80%, 100% and 120%) of 5 µg/ml. The results are given in **Table 3-3.** Recovery greater than 98% with low relative standard deviation (RSD) justified the accuracy of the method.

**Table 3-3: Results for accuracy of the method**

| Sr. No | Concentration Level | Theoretical concentration | Drug recovered | % Recovery ± RSD |
|---|---|---|---|---|
| 1 | 80% | 4 µg/ml | 3.95 µg/ml | 98.71 ± 0.87 |
| 2 | 100% | 5 µg/ml | 4.98 µg/ml | 99.63 ± 1.03 |
| 3 | 120% | 6 µg/ml | 5.94 µg/ml | 99.01 ± 0.91 |

### *3.4.2.4. Sensitivity*

LOD and LOQ were calculated using the formula described above and the values obtained were 0.311 µg/ml and 0.944 µg/ml respectively. As indicated by the results, the concentration range selected for calibration was well above the LOD. LOQ values also indicated the sensitivity of methods for accurate quantification of drug present in standard solutions.

## 3.5. Analytical Method for the Estimation of CBP

Estimation of CBP by HPLC has been reported in USP29-NF 24. The HPLC method was adapted here with a few modifications for estimation of CBP in formulation [7].

### 3.5.1. Chromatographic setup

Method: RPHPLC method

Column specifications: Supelco® C18 [25cm (L) × 4.6mm (ID) with 5µm (PS)]

Mobile Phase: Acetonitrile: Water (20:80)

Flow Rate: 1ml/min

Wavelength for Detection: 230nm

Sample injection volume (loop size): 20µl

Diluent: Water

Run time: 10 min

### 3.5.2. Mobile Phase Preparation

Triple distilled water and acetonitrile were mixed according to proportions described in the chromatographic setup. The solvent mix was filtered through 0.22 µm membrane filter (Ultipor® Nylon 66 membrane filter, Pall Life Sciences, USA), transferred to reagent bottle and degassed using bath sonication for 10 minutes.

### 3.5.3. Preparation of Stock solution

Accurately weighed quantity (10 mg) of CBP was transferred to 10 ml calibrated volumetric flask and dissolved in water. The final volume was made up to 10 ml with water. The resulting solution (1000 µg/ml) was further diluted 10 times to obtain stock solution (100 µg/ml).

### 3.5.4. Preparation of standard solutions

40 to 360 µl of the stock solution was transferred to 2.0 ml microcentrifuge tubes using micropipette. The final volume was made upto 2 ml with water to obtain known final concentrations of 2, 4, 6, 8, 10, 12, 14, 16 and 18 µg/ml.

### 3.5.5. Preparation of calibration plot

The mobile phase was set to a flow rate of 1.0 ml/min at room temperature. 20 µl of each standard solution was injected into the HPLC system through rheodyne injector using Hamilton syringe. The absorption wavelength was set to 230 nm and the chromatogram was run for 10 minutes. The area of the peak was obtained from the software. Calibration graph was drawn by plotting peak area versus drug concentration.

### 3.5.6. Validation of HPLC method

#### *3.5.6.1. Linearity*

Linearity of an analytical method is the capability to produce the test results that are directly or by well-defined transformation, proportional to the concentration of the analyte in the samples within the given range. Different concentrations in the range of 2 to 18 µg/mL were prepared from the stock solution of 100 µg/mL by suitable dilutions using triple distilled water. Linear regression was performed using MS office excel software and the correlation coefficient ($R^2$) was generated to demonstrate linearity.

### *3.5.6.2. Precision*

It refers to the extent of variability of a group of measurements observed under similar conditions. Precision provides an indication of random errors and is generally subdivided into two cases: repeatability and reproducibility, which were determined by calculating RSD (Relative standard deviation) of inter-day and intra-day determinations. The inter-day and intra-day precisions were measured in terms of % Relative Standard Deviation (% RSD). The 3 concentrations (2 µg/ml, 10 µg/ml and 18 µg/ml) were selected and experiment was repeated 3 times in a day for intra-day and on 3 different days for inter-day precision. Criteria: % RSD ≤ 2 % as per the requirements of ICH guidelines.

### *3.5.6.3. Accuracy*

Accuracy refers to the closeness of an individual observation or mean of the observations to true value. Accuracy was performed by standard addition method in which known amounts of standard drug (80%, 100% and 120% of 10 µg/mL) were added to the pre-analyzed samples and the measurements done in triplicate. Accuracy was assessed by calculating the percent recovery of active ingredient.

### *3.5.6.4. Sensitivity*

Calibration plot was repeated for 3 times and the standard deviation (SD) of the intercepts was calculated. The average of the slope of calibration plot was calculated. Then Limit of detection (LOD) and limit of quantification (LOQ) were measured as follows

$LOD = 3.3 * (\sigma/s)$

$LOQ = 10.0 * (\sigma/s)$

Where, $\sigma$ = Standard deviation of intercepts

S = Slope of calibration plot

## 3.6. Results and Discussion

### 3.6.1. Preparation of calibration plot

**Table 3-4: Mean peak area values for HPLC calibration plot for CBP**

| Concentration (µg/ml) | Peak Area |
|---|---|
| 0 | 0.000 |
| 2 | 8.131 ± 0.040 |
| 4 | 17.086 ± 0.031 |
| 6 | 26.292 ± 0.030 |
| 8 | 34.418 ± 0.047 |
| 10 | 44.404 ± 0.043 |
| 12 | 53.334 ± 0.038 |
| 14 | 62.673 ± 0.053 |
| 16 | 71.761 ± 0.047 |
| 18 | 81.594 ± 0.054 |

The experiment was performed in triplicate and the values of area corresponding to the peaks are represented as mean ± SD.

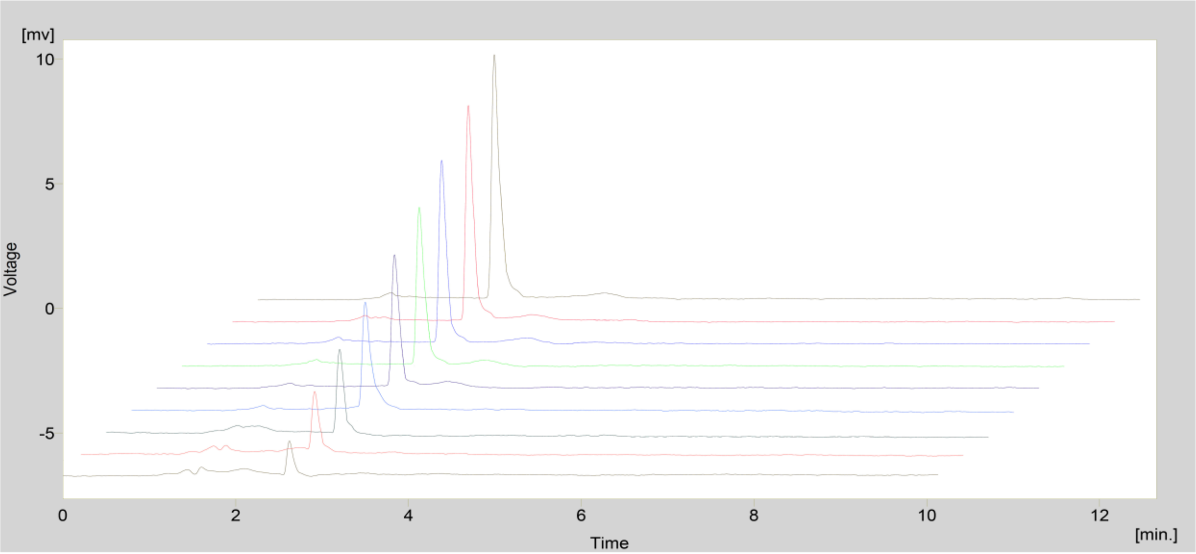

**Fig. 3-3: Overlay chromatogram for CBP**

The chromatograms obtained against standard concentrations were depicted in **Fig. 3-3**. Sharp, symmetric peaks were observed with retention time of 2.6 min at 230 nm detection wavelength and 1 ml/min flow rate.

### 3.6.2. Validation of HPLC Method

#### *3.6.2.1. Linearity*

The mean peak area values with the standard deviation are represented in **Table 3-4** and **Fig. 3-4**. The value of correlation coefficient $R^2$ = 0.9996 indicated that area and concentration of the drug was in significant linear correlation which said to obey beer's law in the range of 2 to 18 µg/ml. The results indicated the linearity of the method for selected concentration range for CBP.

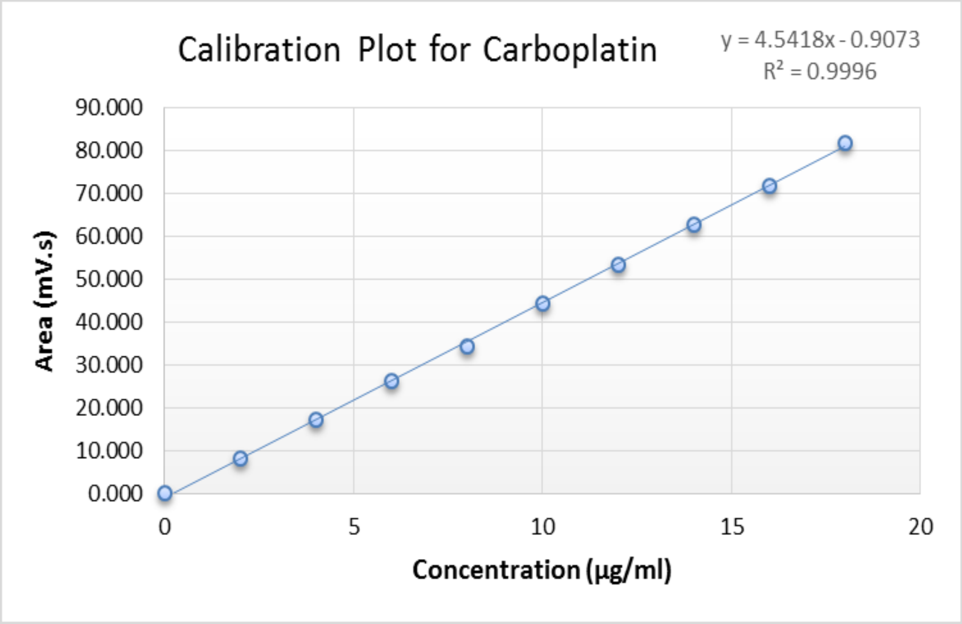

**Fig. 3-4: Calibration Plot for CBP (n=3)**

#### *3.6.2.2. Precision*

The results for intraday and interday precision are summarized in **Table 3-5**. The results were found to be precise under the same operating conditions over the interval of time. Additionally, the RSD values for the analytical methods were within the acceptable range of < 2% indicating that these method is precise.

**Table 3-5: Intraday and interday precision values for CBP**

| Conc. (µg/ml) | Intraday Precision | | | | Interday Precision | | | |
|---|---|---|---|---|---|---|---|---|
| | Peak Area | | | %RSD | Peak Area | | | %RSD |
| | Set 1 | Set 2 | Set 3 | | Set 1 | Set 2 | Set 3 | |
| 2 | 8.011 | 8.137 | 8.153 | 0.96 | 8.045 | 8.011 | 8.153 | 0.92 |
| 10 | 44.445 | 44.368 | 44.371 | 0.10 | 44.524 | 44.21 | 44.405 | 0.36 |
| 18 | 81.558 | 81.693 | 81.502 | 0.12 | 81.698 | 81.514 | 81.584 | 0.11 |

### *3.6.2.3. Accuracy*

**Table 3-6** shows the data of recovery studies. The mean % recoveries for lower, intermediate and higher concentration were presented in the **Table 3-6**. The mean % recovery values, close to 100% with less standard deviation values (SD < 0.7%) indicate high accuracy of the analytical method. These results showed that any small change in the drug concentration could accurately be measured by the present analytical method.

**Table 3-6: Accuracy of the HPLC method for CBP**

| Sr. No | Concentration Level | Theoretical concentration (µg/ml) | Drug recovered (µg/ml) | SD (n=3) | % Recovery ± RSD |
|---|---|---|---|---|---|
| 1 | 80% | 8 | 7.96 | 0.012 | 99.54 ± 0.15 |
| 2 | 100% | 10 | 9.89 | 0.040 | 98.87 ± 0.41 |
| 3 | 120% | 12 | 11.99 | 0.038 | 99.97 ± 0.32 |

### *3.6.2.4. Sensitivity*

LOD and LOQ were calculated using the formula described above and the values obtained were 0.306 µg/ml and 0.926 µg/ml respectively. As indicated by the results, the concentration range selected for calibration was well above the LOD. LOQ values also indicated the sensitivity of methods for accurate quantification of drug present in standard solutions.

## References

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