4. Analytical method development

4.1 Experimental methods

4.1.1 Analytical method development and validation for estimation of CS by UV-Visible spectroscopy

4.1.1.1 Selection of solvent media

The solvent media for the preparation of the calibration curve was selected on the basis of the solubility of CS at room temperature. By understanding the requirement for quantification of CS during different studies, water and phosphate buffer pH 7.4 was selected for the preparation of calibration curve.

4.1.1.2 Standard stock solution preparation

4.1.1.2.1 Standard stock solution preparation in water

Accurately weighed 10 mg of CS was transferred to a 100 ml of volumetric flask and dissolved completely in 70 ml of water. The resultant solution was sonicated and volume was made up to the mark (100 ml) with water to give stock solution containing 100 µg/ml of CS.

4.1.1.2.2 Standard stock solution preparation in phosphate buffer pH 7.4

Accurately weighed 10 mg of CS was transferred to a 100 ml of volumetric flask and dissolved completely in 70 ml of phosphate buffer pH 7.4. The resultant solution was sonicated and volume was made up to the mark (100 ml) with phosphate buffer pH 7.4 to give stock solution containing 100 µg/ml of CS.

4.1.1.3 Identification of λ_{max}

The stock solution was further successively diluted with respective solvent, in order to obtain a final concentration of 10 µg/ml. The resultant solution was subjected to scan

between 200-400 nm using UV-Visible spectrophotometer and wavelength maximum (λ_{max}) was determined.

4.1.1.4 Sample Preparation

From the standard stock solution of CS (100 µg/ml), different volume of aliquots were pipetted out and transferred into a series of volumetric flasks of 10 ml capacity. The volume was adjusted up to the mark with respective solvents to obtain a final concentration of 2, 4, 6, 8, 10 and 12 µg/ml.

4.1.1.5 Calibration curve preparation

The absorbance of each prepared sample solution was measured at 239 nm by using UV-Visible spectrophotometer against respective solvent as a blank. A calibration curve of absorbance vs. concentration (µg/ml) was plotted and straight-line equation was determined by using linear regression analysis. Calibration curves were generated on three consecutive days using freshly prepared samples.

4.1.1.6 Method Validation

The suitability of the developed method for quantitative estimation of CS was confirmed by evaluating the different validation parameters as per ICH Q2B guideline. The developed method was validated for the typical validation characteristics, i.e., accuracy, precision, linearity, range, specificity, selectivity, limit of detection (LOD) and limit of quantification (LOQ) [167].

4.1.1.6.1 Accuracy

Accuracy of the analytical method is the closeness of test results obtained by that method to the true value. Accuracy of the analytical method was determined by performing recovery study using standard addition method. Recovery can be

determined by taking the ratio of observed result to the predicted result and usually expressed as a percentage. Three concentrations of low (4 µg/ml), medium (6 µg/ml) and high (8 µg/ml) of CS were selected. Each sample was scanned in UV-Visible spectrophotometer at same day (n=3) and over three consecutive days (n=3) to access intra-day and inter-day variation, respectively. The accuracy was also expressed as % bias and the acceptable values should be within ± 15% at all concentrations levels [168]. The % bias was calculated as follow (Eq (4.1)):

$$\% \text{ Bias} = \frac{\text{Observed concentration} - \text{Predicted concentration}}{\text{Predicted concentration}} \times 100 \qquad \text{Eq (4.1)}$$

4.1.1.6.2 Precision

Precision is the degree of agreement amongst individual test results, obtained by the series of measurements from multiple sampling of the same homogeneous sample using the developed analytical method. Precision of the analytical method was determined by measuring the relative standard deviations (RSD) of a series of measurement. Three different drug concentrations (4, 6 and 8 µg/ml) of CS were scanned in UV-Visible spectrophotometer on same day (n=3) and three consecutive days (n=3), in order to measure intra-day and inter-day precision, respectively. The acceptable values for % RSD should be within ≤ 15% at all concentrations levels [169]. The % RSD was calculated as follow (Eq (4.2)):

$$\% \text{ RSD} = \frac{\text{Standard deviation}}{\text{Mean observed concentration}} \times 100 \qquad \text{Eq (4.2)}$$

4.1.1.6.3 Linearity and Range

Linearity of an analytical method is its ability to produce test results which are directly proportional to the concentration of analyte in samples. The range is an expression of the lowest and highest levels of analyte concentration, which can be

easily determined, either directly or indirectly with acceptable precision, accuracy and linearity. Linearity and range was determined by measuring the absorbance of the series of samples over the concentration range of 2-12 µg/ml in triplicate over three consecutive days.

4.1.1.6.4 Specificity and Selectivity

Specificity is a procedure to detect quantitatively the analyte in presence of other components, which may be expected to be present in the solvent media. Selectivity is the procedure to detect qualitatively the analyte, in presence of other components in the solvent media. Specificity and selectivity of the analytical method towards CS was determined by the scanning of known concentration (10 µg/ml) at 239 nm in UV-Visible spectrophotometer.

4.1.1.6.5 Limits of detection (LOD)

The limit of detection (LOD) is the lowest concentration of analyte in a sample, which can be detectable, but not necessarily quantifiable as exact value, under the stated experimental conditions. The LOD of CS in different medium was calculated by using following formula (Eq (4.3)):

$$\mathrm{LOD} = \frac{3.3 \times \sigma}{\mathrm{S}} \qquad \text{Eq (4.3)}$$

Where, σ is the standard deviation of y-intercept of the regression equation and S is the slope of the calibration curve.

4.1.1.6.6 Limits of quantification (LOQ)

The limit of quantitation (LOQ) is the lowest concentration of analyte which can be determined with acceptable precision and accuracy under the stated experimental

conditions. The LOQ of CS in different medium was calculated by using following formula (Eq (4.4)):

$$\mathrm{LOQ} = \frac{10 \times \sigma}{S} \qquad \text{Eq (4.4)}$$

Where, σ is the standard deviation of y-intercept of the regression equation and S is the slope of the calibration curve.

4.1.2 Analytical method development and validation for estimation of CS by reverse phase high performance liquid chromatography (RP-HPLC) in blood plasma

4.1.2.1 Instrument and chromatographic conditions

The HPLC system (Shimadzu Corporation, Koyoto, Japan) consisted of a pump (LC-20AD) and a degasser (DGU-20A_3) with UV-Visible detector (SPD-20A) was utilized for the quantification of the CS in plasma samples. A rheodyne manual injector (SIL-20A) attached with 20 µl sample loop was used for injecting the sample. A C_{18} reverse phase column (250 mm × 4.6 mm, particle size 5µ) along with C_{18} guard column was utilized for drug separation and subsequent quantification. The earlier reported chromatographic method with little modification was used for quantification of CS in blood plasma samples [170]. The samples were analyzed by monitoring the elute at 239 nm wavelength, using isocratic elution at a flow rate of 1 ml/min at 25 ^{o}C. Mobile phase consisted of acetonitrile and 20 mM potassium dihydrogen orthophosphate (pH adjusted to 2.0 with orthophosphoric acid) in the ratio of 75:25 was utilized for achieving a satisfactory separation of CS from rat plasma component. The mobile phase was filtered through 0.45 µ Millipore membrane filter and degassed in ultrasonic bath for 10 min prior to use.

4.1.2.2 Standard solutions for preparation of calibration curve

Standard stock solution of CS (100 µg/ml) was prepared by dissolving 10 mg of accurately weighed CS in 100 ml of water. Different working standard solutions of CS were prepared by appropriate dilution of stock solution with acetonitrile (ACN) to yield final concentrations ranging from 50 to 500 ng/ml. Stock and working standard solutions were protected from light and stored at 4 ºC temperature until analyzed. The mean peak area was measured after every injection of sample. A calibration curve was constructed by plotting of mean peak area vs. concentration (ng/ml) and straight-line equation was determined by using linear regression analysis. Calibration curves were generated on six consecutive days using freshly prepared samples.

4.1.2.3 Standard bio analytical solutions for preparation of calibration curve

Similar protocol (as described in section 4.1.2.2) was followed for the preparation of bio analytical stock and working solutions. Calibration standard for rat plasma were prepared by spiking 90 µl of blank rat plasma with 10 µl of the appropriate working standard solutions, in order to obtain different concentrations in the range of 50 to 500 ng/ml. The samples were stored at -20 ºC until analysis.

4.1.2.4 Extraction procedure

A liquid-liquid extraction procedure was employed for the extraction of CS from rat plasma samples. Plasma containing of CS (100 µl) was pipetted into eppendorf tube and 900 µl of methanol was added to precipitate plasma proteins. The resultant mixture was then vortexed for 5 min and centrifuged at 5000 rpm for 10 min at 4 ºC. The supernatant containing organic solvent was separated and transferred into glass tubes for evaporating to dryness in a vacuum oven at 40 ºC. The residue was then reconstituted in 100 µl of mobile phase and further 20 µl of reconstituted samples

were injected into HPLC, for estimation of CS after filtration through 0.2 µm syringe filter.

4.1.2.5 Method validation

4.1.2.5.1 Recovery, accuracy and precision

The recovery of CS from rat plasma was analyzed at three different concentrations 50, 200 and 500 ng/ml (n=6). The drug samples were spiked into drug-free mixture of rat plasma and mobile phase to achieve previously specified drug concentration. Peak area of the bioanalytical samples were compared to that of analytical samples with the same initial concentration. Percentage recovery was calculated as following (Eq(4.5)):

$$\% \text{ Recovery} = \frac{\text{Peak area of bioanalytical sample}}{\text{Peak area of analytical sample}} \times 100 \qquad \text{Eq (4.5)}$$

For the determination of accuracy and precision, the six replicate plasma samples of three different concentration 50, 200 and 500 ng/ml were evaluated at same day and over six consecutive days to access intra-day and inter-day variations, respectively. The accuracy was expressed as % bias and calculated as shown in section 4.1.1.6.1. The acceptable values should be within ± 15% at all concentrations levels [168]. The precision was calculated by measuring RSD as shown in section 4.1.1.6.2. The acceptable values for % RSD should be within ≤ 15% at all concentrations levels [169].

4.1.2.5.2 Linearity

The linearity was determined by measuring the peak area of the different calibration solutions in the range of 50 to 500 ng/ml. Calibration curves were generated on six consecutive days using freshly prepared samples.

4.1.2.5.3 LOD and LOQ

LOD and LOQ were estimated by determining the signal-to-noise ratios as well as calculated using the formula as shown in section 4.1.1.6.5 and 4.1.1.6.6, respectively. The LOD was estimated by measuring the lowest concentration having peak area more than 3.3 times to the base line noise. The LOQ was estimated by measuring the lowest concentration having peak area more than 10 times to the base line noise [171].

4.2 Results and discussions

4.2.1 Analytical method development and validation for estimation of CS by UV-Visible spectroscopy

4.2.1.1 Identification of λ_{max}

CS at concentration of 10 µg/ml was scanned in double beam UV-Visible spectrophotometer in water and phosphate buffer pH 7.4 between the scanning range of 200-400 nm to determine the λ_{max}. UV-Visible spectrums in water and phosphate buffer pH 7.4 are depicted in Figure 4.1 and Figure 4.2, respectively. CS showed three absorption maxima at 221 nm, 239 nm and 327 nm, in both the media. An absorption maximum at 239 nm was distinctly separated from the other two with high intensity and hence, it was chosen for the CS estimation during further studies, in water as well as phosphate buffer pH 7.4.

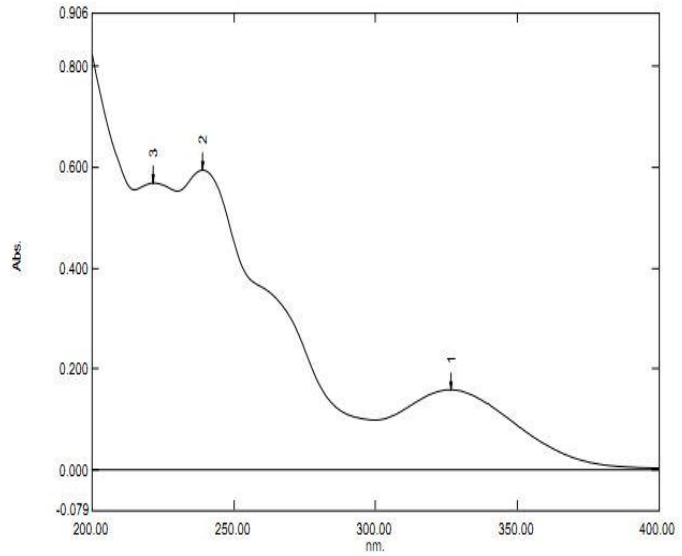

Figure 4.1 UV-Visible absorbance spectra of pure CS in water

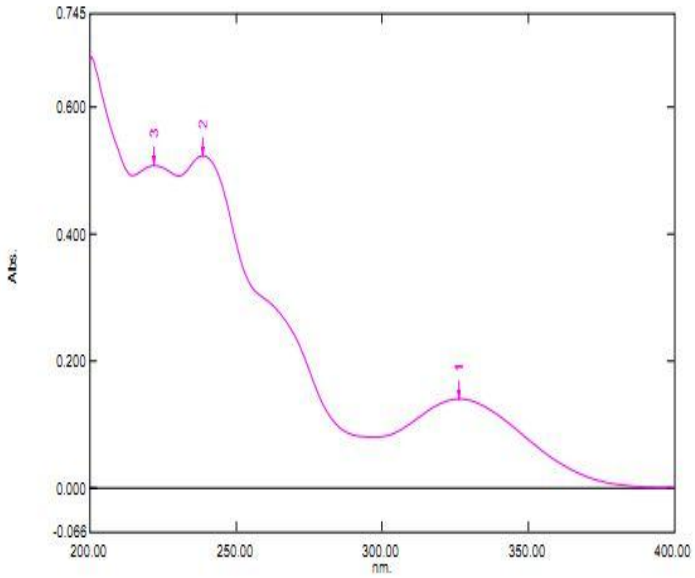

Figure 4.2 UV-Visible absorbance spectra of pure CS in phosphate buffer pH 7.4

4.2.1.2 Calibration curve

The absorbance values for standard solutions of CS were measured at 239 nm by taking respective solvent media as a reference (n=3). A calibration curve of CS in water and phosphate buffer pH 7.4 was constructed by plotting of absorbance vs concentration (µg/ml) as shown in Figure 4.3 and Figure 4.4, respectively. Beer-

Lambert's law was obeyed in the concentration range of 2-10 µg/ml in water and phosphate buffer pH 7.4.

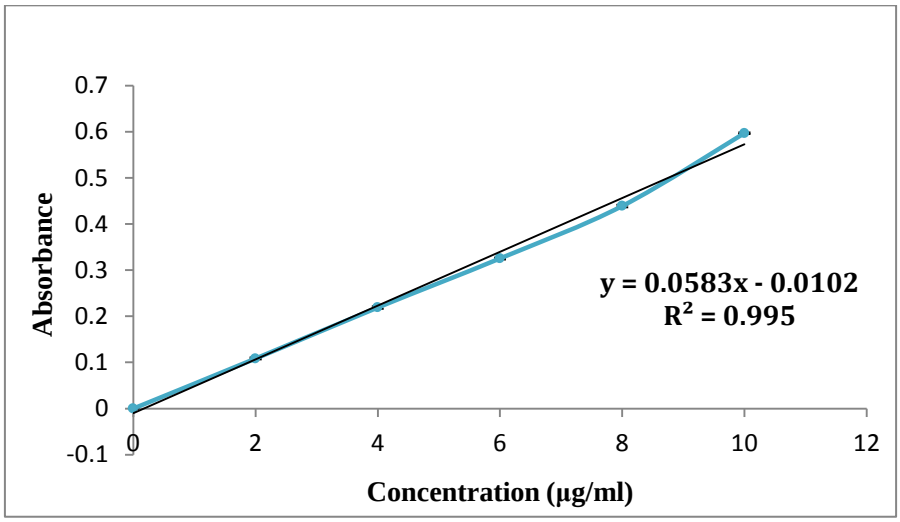

Figure 4.3 Calibration curve of CS by UV-Visible spectroscopy in water (vertical bars represent ± SD; n=3)

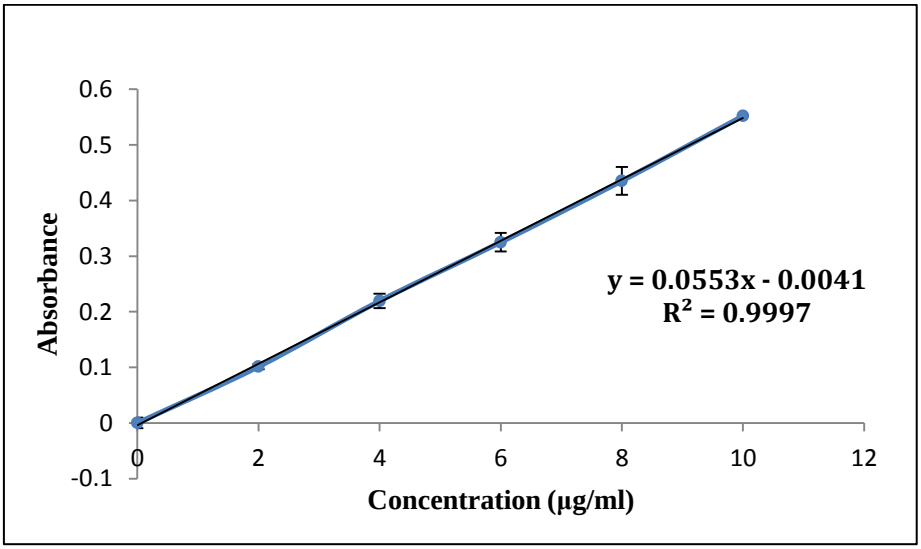

Figure 4.4 Calibration curve of CS by UV-Visible spectroscopy in phosphate buffer pH 7.4 (vertical bars represent ± SD; n=3)

The straight line regression equation obtained by linear regression analysis can be represented as: y = 0.0583x – 0.0102 and y = 0.0553x – 0.0041 for water and phosphate buffer pH 7.4, respectively. The value of correlation coefficient (R^2) was

found to be more than 0.99 in both media, indicating the presence of high linearity between absorbance and concentration in the range of 2-10 µg/ml.

Recovery study was performed for determination of the accuracy of the developed method for the estimation of CS in water and phosphate buffer pH 7.4. The variability associated with the developed analytical method in different media was measured by determining precision (intra-day and inter-day). Three concentrations of low (4 µg/ml), medium (6 µg/ml) and high (8 µg/ml) of CS were selected. Each sample was scanned in UV-Visible spectrophotometer at same day (n=3) and over three consecutive days (n=3) to access intra-day and inter-day variation, respectively. The results of intra-day and inter-day variability in water as well as phosphate buffer pH 7.4 are summarized in Table 4.1 & Table 4.2, respectively. Results were within specified limits of less than 15% for all the three different concentrations [168, 172].

The consistency of results on same day as well as different days confirmed that the developed analytical method is accurate and reproducible for the measurement of CS in water and phosphate buffer pH 7.4. The low variability in the results indicated the suitability of analytical method for consistent and efficient analysis during the *in-vitro* studies in different media, water and phosphate buffer pH 7.4. LOD and LOQ for the developed analytical method in water were found to be 0.06 µg/ml and 0.19 µg/ml, respectively. Whereas, LOD and LOQ for the developed analytical method in phosphate buffer pH 7.4 were found to be 0.25 µg/ml and 0.75 µg/ml, respectively.

Table 4.1 The intra-day and inter-day variability of the analytical method in water

Actual Concentration (µg/ml)	**4**	**6**	**8**
	Intra-day variation		
Calculated concentration (µg/ml)	3.93 ± 0.04	5.72 ± 0.03	7.70 ± 005
Recovery (%)	98.28 ± 1.13	95.92 ± 0.59	96.31 ± 0.64
% RSD	1.15	0.62	0.66
% Bias	-1.71	-4.07	-3.68
	Inter-day variation		
Calculated concentration (µg/ml)	3.94 ± 0.08	5.91 ± 0.14	7.89 ± 0.14
Recovery (%)	98.54 ± 2.19	98.62 ± 2.44	98.68 ± 1.84
% RSD	2.22	2.48	1.86
% Bias	-1.45	-1.37	-1.31

**Values represent mean ± SD; n=3*

Table 4.2 The intra-day and inter-day variability of the analytical method in phosphate buffer pH 7.4

Actual Concentration (µg/ml)	4	6	8
Intra-day variation			
Calculated concentration (µg/ml)	4.03 ± 0.08	6.03 ± 0.06	8.09 ± 0.22
Recovery (%)	100.85 ± 2.22	100.58 ± 1.03	101.14 ± 2.85
% RSD	2.20	1.02	2.82
% Bias	0.85	0.58	1.14
Inter-day variation			
Calculated concentration (µg/ml)	4.12 ± 0.16	6.13 ± 0.17	8.11 ± 0.22
Recovery (%)	103.03 ± 4.21	102.24 ± 2.87	101.38 ± 2.81
% RSD	4.09	2.81	2.77
% Bias	3.03	2.24	1.38

**Values represent mean ± SD; n=3*

4.2.2 Analytical method development and validation for estimation of CS by RP-HPLC in blood plasma

A simple, reproducible and accurate modified RP-HPLC method was proposed as a suitable method for the estimation of CS in the rat plasma samples. The typical chromatograms of blank plasma, pure CS and plasma sample spiked with CS are depicted in Figure 4.5 under the described assay conditions. The chromatogram of pure CS has shown a sharp and symmetrical peak with retention time of 5.2 min, well separated from solvent front. The chromatogram of blank plasma showed numerous small peaks with the retention time between 2 to 3.5 min. However, satisfactory

resolution between CS and endogenous interfering plasma peaks was achieved without any significant change in the retention time, indicating the specificity and selectivity of the developed method, as depicted in Figure 4.5(C).

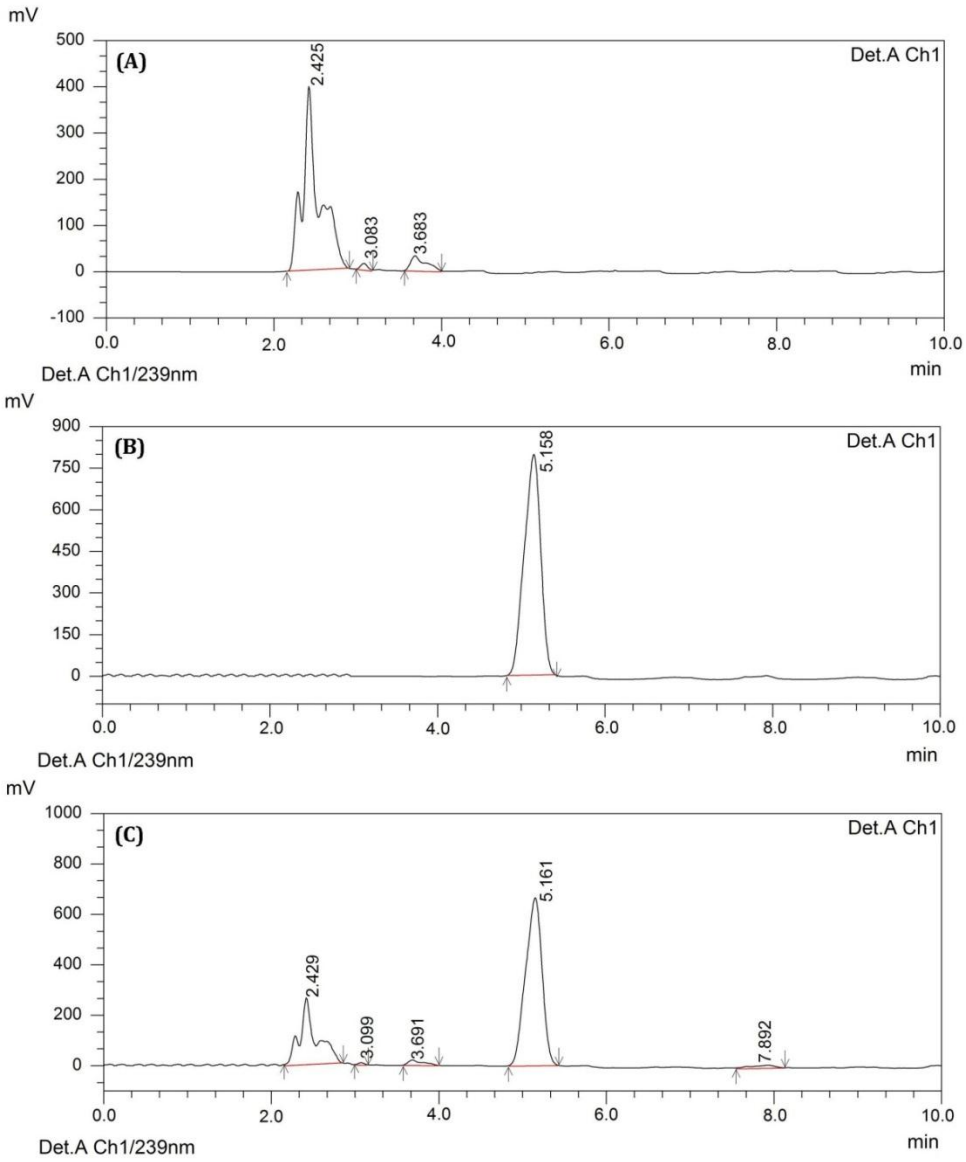

Figure 4.5 Typical chromatograms of (A) blank plasma, (B) pure CS and (C) plasma spiked with CS.

A calibration curve was constructed by plotting of peak area vs. concentration (ng/ml) after evaluating with total of six calibration standard (Figure 4.6). The developed

method was found to be appropriate and has shown linearity over the concentration range of 50-500 ng/ml.

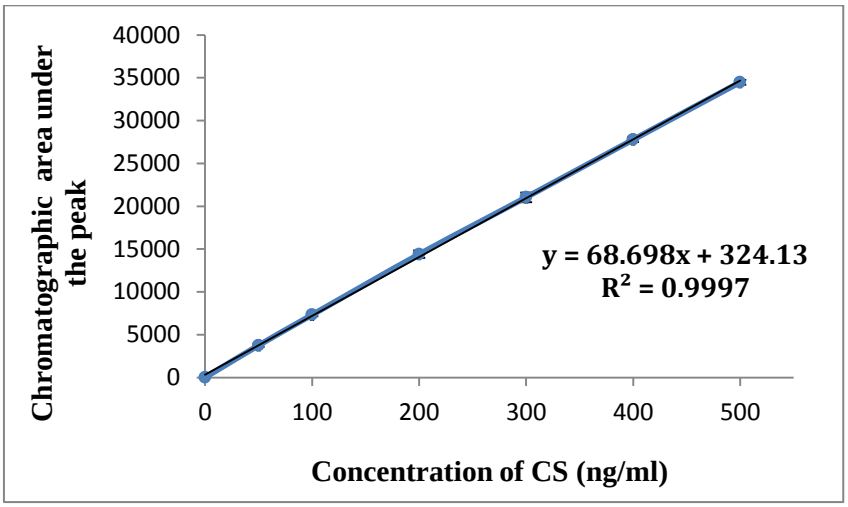

Figure 4.6 Calibration curve of CS by RP-HPLC in rat plasma (vertical bars represent ± SD; n=6).

The straight-line equation was determined by using linear regression analysis, which can be represented as: y = 68.698x + 324.13; where the y axis represents the peak area of CS and x axis represents the concentration (ng/ml). The value of correlation coefficient (R^2) was found to be 0.9997, indicating the high linearity between peak area and concentration of CS (ng/ml) extracted from rat plasma, throughout the concentration range studied. High linearity suggested that the developed method can be easily extrapolated to determine the unknown CS concentration in rat plasma samples.

The accuracy and extraction efficiency of the developed method was evaluated by estimating the recovery of CS from plasma samples. The peak area of CS obtained after extraction of spiked samples was compared to the peak area obtained from direct injection of known amount of analytical standard solution, in order to determine the recovery of CS from plasma samples. The absolute recovery of CS from plasma

samples was found in the range of 93.51-99.67 % at concentrations of 50, 200 and 500 ng/ml (n=6). The results of recovery study are summarized in Table 4.3. Higher values for recovery of CS from plasma samples indicated the suitability of bioanalytical method for the consistent and efficient analysis of the bioanalytical samples [173].

Table 4.3 The intra-day and inter-day variability of the bioanalytical method

Actual Concentration (ng/ml)	**50**	**200**	**500**
Intra-day variation			
Calculated concentration (ng/ml)	48.26 ± 2.77	199.34 ± 4.68	494.09 ± 8.36
Recovery (%)	96.52 ± 5.54	99.67 ± 2.34	98.81 ± 1.67
% RSD	5.74	2.35	1.69
% Bias	-3.47	-0.32	-1.18
Inter-day variation			
Calculated concentration (ng/ml)	46.75 ± 2.95	195.86 ± 2.06	482.36 ± 9.66
Recovery (%)	93.51 ± 5.90	97.93 ± 1.03	96.47 ± 1.93
% RSD	6.31	1.05	2.00
% Bias	-6.48	-2.06	-3.52

**Values represent mean ± SD; n=6*

The variability associated with the developed bioanalytical method for estimation of CS in plasma sample was measured by determining precision (intra-day and inter-day). Table 4.3 summarizes the results of intra-day and inter-day precision of CS in rat plasma. Results were within specified limits of less than 15% for all the three

different concentrations [168, 172, 174]. The consistency of results on same day as well as different days confirmed that the developed bioanalytical method for estimation of CS in plasma samples is accurate and reproducible, which can be further extended to measure CS concentration during pharmacokinetic studies in rats. LOD and LOQ for the developed bioanalytical method were found to be 1.98 ng/ml and 6.022 ng/ml, respectively.

4.3 Summary

The developed analytical method was simple, precise and rapid for the estimation of CS. The analytical method was successfully developed and validated as per ICH guideline. Good linearity over the wide range of concentration indicated the reliability of the method for the estimation of CS during the various *in-vitro* studies. Further, reproducibility over the different time duration with accuracy, confirmed the validity of the analytical method.

The bioanalytical RP-HPLC method for estimation of CS in plasma samples was successfully developed and validated. The method was simple, rapid, precise and highly sensitive. The use of micro volume plasma and simple liquid extraction technique for sample preparation makes it highly suitable for estimation of CS in plasma samples during pharmacokinetic studies. Well resolved peak of CS from plasma components with shorter retention time allows faster estimation in short time period. Moreover, accuracy and precision with good linearity were enough to accept the method for bioanalytical purpose.