CHAPTER 3

ANALYTICAL METHOD DEVELOPMENT

Analytical Methods

In this investigation, lyophilized formulation comprising functionalized liposomes of RGZ and CDS were prepared for the treatment of liver fibrosis. The chemical characterization of liposomes and developed formulation were carried out to determine the percentage drug entrapment and percentage drug retention. The stability studies were conducted to determine percentage drug retained in liposomes over stage of six months period. In vitro drug diffusion studies followed by in vivo pharmacokinetic studies were also carried out. The analytical methods employed in these investigations are discussed below.

3.1 PREPARATION OF CALIBRATION PLOT OF RGZ IN METHANOL

The spectroscopic determination of RGZ is based on the zero order UV spectra of RGZ giving maxima at 311.8 nm in methanol (The Merck Index, 2001; Martindale, 1996; Goyal and Singhvi, 2007; Jagathi et al., 2010).

3.1.1 Reagents

(i) Methanol for spectroscopy Uvasol®.

(ii) Stock solution of RGZ: 1 mg/mL solution of RGZ was prepared in methanol.

3.1.2 Method

Appropriate aliquots of the stock solution of RGZ were transferred to 10 mL volumetric flasks and were diluted up to the mark with methanol. The absorption maxima (λ_{max}) was determined by scanning 10 µg/mL solution against reagent blank on UV-Visible Spectrophotometer (UV-1700, Schimadzu). The absorption of all the prepared solutions was then measured at the absorbance maxima, 311.8 nm against the reagent blank. The readings were recorded in triplicate. Mean value (n=3) along with the standard error of mean (SEM) are recorded in Table 3.1. The regressed values of absorption were plotted graphically against the concentrations, as shown in Figure 3.1.

Stability of the solutions of RGZ in methanol used for preparing the calibration plot was ascertained by observing the changes in the absorbance of the solution at the analytical wavelength, over a period of 48 hr at room temperature.

Table 3.1 Calibration plot of RGZ in methanol for the estimation of RGZ in liposomes (n=3)

Conc.(µg/mL)	Absorbance (±SEM)
10	0.1197 ± 0.0040
15	0.1790 ± 0.0040
20	0.2357 ± 0.0065
25	0.2970 ± 0.0080
30	0.3537 ± 0.0095
40	0.4763 ± 0.0075
50	0.5917 ± 0.0105
60	0.7083 ± 0.0101
70	0.8283 ± 0.0115

Figure 3.1 Calibration plot of RGZ in methanol

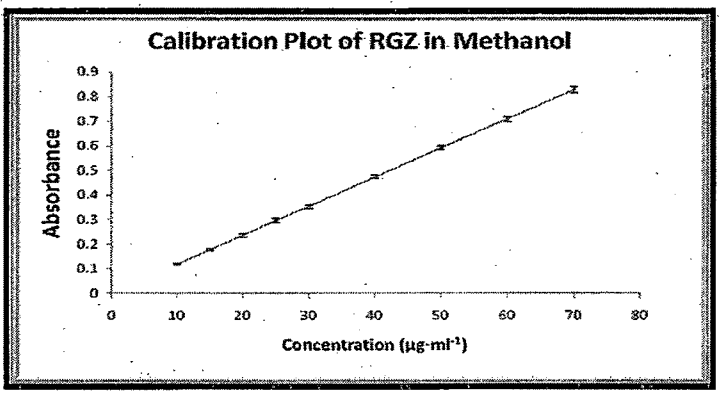


Table 3.2 Optical characteristics of RGZ in methanol

Characteristic	Value
λ_{max}	311.8 nm
Solvent	Methanol
Range	10-70 µg/mL
Regression equation	y = 0.0118x + 0.0012
Regression Coefficient (R^2)	1.0000

Table 3.3 Accuracy and precision of RGZ estimation by UV- specroscopic method in methanol (n=6)

Conc.(µg/mL)	Obtained Conc. (µg/mL) (±SEM)	% Recovery	% RSD
10	10.026 ± 0.082	100.244	1.370
20	20.067 ± 0.115	100.335	0.995
40	40.104 ± 0.237	100.261	1.022
70	69.752 ± 0.398	99.645	0.988

Accuracy is reflected from % Recovery and precision from % RSD

3.2 PREPARATION OF CALIBRATION PLOT OF RGZ IN DIFFUSION MEDIUM [50 mM HPBCD, 20 mM HEPES, pH 7.4]

The spectroscopic determination of RGZ is based on the zero order UV spectra of RGZ giving maxima at 313.8 nm in diffusion medium (50 mM HPBCD, 20 mM HEPES, pH 7.4) (The Merck Index, 2001; Martindale, 1996; Goyal and Singhvi, 2007; Jagathi et al., 2010).

3.2.1 Reagents

(i) Freshly prepared diffusion medium (50 mM HPBCD, 20 mM HEPES, pH 7.4).

(ii) Stock solution of RGZ: 0.5 mg/mL solution of RGZ was prepared in diffusion medium (50 mM HPBCD, 20 mM HEPES, pH 7.4).

3.2.2 Method

Appropriate aliquots of the stock solution of RGZ were transferred to 10mL volumetric flasks and were diluted up to the mark with diffusion medium (50 mM HPBCD, 20 mM HEPES, pH 7.4). The absorption maxima (λ_{max}) was determined by scanning 10 µg/mL solution against reagent blank on UV-Visible Spectrophotometer (UV-1700, Schimadzu). The absorption of all the prepared solutions was then measured at the absorbance maxima, 313.8 nm against the reagent blank. The readings were recorded in triplicate. Mean value (n=3) along with the standard error of mean (SEM) are recorded in Table 3.4. The regressed values of absorption were plotted graphically against the concentrations, as shown in Figure 3.2.

Stability of the solutions of RGZ in diffusion medium (50 mM HPBCD, 20 mM HEPES, pH 7.4) used for preparing the calibration plot, was ascertained by observing

the changes in the absorbance of the solution at the analytical wavelength, over a period of 48 hr at room temperature.

Table 3.4 Calibration plot of RGZ in diffusion medium (50 mM HPBCD, 20 mM HEPES, pH 7.4) for the estimation of RGZ during diffusion (n=3)

Conc.(µg/mL)	Absorbance (±SEM)
10	0.1163 ± 0.0035
15	0.1747 ± 0.0043
20	0.2293 ± 0.0060
25	0.2892 ± 0.0075
30	0.3470 ± 0.0101
40	0.4650 ± 0.0085
50	0.5887 ± 0.0106
60	0.7087 ± 0.0075
70	0.8070 ± 0.0110

Figure 3.2 Calibration plot of RGZ in diffusion medium (50 mM HPBCD, 20 mM HEPES, pH 7.4)

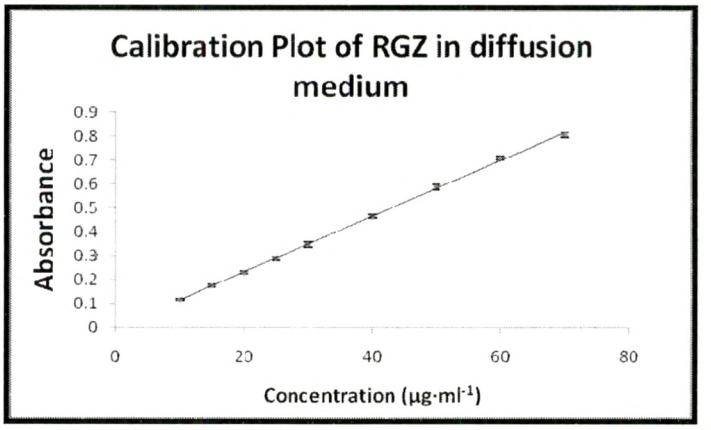


Table 3.5 Optical characteristics of RGZ in diffusion medium (50 mM HPBCD, 20 mM HEPES, pH 7.4)

Characteristic	Value
λ_{max}	313.8 nm
Solvent	50 mM HPBCD, 20 mM HEPES, pH 7.4
Range	10-70 µg/mL
Regression equation	y = 0.0117x - 0.0015
Regression Coefficient (R^2)	0.9995

Table 3.6 Accuracy and precision of RGZ estimation by UV method in diffusion medium (n=6)

Conc.(µg/mL)	Obtained Conc. (µg/mL) (±SEM)	% Recovery	% RSD
10	9.986 ± 0.088	99.858	1.525
20	20.053 ± 0.163	100.267	1.410
40	40.112 ± 0.205	100.280	0.884
70	70.237 ± 0.419	100.339	1.033

Accuracy is reflected from % Recovery and precision from % RSD

3.3 PREPARATION OF CALIBRATION PLOT OF CDS IN METHANOL

The spectroscopic determination of CDS is based on the zero order UV spectra of CDS giving maxima at 304.8 nm in methanol (The Merck Index, 2006; Martindale, 2002; Patil et al., 2011).

3.3.1 Reagents

(i) Methanol for spectroscopy Uvasol®.

(ii) Stock solution of CDS: 1 mg/mL solution of CDS was prepared in methanol.

3.3.2 Method

Appropriate aliquots of the stock solution of CDS were transferred to 10 mL volumetric flasks and were diluted up to the mark with methanol. The absorption maxima (λ_{max}) was determined by scanning 10 µg/mL solution against reagent blank on UV-Visible Spectrophotometer (UV-1700, Schimadzu). The absorption of all the prepared solutions was then measured at the absorbance maxima, 304.8 nm against

the reagent blank. The readings were recorded in triplicate. Mean value (n=3) along with the standard error of mean (SEM) are recorded in Table 3.7. The regressed values of absorption were plotted graphically against the concentrations, as shown in Figure 3.3.

Stability of the solutions of CDS in methanol used for preparing the calibration Plot was ascertained by observing the changes in the absorbance of the solution at the analytical wavelength, over a period of 48 hr at room temperature.

Table 3.7 Calibration Plot of CDS in methanol for the estimation of CDS in liposomes (n=3)

Conc.(µg/mL)	Absorbance (±SEM)
5	0.0467 ± 0.0015
10	0.0933 ± 0.0040
15	0.1437 ± 0.0051
20	0.1983 ± 0.0067
25	0.2393 ± 0.0086
30	0.2890 ± 0.0105
35	0.3313 ± 0.0116
40	0.3807 ± 0.0097
50	0.4803 ± 0.0120
60	0.5677 ± 0.0111
70	0.6723 ± 0.0115
80	0.7663 ± 0.0120

Figure 3.3 Calibration plot of CDS in methanol

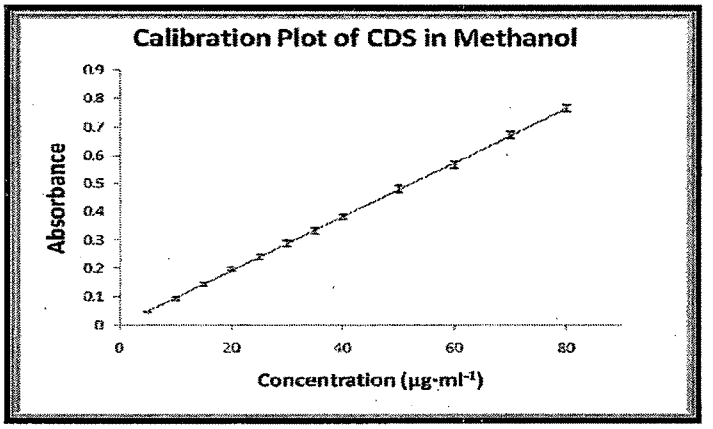


Table 3.8 Optical characteristics of CDS in methanol

Characteristic	Value
λmax	304.8 nm
Solvent	Methanol
Range	5-80 µg/mL
Regression equation	y = 0.0096x + 0.0001
Regression Coefficient (R^2)	0.9998

Table 3.9 Accuracy and precision of CDS estimation by UV- specroscopic method in methanol (n=6)

Conc.(µg/mL)	Obtained Conc. (µg/mL) (±SEM)	% Recovery	% RSD
10	10.026 ± 0.091	100.261	1.578
20	19.954 ± 0.123	99.771	1.070
40	39.897 ± 0.199	99.734	0.863
70	70.227 ± 0.368	100.324	0.906

Accuracy is reflected from % Recovery and precision from % RSD

3.4 PREPARATION OF CALIBRATION PLOT OF CDS IN DIFFUSION MEDIUM (100 mM HPBCD, 20 mM HEPES, pH 7.4)

The spectroscopic determination of CDS is based on the zero order UV spectra of CDS giving maxima at 306.2 nm in diffusion medium (100 mM HPBCD, 20 mM HEPES, pH 7.4) (The Merck Index, 2006; Martindale, 2002; Patil et al., 2011).

3.4.1 Reagents

(i) Freshly prepared diffusion medium (100 mM HPBCD, 20 mM HEPES, pH 7.4).

(ii) Stock solution of CDS: 0.5 mg/mL solution of CDS was prepared in diffusion medium (100 mM HPBCD, 20 mM HEPES, pH 7.4).

3.4.2 Method

Appropriate aliquots of the stock solution of CDS were transferred to 10 mL volumetric flasks and were diluted up to the mark with diffusion medium (100 mM HPBCD, 20 mM HEPES, pH 7.4). The absorption maxima (λ_{max}) was determined by scanning 10 µg/mL solution against reagent blank on UV-Visible Spectrophotometer

(UV-1700, Schimadzu). The absorption of all the prepared solutions was then measured at the absorbance maxima, 306.2 nm against the reagent blank. The readings were recorded in triplicate. Mean value (n=3) along with the standard error of mean (SEM) are recorded in Table 3.10. The regressed values of absorption were plotted graphically against the concentrations, as shown in Figure 3.4.

Stability of the solutions of CDS in diffusion medium (100 mM HPBCD, 20 mM HEPES, pH 7.4) used for preparing the calibration plot, was ascertained by observing the changes in the absorbance of the solution at the analytical wavelength, over a period of 48 hr at room temperature.

Table 3.10 Calibration plot of CDS in diffusion medium (100 mM HPBCD, 20 mM HEPES, pH 7.4) for the estimation of CDS during diffusion (n=3)

Conc. (µg/mL)	Absorbance (±SEM)
5	0.0493 ± 0.0035
10	0.0977 ± 0.0047
15	0.1523 ± 0.0057
20	0.2117 ± 0.0075
25	0.2557 ± 0.0095
30	0.3090 ± 0.0125
35	0.3527 ± 0.0111
40	0.4050 ± 0.0092
50	0.5107 ± 0.0115
60	0.6040 ± 0.0105
70	0.7080 ± 0.0122
80	0.8247 ± 0.0106

Figure 3.4 Calibration plot of CDS in diffusion medium (100 mM HPBCD, 20 mM HEPES, pH 7.4)

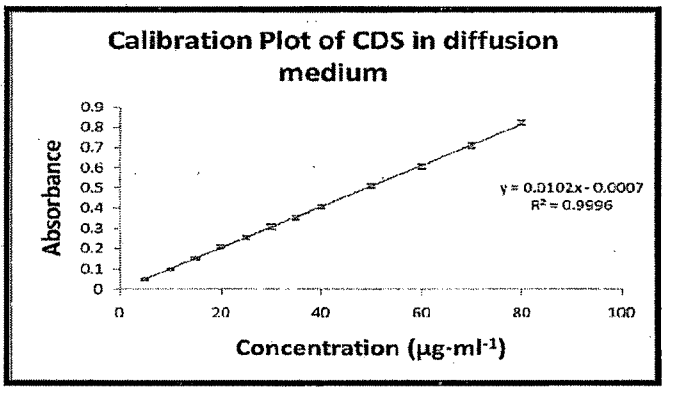


Table 3.11 Optical characteristics of CDS in diffusion medium (100 mM HPBCD, 20 mM HEPES, pH 7.4)

Characteristic	Value
λ_{max}	306.2 nm
Solvent	100 mM HPBCD, 20 mM HEPES, pH 7.4
Range	5-80 µg/mL
Regression equation	y = 0.0102x - 0.0007
Regression Coefficient (R^2)	0.9996

Table 3.12 Accuracy and precision of CDS estimation by UV- specroscopic method in diffusion medium (n=6)

Conc.(µg/mL)	Obtained Conc. (µg/mL) (±SEM)	% Recovery	% RSD
10	9.973 ± 0.094	99.728	1.627
20	19.950 ± 0.194	99.749	1.686
40	40.993 ± 0.248	102.482	1.048
70	69.782 ± 0.427	99.689	1.061

Accuracy is reflected from % Recovery and precision from % RSD

3.5 PREPARATION OF CALIBRATION PLOT OF RGZ IN PLASMA BY HPLC METHOD

The chromatographic determination of RGZ in plasma is based on reversed-phase HPLC method with UV detection (Radhakrishna et al., 2002; Lin et al., 2004; Kang et al., 2009; Mamidi et al., 2003; Kim and Park, 2004; Muxlow, 2001; Kolte et al., 2003; Pedersen et al., 2005; Hruska and Frye, 2004).

3.5.1 Reagents

(i) Sodium acetate solution 10 mM (pH 5) (0.8203 g of monobasic potassium phosphate in 800 mL of water and adjusting to pH 5 with 0.5 N HCl or 0.5 N NaOH and diluting to 1000 mL with water).

(ii) Methanol for liquid chromatography LiChrosolv®

(iii) Acetonitrile for liquid chromatography LiChrosolv®

(iv) Freshly prepared mobile phase (10mM sodium acetate pH 5: acetonitrile: methanol (40 : 40 : 20, v/v/v))

(v) Stock solution of RGZ: 1 mg/mL solution of RGZ was prepared in mobile phase.

3.5.2 Preparation of standard solutions and calibration standards

Stock solutions were prepared by dissolving RGZ in mobile phase to yield primary solutions with a concentration of 1 mg/mL of RGZ. Calibration standards were prepared by spiking working standard solutions into drug-free plasma to yield concentrations of 0.05 µg/mL – 10 µg/mL of RGZ. Triplicate calibration plots were analyzed daily for 3 days.

3.5.3 Instrumentation

The HPLC system Prominence LC-20AT (Shimadzu, Kyoto, Japan) consisted of a LC-20AT pump with a manual injector 20 µL fixed loop, equipped with a UV-VIS detector set at 245.0 nm, with Spinchrom CFR software version 2.4.1.93.

3.5.4 Extraction procedure

RGZ solutions (50 µL) were added and the tube was vortexed for 1 min, was added to plasma samples (200 µL) in microcentrifuge tubes and vortexed briefly. ACN (600 µL) was then added to each sample, vortexed for 2 min, and centrifuged at 3500 g for 10 min. Supernatant was evaporated using nitrogen gas at 45 ◦C. Dried samples were reconstituted with 200 µL of mobile phase and were ready for analysis by HPLC.

3.5.5 HPLC conditions

The chromatographic analysis was carried out on a GRACE Brava™ BDS C18 (5 µm, 25 cm × 4.6 mm) column maintained at 30 ± 0.5°C. RGZ was eluted using a mobile phase composition of 10mM sodium acetate (pH 5): acetonitrile: methanol (40:40:20, v/v/v) at a flow rate 1.0 mL·min^{-1}. The mobile phase was premixed, filtered through a 0.45 mm Nylon 66 membrane filter and degassed before use. RGZ was detected at 245.0 nm and was eluted in 6.7 min after injection.

The calibration plot was linear ($r^2 > 0.9996$) in the concentration range of 0.05-10 µg/mL, and the quantitation limit at 5:1 signal to noice ratio was 0.05 µg/mL.

Table 3.13 Calibration plot of RGZ in plasma by HPLC method (n=3)

Conc. (µg/mL)	Peak area (mV·s) (±SEM)
0.05	1.854 ± 0.317
0.1	4.192 ± 0.485
0.2	7.014 ± 1.808
0.4	14.819 ± 2.059
0.8	26.828 ± 1.904
1	34.326 ± 2.151
2	68.254 ± 2.502
4	140.764 ± 3.513
5	177.454 ± 3.822
8	284.562 ± 3.541
10	364.511 ± 3.433

Figure 3.5 Calibration plot of RGZ in plasma by HPLC Method

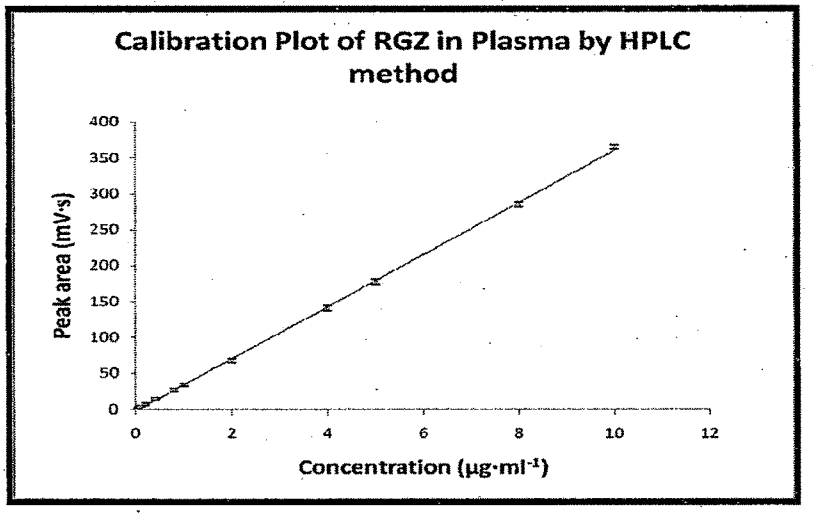


Table 3.14 Chromatographic conditions

Characteristic	Condition
Column	GRACE Brava™ BDS C18 (5 µm, 25 cm × 4.6 mm)
Column temperature	30 ± 0.5°C
Mobile phase	10mM sodium acetate (pH 5): acetonitrile: methanol (40:40:20, v/v/v)
Flow rate	1.0 mL·min^{-1}
Injection volume	20 µL
Detection wavelength	245.0
Range	0.05-10 µg/mL
Regression equation	y = 36.1473x - 1.5747
Regression Coefficient (R^2)	0.9996

Table 3.15 Accuracy and precision of RGZ estimation by HPLC method (n=6)

Conc.(µg/mL)	Obtained Conc. (µg/mL) (±SEM)	% Recovery	% RSD
0.8	0.802 ± 0.0073	100.276	1.576
2	1.992 ± 0.0173	99.585	1.506
4	3.916 ± 0.0219	97.910	0.970
8	8.029 ± 0.0399	100.357	0.861

Accuracy is reflected from % Recovery and precision from % RSD

3.6 PREPARATION OF CALIBRATION PLOT OF CDS IN PLASMA BY HPLC METHOD

The chromatographic determination of CDS in plasma is based on reversed-phase HPLC method with UV detection (Gonzalez et al., 2000; Daneshtalab et al., 2002; Qutab et al., 2007; Subba Rao et al., 2007; Stenhoff et al., 1999; Erk, 2003).

3.6.1 Reagents

(i) Potassium dihydrogen phosphate 10 mM (1.3609 g of Potassium dihydrogen phosphate in 1000 mL of water).

(ii) Methanol for liquid chromatography LiChrosolv®

(iii) Acetonitrile for liquid chromatography LiChrosolv®

(iv) Freshly prepared mobile phase (methanol: acetonitrile: 10mM sodium acetate pH 5 (74 : 16 : 10, v/v/v) pH 2.5 adjusted with 0.5 N HCl or 0.5 N NaOH)

(v) Stock solution of CDS: 1 mg/mL solution of CDS was prepared in mobile phase.

3.6.2 Preparation of standard solutions and calibration standards

Stock solutions were prepared by dissolving CDS in mobile phase to yield primary solutions with a concentration of 1 mg/mL of CDS. Calibration standards were prepared by spiking working standard solutions into drug-free plasma to yield concentrations of 0.05 µg/mL – 10 µg/mL of CDS. Triplicate calibration plots were analyzed daily for 3 days.

3.6.3 Instrumentation

The HPLC system Prominence LC-20AT (Shimadzu, Kyoto, Japan) consisted of a LC-20AT pump with a manual injector 20 µL fixed loop, equipped with a UV-VIS detector set at 260.0 nm, with Spinchrom CFR software version 2.4.1.93.

3.6.4 Extraction procedure

CDS solutions (50 µL) were added and the tube was vortexed for 1 min, was added to plasma samples (200 µL) in microcentrifuge tubes and vortexed briefly. ACN (600 µL) was then added to each sample, vortexed for 2 min, and centrifuged at 3500 g for 10 min. Supernatant was evaporated using nitrogen gas at 45 °C. Dried samples were reconstituted with 200 µL of mobile phase and were ready for analysis by HPLC.

3.6.5 HPLC conditions

The chromatographic analysis was carried out on a GRACE Brava™ BDS C18 (5 µm, 25 cm × 4.6 mm) column maintained at 30 ± 0.5°C. CDS was eluted using a mobile phase composition of methanol: acetonitrile: 10mM sodium acetate pH 5 (74 : 16 : 10, v/v/v) (pH 2.5) at a flow rate 1.0 $mL \cdot min^{-1}$. The mobile phase was premixed, filtered through a 0.45 mm Nylon 66 membrane filter and degassed before use. CDS was detected at 260.0 nm and was eluted in 4.5 min after injection.

The calibration plot was linear ($r^2 > 0.9997$) in the concentration range of 0.05-10 µg/mL, and the quantitation limit at 5:1 signal to noice ratio was 0.05 µg/mL.

Table 3.16 Calibration plot of CDS in plasma by HPLC method (n=3)

Conc. (µg/mL)	Peak area (mV·s) (±SEM)
0.05	1.247 ± 0.266
0.1	2.554 ± 0.433
0.2	4.645 ± 0.884
0.4	7.902 ± 1.083
0.8	14.958 ± 1.317
1	19.145 ± 1.857
2	36.057 ± 2.504
4	73.039 ± 2.837
5	92.977 ± 3.054
8	143.811 ± 3.268
10	184.242 ± 2.573

Figure 3.6 Calibration plot of CDS in plasma by HPLC

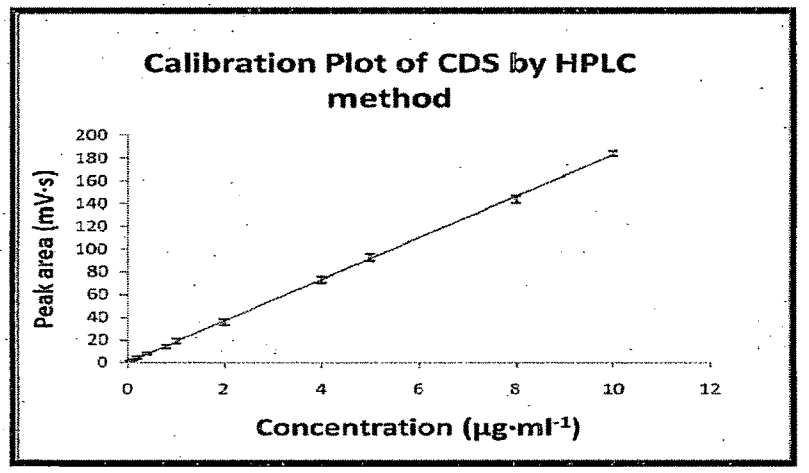


Table 3.17 Chromatographic conditions

Characteristic	Condition
Column	GRACE Brava™ BDS C18 (5 µm, 25 cm × 4.6 mm)
Column temperature	30 ± 0.5°C
Mobile phase	Methanol: acetonitrile: 10mM sodium acetate pH 5 (74 : 16 : 10, v/v/v) (pH 2.5)
Flow rate	1.0 mL·min^{-1}
Injection volume	20 µL
Detection wavelength	260.0
Range	0.05-10 µg/mL
Regression equation	y = 18.2094x + 0.5733
Regression Coefficient (R^2)	0.9997

Table 3.18 Accuracy and precision of CDS estimation by HPLC method (n=6)

Conc.(µg/mL)	Obtained Conc. (µg/mL) (±SEM)	% Recovery	% RSD
0.8	0.799 ± 0.0081	99.874	1.756
2	1.995 ± 0.0152	99.749	1.320
4	4.100 ± 0.0229	102.488	0.966
8	7.966 ± 0.0319	99.571	0.694

Accuracy is reflected from % Recovery and precision from % RSD

3.7 DETERMINATION OF D-MANNOSE (Dubois et al., 1956)

3.7.1 Reagents

Reagent 1: Sulfuric acid

Reagent 2: Phenol (80% by weight in water)

3.7.2 Procedure

2 mL of sugar solution containing between 10-70 µg of D-Mannose is pipette into a colorimetric tube, and 0.05 mL of 80% phenol is added. Then 5 mL of concentrated sulfuric acid is added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes are allowed to stand for 10 minutes, and then they are shaken and placed for 10 to 20 minutes in a water bath at 25° to 30°C before readings are taken. The color is stable for several hours and readings may be made later if necessary. The absorbance

of the characteristic yellow-orange color is measured at 490 nm. Blank is prepared by substituting distilled water for sugar solution. All solutions were prepared in triplicate to minimize errors.

Table 3.19 Calibration plot of D-Mannose (n=3)

Conc. (µg/mL)	Absorbance (±SEM)
10	0.119 ± 0.013
20	0.237 ± 0.010
30	0.347 ± 0.012
40	0.438 ± 0.019
50	0.548 ± 0.019
60	0.677 ± 0.020
70	0.768 ± 0.015
80	0.893 ± 0.018

Figure 3.7 Calibration plot of D-Mannose

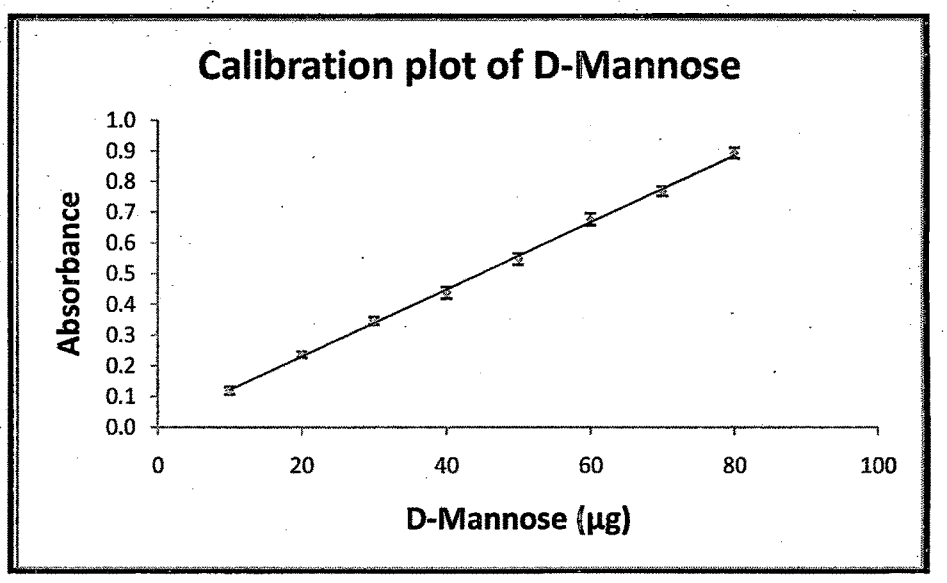


Table 3.20 Optical characteristics

Characteristic	Value
λ_{max}	490 nm
Range	10-80 µg
Regression equation	y = 0.0109x - 0.0122
Regression Coefficient (R^2)	0.9989

Table 3.21 Accuracy and precision of D-Mannose estimation by Colorimetric method (n=6)

Conc.(µg/mL)	Obtained Conc. (µg/mL) (±SEM)	% Recovery	% RSD
10	10.030 ± 0.157	100.299	2.597
20	19.936 ± 0.305	99.682	2.646
40	39.883 ± 0.464	99.706	2.014
70	69.787 ± 0.870	99.695	2.160

Accuracy is reflected from % Recovery and precision from % RSD

3.8 DETERMINATION OF PHOSPHORUS.

3.8.1 Principle

In this method phosphorus in the sample is first acid hydrolyzed inorganic phosphate. This is converted to phosphor-molybdic acid by the addition of ammonium molybdate, and the pho-molybdic acid is quantitatively reduced to a blue colored compound by amino-naphthyl-sulfonic cid. The intensity of the blue color is measured spectroscopically, and is compared with calibration standards to give phosphorus content (Bottcher et al., 1961; Bartlett, 1959).

3.8.2 Reagents

1. Reagent 1: Sulfuric acid (5M) reagent
2. Reagent 2: Phosphate standard solutions: Dry a sample of solid anhydrous potassium dihydrogen phosphate at 105°C for 4 hours in a vacuum oven. Weigh out 43.55 mg of dried solid and transfer to a 100 mL volumetric flask. Dissolve in double distilled water, and make up to 100 mL. The final concentration should be 3.2 µmol phosphorus mL^{-1}. For working standard solutions transfer 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5 mL of stock phosphate solution into separate 100 mL volumetric flasks and make up to the mark with double distilled water to give solutions with concentration of 0.016, 0.032, 0.048, 0.064, 0.080, 0.096, 0.112, 0.128, 0.144, 0.160 and 0.176 µmol phosphorus mL^{-1} respectively (Equivalent to 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5 µg of phosphorus).
3. Reagent 3: Ammonium molybdate – sulfuric acid reagent: Add 5 mL of the sulfuric acid reagent to approximately 50 mL of distilled water and add 0.44 g

of ammonium molybdate. Mix well and make up to 200 mL with distilled water.

4. Reagent 4: 1-Amino 2-naphthyl 4-sulfonic acid reagent: Weigh out 0.8 g and dissolve in 5 mL of double distilled water. Prepare fresh on day of use.
5. Reagent 5: Hydrogen peroxide: Add 1 mL of 100 % hydrogen peroxide to 9 mL of distilled water and mix well. Prepare fresh immediately before use.

3.8.3 Procedure

1. Equilibrate heating block by pre-heating at 200°C for 30 min.
2. Set up calibration curve by pipetting, into separate 16 × 150 mm disposable borosilicate tubes, 0.5 mL of each working standard solution, together with a blank (0.5 mL of double distilled water).
3. Prepare sample tube in triplicate by adding 50 µL of the sample to each three empty tubes. Dry down and resuspend in 0.5 mL of distilled water.
4. Add 0.4 mL of sulfuric acid reagent to each tube, cover and incubate in a heating block in a fume hood at 180 – 200 °C for an hour.
5. Allow the tubes to cool by standing them at room temperature.
6. Prepare diluted hydrogen peroxide (10%) fresh.
7. To each tube add 0.1 mL of diluted hydrogen peroxide and incubate on the heating block at 180 – 200 for 30 min to achieve a clear solution. (If necessary, repeat addition and heating until solution is clear.)
8. Cool the tubes by standing them at room temperature.
9. Add 4.6 mL of acid – molybdate solution to each tube and mix thoroughly by vortexing.
10. Add 0.2 mL of reagent 4 to each tube and mix thoroughly by vortexing after addition.
11. Cover the tubes and place them in boiling water bath.
12. Leave the tubes in the bath for 7 min after boiling recommences.
13. Cool the tubes to room temperature.
14. Measure absorbance of all tubes against distilled water at 830 nm. The concentration in the starting sample is ten times that read off the graph of the standard curve.

Table 3.22 Calibration plot of Phosphorus (n=3)

Conc. (µmol phosphorus/mL) [µg of phosphorus]	Absorbance (±SEM)
0.016 [0.5]	0.079 ± 0.0051
0.032 [1.0]	0.172 ± 0.0090
0.048 [1.5]	0.238 ± 0.0070
0.064 [2.0]	0.341 ± 0.0080
0.080 [2.5]	0.406 ± 0.0075
0.096 [3.0]	0.514 ± 0.0099
0.112 [3.5]	0.582 ± 0.0111
0.128 [4.0]	0.678 ± 0.0074
0.144 [4.5]	0.749 ± 0.0115
0.160 [5.0]	0.846 ± 0.0086
0.176 [5.5]	0.904 ± 0.0132

Figure 3.8 Calibration plot of Phosphorus

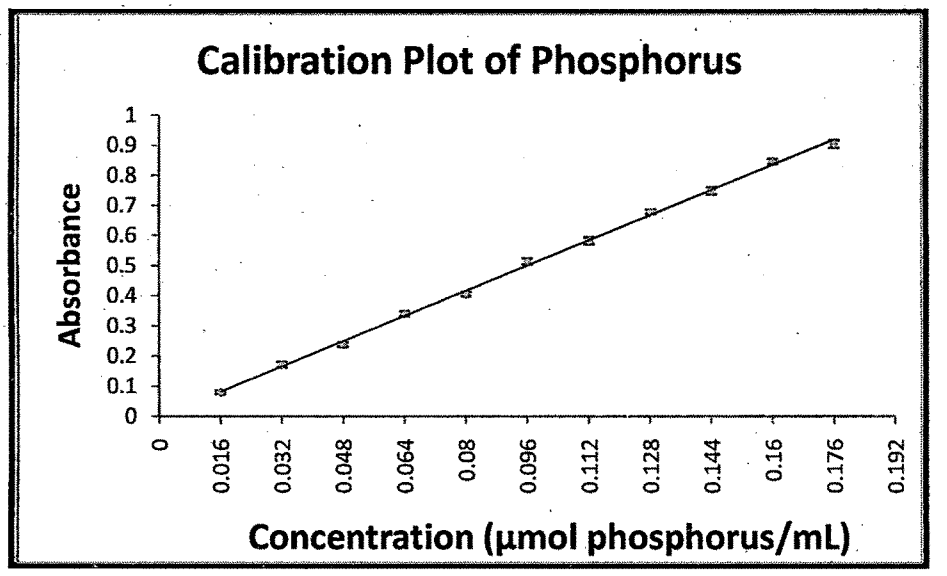


Table 3.23 Optical characteristics

Characteristic	Value
λ_{max}	830 nm
Range	0.5-5.5 µg of phosphorus
Regression equation	y = 5.2302x - 0.0014
Regression Coefficient (R^2)	0.9987

Table 3.24 Accuracy and precision of Phosphorus by spectroscopic method (n=6)

Conc.(µmol/mL)	Obtained Conc. (µmol/mL) (±SEM)	% Recovery	% RSD
0.032	0.0320 ± 0.0008	100.125	4.207
0.064	0.0646 ± 0.0014	100.953	3.753
0.128	0.1264 ± 0.0022	98.719	3.016
0.176	0.1771 ± 0.0026	100.648	2.542

Accuracy is reflected from % Recovery and precision from % RSD

3.9 DETERMINATION OF TOTAL PROTEINS

3.9.1 Principle

Proteins react with copper of Biuret reagent in alkaline medium to form a blue purple complex with absorption maximum at 550 nm (Gornall et al., 1949; Smith et al., 1985.

3.9.2 Reagents

Reagent 1: Biuret reagent

Reagent 2: Protein standard

3.9.3 Procedure

Table 3.25 Procedure for determination of total protein

	Blank (B)	Standard (S)	Test (T)
Sample	---	---	0.1 mL
Reagent 2: Protein standard	---	0.1 mL	---
Reagent 1: Biuret reagent	2.5 mL	2.5 mL	2.5 mL

Mix well. Allow tubes to stand at R.T. for 5 min. measure the O.D. of Standard(S) and Test(T) at 550 nm on spectrophotometer.

3.9.4 Calculation

$$Total\ protein\ (\tfrac{g}{100ml}) = \frac{O.D.Test}{O.D.Std.} \times Conc.of\ total\ protein\ (Std.)$$

3.10 DETERMINATION OF ALBUMIN IN SERUM

3.10.1 Principle

Determination of albumin in serum or plasma is based on the binding behavior of the protein with the dye bromocresol green. At pH 4.0, albumin acts as a cation and binds to the anionic dye, forming a green complex, the absorbance of which is measured at 630 nm against reagent blank(B) (Rodkey, 1965; McDonald and Gerarde, 1963; Varley, 1980; Corcoran and Durnan, 1977; Gustafsson, 1976).

3.10.2 Reagents

Reagent 1: Albumin reagent (Bromocresol green, Buffer pH 3.8, preservative, surfactant)

Reagent 2: Albumin standard 4.0 gm/dL (Bovine serum albumin, Buffer, Preservatives)

3.10.3 Procedure

Table 3.26 Basic parameters for determination of Albumin in serum

Parameter	Value
Detection wavelength	630 nm
Temperature	Room temperature
Blank	Reagent blank
Incubation time	1 min
Sample volume	10 µL
Reagent volume	1000 µL
Concentration of standard	4.0 gm/dL
Stability of color	2 hours
Unit	gm/dL

Table 3.27 Procedure for determination of Albumin in serum

	Blank (B)	Standard (S)	Test (T)
Sample	---	---	0.1 µL
Reagent 2: Albumin standard	---	10 µL	---
Reagent 1: Albumin reagent	1.0 mL	1.0 mL	1.0 mL

Mix well. Allow tubes to stand at R.T. for 1 min. measure the O.D. of Standard(S) and Test(T) at 630 nm on colorimeter against reagent blank(B).

3.10.4 Calculation

$$Albumin\ (\frac{g}{dl}) = \frac{Absorbance\ of\ Test}{Absorbance\ of\ Std.} \times 4.0$$

3.11 DETERMINATION OF GLOBULIN IN SERUM

Amount of globulin in serum was derived from the amount of total protein and albumin in serum according to following formula.

$$Globulin\ \left(\frac{g}{100\ ml}\right) = Serum\ total\ protein\ \left(\frac{g}{100\ ml}\right) - Serum\ albumin\ \left(\frac{g}{100\ ml}\right)$$

3.12 DETERMINATION OF ASPARTATE AMINOTRANSFERASE (AST) IN SERUM

3.12.1 Principle

Aspartate aminotransferase (AST) catalyses the transamination of L- Aspartate and α-ketoglutarate (α-KG) to form Oxaloacetate and L- Glutamate. Oxaloacetate so formed is coupled with 2,4-Dinitrophenyl hydrazine (2,4-DNHP) to form a corresponding hydrazone, a brown colored complex in alkaline medium and this can be measured colorimetrically.

$$\alpha\ KG + L\ Aspartate \leftrightharpoons Oxaloacetate + L\ Glutamate$$

$$Oxaloacetate + 2,4\ DNPH \leftrightharpoons Corresponding\ Hydrazone\ (Brown\ color)$$

Reitman and Frankel method is an end point colorimetric method for the estimation of enzyme activity. To obtain accurate results, method has been standardized with kinetic method (standard karmen unit assay). The method used here is single point calibration version of original method for maximum ease of use and convenience (Henry et al., 1960; Karmen, 1955; Reitman and Frankel, 1957; Feri et al., 1995).

3.12.2 Reagents

Reagent 1: Buffered Aspartate - α-KG substrate, pH 7.4 (Phosphate buffer, L-Aspartic acid, α-KG, Stabilizer, Preservative)

Reagent 2: 2,4-DNHP color reagent (2,4-Dinitrophenyl hydrazine, Stabilizer, Preservative)

Reagent 3: Sodium hydroxide, 4N

Reagent 4: Working Pyruvate standard, 6 mM (114 IU/L) (Sodium pyruvate, Stabilizer, Preservative)

Solution I: Dilute 1 mL of reagent 3 to 10 mL with purified water.

3.12.3 Procedure

Table 3.28 Procedure for determination of AST

	Blank	Standard	Test	Control
	Volume in mL			
Regent 1	0.25	0.25	0.25	0.25
Serum	--	--	0.05	--
Reagent 4	--	0.05	--	--
Mix well and incubate at 37°C for 60 minutes				
Reagent 2	0.25	0.25	0.25	0.25
Deionized water	0.05	--	--	--
Serum	--	--	--	0.05
Mix well and allow to stand at room temperature (15-30°C) for 20 minutes				
Solution I	2.5	2.5	2.5	2.5

Mix well and read the O.D. against purified water in a spectrophotometer at 505 nm, within 15 minutes.

3.12.4 Calculation

$$AST\ (SGOT) activity\ \left(in \frac{IU}{L}\right) = \frac{Absorbance\ of\ Test - Absorbance\ of\ control}{Absorbance\ of\ Standard - Absorbance\ of\ Blank} \times Conc.\ of\ Standard$$

3.13 DETERMINATION OF ALANINE AMINOTRANSFERASE (ALT) IN SERUM

3.13.1 Principle

Alanine aminotransferase (AST) catalyses the transamination of L- Alanine and α-ketoglutarate (α-KG) to form Pyruvate and L- Glutamate. Pyravate so formed is coupled with 2,4-Dinitrophenyl hydrazine (2,4-DNHP) to form a corresponding hydrazone, a brown colored complex in alkaline medium and this can be measured colorimetrically.

$$\alpha \text{ KG} + \text{L Alanine} \leftrightharpoons \text{Pyruvate} + \text{L Glutamate}$$

$$\text{Pyruvate} + \text{2,4 DNPH} \leftrightharpoons \text{Corresponding Hydrazone (Brown color)}$$

Reitman and Frankel method is an end point colorimetric method for the estimation of enzyme activity. To obtain accurate results, method has been standardized with kinetic method (standard karmen unit assay). The method used here is single point calibration version of original method for maximum ease of use and convenience (Henry et al., 1960; Karmen, 1955; Reitman and Frankel, 1957; Feri et al., 1995).

3.13.2 Reagents

Reagent 1: Buffered Alanine - α-KG substrate, pH 7.4 (Phosphate buffer, L-Alanine, α-KG, Stabilizer, Preservative)

Reagent 2: 2,4-DNHP color reagent (2,4-Dinitrophenyl hydrazine, Stabilizer, Preservative)

Reagent 3: Sodium hydroxide, 4N

Reagent 4: Working Pyruvate standard, 8 mM (150 IU/L) (Sodium pyruvate, Stabilizer, Preservative)

Solution I: Dilute 1 mL of reagent 3 to 10 mL with purified water.

3.13.3 Procedure

Table 3.29 Procedure for determination of ALT

	Blank	Standard	Test	Control
	Volume in mL			
Regent 1	0.25	0.25	0.25	0.25
Serum	--	--	0.05	--
Reagent 4	--	0.05	--	--
Mix well and incubate at 37°C for 60 minutes				
Reagent 2	0.25	0.25	0.25	0.25
Deionized water	0.05	--	--	--
Serum	--	--	--	0.05
Mix well and allow to stand at room temperature (15-30°C) for 20 minutes				
Solution I	2.5	2.5	2.5	2.5

Mix well and read the O.D. against purified water in a spectrophotometer at 505 nm, within 15 minutes.

3.13.4 Calculation

$$ALT\ (SGPT) activity\ \left(in \frac{IU}{L}\right) = \frac{Absorbance\ of\ Test - Absorbance\ of\ control}{Absorbance\ of\ Standard - Absorbance\ of\ Blank} \times Conc.\ of\ Standard$$

3.14 DETERMINATION OF HYDROXYPROLINE IN TISSUE (Switzer, 1991)

3.14.1 Reagents:

I. **Hydroxyproline standard, 0.1 mg/mL.** Dissolve 250 mg of vacuum-dried L-hydroxyproline (Sigma, Cat. No. H-6002) in 25 mL 0.001 N HC1. One mL of this solution is diluted to 100 mL with 0.001 N HC1.

II. **Hydroxyproline working standard, 10 µg/mL.** Dilute 10 mL 0.I mg/mL hydroxyproline standard with deionized water to 100 mL.

III. **Potassium borate buffer, pH 8.7.** Mix 61.84 g boric acid and 225 g KCI in about 800 mL deionized water. Adjust the pH to 8.7 with I0 N and 1 N KOH and make the final volume up to 1 liter. Prepare a 1:5 dilution of buffer as needed.

IV. **Chloramine T solution.** Prepare fresh daily a solution of 536.4 mg of chloramine T (Sigma, Cat. No. C-9887) in l0 mL methyl cellosolve (ethylene glycol monomethyl ether, Fisher, Cat. No. E-182).

V. **Sodium thiosulfate, 3.6 M.** Dissolve 893.4 g sodium thiosulfate in about 900 mL deionized water and bring the final volume to 1 liter. Store under toluene at room temperature for several weeks.

VI. **Ehrlich's reagent.** Add 27.4 mL concentrated sulfuric acid to 200 mL absolute ethanol in a beaker and cool the mixture. In another beaker, place 120 g p-dimethylaminobenzaldehyde (Fisher, Cat. No. D7 l- 100) and 200 mL absolute ethanol and then add slowly with stirring the acid-ethanol mixture from the first beaker. The solution can be stored in the refrigerator for several weeks and the crystals that form can be redissolved by warming the solution.

VII. **Glass culture tubes, 150×16 ram, screw-capped with Teflon liners** (Fisher, Cat. No. 14-930-10E) are used both for hydrolysis and for hydroxyproline oxidation.

3.14.2 Procedure

3.14.2.1 Tissue hydrolysis

1. Place 25-350 mg wet weight of tissue in dry culture tube of known weight.
2. Dry the samples in an oven at 65 ° C for 18-24 hours and then allow the tubes to cool to room temperature in a desiccator.
3. Weigh the tubes.
4. Add 2.0 mL 6 N HCI, cap and hydrolyze the samples at 110 ° C for 24 hr.
5. Evaporate the samples to dryness with a stream of nitrogen.
6. Add 10.0 mL deionized water to each tube and mix well.

3.14.2.2 Hydroxyproline determination

1. Transfer 0.2 mL hydrolyzate to a clean, labeled culture tube followed by 1.6 mL deionized water.
2. Prepare a set of tubes containing known amounts of hydroxyproline (1.0-8.0 µg) and water as reagent blank.
3. Add 1.0 mL 1:5 diluted borate buffer to all tubes.
4. Add 0.3 mL chloramine T to each tube in a timed sequence to oxidize the hydroxyproline and mix well.
5. After 20 min, add 1.0 mL sodium thiosulfate and mix well.
6. Add about 1.5 g potassium chloride to saturate all tubes. If indole, dehydroproline, or similar compounds are anticipated in the samples, extract with 2.5 mL toluene and discard the toluene extract.
7. Cap and heat the tubes in boiling water for 20 min.
8. Cool tubes to room temperature, add 2.5 mL toluene, and cap all tubes tightly. Invert the tubes 100 times or shake them about 5 min.

9. Centrifuge the tubes at low speed briefly and transfer 1.0 mL toluene extract to labeled 12 × 75 mm test tubes.
10. Add 0.4 mL Ehrlich's reagent and allow the color to develop by 30 min.
11. Read the absorbances at 565 nm against a reagent blank.
12. A linear regression of absorbance versus µg hydroxyproline standard can be used to calculate the hydroxyproline content of each unknown sample. Multiply the values by the dilution factor of 50 to determine the hydroxyproline content of the original hydrolyzate.

3.14.2.3 Special considerations

1. The toluene extract in step 6 of hydroxyproline determination can be used to determine the concentration of proline by a periodate oxidation method.
2. The potassium chloride can be added at any point before oxidation with chloramine T without any effect on the color yield.
3. Toluene may be added before heating the samples in order to save time in removing caps and recapping tubes in step 8. Any toluene lost appears to be proportional in all tubes, but the caps need to be checked for tightness since they frequently become loose on cooling after heating.
4. Centrifugation in step 9 may not be necessary if no emulsion is present in any of the samples.
5. Hydroxyproline can be determined in 48-60 samples in about 4 hours.

Table 3.30 Calibration plot of Hyroxyproline (n=3)

Conc. (µg/mL)	Absorbance (±SEM)
0.5	0.0499 ± 0.0028
1	0.1209 ± 0.0047
2	0.2078 ± 0.0083
3	0.3361 ± 0.0071
4	0.4483 ± 0.0100
5	0.5579 ± 0.0127
6	0.6587 ± 0.0150
7	0.7867 ± 0.0148
8	0.8997 ± 0.0160

Figure 3.9 Calibration plot of Hydroxyproline

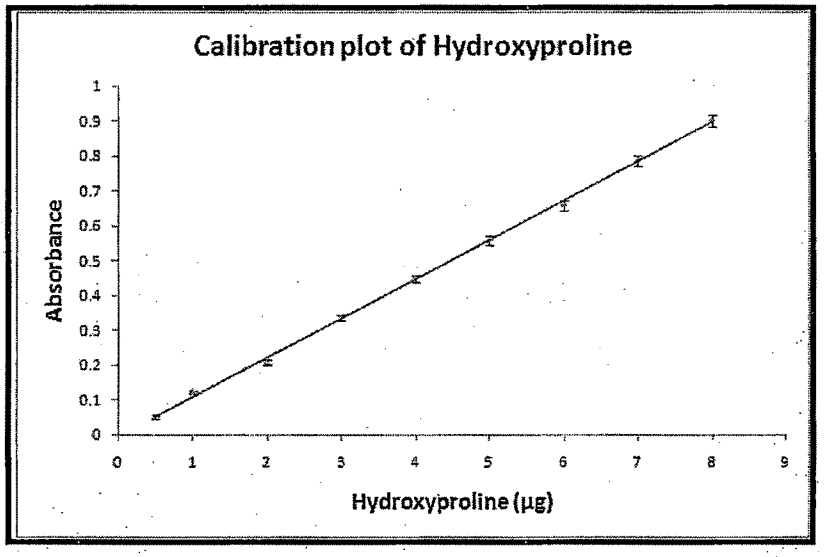


Table 3.31 Calibration parameters

Characteristic	Value
λ	565 nm
Range	0.5-8 µg
Regression equation	y = 0.1124x - 0.0040
Regression Coefficient (R^2)	0.9993

Table 3.32 Accuracy and precision of Hydroxyproline by specroscopic method (n=6)

Amount (µg)	Obtained Amount (µg) (±SEM)	% Recovery	% RSD
1	1.004 ± 0.0256	100.435	4.290
2	2.007 ± 0.0377	100.335	3.252
4	4.010 ± 0.0668	100.261	2.886
8	7.972 ± 0.1089	99.645	2.366

Accuracy is reflected from % Recovery and precision from % RSD

3.15 DETERMINATION OF HYALURONIC ACID IN SERUM

3.15.1 Background

Hyaluronan (HA) is a high molecular weight (1000-5000 kD) anionic polysaccharide composed of repeating disaccharides of glucuronate acetylglucosamine. The HA-

ELISA is a quantitative enzyme-linked immunoassay designed for the in vitro measurement of HA levels in human or animal biological fluids (blood, serum, urine, diffusate, synovial fluid) or cell-culture supernatant.

The HA-ELISA is a competitive ELISA assay in which the colorimetric signal is inversely proportional to the amount of HA present in the sample. Samples to be assayed are first mixed with the Detector, then added to the HA ELISA Plate for competitive binding. An enzyme-linked antibody and colorimetric detection is used to detect the HA detector bound to the plate. The concentration of HA in the sample is determined using a standard plot of known amounts of HA (Kongtawelert and Ghosh, 1989; Plevris et al., 2000; McHutchison et al., 2000).

3.15.2 Reagent Preparation

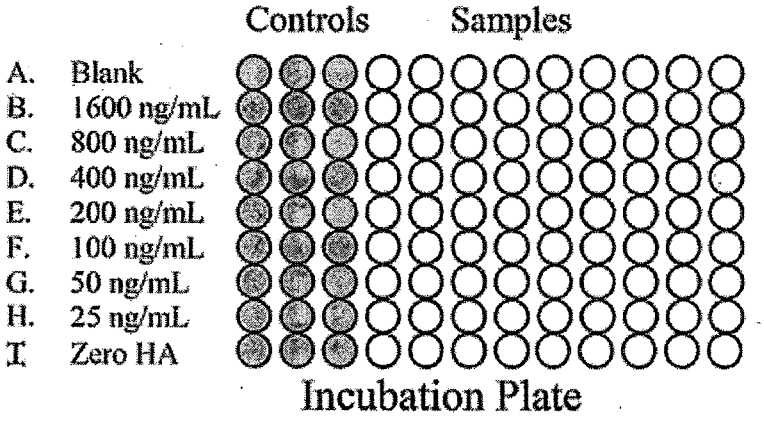
I. **HA Standards:** Make 1:2 serial dilutions of the HA Standard using the Diluent to obtain standards of 1600, 800, 400, 200, 100, and 50 ng/mL (Controls may be diluted in the plate, following the diagram below).

II. **Working Detector:** Dilute Detector with 5 mL Diluent.

III. **Working Enzyme:** Dilute Enzyme with 10 mL Diluent.

IV. **Wash Buffer:** Make a 1:10 dilution of Wash Buffer in distilled water.

V. **Working Substrate Solution:** Dissolve Substrate Pellet in 10.5 mL Substrate Buffer.



3.15.3 Assay Procedure

1. Set up the incubation plate as illustrated above. We suggest the HA Standard dilution series be run in triplicate for best results. Add 100 µL of Standards and samples into corresponding wells. Add 150 µL of Diluent to Blank Control and 100 µL of Diluent to Zero HA Control wells. Add 50 µL of Working Detector to all wells except the Blank. Mix well. Cover plate and incubate for one hour at 37°C.
2. Following the incubation, transfer 100 µL of controls and samples to the corresponding wells of the HA ELISA plate. Cover plate and incubate for 30 minutes at 4°C.
3. Discard the solution and wash the wells four times with 300 µL of 1X Wash Buffer.
4. Add 100 µL of Working Enzyme to each well. Cover plate and incubate at 37°C for 30 minutes.
5. Repeat wash step 3.
6. Add 100 µL Working Substrate Solution to each well. Incubate the plate in the dark at room temperature for 30-45 minutes
7. Measure the absorbance of each well at 405 nm. The Blank should have an absorbance of < 0.10 and the ratio of the Zero HA Control to the 1600 ng/mL HA Standard should be > 4.0. If the ratio is < 4.0, continue incubation and read plate every 15 minutes until ratio is reached.
8. Stop the reaction by adding 50µL Stop Solution to each well.
9. Calculate the binding percentage for each sample using the formula:

$$\%\,Binding = 100 \times \frac{[A_{405}(Sample) - A_{405}(Blank)]}{[A_{405}(Zero\ HA) - A_{405}(Blank)]}$$

Using linear or nonlinear regression, plot a standard plot of percent binding versus concentrations of HA standards. A Log2 plot with linear regression is shown as an example. Determine HA levels of unknowns by comparing their percentage of binding relative to the standard plot.

Table 3.33 Calibration plot of Hyaluronic acid by ELISA method (n=3)

Conc. (ng/mL)	% Binding (±SEM)
25	94.331 ± 3.214
50	82.085 ± 2.973
100	63.911 ± 2.768
200	48.637 ± 1.442
400	34.772 ± 2.095
800	16.094 ± 1.978
1600	7.003 ± 1.283

Figure 3.10 Calibration plot of Hyaluronic acid by ELISA method

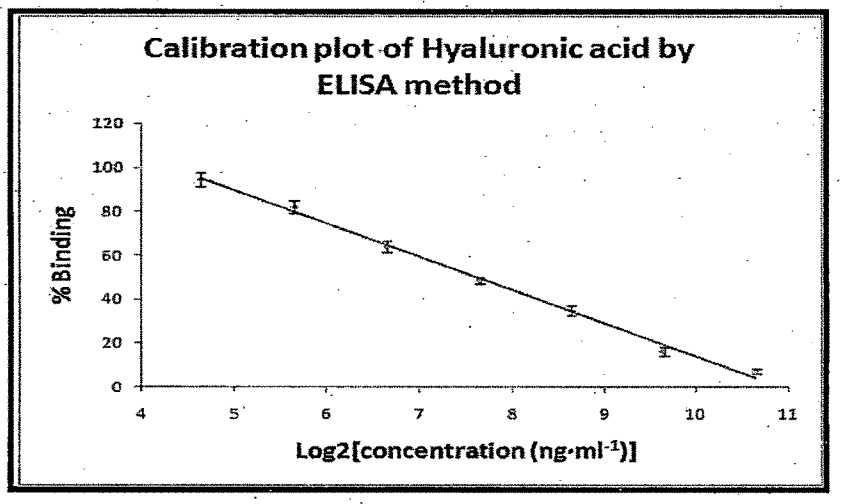


Table 3.34 ELISA parameters

Characteristic	Value
λ	405 nm
Range	25-1600 ng/mL
Regression equation	y = -15.111x + 165.05
Regression Coefficient (R^2)	0.9960

Table 3.35 Accuracy and precision of Hyaluronic acid estimation by ELISA assay (n=6)

Conc.(ng/mL)	Obtained Conc. (ng/mL) (±SEM)	% Recovery	% RSD
50	50.118 ± 1.892	100.236	6.360
200	199.027 ± 5.011	99.514	4.361
800	803.846 ± 16.689	100.481	3.596
1600	1609.710 ± 38.490	100.607	4.142

Accuracy is reflected from % Recovery and precision from % RSD

3.15.4 Reference Values

Normal HA levels in serum from healthy blood donors are less than 120 ng/mL. Serum HA levels are elevated in several disease states including hepatitis (greater than 160ng/mL) and cirrhosis (greater than 250ng/mL).

3.16 DETERMINATION OF TOTAL BILIRUBIN IN SERUM (Dangerfield, 1953)

3.16.1 Principle

In the method the serum is treated with diazo reagent- and a mixture of caffeine sodium benzoate and a phosphate buffer; the azobilirubin is formed rapidly and it is measured in a photo-electric colorimeter and compared with that of an azobilirubin standard previously prepared. A blank is used to compensate for any slight cloudiness or any color other than bilirubin, which may be present in the test serum.

3.16.2 Reagents

Reagent 1: Diazo Reagent

Diazo A:

Sulphanilic acid _____ 1 g.

Concentrated hydrochloric acid _____ 15 mL.

Distilled water to _____1000 mL.

Diazo B:

Sodium nitrite _____ 0.5 g.

Distilled water to_____ 100 mL.

For use 10 mL of diazo A is mixed with 0.3 mL. of diazo B.

Reagent 2: Diazo Blank

Concentrated hydrochloric acid _____15 mL.

Distilled water to _____ 1000 mL.

Reagent 3: Caffeine Buffer Mixture

Caffeine (25 g) and sodium benzoate (25 g) are dissolved in about 400 mL warm distilled water then cooled and potassium dihydrogen phosphate (4.1 g) and 1N sodium hydroxide (3.4 mL) are added. The mixture is diluted to 500 mL with water. The mixture, which should be filtered if not clear, will keep for at least a month.

Reagent 4: Bilirubin Solution

About 20 mg of pure bilirubin is weighed accurately and 0.2 mL wetting agent added (10% lissapol or 10% teepol). When wetting is complete 7 mL of 0.2N sodium hydroxide is added and the mixture is stirred until the bilirubin is completely dissolved. It is diluted with 0.1N sodium carbonate to a volume of 500 mL. It is important that this preparation should be carried out rapidly (within five minutes). Immediately after this solution has been made it should be used for the preparation of the standard azobilirubin and standard blank solution as below.

Reagent 5: Azobilirubin Standard

This standard solution should be prepared in duplicate. To 50 mL of caffeine buffer mixture 5 mL of diazo solution is added, followed by 10 mL of bilirubin solution, mixed, and allowed to stand for 10 minutes. Then its optical density (S) is measured using a green filter. If stored in the dark this solution can be used for checking the photometer in the subsequent serum determinations made during the following two weeks.

Reagent 6: Standard Blank Solution

This solution also should be prepared in duplicate. To 10 mL of caffeine buffer mixture, 1 mL water is added, followed by 2 mL of bilirubin solution. The solution is mixed and allowed to stand for 10 minutes, and then its optical density (SB) is measured using the same green filter as employed in measuring the density of the standard.

3.16.3 Procedure

Fresh serum (1 mL) is pipetted into each of two test tubes. To one tube (the test) 0.5 mL diazo reagent is added and to the other tube (the blank) 0.5 mL diazo blank. The tubes are shaken and stood for approximately one minute and it is noted if a red develops in the test solution indicating a positive direct van den Bergh reaction. To both tubes 5 mL of caffeine buffer mixture is added. The contents are mixed well and stood for 15 minutes to allow for full color development. The color is stable for at least one hour.

The optical densities of the test (T) and the blank (B) are measured in a photoelectric colorimeter using a green light filter or in a spectrophotometer at 525 nm, making the zero setting with water.

3.16.4 Calculation

$$Concentration\ of\ bilirubin\ in\ serum\ \left(in\frac{mg}{100\ ml}\right) = \frac{T-B}{S-SB} \times \frac{W}{5}$$

Where W= weight of bilirubin in mg. taken in preparing the bilirubin solution, and T, B, S, and SB, the optical densities of the solutions indicated above.

3.17 CONCLUSIONS

From above mentioned experimentation it was found that analytical methods for estimation of both RGZ and CDS in the dosage forms, in diffusion medium and in plasma as well as estimation of D-mannose, phosphorus, total protein, albumin, globulin, aspartate aminotransferase, alanine aminotransferase, hydroxyproline and hyaluronic acid showed good linearity, accuracy and precision. So these methods can be used for further study.

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