Chapter 3

ANALYTICAL METHODS

3.1 INTRODUCTION

Naturally occurring gums, which impart many advantageous properties to their aqueous solutions/dispersions, can be linear or branched neutral polysaccharides, polysaccharides with carboxyl groups, polysaccharides with strong acid groups or polysaccharides with basic groups. Chemical modification of polysaccharides is useful for alteration of chemical and physical properties to give the polysaccharide new applications. GG, a naturally occurring galactomannan polysaccharide, was modified chemically by acid hydrolysis, methylation, oxidation and carboxymethylation, in the present investigation. The chemically modified GGs were characterised using suitable analytical techniques. GG and chemically modified GGs were characterised by the physical parameters like bulk density and tapped density, viscosity and by instrumental methods like I.R. spectra and differential scanning calorimetry. The chemical constituents in the respective modified GG products such as the content of reducing sugars in hydrolysed GG products, methoxy content in methylated GG products and carboxy content in oxidised GG products were estimated. The analytical methods employed in this investigation are as discussed below.

3.2 MATERIALS

Galactose A.R. grade, phenol I.P. (Sarabhai M. Chemicals, Baroda), sulphuric acid A.R. (Qualigens Fine Chemicals, Mumbai), hydroiodic acid, bromine (National Chemicals, Baroda), sodium thiosulphate, sodium hydroxide A.R., sodium chloride A.R. (S.D. Fine Chem Pvt. Ltd., Boisar), hydrochloric acid A.R. (Emerck (India) Ltd., Mumbai), bromocresol purple (B.D.H. Laboratory Chemicals Division, Poole, England), chlorpheniramine maleate, phenylpropanolamine hydrochloride (courtesy Alembic Chemical Works Co. Ltd., Baroda), diltiazem hydrochloride (gifted by M.J. Institute of Research, Baroda).

3.3 EQUIPMENTS

Measuring Cylinder, Densitometer (Electrolab Instruments, Ahmedabad), Overhead mechanical stirrer (Remi Scientific Instruments, Mumbai), Brookefield synchro-lectric viscometer LVT model (Brookefield Engineering Equipments, Stoughton, Massuchetts, U.S.A.), Carver Laboratory Press (Fred S. Carver, Inc., U.S.A.), Infrared Spectrophotometer (IR460, Shimadzu, Japan), Differential Scanning Calorimeter(DSC20, Mettler, Switzerland), UV-visible Spectrophotometer (Hitachi U-2000, Japan). Analytical balance (National Scientific Works Ltd., India, 1.00 mg accuracy), calibrated pipettes 0.1ml, 0.2ml, 0.5ml, 1.0ml, and 10ml, volumetric flasks of 10ml and 100ml capacity and other requisite glasswares (Corning (India) Ltd., Bombay). High Performance Liquid Chromatograph (Waters 501, Bangalore).

3.4 ANALYTICAL METHODS FOR CHARACTERISATION OF GG AND MODIFIED GGs :

The present study involves chemical modification of GG to overcome the limitations in its use as a hydrophilic matrix for controlled release drug delivery systems. GG and modified GGs, prepared in the present work, were characterised using the following analytical techniques.

3.4.1 Bulk Density and Tapped Density :

5g of powder was transferred carefully through a glass funnel into a 25ml measuring cylinder. The volume occupied by the powder was noted. The bulk density of the product was calculated as the ratio of sample weight to sample volume. The measuring cylinder was then dropped from a height of one inch on a plain hard surface at the rate of 20 cycles/min and the tapping continued till the powder occupied a constant volume (100 taps). Tapped density was calculated as the ratio of sample weight to this constant volume.

The determinations of densities were done in triplicate for three batches of each type of modification and the average (n = 9) was recorded alongwith the standard error values.

3.4.2 Viscosity :

1g of accurately weighed sample (GG /modified GGs) was gradually added to a beaker, containing about 85ml of cold purified water, under continuous stirring at the rate of 600 ± 25 rpm, using a mechanical stirrer. The volume of the dispersion was made upto 100ml with additional amount of purified water and the dispersion was allowed to hydrate for a period of 2 h at room temperature (33°C ± 2°C). The viscosity of the dispersion/solution was measured using spindle no. 2 at 12 rpm of Brookefield synchro-lectric viscometer LVT model. In all cases the dial reading was noted after 3 minutes at room temperature (33°C ± 2°C) and viscosity was calculated.

All viscosity measurements were made in triplicate for each of the three batches of all the products. Mean values (n = 9) alongwith the standard error values were recorded.

3.4.3 Infra-red Spectroscopy :

Pellets of GG and modified GGs with dried potassium bromide were made on Carver Laboratory press at 100 kg/cm^2 pressure applied for 30 seconds. Infrared spectra of these pellets were recorded between 400 cm^{-1} to 4000cm^{-1} on IR460 , Shimadzu Infrared Spectrophotometer.

3.4.4 Differential Scanning Calorimetry :

A weighed amount of sample was placed at the bottom of the sample pan and the thermogram was recorded at following experimental conditions.

$T_{start} = 0°C$

$T_{final} = 300°C$

Scanning Rate = 10°C/minute

The DSC thermograms of GG and modified GGs were recorded when the scanning run was complete.

3.5 QUANTITATIVE EVALUATION OF MODIFIED GGs AND DRUGS

Modified GGs were characterised for their respective chemical constituents such as total amount of reducing sugars in hydrolysed GG products, methoxy content in methylated GG products and carboxy content in oxidised GG products.

3.5.1 Estimation of Reducing Sugars in terms of Galactose :

Colorimetric estimation of reducing sugars in hydrolysed GG products, in terms of galactose, was done using phenol-sulphuric acid method as described by Dubois,M. *et al* (1).

3.5.1.1 Reagents:

(i) Water : Purified water I.P.

(ii) Phenol, 5% w/v: 5g of phenol was accurately weighed into a 100ml volumetric flask and dissolved in water. Finally the volume was made up with water.

(iii)Sulphuric acid, 96% w/w : Analytical Reagent grade sulphuric acid was used.

(iv)Standard solutions of galactose : 100mg of accurately weighed galactose was dissolved in water and the volume was made to 100ml with water in a volumetric flask. 10ml of this solution was further diluted to 100ml in a volumetric flask using water. Aliquots of 0.4ml, 0.6ml, 0.8ml, 1.0ml, 1.2ml and 1.4ml were accurately pippetted using 1ml graduated pippette into 10 ml volumetric flasks separately. The volume was made upto 10ml with water.

3.5.1.2 Preparation of Calibration curve for estimation of Galactose:

1ml standard solution of galactose and 1ml of 5% w/v phenol solution were transferred into colorimetric tube using 1ml volumetric pippette. Blank was prepared with water instead of sugar solution. 5ml of 96% w/w sulphuric acid was added to each tube, using a fast flow pippette, so that the stream hit the liquid surface directly to produce good mixing and an even heat distribution. The solutions were kept aside for at least 20 minutes to cool at room temperature (32°C ± 2°C). The absorbance of the developed yellow orange colour was measured at 490nm against reagent blank using Hitachi U-2000 UV-Visible Spectrophotometer.

All readings were taken six times using freshly prepared reagents and standard solutions. Mean values of absorbance alongwith the standard error are recorded in Table 3.1. The data was plotted graphically as shown in Fig 3.1.

3.5.2 Determination of Methoxy Content :

Quantitative methoxy determination was carried out using semi-micro Zeisel method (2). The methylated derivative is hydrolysed with hydroiodic acid, the resulting methyliodide is distilled in a stream of nitrogen, trapped in bromine and titrated against sodium thiosulphate as per the following equation -

$$R\text{-}OCH_3 + HI \rightarrow CH_3I + ROH$$

$$CH_3I + Br_2 \rightarrow CH_3Br + IBr$$

$$IBr + 2Br_2 + 3\,H_2O \rightarrow HIO_3 + 5HBr$$

Excess Br_2 destroyed by HCOOH

$$5H^+ + HIO_3 + 5\,I^- \rightarrow 3\,H_2O + 3\,I_2$$

$$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaI$$

$$2S_2O_3 \equiv 0.0517 \text{ mg of } OCH_3.$$

3.5.2.1 Reagents :

(i) Hydroiodic acid : Reagent grade hydroiodic acid was used.

(ii) Receiver solution: 10g of accurately weighed potassium acetate was dissolved in glacial acetic acid in 100ml volumetric flask, 0.2ml bromine was added to this and the volume was made up with glacial acetic acid.

(iii)0.1M Sodium thiosulphate solution: Sodium thiosulphate was dissolved in water to contain 2.482g in 100ml (3).

3.5.2.2 Method :

200mg of the product was placed in a 250ml round bottom flask (Fig 3.2). 2.5ml of liquefied phenol and 5 ml of hydroiodic acid were added to the flask after addition of 2-3 porcelain pieces. The flask was connected with the remainder of the apparatus. The first receiver contained about 6ml and the second about 4ml of 10% w/v solution of potassium acetate in glacial acetic acid to which 0.2ml of bromine was added. A slow uniform stream of nitrogen was passed through the side arm of the round bottom flask. The liquid was gradually heated in the thermostatically controlled heating mantle at such a rate that the vapours of the boiling liquid rose to half-way up the condenser. The reaction was continued for about half an hour. The apparatus was then dismantled and the contents of the receivers were emptied into a 250ml glass stoppered conical flask containing 5ml of 25% w/v solution of sodium acetate. The volume of the solution was adjusted to 125ml and 1.0ml of formic acid was added. The flask was stoppered and rotated till the colour due to bromine disappeared. After allowing to stand for 5 minutes 1g of potassium iodide and 1 - 1.5 ml of 1M sulphuric acid were added to the conical flask. The liberated iodine was titrated with 0.1M sodium thiosulphate solution.

A blank titration was performed to make the necessary correction. The methoxy content was calculated using the equation-

1ml of 0.1M sodium thiosulphate ≡ 0.0005172 g of methoxy (OCH_3).

Each methoxy content determination was repeated six times for all the three batches of each type of methylated GG product. The average of these determinations (n = 18) were recorded alongwith the standard error values.

3.5.3 Determination of Carboxy Content :

The carboxy content of oxidised GG products was determined by treating them with excess alkali and then back titrating the excess alkali with an acid (4). The amount of alkali consumed by the product was determined and the carboxy content of the product was calculated in terms of milliequivalents of alkali per gram of the product.

3.5.3.1 Reagents :

(i) Sodium hydroxide, 0.01M, containing sodium chloride, 25 g/lit: 25g of sodium chloride was accurately weighed and transferred to a clean 1000ml volumetric flask and dissolved in sufficient quantity of water (about 600ml). 10ml of 40% w/v solution of sodium hydroxide was added to it and finally the volume was made to 1000ml with water.

(ii) Hydrochloric acid,0.02N : As per the Indian Pharmacopoeia. (5)

3.5.3.2 Method :

40 ml of 0.01M sodium hydroxide solution containing 25 g/lit of sodium chloride was transferred to a 250ml titration flask and 1g of powder GG/Oxidised GG products was dispersed in it. The reaction was allowed to continue at room temperature (33°C ± 2°C) for 18h and then excess alkali was back titrated with 0.02N hydrochloric acid using bromocresol purple as indicator. A blank titration was also run in parallel to make necessary correction.

Each titration was done in triplicate and the mean values of carboxy content in meq/g were calculated and recorded alongwith the standard error values.

3.5.4 Preparation of Calibration Curve for *In Vitro* Estimation of CPM :

Estimation of CPM in the dissolution media and the dosage form was done by direct uv-spectroscopy.

3.5.4.1 Reagents :

(i) Water : Purified water.

(ii) Hydrochloric acid, 0.1N : It was prepared as per the Indian Pharmacopoeia (5).

(iii)Phosphate buffer, pH 5.4 : 6.80g of accurately weighed potassium dihydrogen phosphate was dissolved in 1000ml of water and the pH was adjusted to 5.4 with 10M potassium hydroxide solution.

(iv)Phosphate buffer, pH 6.8 : Accurately weighed 28.80g of disodium hydrogen phosphate and 11.45g of potassium dihydrogen phosphate were dissolved in sufficient amount of water and the volume was finally adjusted to 1000ml with water.

(v) Phosphate buffer, pH 7.4: 50.0ml of 0.2M potassium hydrogen phosphate was placed in a 200ml volumetric flask. 39.1ml of 0.2M sodium hydroxide solution was added to it and the volume was made up with water.

(vi)Standard solutions of CPM :

- ***In water*** : 100mg of CPM was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of water (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with water. 10ml of this solution was pipetted into a 100ml volumetric flask and was diluted to 100ml with water. Aliquots of 0.8ml, 1.0ml, 1.4ml, 1.8ml, 2.2ml, 2.6ml and 3.0ml from the stock solution of CPM were accurately measured using suitable calibrated pipettes and transferred to clean and

dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with water in each flask.

- ***In 0.1N Hydrochloric acid***: 100mg of CPM was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of 0.1N hydrochloric acid (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with 0.1N hydrochloric acid. 10ml of this solution was pipetted into 100ml volumetric flask and was diluted to 100ml with 0.1N hydrochloric acid. Aliquots of 0.8ml, 1.0ml, 1.4ml, 1.8ml, 2.2ml, 2.6ml and 3.0ml from the stock solution of CPM were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with 0.1N hydrochloric acid in each flask.
- ***In pH 5.4 phosphate buffer***: 100mg of CPM was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of pH 5.4 phosphate buffer (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with the buffer. 10ml of this solution was pipetted into 100ml volumetric flask and was diluted to 100ml with pH 5.4 phosphate buffer. Aliquots of 0.8ml, 1.0ml, 1.4ml, 1.8ml, 2.2ml, 2.6ml and 3.0ml from the stock solution of CPM were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with the buffer in each flask.
- ***In pH 6.8 phosphate buffer***: 100mg of CPM was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of pH 6.8 phosphate buffer (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with the buffer. 10ml of this solution was pipetted into 100ml volumetric flask and was diluted to 100ml with pH

6.8 phosphate buffer. Aliquots of 0.8ml, 1.0ml, 1.4ml, 1.8ml, 2.2ml, 2.6ml and 3.0ml from the stock solution of CPM were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with the buffer in each flask.

- ***In pH 7.4 phosphate buffer***: 100mg of CPM was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of pH 7.4 phosphate buffer (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with the buffer. 10ml of this solution was pipetted into 100ml volumetric flask and was diluted to 100ml with pH 7.4 phosphate buffer. Aliquots of 0.8ml, 1.0ml, 1.4ml, 1.8ml, 2.2ml, 2.6ml and 3.0ml from the stock solution of CPM were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with the buffer in each flask.

3.5.4.2 Method for Preparation of Calibration Curve of CPM :

The absorbance maxima (λ_{max}) was determined by scanning 10 mcg/ml standard solutions of CPM against solvent blank in each case on Hitachi U-2000 uv-visible spectrophotometer. The absorbance of the standard solutions of CPM was measured at the absorbance maxima. The readings were recorded in triplicate on 3 consecutive days using freshly prepared stock solutions each time. Mean values (n = 9) alongwith the standard error are recorded in Table 3.2. The values of absorbance were plotted graphically against the concentration of the standard solutions, as shown in Figure 3.3.

3.5.5 Preparation of Calibration Curve for *In Vitro* Estimation of DIL :

Estimation of DIL in the dissolution media and the dosage form was done by direct uv-spectroscopy.

3.5.5.1 Reagents :

(i) Water : Purified water.

(ii) Hydrochloric acid, 0.1N : It was prepared as per the Indian Pharmacopoeia (5).

(iii)Phosphate buffer, pH 5.4 : 6.8g of accurately weighed potassium dihydrogen phosphate was dissolved in 1000ml of water and the pH was adjusted to 5.4 with 10M potassium hydroxide solution.

(iv)Phosphate buffer, pH 6.8 : Accurately weighed 28.80g of disodium hydrogen phosphate and 11.45g of potassium dihydrogen phosphate were dissolved in sufficient amount of water and the volume was finally adjusted to 1000ml with water.

(v) Phosphate buffer, pH 7.4: 50.0 ml of 0.2M potassium hydrogen phosphate was placed in a 200ml volumetric flask. 39.1ml of 0.2M sodium hydroxide solution was added to it and the volume was made up with water.

(vi)Standard solutions of DIL:

- ***In water*** : 100mg of DIL was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of water (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with water. 10ml of this solution was pipetted into 100ml volumetric flask and was diluted to 100ml with water. Aliquots of 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml and 1.2ml from the stock solution of DIL were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with water in each flask.
- ***In 0.1N Hydrochloric acid***: 100mg of DIL was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of 0.1N hydrochloric acid (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The

volume was made upto 100ml with 0.1N hydrochloric acid. 10ml of this solution was pipetted into 100ml volumetric flask and was diluted to 100ml with 0.1N hydrochloric acid. Aliquots of 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml and 1.2ml from the stock solution of DIL were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with 0.1N hydrochloric acid in each flask.

- ***In pH 5.4 phosphate buffer***: 100mg of DIL was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of pH 5.4 phosphate buffer (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with the buffer. 10ml of this solution was pipetted into 100ml volumetric flask and was diluted to 100ml with pH 5.4 phosphate buffer. Aliquots of 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml and 1.2ml from the stock solution of DIL were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with the buffer in each flask.
- ***In pH 6.8 phosphate buffer***: 100mg of DIL was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of pH 6.8 phosphate buffer (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with the buffer. 10ml of this solution was pipetted into 100ml volumetric flask and was diluted to 100ml with pH 6.8 phosphate buffer. Aliquots of 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml and 1.2ml from the stock solution of DIL were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with the buffer in each flask.

- ***In pH 7.4 phosphate buffer***: 100mg of DIL was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of pH 7.4 phosphate buffer (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with the buffer. 10ml of this solution was pipetted into 100ml volumetric flask and was diluted to 100ml with pH 7.4 phosphate buffer. Aliquots of 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml and 1.2ml from the stock solution of DIL were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with the buffer in each flask.

3.5.5.2 Method for Preparation of Calibration Curve of DIL:

The absorbance maxima (λ_{max}) was determined by scanning 10 mcg/ml standard solution of DIL against solvent blank in each case on Hitachi U-2000 uv-visible spectrophotometer. The absorbance of the standard solutions of DIL was measured at the absorbance maxima. The readings were recorded in triplicate on 3 consecutive days using freshly prepared stock solutions each time. Mean values (n = 9) alongwith the standard error are recorded in Table 3.3. The values of absorbance were plotted graphically against the concentration of the standard solutions, as shown in Figure 3.4.

3.5.6 Preparation of Calibration Curve for *In Vitro* Estimation of PPA :

Direct uv-spectroscopic method was used for estimation of PPA in the dissolution media and the dosage form.

3.5.6.1 Reagents :

(i) Water : Purified water.

(ii) Hydrochloric acid, 0.1N : It was prepared as per the Indian Pharmacopoeia (5).

(iii)Phosphate buffer, pH 5.4 : 6.8g of accurately weighed potassium dihydrogen phosphate was dissolved in 1000ml of water and the pH was adjusted to 5.4 with 10M potassium hydroxide solution.

(iv)Phosphate buffer, pH 6.8 : Accurately weighed 28.80g of disodium hydrogen phosphate and 11.45g of potassium dihydrogen phosphate were dissolved in sufficient amount of water and the volume was finally adjusted to 1000ml with water.

(v) Phosphate buffer, pH 7.4 : 50.0ml of 0.2M potassium hydrogen phosphate was placed in a 200ml volumetric flask. 39.1ml of 0.2M sodium hydroxide solution was added to it and the volume was made up with water.

(vi)Standard solutions of PPA:

- ***In water*** : 100mg of PPA was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of water (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with water. Aliquots of 0.5ml, 1.0ml, 1.5ml, 2.0ml, 3.0ml, 4.0ml, 5.0ml and 6.0ml from the stock solution of PPA were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with water in each flask.
- ***In 0.1N Hydrochloric acid***: 100mg of PPA was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of 0.1N hydrochloric acid (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with 0.1N hydrochloric acid. Aliquots of 0.5ml, 1.0ml, 1.5ml, 2.0ml, 3.0ml, 4.0ml, 5.0ml and 6.0ml from the stock solution of PPA were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with 0.1N hydrochloric acid in each flask.

- ***In pH 5.4 phosphate buffer***: 100mg of PPA was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of pH 5.4 phosphate buffer (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with the buffer. Aliquots of 0.5ml, 1.0ml, 1.5ml, 2.0ml, 3.0ml, 4.0ml, 5.0ml and 6.0ml from the stock solution of PPA were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with the buffer in each flask.
- ***In pH 6.8 phosphate buffer***: 100mg of PPA was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of pH 6.8 phosphate buffer (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with the buffer. Aliquots of 0.5ml, 1.0ml, 1.5ml, 2.0ml, 3.0ml, 4.0ml, 5.0ml and 6.0ml from the stock solution of PPA were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with the buffer in each flask.
- ***In pH 7.4 phosphate buffer***: 100mg of PPA was accurately weighed and transferred to a clean and dry 100ml volumetric flask. Sufficient quantity of pH 7.4 phosphate buffer (about 70ml) was added and the flask was gently shaken by circular motion till all the powder dissolved. The volume was made upto 100ml with the buffer. Aliquots of 0.5ml, 1.0ml, 1.5ml, 2.0ml, 3.0ml, 4.0ml, 5.0ml and 6.0ml from the stock solution of PPA were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. The volume was made upto 10ml with the buffer in each flask.

3.5.6.2 Method for Preparation of Calibration Curve of PPA :

The absorbance maxima (λ_{max}) was determined by scanning 200mcg/ml standard solutions of PPA against solvent blank in each

case on Hitachi U-2000 uv-visible spectrophotometer. The absorbance of the standard solutions of PPA was measured at the absorbance maxima. The readings were recorded in triplicate on 3 consecutive days using freshly prepared stock solutions each time. Mean values (n = 9) alongwith the standard error are recorded in Table 3.4. The values of absorbance were plotted graphically against the concentration of the standard solutions, as shown in Figure 3.5.

3.5.7 Interference of Formulation Components :

The absorbance values of standard solutions of the drugs (10 mcg/ml of CPM and DIL; 200 mcg/ml of PPA) were determined in presence of the maximum used concentrations of each of the formulation components used. The observations are recorded in Table 3.5.

3.6 ESTIMATION OF PPA IN URINE

Various methods of high performance liquid chromatographic analysis of PPA in human serum and urine have been reported. Colour reaction with sodium β-napthoquinone-4-sulfonate after extraction from alkaline urine and α-phthalicdicarboxaldehyde derivatization (6) have been reported for determination of PPA by HPLC. In the present study a HPLC method for determination of PPA without derivatization has been used (7).

3.6.1 Reagents :

(i) Acetic Acid solution, 5% v/v : 5ml of glacial acetic acid was accurately measured and transferred to a clean and dry 100ml volumetric flask. The volume was made up to 100ml with water .

(ii) Saturated Solution of Sodium Carbonate : Excess amount of sodium carbonate was added to 100ml water in a beaker. The dispersion was shaken at 200 rpm for a period of 2h and the solution was filtered through Whatman No. 1 filter under vacuum.The filtered solution was used.

3.6.2 Method :

25 mg of PPA was accurately weighed and transferred to a clean and dry 10ml volumetric flask. PPA was dissolved in urine and the volume was adjusted to 10ml with urine. This solution of drug in urine was transferred to a 50ml separating funnel. The pH of the solution was adjusted to about 10 using saturated solution of sodium carbonate. 10ml of chloroform was added and funnel was shaken vigorously, manually, for about 3 minutes. The chloroform and aqueous layers were separated by centrifugation at 2000 X g for 5 minutes. Extraction was repeated with additional 10 ml and 5 ml quantities of chloroform. The chloroform layers were collected and extracted twice using 10ml of 5 % v/v acetic acid each time. The acetic acid layers were collected in 25 ml volumetric flasks and the volume was made up with 5%v/v acetic acid. Further dilutions of these acetic acid extract were made and injected into the column of High Performance Liquid Chromatograph of Waters 501, fitted with C18 column and 254 nm detector. The area of the peak as a function of concentration of PPA was noted. Calibration curve of peak area was plotted against the concentration of PPA.

Each determination was repeated six times using freshly prepared stock solutions for extraction and the peak area was measured. Mean (n = 6) values alongwith the standard error are recorded in Table 3.6.

3.7 RESULTS AND DISCUSSION

Powder density, defined as the ratio of mass to volume, is an extremely important micromeritic parameter. Bulk density was obtained by measuring the volume of a known mass of powder (5g) into a suitable volume-

measuring apparatus and by dividing the mass of solid by the unsettled apparent volume. A measurement of tapped density was obtained at the same time, with volume of solid being measured after subjecting the system to a number of controlled shocks. The repeated mechanical stress causes the powder bed to pack into smaller volume. The bulk density and tapped density of GG and modified GGs were determined in triplicate for each of the 3 batches and the mean alongwith the standard error values were recorded.

GG forms viscous, colloidal dispersions(solutions) when hydrated in cold water and the viscosities can be measured with a rotational, shear type viscometer. Viscosity of 1% w/v aqueous solutions of GG and modified GGs was determined using Brookefield synchro-lectric viscometer. Mean (n = 9) and the standard error values of viscosities of GG and modified GGs were recorded. Viscosity of the polymer is related to its molecular weight by Mark-Houwink equation (8)given as -

$$[\eta] = K M^{a}$$

Thus change in viscosity indirectly reflects change in the molecular weight of GG on modification.

The I.R. spectra of the GG and modified GGs were recorded on IR460 Shimadzu Infrared Spectrophotometer between 400 cm^{-1} and 4000 cm^{-1} to identify any changes in the functional groups of the molecule.

Differential scanning calorimetry (DSC) is a technique to measure the uptake of heat by a specimen as it is heated. This technique provides a record of temperature at which phase changes, glass transition or chemical reactions occur. These temperatures are useful for characterising substances. DSC thermograms of GG and modified GGs were recorded on DSC 20 Mettler Differential Scanning Calorimeter.

Estimation of sugars, in terms of galactose, liberated on hydrolysis of GG was done by using phenol-sulphuric acid method as reported by Dubois M. *et al*(1). This method involves the use of low-priced, easily available reagents and is very sensitive. Galactose solution is mixed with phenol solution and treated with concentrated sulphuric acid for colour development. The developed yellow orange colour is stable for more than 2h. Absorbance of the colour solutions was measured at 490nm using Hitachi U-2000 UV-Visible spectrophotometer. Mean values (n = 9) are recorded in Table 3.1 and plotted graphically against concentration, as shown in Figure 3.1. Regression analysis proved the linearity of the curve ($r > 0.995$) in the concentration range of 40 mcg/ml to 140 mcg/ml.

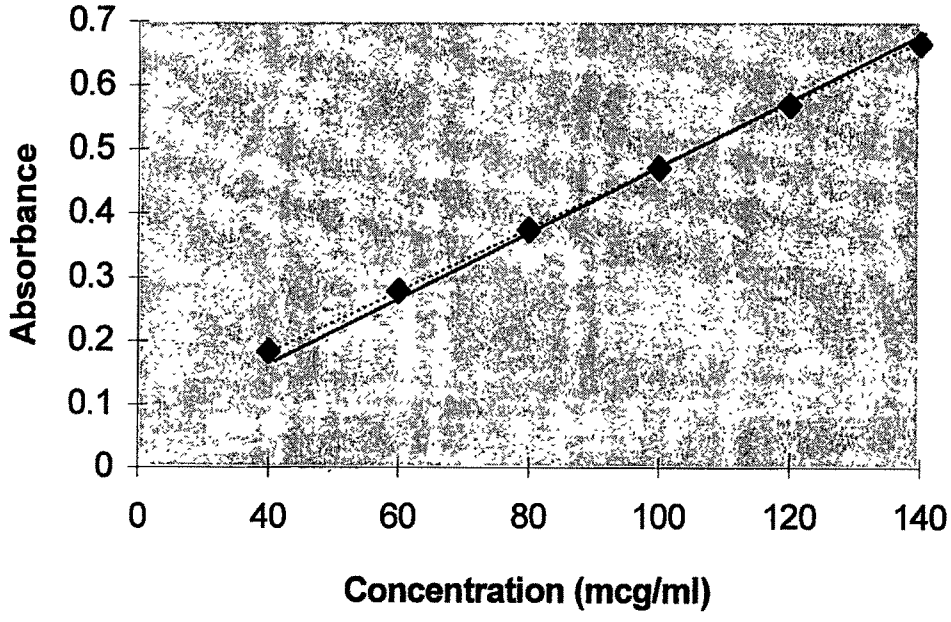
A quantitative methoxy determination was carried out by hydrolysing the derivative with hydroiodic acid (2). The resulting methyliodides distilled in a stream of nitrogen trapped in bromine were titrated against sodium thiosulphate. Methoxy content of MGGs were determined using the relation - 1ml of 0.1M sodium thiosulphate ≡ 0.0005172 g of methoxy (OCH_3). Mean (n = 18) was recorded alongwith the standard error .

Consumption of alkali by free carboxy groups of the oxidised GGs was determined using the method described by David *et al*(4). The oxidised GGs were dispersed in 0.01M sodium hydroxide solution containing sodium chloride and it was allowed to stand for 18 h. The excess alkali was titrated against 0.02N hydrochloric acid using bromocresol purple as an indicator. The carboxy content of GG and oxidised GGs were determined in triplicate for each batch and the mean (n = 9) alongwith the standard error values were recorded.

Table 3.1 Calibration curve for estimation of Galactose.

Sr. No.	Concentration (mcg/ml)	Absorbance
1.	40.00	0.184 ± 0.004
2.	60.00	0.278 ± 0.002
3.	80.00	0.374 ± 0.002
4.	100.00	0.470 ± 0.002
5.	120.00	0.568 ± 0.003
6.	140.00	0.664 ± 0.002
r squared		0.9995
Std error of Y est		0.0039
X coefficient (S.E.)		0.0047 (0.0000)

Fig 3.1 Calibration curve for estimation of galactose



Standard solutions of CPM were prepared in water, 0.1N hydrochloric acid and phosphate buffers of pH 5.4, 6.8 and 7.4. The absorbance maxima determined in each case by scanning a standard solution of concentration 10mcg/ml on Hitachi-U-2000 UV-Visible Spectrophotometer was found to be 261.2 ± 0.05 nm. Absorbance of the standard solutions of CPM were measured at the absorbance maxima. The results are recorded in Table 3.2 and shown graphically in Fig 3.3. The data reveal that there is no significant difference (< 0.005) in the absorbance values of standard solutions of CPM made in water, 0.1N hydrochloric acid and phosphate buffers of pH 5.4, 6.8 and 7.4. Regression analysis proved the linearity of the data ($r > 0.998$) in the concentration range of 8 - 30 mcg/ml.

Standard solutions of DIL were prepared in water, 0.1N hydrochloric acid and phosphate buffers of pH 5.4, 6.8 and 7.4. The absorbance maxima determined in each case by scanning a standard solution of concentration 10mcg/ml on Hitachi-U-2000 UV-Visible Spectrophotometer was found to be 236.0 ± 0.05 nm. Absorbance of the standard solutions of DIL were measured at the absorbance maxima. The results are recorded in Table 3.3 and shown graphically in Fig 3.4. The data reveal that there is no significant difference (< 0.005) in the absorbance values of standard solutions of DIL made in water, 0.1N hydrochloric acid and phosphate buffers of pH 5.4, 6.8 and 7.4. Regression analysis proved the linearity of the data ($r > 0.998$) in the concentration range of 2 - 12 mcg/ml.

Standard solutions of PPA were prepared in water, 0.1N hydrochloric acid and phosphate buffers of pH 5.4, 6.8 and 7.4. The absorbance maxima determined in each case by scanning standard solution of concentration 200mcg/ml on Hitachi-U-2000 UV-Visible spectrophotometer was found to be 257.2 ± 0.05 nm. Absorbance of the standard solutions of PPA were measured at the absorbance maxima. The results recorded in Table 3.4 and

Table 3.2 Calibration curve for estimation of CPM.

Sr. No.	Concentration (mcg/ml)	Absorbance ± S.E. of CPM solution in Water	0.1N HCl	pH5.4 buffer	pH6.8 buffer	pH 7.4 buffer
1.	8.00	0.164±0.002	0.165±0.003	0.164±0.002	0.164±0.003	0.163±0.003
2.	10.00	0.204±0.002	0.204±0.002	0.202±0.003	0.202±0.003	0.204±0.002
3.	14.00	0.284±0.003	0.287±0.003	0.285±0.002	0.287±0.003	0.284±0.003
4.	18.00	0.364±0.003	0.365±0.002	0.363±0.002	0.365±0.004	0.364±0.002
5.	22.00	0.443±0.004	0.445±0.003	0.444±0.003	0.443±0.003	0.444±0.003
6.	26.00	0.523±0.003	0.524±0.002	0.523±0.002	0.523±0.002	0.524±0.002
7.	30.00	0.600±0.003	0.602±0.002	0.600±0.002	0.600±0.002	0.600±0.002
r squared		0.9997	0.9997	0.9996	0.9996	0.9998
Std error of Y est		0.0030	0.0030	0.0031	0.0031	0.0024
X coefficient (S.E.)		0.0202 (0.0001)	0.0202 (0.0000)	0.0201 (0.0001)	0.0201 (0.0001)	0.0201 (0.0000)

Fig 3.3 Calibration curve for estimation of CPM.

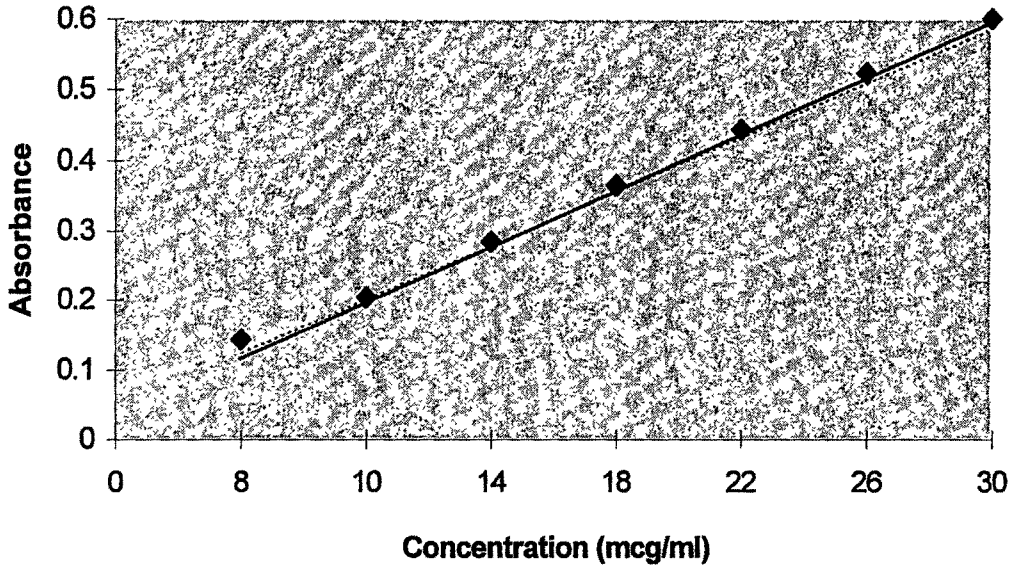


Table 3.3 Calibration curve for estimation of DIL.

Sr. No.	Concentration (mcg/ml)	Absorbance ± S.E. of DIL solution in				
		Water	0.1N HCl	pH5.4 buffer	pH6.8 buffer	pH 7.4 buffer
1.	2.00	0.108±0.003	0.110±0.002	0.108±0.003	0.108±0.002	0.110±0.003
2.	4.00	0.230±0.002	0.232±0.003	0.232±0.003	0.230±0.002	0.230±0.002
3.	6.00	0.348±0.003	0.350±0.002	0.348±0.004	0.348±0.002	0.348±0.003
4.	8.00	0.470±0.003	0.472±0.002	0.472±0.002	0.472±0.003	0.472±0.002
5.	10.00	0.590±0.004	0.592±0.003	0.590±0.003	0.590±0.002	0.592±0.003
6.	12.00	0.710±0.003	0.712±0.002	0.710±0.002	0.710±0.003	0.712±0.002
r squared		0.9982	0.9995	0.9994	0.9993	0.9994
Std error of Y est		0.0095	0.0048	0.0055	0.0058	0.0056
X coefficient (S.E.)		0.0586 (0.0005)	0.0590 (0.0003)	0.0589 (0.0003)	0.0588 (0.0003)	0.0590 (0.0003)

Fig 3.4 Calibration curve for estimation of DIL.

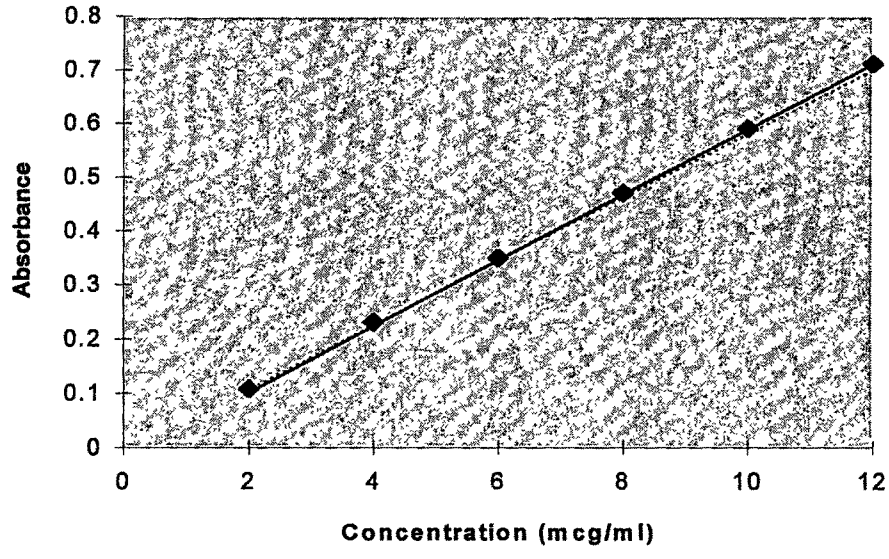
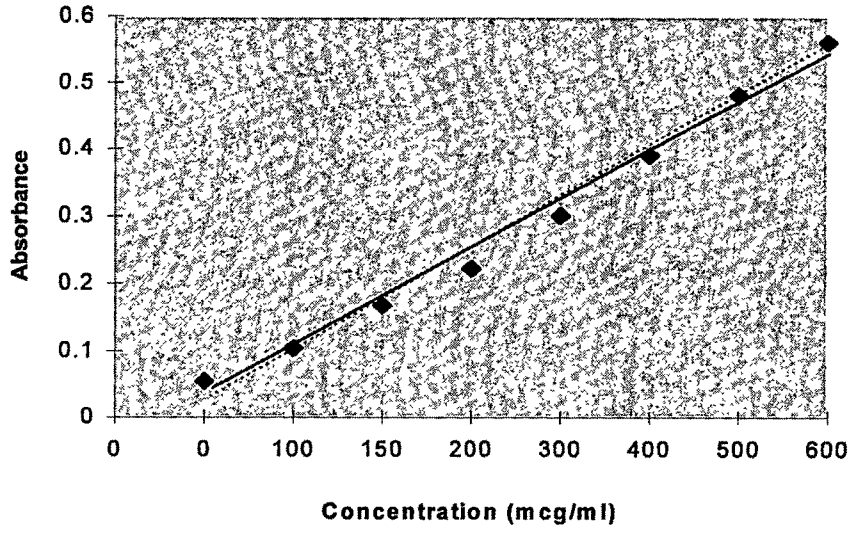


Table 3.4 Calibration curve for estimation of PPA.

Sr. No.	Concentration (mcg/ml)	Absorbance ± S.E. of PPA solution in Water	0.1N HCl	pH5.4 buffer	pH6.8 buffer	pH 7.4 buffer
1.	50.00	0.061±0.002	0.063±0.003	0.061±0.003	0.060±0.003	0.061±0.003
2.	100.00	0.113±0.002	0.115±0.002	0.113±0.003	0.113±0.002	0.113±0.002
3.	150.00	0.167±0.003	0.168±0.003	0.168±0.003	0.167±0.003	0.167±0.003
4.	200.00	0.218±0.003	0.220±0.002	0.220±0.002	0.218±0.002	0.220±0.002
5.	300.00	0.320±0.003	0.321±0.003	0.320±0.002	0.320±0.003	0.320±0.002
6.	400.00	0.418±0.003	0.420±0.002	0.418±0.002	0.418±0.003	0.418±0.003
7.	500.00	0.520±0.002	0.522±0.002	0.522±0.003	0.520±0.002	0.520±0.002
8.	600.00	0.622±0.002	0.621±0.002	0.620±0.003	0.622±0.003	0.622±0.002
r squared		0.9986	0.9981	0.9984	0.9985	0.9984
Std error of Y est		0.0075	0.0088	0.0080	0.0077	0.0079
X coefficient (S.E.)		0.0010 (0.0000)	0.0011 (0.0000)	0.0010 (0.0001)	0.0010 (0.0001)	0.0010 (0.0000)

Fig 3.5 Calibration curve for estimation of PPA.



shown graphically in Fig 3.5. The data reveal that there is no significant difference (< 0.005) in the absorbance values of standard solutions of PPA made in water, 0.1N hydrochloric acid and phosphate buffers of pH 5.4, 6.8 and 7.4. Regression analysis proved the linearity of the data ($r > 0.998$) in the concentration range of 50 - 600 mcg/ml.

The interference of the formulation components was determined by measuring the absorbance of standard solutions in presence of the maximum concentration of the excipients. The differences greater than 0.005 were considered statistically significant. The results (Table 3.5) indicate no significant differences in the absorbance values of the standard drug solutions suggesting no interference from the excipients in the used concentrations.

Estimation of PPA in urine was done by HPLC method(8), which involves a rapid and relatively simple extraction of PPA from urine. High performance liquid chromatograph, Waters 501, equipped with C18 column and 254nm detector, was employed for the estimation. Calibration curve was plotted between concentration range of 20mcg/ml to 200mcg/ml. The results are recorded in Table 3.6 and shown graphically in Fig 3.6. Regression analysis of the data show that the curve is linear in the concentration range of 20 - 200 mcg/ml ($r > 0.998$). This curve was used for estimation of PPA excreted in urine.

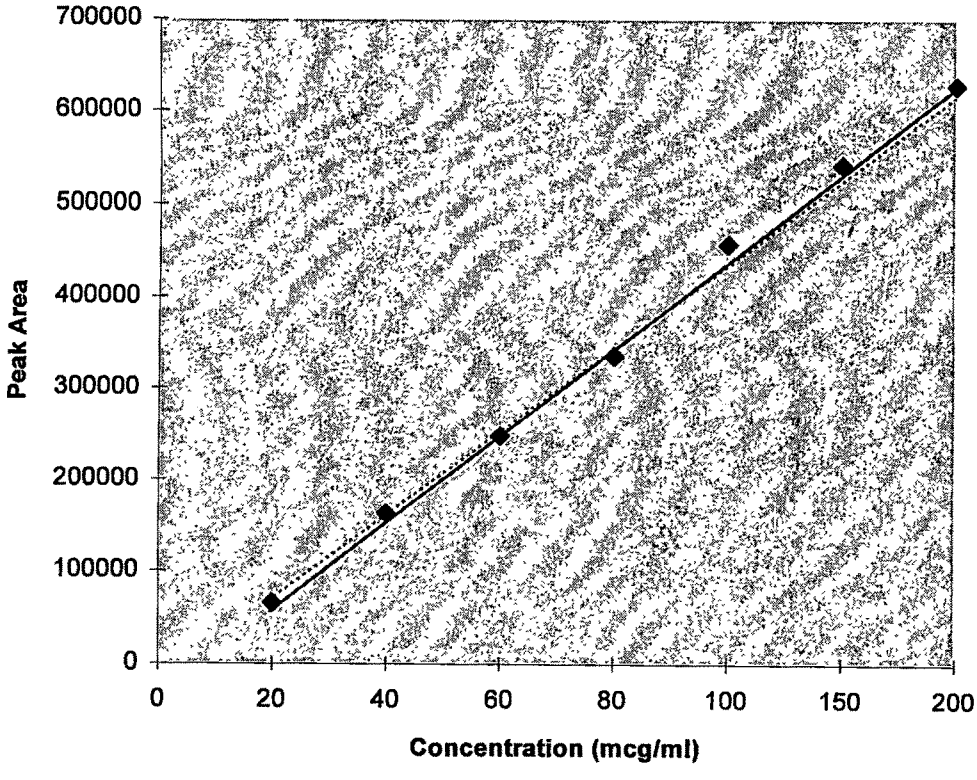
Table 3.5 Interference of excipients used in the formulations.

Sr. No.	Excipients	Absorbance of standard solutions of		
		CPM (10mcg/ml)	DIL (10mcg/ml)	PPA (200mcg/ml)
1.	Lactose	0.204 ± 0.002	0.590 ± 0.003	0.220 ± 0.002
2.	Microcrystalline Cellulose	0.202 ± 0.003	0.588 ± 0.002	0.222 ± 0.003
3.	Dicalcium phosphate	0.203 ± 0.002	0.588 ± 0.003	0.220 ± 0.003
4.	Magnesium stearate	0.204 ± 0.003	0.590 ± 0.002	0.220 ± 0.002
5.	Talc	0.202 ± 0.002	0.590 ± 0.002	0.222 ± 0.001
6.	Guar gum	0.206 ± 0.003	0.592 ± 0.003	0.222 ± 0.002
7.	Hydroxypropyl methylcellulose K4M	0.205 ± 0.003	0.592 ± 0.002	0.222 ± 0.003
8.	Polyvinyl pyrrolidone K-30	0.204 ± 0.002	0.592 ± 0.002	0.220 ± 0.002

Table 3.6 Calibration curve of PPA in urine by HPLC.

Sr. No.	Concentration (mcg/ml)	Peak Area ± S.E.
1.	20.00	65993 ± 20.44
2.	40.00	162658 ± 27.28
3.	60.00	247748 ± 30.33
4.	80.00	335019 ± 33.69
5.	100.00	456759 ± 32.43
6.	150.00	542029 ± 35.42
7.	200.00	627299 ± 34.48

Fig 3.6 Calibration curve for estimation of PPA in urine



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