Chapter 3

ANALYTICAL METHODS

3.1. INTRODUCTION

The analytical methods used in the studies of drug containing formulations should, in addition to possessing the desired characteristics of accuracy, precision, reproducibility, ruggedness etc. should also possess the ability to be used in conjunction with techniques common to microspheres. The methods used should preferably be stability indicating which would when used, draw attention to any potential incompatibility between the various components of the formulation.

3.2. EXPERIMENTAL

3.2.1 Drugs

Celecoxib was a gift sample from Sun Pharmaceutical Advanced Research Centre, Baroda. Rofecoxib was a gift sample from Torrent pharmaceuticals limited and Valdecoxib was gifted by Lyka laboratories limited, Ankleshwar.

3.2.2 Reagents

Methanol, Dichloromethane, Hydrochloric acid, Sodium hydroxide, disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from S.D.Fine Chem. Ltd. Boisar, Thane. Glutaraldehyde was purchased from E.Merck India limited. Formaldehyde was purchased from S.D.fine chem.limited.

3.2.3 Apparatus

Ultrasonicator, UV Spectrophotometer (Shimadzu Corporation, Japan).

3.2.4 Estimation of celecoxib in methanol and 0.1N sodium hydroxide

Celecoxib in methanol and 0.1N sodium hydroxide yields characteristic curves when scanned in the ultraviolet region between 200-330 nm. The λmax of celecoxib in both 0.1N sodium hydroxide (ME1) and methanol (ME2) is at 250 nm.

3.2.4.1 Solutions

(1) 0.1N sodium hydroxide was prepared as per the method given in Indian

Pharmacopoeia (1996).

(2) Stock solutions of celecoxib (50 μg/ml) was prepared by dissolving 50 mg of celecoxib in 100 ml of either methanol or 0.1N sodium hydroxide and diluting 5 ml of this solution to 50 ml with methanol or 0.1N sodium hydroxide respectively.

3.2.4.2 Preparation of calibration curve

Suitable aliquots of the stock solution were pipetted and transferred to separate 10 ml volumetric flasks. The volume was made up with methanol or 0.1N Sodium Hydroxide to give final concentrations of 5, 7.5, 10, 12.5,15, 20 and 25μg/ml. The solutions were shaken well and the absorbance of the resulting solutions was measured at 250 nm using methanol or 0.1N sodium hydroxide as blank respectively. The above procedure was repeated six times. Mean absorbance values along with the regressed values (method of least squares) and statistical data for the methods ME1 and ME2 are shown in Tables 3.1 and 3.3, respectively. The optical characteristics for the solution of celecoxib in 0.1N Sodium hydroxide and Methanol are given in Table 3.2 and 3.4 respectively. Absorptivity scans of celecoxib in methanol and 0.1N sodium hydroxide are shown in figure 3.1 and figure 3.3 respectively. The calibration curves of celecoxib in 0.1N sodium hydroxide and methanol are shown in figure 3.2 and 3.4 respectively.

3.2.4.3 Stability and selectivity

Stability of the solutions of celecoxib, used for preparing the calibration curves in both the methods, was ascertained by observing the changes in the absorbance at 250nm over a period of 24 hours, at room temperature.

The selectivity of the method for the estimation of celecoxib was ascertained by carrying out the procedure in the presence of the various ingredients used in the

preparation of microspheres at the concentrations in which they are present in the microspheres.

3.4.2.4 Accuracy and precision

In order to determine the accuracy and precision of the method, solutions containing known amounts of celecoxib were prepared and analyzed in three replicates. The analytical results obtained from these investigations are summarized in table 3.5.

Table 3.1: Mean absorbance values, Regressed values and statistical data of the Calibration curve for the estimation of celecoxib in 0.1N Sodium Hydroxide

Concentration (µg/ml)	Mean ABS* (±S.E.)	Regressed values**
5	0.271± 0.0019	0.275
7.5	0.413± 0.0043	0.409
10	0.549± 0.0045	0.544
12.5	0.678± 0.0049	0.679
15	0.813± 0.0046	0.814
20	1.085±0.0065	1.084
25	1.354±0.0071	1.354

Regression equation: Y=0.05396x + 0.0052

Correlation coefficient= 0.999

*Mean of 6 values

**Using regression equation

n= 42

Table 3.2: Optical characteristics for Celecoxib in 0.1N Sodium Hydroxide

Characteristic	Value
Absorption maxima (nm)	216nm, 250nm[a]
Beer's law limit (µg/ml)[b]	5-25
Apparent molar absorptivity[b] ($L\ mol^{-1}\ cm^{-1}$)	20937.762
Sandell's sensitivity coefficient ($\mu g/cm^2$/0.001 abs unit)	1.82×10^{-5}
Limit of detection(µg/ml)	0.137
Limit of quantitation(µg/ml)	0.458

a =Analytical wavelength for the proposed method

b= At analytical wavelength

Table 3.3: Mean absorbance values, Regressed values and statistical data of the Calibration curve for the estimation of celecoxib in methanol

Concentration (µg/ml)	Mean ABS* (±S.E.)	Regressed values**
5	0.265±0.003	0.264
7.5	0.392±0.0021	0.393
10	0.524±0.0032	0.522
12.5	0.647±0.0033	0.650
15	0.782±0.0028	0.779
20	1.038±0.0041	1.037
25	1.295±0.0056	1.295

Regression equation: Y= 0.05156x + 0.0064

Correlation coefficient= 0.999

*Mean of 6 values

**Using regression equation

n= 42

Table 3.4: Optical characteristics for Celecoxib in methanol

Characteristic	Value
Absorption maxima (nm)	205nm, 250nm[a]
Beer's law limit (µg/ml)[b]	5-25
Apparent molar absorptivity[b] ($L\ mol^{-1}\ cm^{-1}$)	19964.4
Sandell's sensitivity coefficient ($\mu g/cm^2$/0.001 abs unit)	$1.91x10^{-5}$
Limit of detection(µg/ml)	0.132
Limit of quantitation(µg/ml)	0.440

a =Analytical wavelength for the proposed method

b= At analytical wavelength

Table 3.5: Evaluation of accuracy and precision of the method for estimation of celecoxib in methanol and 0.1N sodium hydroxide

Exact amount of the drug added (mg)		Individual amounts found (mg); mean (S.D.)[a]		Coefficient of variation (CV)		Relative mean error (RME)		Confidence limits[b]	
ME1	ME2	ME1	ME2	ME1	ME2	ME1	ME2	ME1	ME2
40.2	42.0	40.01	42.24	1.7	4.0	0.001	0.00074	40.22±1.739	40.33±4.20
39.9	39.8	39.64	39.76						
40.7	39.2	41.01	39.01						
		40(0.70)	40.33(1.69)						
49.7	50.4	49.71	50.21	1.5	4.4	0.003	0.012	49.55 ±1.966	50.06 ±3.01
49.4	49.8	48.70	51.2						
49.1	48.2	50.26	48.79						
		49.55(0.79)	50.06(1.21)						
59.0	61.6	59.87	62.68	1.5	0.76	0.0067	0.00032	59.93 ±0.917	62.35±1.183
59.6	62.4	59.6	62.58						
60.0	63.0	60.33	61.81						
		59.93(0.369)	62.35(0.476)						

[a]n=3

[b]Confidence limits at P=0.95 and two degrees of freedom.

Figure 3.1: Absorptivity scan of celecoxib in 0.1N Sodium hydroxide

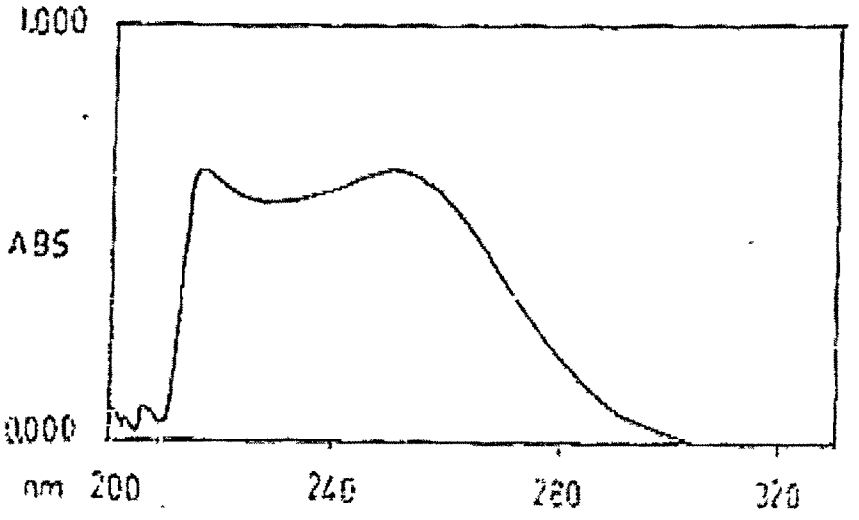


Figure 3.2: Calibration curve of celecoxib in 0.1N Sodium hydroxide

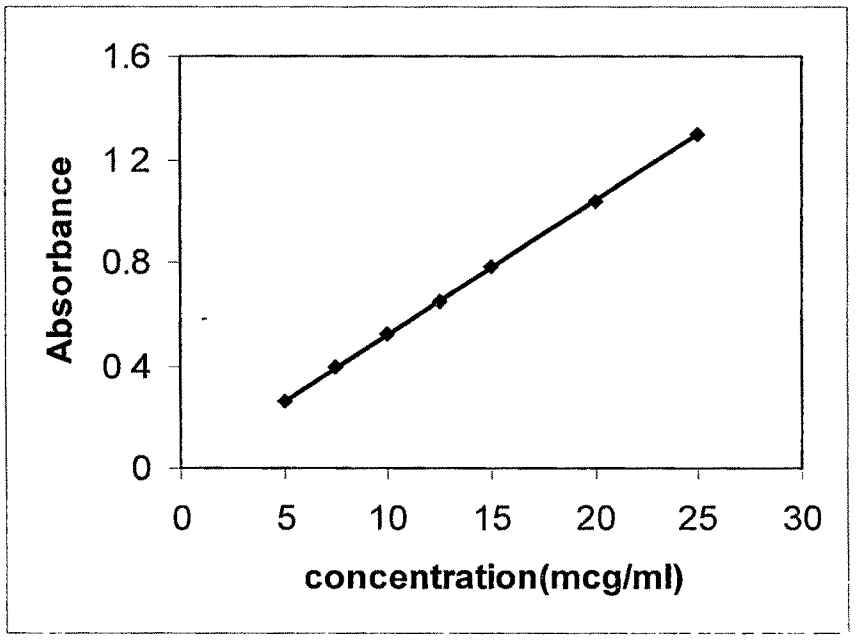


Figure 3.3 Absorptivity scan of celecoxib in methanol

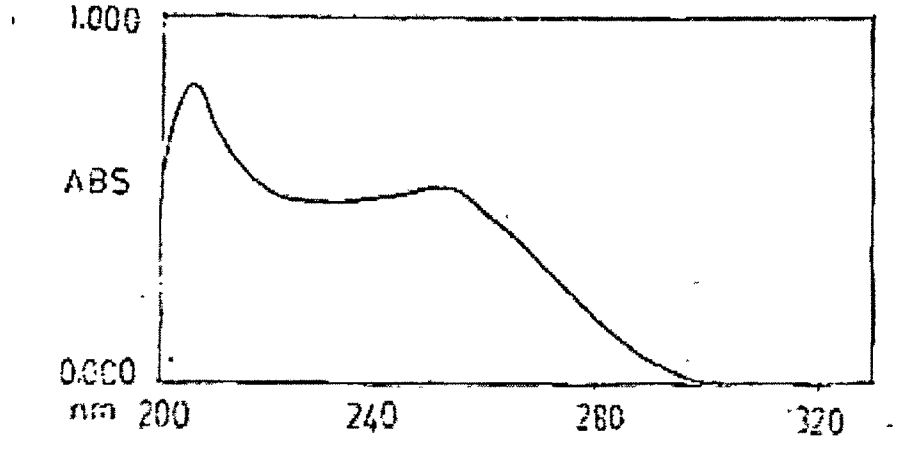
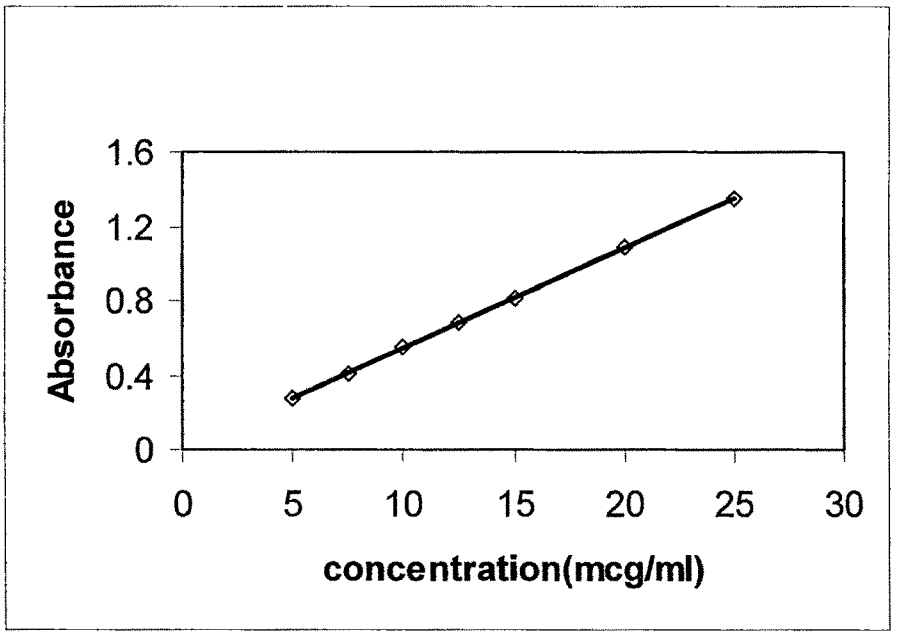


Figure 3.4: Calibration curve of celecoxib in methanol



3.2.5 Estimation of celecoxib in phosphate buffer with 2.0% tween-80.

Since celecoxib is not soluble in phosphate buffer pH-7.4, tween-80 was added at a concentration of 2% as a surfactant to solubilize celecoxib. Celecoxib in this

dissolution media yield a characteristic curve when scanned in the ultraviolet region between 200-330 nm. The value of λmax of celecoxib in this media is 261 nm.

3.2.5.1 Solutions

(1) Phosphate buffer pH-7.4 was prepared as per Indian pharmacopoeia. 2% w/v Tween-80 was dissolved in this solution.

(2) Stock solution of celecoxib (50µg/ml) in the dissolution medium was prepared by dissolving 5 mg of celecoxib in 100 ml of the dissolution medium.

3.2.5.2 Preparation of calibration curve

Suitable aliquots of the stock solution were pipetted and transferred to separate 10 ml volumetric flasks and volume was made up with the dissolution medium to give the final concentrations of 5, 10, 15 and 20µg/ml. The absorbance of the resulting solutions was measured at 261 nm using the dissolution medium as blank. The above procedure was repeated six times.

3.2.5.3 Stability and selectivity

Stability of the solutions of celecoxib, used for preparing the calibration curves in both the methods, was ascertained by observing the changes in the absorbance at 250nm over a period of 24 hours, at room temperature. The selectivity of the method for the estimation of celecoxib was ascertained by carrying out the procedure in the presence of the various ingredients used in the preparation of microspheres at the concentrations in which they are present in the microspheres.

3.2.5.4 Accuracy and precision

In order to determine the accuracy and precision of the method, solutions containing known amounts of celecoxib were prepared and analyzed in three

replicates. The analytical results obtained from these investigations are summarized in table 3.8.

Table 3.6: Mean absorbance values, regressed values and statistical data of the Calibration curve for the estimation of celecoxib in phosphate buffer with 2.0% tween-80

Concentration µg/ml	Mean ABS* (± S.E.)	Regressed values**
5	0.255±0.006	0.261
10	0.55±0.008	0.546
15	0.843±0.007	0.832
20	1.109±0.008	1.117

Regression equation: Y= 0.0571x - 0.0245
Correlation coefficient = 0.999
*Mean of six values
** Using regression equation
n=24

Table 3.7 Optical characteristics of celecoxib in phosphate buffer pH-7.4 with 2.0% tween-80

Characteristic	Value
Absorption maxima (nm)	261[a]
Beer's law limit[b] (µg/ml)	5-20
Apparent molar absorptivity[b] ($L\ mol^{-1}\ cm^{-1}$)	20955
Sandell's sensitivity coefficient ($\mu g/cm^2$/0.001 abs unit)	1.5×10^{-5}
Limit of detection (µg/ml)	0.121
Limit of quantitation (µg/ml)	0.405

a = Analytical wave length for the proposed method
b = At analytical wave length

Table 3.8: Evaluation of accuracy and precision of the method for the estimation of celecoxib in phosphate buffer pH 7.4 with 2.0% tween-80

Exact amount of the drug added (mg)	Individual amounts found (mg); mean (S.D.)[a]	Coefficient of variation (CV)	Relative mean error (RME)	Confidence limits[b]
4.2	4.15	4.04	0.019	4.02±0.397
4.1	4.08			
4.0	3.84			
	4.02(0.16)			
5.2	5.17	2.45	0.013	5.03±0.297
5.1	5.0			
5.0	4.93			
	5.03(0.12)			
6.0	5.82	4.02	0.010	6.10±0.595
6.2	6.25			
6.3	6.24			
	6.10(0.24)			

[a]n=3

[b]Confidence limits at P=0.95 and two degrees of freedom.

Figure 3.5: Absorptivity scan of celecoxib in phosphate buffer pH 7.4 with 2.0% tween-80

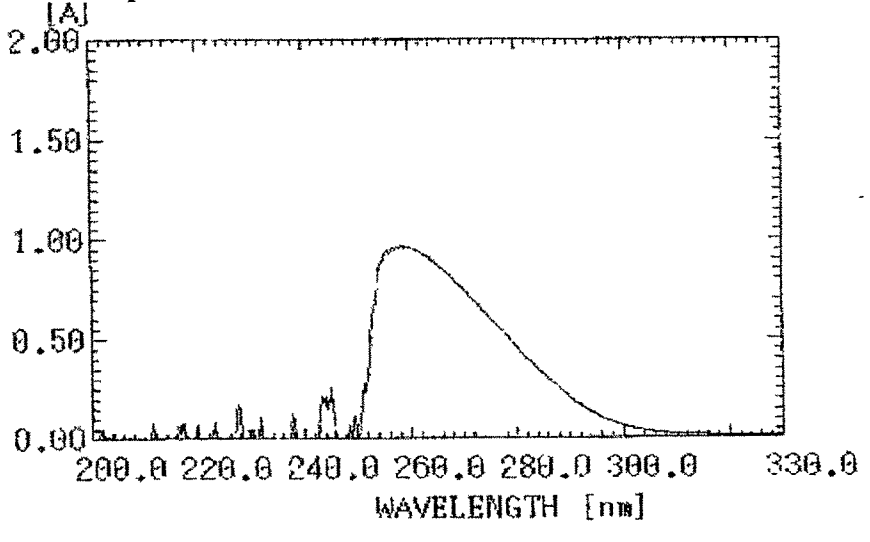
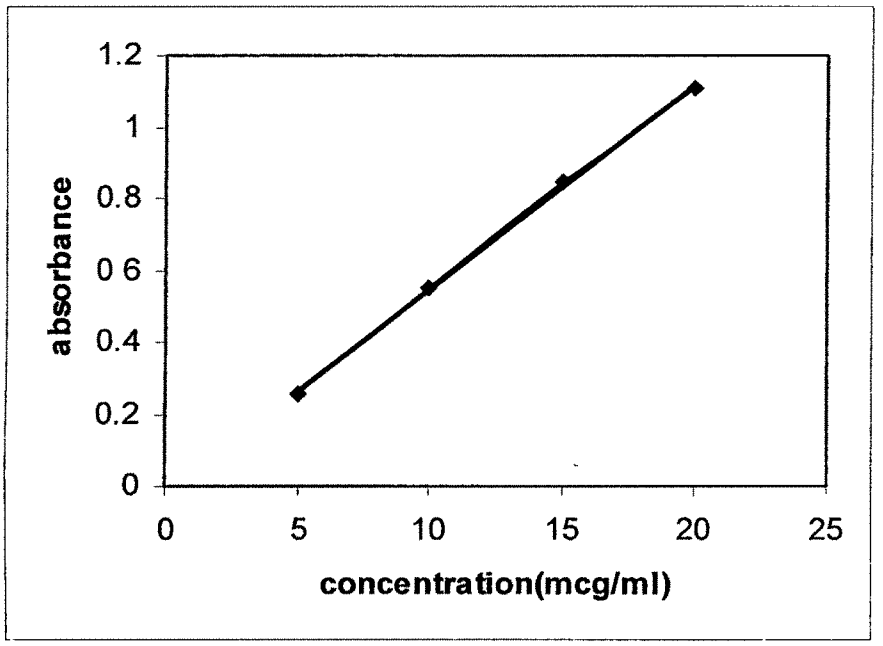


Figure 3.6: Calibration curve of celecoxib in phosphate buffer pH 7.4 with 2.0% tween-80



3.2.6 Estimation of rofecoxib in methanol

Rofecoxib in methanol yields a characteristic curve when scanned in the ultra-violet region between 200-330 nm. Rofecoxib exhibits three λmax values in methanol: 206nm, 222nm and 275 nm.

3.2.6.1 Solutions

Stock solution (100μg/ml) was prepared by dissolving 10 mg of rofecoxib in 100 ml of methanol.

3.2.6.2 Preparation of the calibration curve

Suitable aliquots of the stock solution were transferred into separate 10 ml volumetric flasks and volume was made up with methanol to get the final concentrations of 10, 20, 30 and 40 μg/ml. Absorbance of these solutions was measured at 275 nm using methanol as blank. The above procedure was repeated six times.

3.2.6.3 Stability and selectivity

Stability of the solutions of rofecoxib, used for preparing the calibration curve, was ascertained by observing the changes in the absorbance at 275nm over a period of 24 hours, at room temperature. The solutions were protected from light by performing the analysis in amber colored volumetric flasks.

The selectivity of the method for the estimation of rofecoxib was ascertained by carrying out the procedure in the presence of the various ingredients used in the preparation of microspheres at the concentrations in which they are present in the microspheres.

3.2.6.4 Accuracy and precision

In order to determine the accuracy and precision of the method, solutions containing known amounts of rofecoxib were prepared and analyzed in three replicates. The analytical results obtained from these investigations are summarized in table 3.11.

Table 3.9: Mean absorbance values, regressed values and statistical data of the calibration curve for the estimation of rofecoxib in methanol

Concentration (µg/ml)	Mean Abs* ±S.E	Regressed values**
10	0.382±0.007	0.385
20	0.79±0.009	0.785
30	1.179±0.007	1.183
40	1.582±0.009	1.582

Regression equation: Y= 0.0399x – 0.014
Correlation coefficient = 0.999
** Using regression equation
*mean of 6 values
n= 24

Table 3.10 Optical characteristics of rofecoxib in methanol

Characteristic	Value in methanol
Absorption maxima (nm)	206, 222, 275[a]
Beer's law limit[b] (µg/ml)	10-40
Apparent molar absorptivity[b] ($L\ mol^{-1}\ cm^{-1}$)	12008.55
Sandell's sensitivity coefficient ($\mu g/cm^2$/0.001 abs unit)	2.61×10^{-5}
Limit of detection(µg/ml) Limit of quantitation(µg/ml)	0.129 0.431

a= Analytical wave length for the proposed method

b= At analytical wave length

Table 3.11: Evaluation of accuracy and precision of the method for estimation of rofecoxib in methanol

Exact amount of the drug added (mg)	Individual amounts found (mg); mean (S.D.)[a]	Coefficient of variation (CV)	Relative mean error (RME)	Confidence limits[b]
8.3	8.37	2.41	0.0025	8.29±0.496
8.2	8.06			
8.3	8.44			
	8.29(0.20)			
10.0	9.85	2.38	0.0062	10.07±0.595
10.2	10.03			
10.2	10.34			
	10.07(0.24)			
12.3	12.15	0.76	0.0037	12.21±0.230
12.2	12.32			
12.0	12.17			
	12.21(0.093)			

[a]n=3

[b]Confidence limits at P=0.95 and two degrees of freedom.

Figure 3.7 Absorptivity scan of rofecoxib in methanol

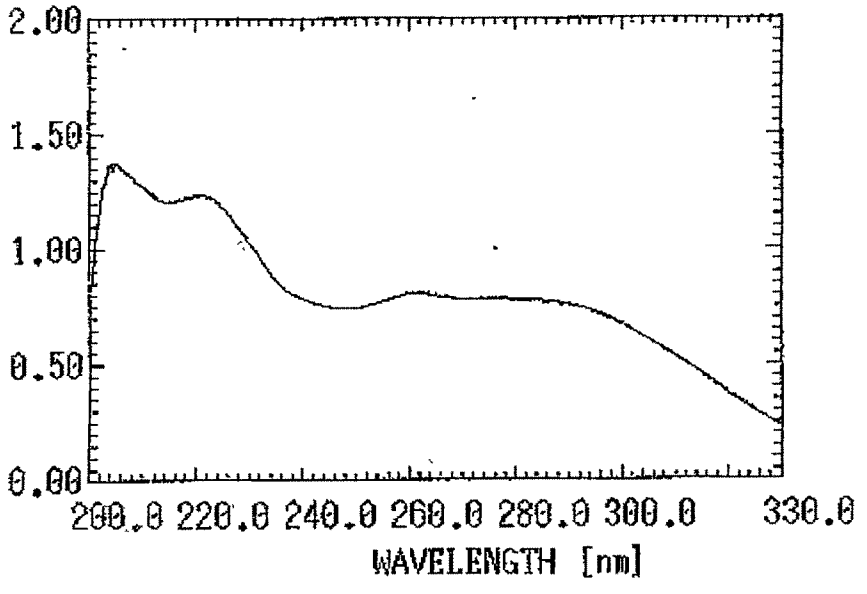
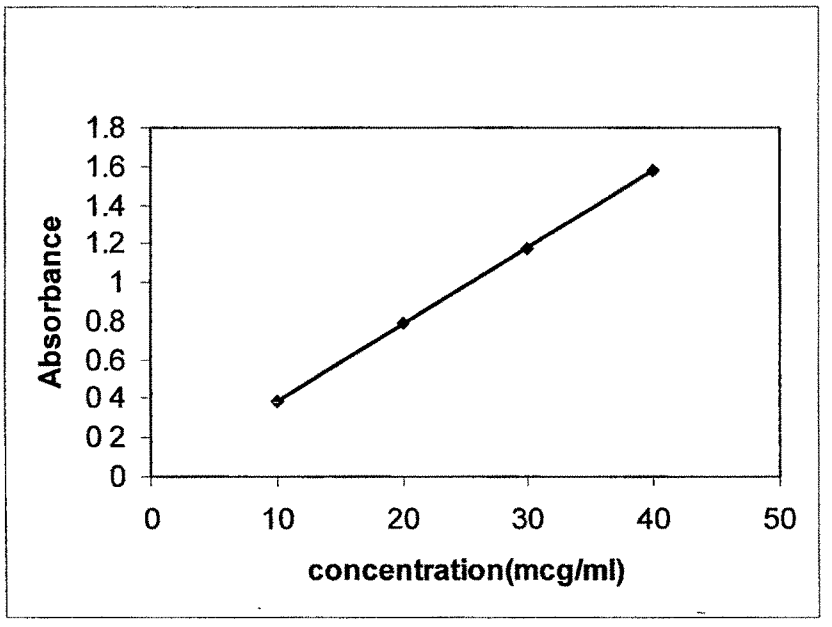


Figure 3.8: Calibration curve of rofecoxib in methanol



3.2.7 Estimation of rofecoxib in phosphate buffer with 2.5% tween-80

Since rofecoxib is not soluble in phosphate buffer pH-7.4, tween-80 at a concentration of 2.5% was added to solubilize rofecoxib. Rofecoxib in this media

yields a characteristic curve when scanned in the ultra-violet region between 200-330 nm. In this media, the λmax of rofecoxib is 294nm.

3.2.7.1 Solutions

Stock solution of rofecoxib (50µg/ml) in phosphate buffer pH 7.4 with 2.5% tween-80 was prepared by dissolving 5 mg of rofecoxib in 100 ml of phosphate buffer pH 7.4 with 2.5% tween-80.

3.2.7.2 Preparation of calibration curve

Suitable aliquots of the stock solution were transferred to separate amber colored 10 ml volumetric flasks to get final concentrations of 10, 20, 30 and 40µg/ml. Absorbance of the resulting solutions was measured at 294 nm using the dissolution medium as the blank. The above procedure was repeated six times.

3.2.7.3 Stability and selectivity

Stability of the solutions of rofecoxib, used for preparing the calibration curve, was ascertained by observing the changes in the absorbance at 294 nm over a period of 96 hours, at room temperature. The samples were stored in amber coloured volumetric flasks to protect from light.

The selectivity of the method for the estimation of rofecoxib was ascertained by carrying out the procedure in the presence of the various ingredients used in the preparation of microspheres at the concentrations in which they are present in the microspheres.

3.2.7.4 Accuracy and precision

In order to determine the accuracy and precision of the method, solutions containing known amounts of rofecoxib were prepared and analyzed in three

replicates. The analytical results obtained from these investigations are summarized in table 3.14.

Table 3.12: Mean absorbance values, regressed values and statistical data of the calibration curve for the estimation of rofecoxib in phosphate buffer pH 7.4 with 2.5% tween-80

Concentration (µg/ml)	Mean Abs* ±S.E	Regressed values**
10	0.365±0.007	0.365
20	0.772±0.017	0.765
30	1.158±0.026	1.165
40	1.571±0.011	1.565

Regression equation: Y=0.04x – 0.0345
Correlation coefficient= 0.999
*mean of six values
** Using regression equation
n= 24

Table 3.13: Optical characteristics of rofecoxib in phosphate buffer pH 7.4 with 2.5% tween-80

Characteristic	Value
Absorption maxima (nm)	294[a]
Beer's law limit[b] (µg/ml)	10-40
Apparent molar absorptivity[b] ($L\ mol^{-1}\ cm^{-1}$)	11474.14
Sandell's sensitivity coefficient ($\mu g/cm^2$/0.001 abs unit)	2.73×10^{-5}
Limit of detection(µg/ml)	0.120
Limit of quantitation(µg/ml)	0.401

a= Analytical wave length for the proposed method

b= At analytical wave length

Table 3.14: Evaluation of accuracy and precision of the proposed method for the estimation of rofecoxib in phosphate buffer with 2.5% tween-80

Exact amount of the drug added (mg)	Individual amounts found (mg); mean (S.D.)[a]	Coefficient of variation (CV)	Relative mean error (RME)	Confidence limits[b]
4.1	4.25	3.92	0.003	4.08±0.397
4.1	4.07			
4.0	3.93			
	4.08(0.16)			
5.1	5.16	2.03	0.0135	5.17±0.260
5.0	5.07			
5.2	5.28			
	5.17(0.105)			
6.1	6.27	3.94	0.0072	6.09±0.595
6.3	6.19			
6.0	5.82			
	6.09(0.24)			

[a]n=3

[b]Confidence limits at P=0.95 and two degrees of freedom.

Figure 3.9: Absorptivity scan of rofecoxib in phosphate buffer pH 7.4 with 2.5% tween-80

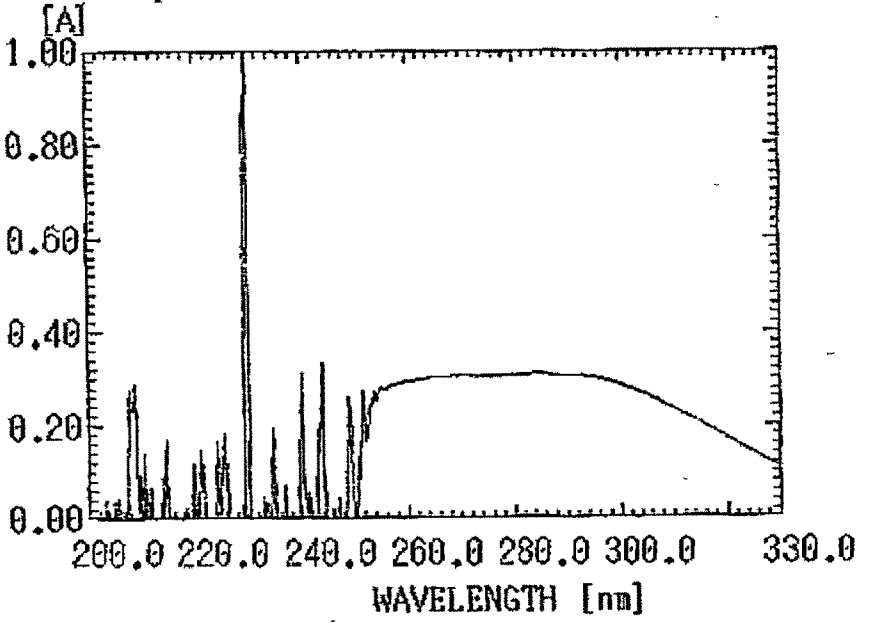
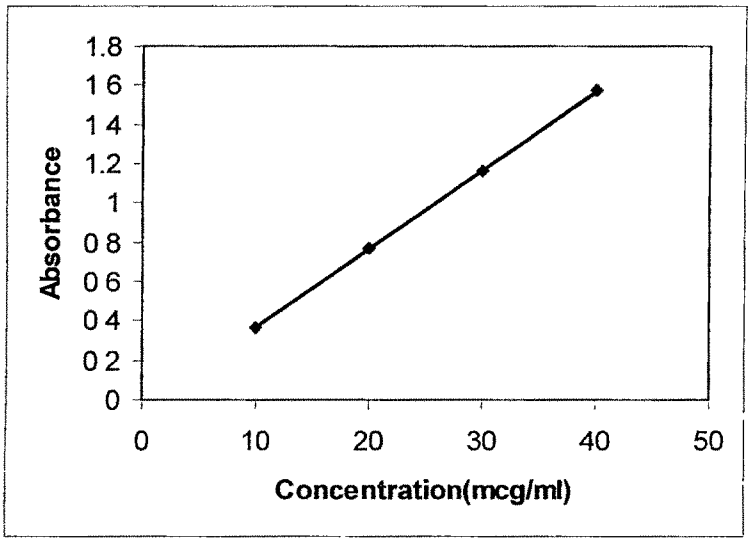


Figure 3.10: Calibration of rofecoxib in phosphate buffer pH 7.4 with 2.5% tween-80



3.2. 8 Estimation of valdecoxib in methanol

Valdecoxib in methanol yields a characteristic curve when scanned in the ultra-violet region between 200-330 nm. It exhibits two λmax values: 214nm and 237 nm.

3.2.8.1 Solutions

Stock solution of valdecoxib (100µg/ml) was prepared by dissolving 5 mg of valdecoxib in 50 ml of methanol.

3.2.8.2 Preparation of calibration curve

Suitable aliquots of the stock solution were transferred to separate 10 ml volumetric flasks to give the final concentrations of 5,10,15,20 and 25 µg/ml. Absorbance of the resulting solutions was measured at 237 nm using methanol as blank. The above procedure was repeated six times.

3.2.8.3 Stability and selectivity

Stability of the solutions of valdecoxib, used for preparing the calibration curve, was ascertained by observing the changes in the absorbance at 237nm over a period of 24 hours, at room temperature.

The selectivity of the method for the estimation of valdecoxib was ascertained by carrying out the procedure in the presence of the various ingredients used in the preparation of microspheres at the concentrations in which they are present in the microspheres.

3.2.8.4 Accuracy and precision

In order to determine the accuracy and precision of the method, solutions containing known amounts of valdecoxib were prepared and analyzed in three replicates. The analytical results obtained from these investigations are summarized in table 3.17.

Table 3.15: Mean absorbance values, regressed values and statistical data of the calibration curve for the estimation of valdecoxib in methanol

Concentration µg/ml	Mean ABS* (±S.E.)	Regressed values**
5	0.336±0.007	0.335
10	0.679±0.017	0.664
15	0.993±0.026	0.993
20	1.310±0.011	1.322
25	1.655±0.014	1.650

Regression equation: Y= 0.06538x + 0.0139
Correlation coefficient= 0.999
* mean of six values
** Using regression equation
n=30

Table 3.16: Optical characteristics of valdecoxib in methanol

Characteristic	Value
Absorption maxima (nm)	207, 237[a]
Beer's law limit[b] (µg/ml)	5-25
Apparent molar absorptivity ($L\ mol^{-1}\ cm^{-1}$)	21345.044
Sandell's sensitivity coefficient ($\mu g/cm^2$/0.001 abs unit)	1.47×10^{-5}
Limit of detection(µg/ml)	0.046
Limit of quantitation (µg/ml)	0.153

a= Analytical wave length for the proposed method

b = At analytical wave length

Table 3.17: Evaluation of accuracy and precision of the method for estimation of valdecoxib in methanol

Exact amount of the drug added (mg)	Individual amounts found (mg); mean (S.D.)[a]	Coefficient of variation (CV)	Relative mean error (RME)	Confidence limits[b]
4.2	4.17	4.0	0.005	4.12±0.409
4.1	4.26			
4.0	3.94			
	4.12(0.165)			
4.9	5.06	3.18	0.0122	5.03±0.397
5.1	5.18			
4.9	4.85			
	5.03(0.16)			
6.1	6.19	2.97	0.014	6.06±0.446
6.0	5.85			
5.8	6.14			
	6.06(0.18)			

[a]n=3

[b]Confidence limits at P=0.95 and two degrees of freedom.

Figure 3.11: Absorptivity scan of valdecoxib in methanol

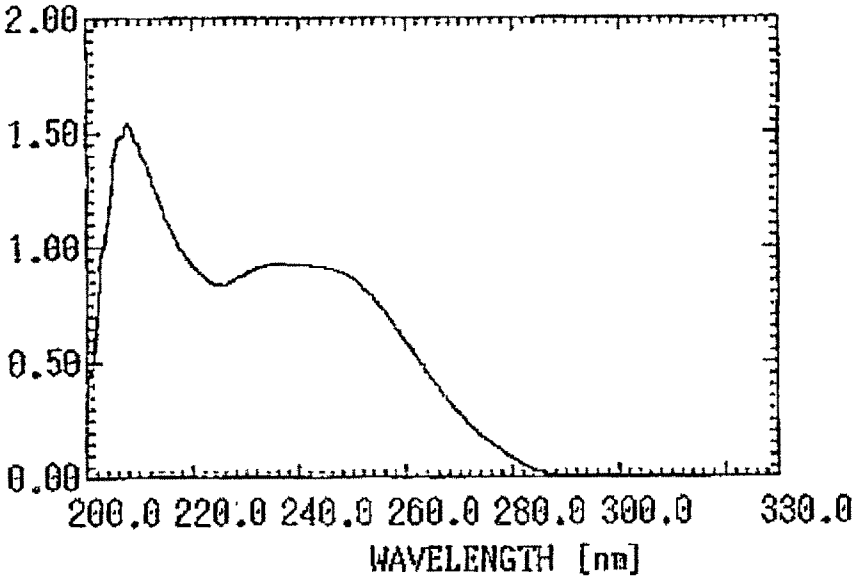
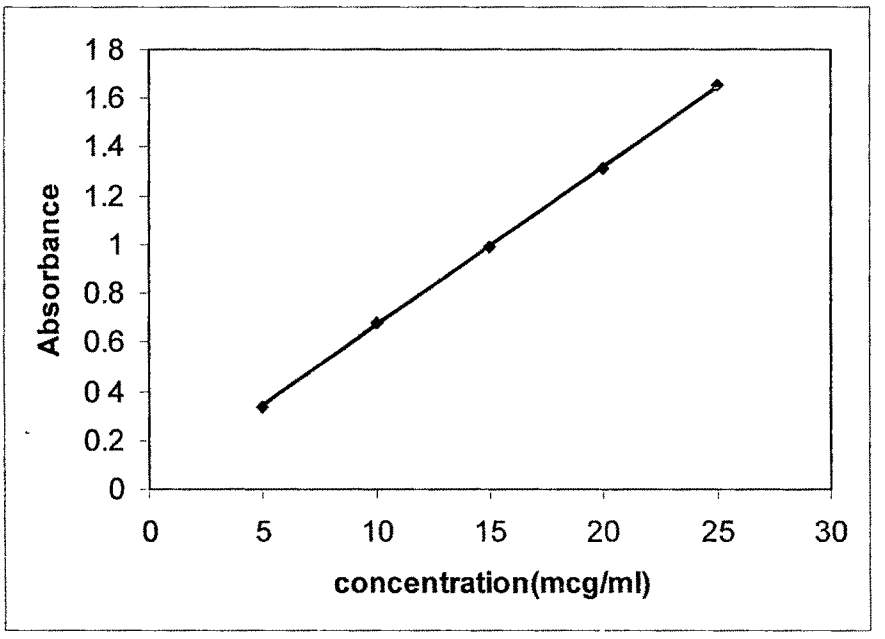


Figure 3.12: Calibration of valdecoxib in methanol



3.2.9 Estimation of valdecoxib in phosphate buffer with 2.0% tween-80

Valdecoxib is not soluble in phosphate buffer pH-7.4. So tween-80 is added in order to solubilize valdecoxib. Valdecoxib in this media yields a characteristic curve when scanned in the ultra-violet region between 200-330 nm. The λmax of valdecoxib in this media is 259 nm.

3.2.9.1 Solutions

Stock solution of valdecoxib (100μg/ml) was prepared by dissolving 5 mg of valdecoxib in phosphate buffer pH-7.4 with 2.0% tween-80 to produce 50 ml.

3.2.9.2 Preparation of calibration curve

Suitable aliquots of the stock solution were transferred to a separate 10 ml volumetric flasks and the volume was made up with phosphate buffer with 2.0% tween-80 to get the final concentration of 5, 10, 15 and 20 μg/ml. Absorbance of the resulting solutions was measured at 259 nm using the dissolution medium as the blank. The above procedure was repeated six times.

3.2.9.3 Stability and selectivity

Stability of the solutions of valdecoxib, used for preparing the calibration curve, was ascertained by observing the changes in the absorbance at 259nm over a period of 24 hours, at room temperature.

The selectivity of the method for the estimation of valdecoxib was ascertained by carrying out the procedure in the presence of the various ingredients used in the preparation of microspheres at the concentrations in which they are present in the microspheres.

3.2.9.4 Accuracy and precision

In order to determine the accuracy and precision of the method, solutions containing known amounts of valdecoxib were prepared and analyzed in three replicates. The analytical results obtained from these investigations are summarized in table 3.20.

Table 3.18: Mean absorbance values, regressed values and statistical data of the calibration curve for the estimation of valdecoxib in phosphate buffer pH 7.4 with 2.0% tween-80

Concentration µg/ml	Mean ABS* (± S.E.)	Regressed values**
5	0.195±0.005	0.198
10	0.441±0.007	0.423
15	0.676±0.006	0.679
20	0.932±0.011	0.919
25	1.152±0.014	1.160

Regression equation: Y= 0.0481x -0.423
Correlation coefficient= 0.999
*mean of 6 values
** using regression equation
n=30

Table 3.19: Optical characteristics of valdecoxib in phosphate buffer pH 7.4 with 2% tween-80

Characteristic	Value
Absorption maxima (nm)	259[a]
Beer's law limit[b](µg/ml)	5-20
Apparent molar absorptivity[b] (L mol^{-1} cm^{-1})	13863.27
Sandell's sensitivity coefficient (µg/cm^2/0.001 abs unit)	2.26 x 10^{-5}
Limit of detection(µg/ml) Limit of quantitation(µg/ml)	0.100 0.333

a=analytical wave-length for the proposed method

b=At analytical wave-length

Table 3.20: Evaluation of accuracy and precision of the proposed method for the estimation of valdecoxib in phosphate buffer with 2.0% tween-80

Exact amount of the drug added (mg)	Individual amounts found (mg); mean (S.D.)[a]	Coefficient of variation (CV)	Relative mean error (RME)	Confidence limits[b]
4.0	3.83	2.83	0.029	3.88±0.273
4.1	4.02			
3.9	3.81			
	3.88(0.11)			
5.1	5.18	3.59	0.008	5.01±0.446
4.9	4.82			
4.9	5.03			
	5.01(0.18)			
6.2	6.01	3.47	0.002	6.05±0.521
6.1	6.29			
5.9	5.87			
	6.05(0.21)			

[a]n=3

[b]Confidence limits at P=0.95 and two degrees of freedom.

Figure 3.13 Absorptivity scan of valdecoxib in phosphate buffer pH 7.4 with 2.0% tween-80

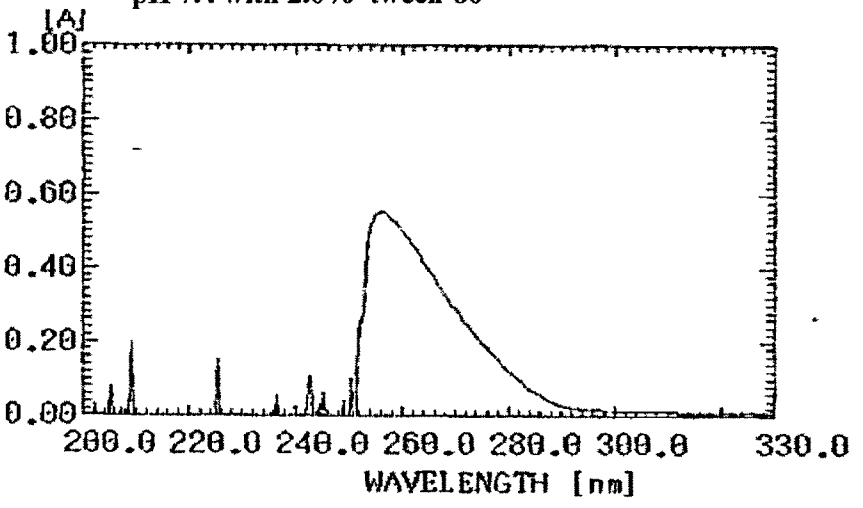
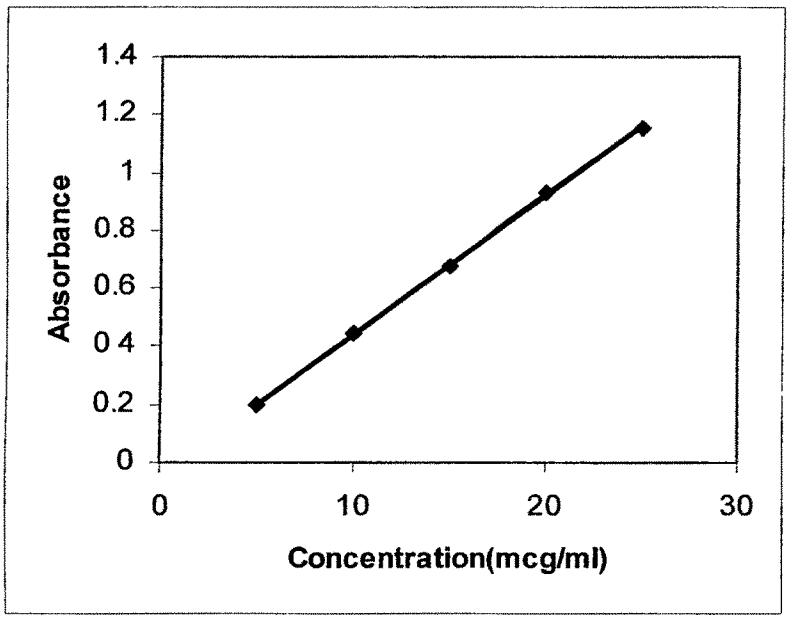


Figure 3.14: Calibration curve of valdecoxib in Phosphate buffer with 2.0% tween-80



3.2.10 Estimation of glutaraldehyde

The estimation of glutaraldehyde was done as per the method of United States Pharmacopoeia (1990). It is based on the reaction of glutaraldehyde with hydroxylamine hydrochloride.

3.2.10.1 Reagents and solutions

(1) Buffer: Dissolve 2.59 gm of potassium dihydrogen phosphate and 6.77 gm of disodium hydrogen phosphate in sufficient distilled water to produce 1000ml.

(2) Hydroxylamine hydrochloride solution: Dissolve 70 mg of hydroxylamine hydrochloride in sufficient buffer to produce 100ml. 10 ml of this solution is further diluted to 100 ml with the buffer.

(3) Reagent blank: 10 ml of the buffer was mixed with 10 ml of hydroxylamine hydrochloride solution and volume was made up to 50 ml with distilled water.

(4) Stock solution of glutaraldehyde (100µg/ml) was prepared by diluting 0.4 ml of glutaraldehyde (25%) to 100 ml with distilled water and further diluting 10 ml of this to 100 ml with distilled water. Suitable aliquots of the stock solution were transferred to separate 100 ml volumetric flasks and volume made up to give the final concentrations of 10,25,50,75 and 100 µg/ml.

3.2.10.2 Preparation of calibration curve

10 ml of the standard solutions were transferred to separate 50 ml volumetric flasks. 10 ml of the hydroxylamine hydrochloride solution was added and volume was made up to 50 ml with distilled water. The solution was allowed to stand for 25 minutes and the absorbance was measured at 237 nm using the reagent blank as the blank. The above procedure was repeated six times.

The measured and calculated parameters for the method are shown in table 3.21. The optical characteristics of the solution prepared for estimation of glutaraldehyde is shown in table 3.22. The calibration curve for glutaraldehyde estimation is shown in figure 3.14.

3.2.10.4 Selectivity

In order to determine the selectivity of the method, glutaraldehyde estimation was done in the presence of the ingredients which are present in the microspheres.

Table 3.21: Mean absorbance values, regressed values and statistical data of the calibration curve for estimation of glutaraldehyde

Concentration µg/ml	Mean ABS* (±S.E.)	Regressed values**
10	0.102±0.002	0.100
25	0.263±0.003	0.301
50	0.604±0.007	0.636
75	1.00±0.002	0.971
100	1.311±0.005	1.307

Regression equation: Y= 0.0137x – 0.0594
Correlation coefficient = 0.996
*mean of six values
** Using regression equation
n=30

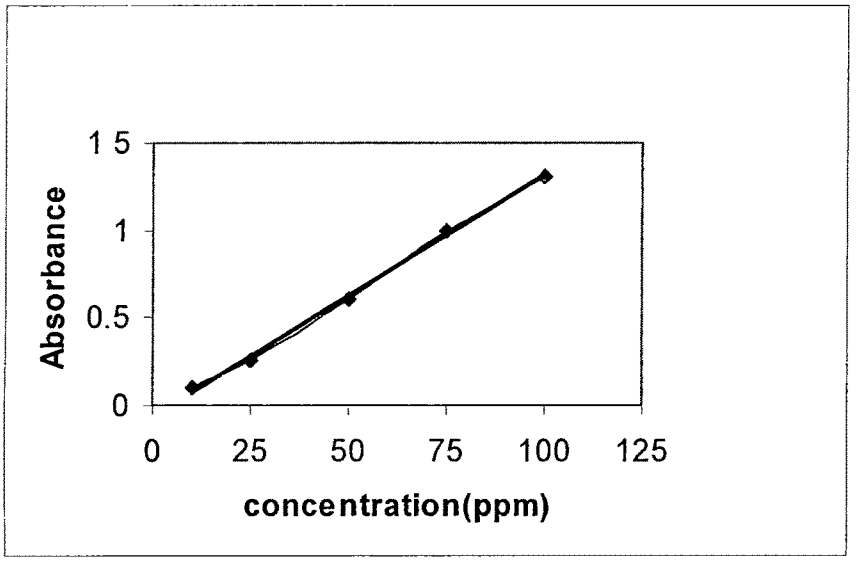
Table 3.22: Optical characteristics of solutions prepared for glutaraldehyde estimation

Characteristic	Value
Absorption maxima (nm)	237[a]
Beer's law limit[b](µg/ml)	10-100
Apparent molar absorptivity[b] ($L\ mol^{-1}\ cm^{-1}$)	1020
Sandell's sensitivity coefficient ($\mu g/cm^2$/0.001 abs unit)	9.8×10^{-5}
Limit of detection(µg/ml)	0.275
Limit of quantitation(µg/ml)	0.918

a=analytical wave-length for the proposed method

b=At analytical wave-length

Figure 3.14: Calibration curve for glutaraldehyde estimation



3.2.11 Estimation of formaldehyde

The estimation of formaldehyde was done by slight modification in the limit test for residual formaldehyde as mentioned in Indian Pharmacopoeia (1996). The test is based on the formation of deep red colour by reaction of formaldehyde with phenyl hydrazine hydrochloride and potassium ferricynide in acidic medium.

3.2.11.1 Reagents and solutions

(1) Phenyl hydrazine hydrochloride solution (1%w/v): Dissolve 100 mg of phenylhydrazine hydrochloride in sufficient distilled water to produce 10 ml.

(2) Potassium ferricynide solution (5%w/v): Dissolve 500 mg of potassium ferricynide in sufficient distilled water to produce 10 ml.

(3) Concentrated Hydrochloric acid.

(4) Standard solution: A volume equivalent to 0.1 gm of formaldehyde was diluted to 100 ml with distilled water. 1 ml of this solution was diluted to 200 ml with distilled water.

3.2.11.2 Procedure

The microspheres were shaken with 10 ml of distilled water and filtered. 1.0 ml of the filtrate was taken into a 10 ml test tube. 1 ml of 1%w/v solution of phenyl hydrazine hydrochloride and 0.5 ml of 5%w/v solution of potassium ferricynide and 1 ml of concentrated hydrochloric acid were added and allowed to stand for 15 minutes. The solution is not more intensely coloured than the standard solution treated similarly.

3.3 Results and Discussion

3.3.1 Estimation of celecoxib in 0.1N sodium hydroxide and methanol

Celecoxib in 0.1N Sodium Hydroxide yields a characteristic curve when scanned in the ultraviolet range between 200nm and 330 nm. The scan (Figure 3.1) shows absorption maxima at 216 nm and 250nm. Celecoxib in methanol yields a characteristic curve similar to that obtained with 0.1N sodium Hydroxide with absorption maxima at 205nm and 250nm (Figure 3.3). Correlation coefficients for both the methods were found to be 0.999, signifying that a linear relation exists between absorbance and concentration of the drug.

Beer's law was found to be obeyed between 5 and 25 µg/ml for both the methods. Regression analysis was performed on the experimental data. The raw data along with the results of regression anlaysis (method of least squares) is shown in Tables 3.1 and 3.3 for ME1 and ME2, respectively. The variance of the response variable, $S^2_{y,x}$ for the method ME1 was calculated to be 1.2×10^{-5} and for ME2 was 5×10^{-6}.

These low values indicate the closeness of the experimental points to the least square line. The fact is in concurrence with the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve. The variances of both the methods were compared using 'F' distribution to determine whether they are significantly different from each other. The calculated 'F'value was found to be 2.4 whereas the tabulated 'F' value was 5.8 for six degrees of freedom in both the denominator and numerator. This indicates that there is no significant difference in the variances and hence no difference in variability exists between the two methods. The variance of the slope S^2_b, was calculated to be 4.02 $x10^{-8}$ for ME1 and 1.6 x 10^{-8} for ME2. The variance of the intercept,S^2_a, determined for ME1 is 9.03 $x10^{-6}$ and for ME2 is 3.76 x 10^{-6}. To examine whether these intercepts were significantly different from zero, the intercepts were subjected to a 't' test. The values of 't' obtained for ME1 was 0.966 and for ME2 it was 1.68. The corresponding tabulated value of 't' was 4.03 at 5 degrees of freedom at 1% level. Thus acceptance of null hypothesis indicates that the intercepts were not significantly different from zero. Therefore, there are no interferences from the solvents i.e. 0.1N Sodium Hydroxide and Methanol.

The stability of celecoxib in 0.1N Sodium Hydroxide and Methanol was monitored over a period of 24 hours, at room temperature. ANOVA studies of the mean absorbance values of the solutions of different concentrations at preselected time intervals indicated that no significant difference exists between the readings. Thus, celecoxib is stable over a period of 24 hours in 0.1N Sodium Hydroxide and in Methanol.

The presence of various constituents of the microspheres at the levels in which they are present, did not interfere with the estimation of celecoxib in case of ME2. In case of ME1, the gelatin and glutaraldehyde interferes with the estimation of celecoxib. Thus, for the estimation of celecoxib in microspheres, ME2 is more suitable.

The results summarized in table 3.5 shows that the amount added and amount estimated by the proposed methods are very close. The accuracy of the method to estimate the correct amount of the drug added was ascertained by using 't' test at each level. The computed 't' values at 40mg, 50mg and 60 mg for ME1 are 0.279, 0.291 and 1.58 respectively and for ME2 are 0.081, 1.32 and 0.015 respectively. These values are lower than the tabulated 't' value of 4.30 ($P \leq 0.05$) indicating no significant difference exists between the added and estimated quantities Thus the methods are accurate in estimating the celecoxib content. This is further indicated by the low values of the relative mean error for both the methods.

3.3.2 Estimation of celecoxib in phosphate buffer with 2.0% tween-80

Since celecoxib is not soluble in phosphate buffer pH 7.4 which is the drug release medium for the microspheres, tween-80 was added at a concentration of 2%w/w. Celecoxib in this media yields a characteristic curve when scanned in the ultraviolet range between 200nm and 330 nm. The scan (Figure 3.5) shows absorption maxima at 261 nm.

Correlation coefficient was found to be 0.999, signifying that a linear relation exists between absorbance and concentration of the drug. Beer's law was found to be obeyed between 5 and 20 µg/ml. The variance of the response variable, $S^2_{y.x}$ was calculated to be 1.19×10^{-4} This low value indicates the closeness of the

experimental points to the least square line. The fact is in concurrence with the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve. The value of the Sandell's Sensitivity coefficient was found to be 1.5 x 10^{-5} μg cm^{-2} per 0.001 abs unit. The variance of the slope S^2_b, was calculated to be 9.52 x10^{-8}. The variance of the intercept,S^2_a, is 1.78 x10^{-4}. To examine whether the intercepts is significantly different from zero, the intercepts were subjected to a 't' test. The values of 't' obtained was 1.842. The corresponding tabulated value of 't' was 9.92 at 2 degrees of freedom at 1% level. Thus acceptance of null hypothesis indicates that the intercepts were not significantly different from zero. Therefore, there is no interference from the solvent i.e. Phosphate buffer with 2.0% tween-80.

ANOVA studies of the mean absorbance values of the solutions of different concentrations at preselected time intervals indicated that no significant difference exists between the readings. Thus, celecoxib is stable over a period of 96 hours in this media.

The presence of various ingredients of microspheres did not influence the estimation of celecoxib from the microspheres. The results summarized in table 3.8 shows that the amount added and amount estimated by the proposed methods are very close. The accuracy of the method to estimate the correct amount of the drug added was ascertained by using 't' test at each level. The computed 't' values at 4mg, 5mg and 6 mg are 0.216, 0.434 and 0.724 respectively. These values are lower than the tabulated 't' value of 4.30 ($P \leq 0.05$) indicating no significant difference exists between the added and estimated quantities Thus the methods are accurate in estimating the celecoxib content.

3.3.3 Estimation of rofecoxib in methanol

Rofecoxib in methanol yields a characteristic curve when scanned in the ultraviolet range between 200nm and 330 nm. The scan (Figure 3.7) shows three absorption maxima: 206nm, 222nm and 275 nm. 275 nm was chosen as the analytical wave-length.

Correlation coefficient was found to be 0.999, signifying that a linear relation exists between absorbance and concentration of the drug. Beer's law was found to be obeyed between 10 and 40 μg/ml. The variance of the response variable, $S^2_{y,x}$ was calculated to be 2.5×10^{-5} This low value indicates the closeness of the experimental points to the least square line. The fact is in concurrence with the low values of the standard error of the mean absorbance of the solutions used for preparing the calibration curve. The variance of the slope S^2_b, was calculated to be 5.0×10^{-8}. The variance of the intercept, S^2_a was 1.12×10^{-5}. To examine whether the intercepts is significantly different from zero, the intercepts were subjected to a 't' test. The value of 't' obtained was 4.173. The corresponding tabulated value of 't' was 9.92 at 2 degrees of freedom at 1% level. Thus acceptance of null hypothesis indicates that the intercepts were not significantly different from zero. Therefore, there is no interference from the solvent i.e. Methanol.

The mean absorbance values of rofecoxib solutions in methanol at different time intervals were subjected to ANOVA studies that indicated that no significant difference exists between the readings. Thus, rofecoxib is stable over a period of 24 hours in methanol.

The presence of various ingredients of microspheres did not influence the estimation of rofecoxib from the microspheres. The results summarized in table

3.11 shows that the amount added and amount estimated by the proposed methods are very close.

The accuracy of the method to estimate the correct amount of the drug added was ascertained by using 't' test at each level. The computed't' values at 8mg, 10mg and 12 mg are 2.52, 0.507 and 3.96 respectively. These values are lower than the tabulated 't' value of 4.30 ($P \leq 0.05$) indicating no significant difference exists between the added and estimated quantities Thus the methods are accurate in estimating the rofecoxib content.

3.3.3 Estimation of rofecoxib in phosphate buffer with 2.5% tween-80

Rofecoxib in phosphate buffer with 2.5% tween-80 yields a characteristic curve when scanned in the ultraviolet range between 200nm and 330 nm. The scan (Figure 3.9) shows absorption maxima at 294 nm.

Correlation coefficient was found to be 0.999, signifying that a linear relation exists between absorbance and concentration of the drug. Beer's law was found to be obeyed between 10 and 40 μg/ml. The variance of the response variable, $S^2_{y,x}$ was calculated to be 6.7×10^{-5} This low value indicates the closeness of the experimental points to the least square line. The fact is in concurrence with the low values of the standard error of the mean absorbance of the solutions used for preparing the calibration curve. The variance of the slope S^2_b, was calculated to be 1.34×10^{-7}. The variance of the intercept, S^2_a was 3.01×10^{-5}. To examine whether the intercepts is significantly different from zero, the intercepts were subjected to a 't' test. The values of 't' obtained was 6.28. The corresponding tabulated value of 't' was 9.92 at 2 degrees of freedom at 1% level. Thus acceptance of null hypothesis indicates that the intercepts were not significantly different from zero.

Therefore, there is no interference from the solvent i.e. phosphate buffer with 2.5% tween-80.

The mean absorbance values of rofecoxib solutions in phosphate buffer with 2.5% tween-80 at different time intervals were subjected to ANOVA studies which indicated that no significant difference exists between the readings. Thus, rofecoxib is stable over a period of 96 hours in phosphate buffer with 2.5% tween-80. The presence of various ingredients of microspheres did not influence the estimation of rofecoxib from the microspheres. The results summarized in table 3.14 shows that the amount added and amount estimated by the proposed methods are very close.

The accuracy of the method to estimate the correct amount of the drug added was ascertained by using 't' test at each level. The computed 't' values at 4mg, 5mg and 6 mg are 0.869, 2.83 and 0.652 respectively. These values are lower than the tabulated 't' value of 4.30 ($P \leq 0.05$) indicating no significant difference exists between the added and estimated quantities Thus the methods are accurate in estimating the rofecoxib.

3.3.4 Estimation of valdecoxib in methanol

Valdecoxib in methanol yields a characteristic curve when scanned in the ultraviolet range between 200nm and 330 nm. The scan (Figure 3.11) shows absorption maxima at 237 nm.

Correlation coefficient was found to be 0.999, signifying that a linear relation exists between absorbance and concentration of the drug. Beer's law was found to be obeyed between 5 and 25 μg/ml. The variance of the response variable, $S^2_{y.x}$ was calculated to be 1.31×10^{-4} This low value indicates the closeness of the

experimental points to the least square line. The fact is in concurrence with the low values of the standard error of the mean absorbance of the solutions used for preparing the calibration curve. The variance of the slope S^2_b, was calculated to be 5.26×10^{-7}. The variance of the intercept,S^2_a was 7.9×10^{-5}. To examine whether the intercepts is significantly different from zero, the intercepts were subjected to a 't' test. The values of 't' obtained was 1.56. The corresponding tabulated value of 't' was 5.84 at 3 degrees of freedom at 1% level. Thus acceptance of null hypothesis indicates that the intercepts were not significantly different from zero. Therefore, there is no interference from the solvent i.e. Methanol.

The mean absorbance values of valdecoxib solutions in methanol at different time intervals were subjected to ANOVA studies, which indicated that no significant difference exists between the readings indicating its stability in methanol.

The presence of various ingredients of microspheres did not influence the estimation of valdecoxib from the microspheres. The results summarized in table 3.17 shows that the amount added and amount estimated by the proposed methods are very close.

The accuracy of the method to estimate the correct amount of the drug added was ascertained by using't' test at each level. The computed't' values at 4mg, 5mg and 6 mg are 1.26, 0.326 and 0.582 respectively. These values are lower than the tabulated 't' value of 4.30 ($P \leq 0.05$) indicating no significant difference exists between the added and estimated quantities Thus the methods are accurate in estimating the valdecoxib content. This is further indicated by the low values of the relative mean error for the method.

3.3.5 Estimation of valdecoxib in phosphate buffer with 2.0% tween-80

Valdecoxib in phosphate buffer with 2.0% tween-80 yields a characteristic curve when scanned in the ultraviolet range between 200nm and 330 nm. The scan shows absorption maxima at 259 nm (figure 3.13).

Correlation coefficient was found to be 0.999, signifying that a linear relation exists between absorbance and concentration of the drug. Beer's law was found to be obeyed between 5 and 25 μg/ml. The variance of the response variable, $S^2_{y,x}$ was calculated to be 6.7 $x10^{-5}$ This low value indicates the closeness of the experimental points to the least square line. The fact is in concurrence with the low values of the standard error of the mean absorbance of the solutions used for preparing the calibration curve. The variance of the slope S^2_b, was calculated to be 1.34 $x10^{-7}$. The variance of the intercept, S^2_a was 3.01 $x10^{-5}$. To examine whether the intercepts is significantly different from zero, the intercepts were subjected to a 't' test. The value of 't' calculated was 6.28. The corresponding tabulated value of 't' was 9.92 at 2 degrees of freedom at 1% level. Thus acceptance of null hypothesis indicates that the intercepts were not significantly different from zero. Therefore, there is no interference from the solvent i.e. phosphate buffer with 2.0% tween-80.

The mean absorbance values of valdecoxib solutions in phosphate buffer with 2.0% tween-80 at different time intervals were subjected to ANOVA studies, which indicated that no significant difference exists between the readings indicating its stability in phosphate buffer with 2.5% tween-80.

The presence of various ingredients of microspheres did not influence the estimation of valdecoxib from the microspheres. The results summarized in table

3.20 shows that the amount added and amount estimated by the proposed methods are very close.

The accuracy of the method to estimate the correct amount of the drug added was ascertained by using 't' test at each level. The computed 't' values at 4mg, 5mg and 6 mg are 1.89, 0.096 and 0.413 respectively. These values are lower than the tabulated 't' value of 4.30 ($P \leq 0.05$) indicating no significant difference exists between the added and estimated quantities Thus the methods are accurate in estimating the valdecoxib content. This is further indicated by the low values of the relative mean error for both the methods.

3.3.6 Estimation of glutaraldehyde

Glutaraldehyde, by the method of United States Pharmacopoeia (1990) on reaction with hydroxylamine hydrochloride, gives a compound having a λmax at 237 nm. The molar absorptivity of the compound is 1020 L mol^{-1} cm^{-1}. The limit of detection and limit of quantitation were found to be 0.275 and 0.918 respectively. Correlation coefficient was found to be 0.996 signifying a linear relationship between concentration and absorbance. Beer's law was found to be obeyed between 10µg/ml and 100µg/ml. Regression equation was Y= 0.0137x - 0.0594.The variance of the response variable,$S^2_{y,x}$ was calculated to be 1.1 $x10^{-3}$ This low value indicates the closeness of the experimental points to the least square line. The fact is in concurrence with the low values of the standard error of the mean absorbance of the solutions used for preparing the calibration curve. The value of the Sandell's Sensitivity coefficient was found to be 9.8 x 10^{-5} µg cm^{-2} per 0.001 abs unit. The variance of the slope S^2_b, was calculated to be 2.06 $x10^{-7}$. The variance of the intercept,S^2_a was 7.78 $x10^{-4}$. To examine whether the intercepts is

significantly different from zero, the intercepts were subjected to a't' test. The values of 't' obtained was 2.13. The corresponding tabulated value of 't' was 5.84 at 3 degrees of freedom at 1% level. Thus acceptance of null hypothesis indicates that the intercepts were not significantly different from zero.

The presence of various ingredients of microspheres did not influence the estimation of glutaraldehyde from the microspheres.

Since the method was pharmacopoeial, the accuracy and precision studies were not carried out.

3.3.7 Estimation of formaldehyde

The formaldehyde estimation was done by the method of Indian Pharmacopoeia (1996). The intensity of the colour obtained by reaction of residual formaldehyde in the microspheres with phenyl-hydrazine hydrochloride and potassium ferricyanide in acidic media was compared with that of the colour obtained by similarly treating the 5 ppm solution of formaldehyde. The low colour intensity of the sample solution as compared to that of the standard solution indicates that the residual formaldehyde in the sample is less than 5 ppm.

3.4 References

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