SECTION II
Analytical methods and permeation mechanism

Chapter 3
Analytical methods

3a. ESTIMATION OF SUMATRIPTAN SUCCINATE USING UV-VISIBLE SPECTROSCOPY

This method of estimation is based on the observation that sumatriptan succinate in 0.1N HCl , distilled water, phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution shows strong absorbance in the UV region of the electromagnetic spectrum.

3a.1 EXPERIMENTAL

3a.1.1.Reagents and solutions

(1) 0.1N HCl was prepared as per the method given in Indian Pharmacopoeia (1996).

(2) Phosphate buffer saline (pH 6.4): Dissolve 1.79 gm of disodium hydrogen phosphate, 1.36gm of potassium dihydrogen phosphate and 7.02 gm of sodium chloride in sufficient distilled water to produce 1000 ml.

(3) Kreb's solution: The composition of the Krebs solution (mM) was NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 22.0, and glucose 11.0.

(4) Sodium free Kreb's solution: Sodium chloride and sodium bicarbonate in the kreb's solution were replaced by equimolar NMDG(N-methyl D-glucamine)

(5) Calcium free Kreb's solution: Calcium chloride was omitted in the Kreb's solution and 2.5mM EGTA was added

3a.1.2. Preparation of calibration curve

Stock solution of sumatriptan succinate in the solvent in which calibration curve is to be prepared (0.1N HCl , distilled water, phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution) was prepared by dissolving 10 mg of sumatriptan in 100 ml solvent with sonication. Suitable aliquots of 100μg/ml stock solution of drug were pipetted into 10 ml volumetric flasks. The volume was made up with the same solvent, the contents shaken well and the absorbance measured at 283 nm using a Shimadzu UV, 1601 UV-Visible spectrophotometer with cells of 10mm path length against the same solvent used as blank. The above procedure was repeated six times. Mean absorbance values and the regressed values(method of least squares) of the calibration curve in 0.1N HCl , distilled water, phosphate buffer saline(pH

6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution are shown in Table 3.1, 3 4, 3.7, 3.10 , 3.13 and 3.16 respectively. The calibration curve in 0 1N HCl , distilled water, phosphate buffer saline(pH 6 4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution is shown in Figure 3.1,3 3,3.5,3 7,3.9 and 3.11 respectively The optical characteristics for the solution of drug in 0.1N HCl , distilled water, phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution are shown in Table 3.2, 3.5, 3.8, 3.11, 3 14 and 3.17 respectively. Absorptivity scans over the wavelength range of 200 to 400 nm of the solution of sumatriptan succinate in 0.1N HCl , distilled water, phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution are shown in Figure 3.2,3.4, 3.6, 3.8, 3 10 and 3.12 respectively.

3a.1.3 Stability and selectivity

Changes in absorbance of the solutions of sumatriptan succinate in 0.1N HCl , distilled water, phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution used for preparing calibration curve at analytical wavelength over a period of 72 hours, was used as a means to study the stability of these solutions with time.

Sumatriptan succinate was estimated in presence of other constituents of the formulations (chitosan glutamate, carbopol 934P, pluronic-F127), in the same concentration in which they were included in the formulations, to obtain an understanding of the selectivity of the developed method for estimation of sumatriptan succinate.

3a.1.4. Accuracy and Precision

Known amounts of sumatriptan succinate in each of the above mentioned solvents were analysed using the procedure described above, in three replicates, to determine the accuracy and precision of the method The analytical results obtained from these investigations are summarized in Table 3.3, 3.6, 3 9, 3.12 , 3.15 and 3.18 for 0.1N HCl , distilled water, phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution respectively.

Table 3.1 Mean absorbance values, regressed values and statistical data of the calibration curve for estimation of Sumatriptan succinate in 0.1 N hydrochloric acid

Concentration (μg/ml)	Mean Absorbance ± SEM*	Regressed Values
2.5	0 025 ± 0 001	0 027
5	0 061 ± 0.002	0.052
10	0.108 ± 0 002	0.101
20	0.200 ± 0.001	0 199
30	0 295 ± 0.005	0 297
40	0.388 ± 0.001	0 395
50	0.471 ± 0.003	0.493
75	0.747 ± 0.009	0.738
100	0.988 ± 0.013	0.983

Regression equation: Y=0 0098X + 0.0029, Correlation coefficient = 0.9991

* Mean of six values

Table 3.2 Optical Characteristics for Sumatriptan succinate in 0.1 N hydrochloric acid

Characteristic	Value
Absorption maxima	283
Beer's law limit at 283 nm (μg/ml)	2.5-100
Apparent molar absorptivity at 283 nm(1 mol^{-1} cm^{-1})	4.46×10^3
Sandell's sensitivity coefficient (S) at 283 nm (μg/cm^2/0.001 abs unit)	9.27×10^{-2}

Table 3.3 Evaluation of accuracy and precision of the estimation method of Sumatriptan succinate in 0.1 N hydrochloric acid

Added (µg/ml)	Found(µg/ml) ± SD[a]	Coefficient of variation (CV)	% Relative mean error	Confidence limits[b]
5	5 52 ± 0 10	2.00	2 245	5 52 ± 0 253
20	19 64 ± 0 33	1 67	1 820	19 64 ± 0 816
40	38 85 ± 0 46	1 18	2 866	38 85 ± 1.144
75	74 57 ± 1 17	1 57	0 576	74 57 ± 2 918

[a] n=3, [b] Confidence limits at P=0 95 and two degrees of freedom

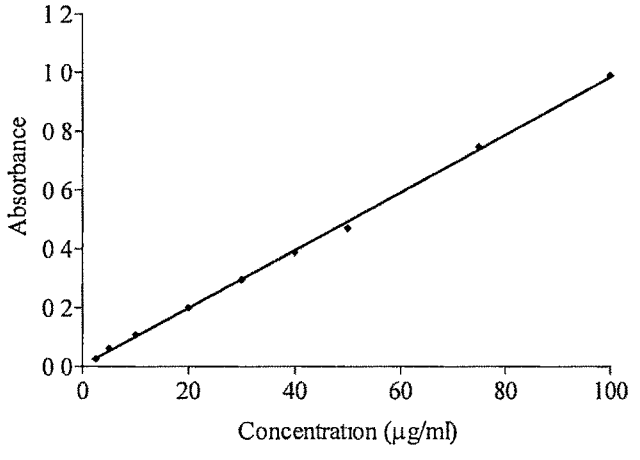

Figure 3.1 Calibration curve of sumatriptan succinate in 0.1N HCl

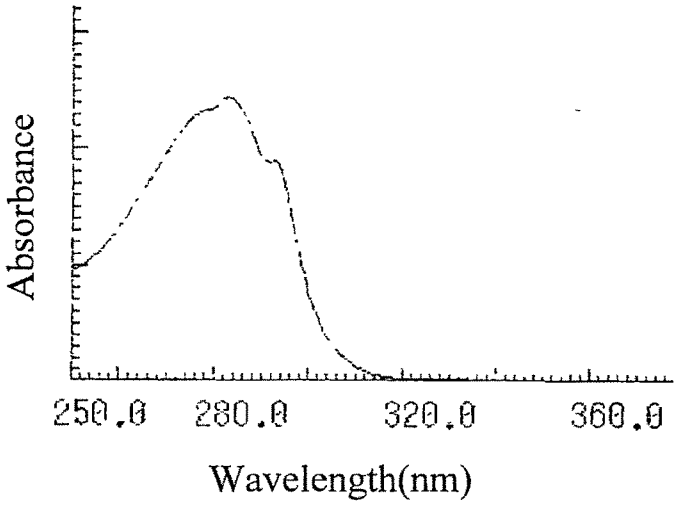

Figure 3.2 Absorptivity scan of sumatriptan succinate in 0.1N HCl

Table 3.4 Mean absorbance values, regressed values and statistical data of the calibration curve for estimation of Sumatriptan succinate in Distilled water

Concentration (μg/ml)	Mean Absorbance ± SEM*	Regressed Values
2.5	0 032 ± 0 003	0 028
5	0 054 ± 0 002	0 052
10	0.115 ± 0.006	0.101
20	0.185 ± 0.004	0.198
30	0.282 ± 0.004	0.295
40	0.383 ± 0.008	0.392
50	0.487 ± 0.008	0.489
75	0.736 ± 0.014	0.731
100	0 971 ± 0 020	0 974

Regression equation: Y=0.0097X + 0.0037, Correlation coefficient = 0.9993

* Mean of six values

Table 3.5 Optical Characteristics for Sumatriptan succinate in distilled water

Characteristic	Value
Absorption maxima	283
Beer's law limit at 283 nm (μg/ml)	2.5-100
Apparent molar absorptivity at 283 nm(1 mol^{-1} cm^{-1})	4.75×10^{3}
Sandell's sensitivity coefficient (S) at 283 nm (μg/cm^2/0.001 abs unit)	8.71×10^{-2}

Table 3.6 Evaluation of accuracy and precision of the estimation method of Sumatriptan succinate in distilled water

Added (μg/ml)	Found(μg/ml) ± SD[a]	Coefficient of variation (CV)	% Relative mean error	Confidence limits[b]
5	5 08 ± 0 10	2 03	1 649	5 08 ± 0 2565
20	19 52 ± 0 47	2.42	2 423	19 52 ± 1 1755
40	39 58 ± 0 53	1.34	1.040	39.58 ± 1 3142
75	74 98 ± 0 82	1 10	0 027	74.98 ± 2 0494

[a] n=3, [b] Confidence limits at P=0.95 and two degrees of freedom

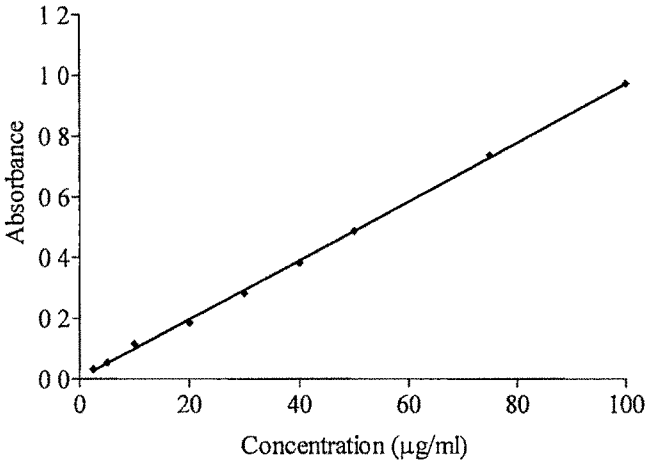

Figure 3.3 Calibration curve of sumatriptan succinate in Distilled water

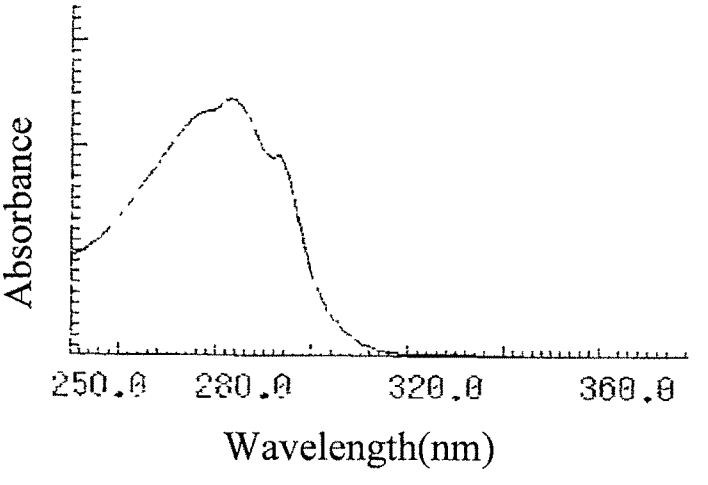

Figure 3.4 Absorptivity scan of sumatriptan succinate in Distilled water

Table 3.7 Mean absorbance values, regressed values and statistical data of the calibration curve for estimation of Sumatriptan succinate in Phosphate buffered saline (pH=6.4)

Concentration (μg/ml)	Mean Absorbance ± SEM*	Regressed Values
1.25	0.020 ± 0.001	0.019
2.5	0.034 ± 0.001	0.034
5	0.068 ± 0.002	0.064
10	0.130 ± 0 002	0 124
20	0.245 ± 0.003	0.244
30	0 356 ± 0 006	0.364
40	0.487 ± 0.004	0.484
50	0.599 ± 0.003	0 604
75	0.896 ± 0.013	0.904
100	1.218 ± 0 002	1 204

Regression equation· Y=0.012X + 0 0038, Correlation coefficient = 0 9997

*Mean of six values

Table 3.8 Optical Characteristics for Sumatriptan succinate in Phosphate buffered saline(pH=6.4)

Characteristic	Value
Absorption maxima	283
Beer's law limit at 283 nm (μg/ml)	1.25-100
Apparent molar absorptivity at 283 nm(1 mol^{-1} cm^{-1})	5.38 x 10^3
Sandell's sensitivity coefficient (S) at 283 nm ($\mu g/cm^2$/0.001 abs unit)	7 68 x 10^{-2}

Table 3.9 Evaluation of accuracy and precision of the estimation method of Sumatriptan succinate in Phosphate buffered saline (pH=6.4)

Added (μg/ml)	Found(μg/ml) ± SD[a]	Coefficient of variation (CV)	% Relative mean error	Confidence limits[b]
5	5 10 ± 0 14	2 83	2 0	5.10 ± 0 359
20	20.21 ± 0 63	3 12	1.056	20 21 ± 1 571
40	40 16 ± 0 17	0 43	0.389	40 16 ± 0 436
75	75 43 ± 0 46	0 62	0 578	75.43 ± 1 153

[a] n=3, [b] Confidence limits at P=0.95 and two degrees of freedom

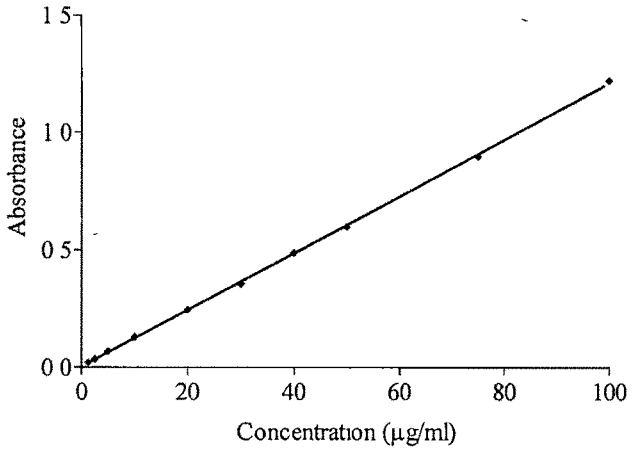

Figure 3.5 Calibration curve of sumatriptan succinate in PBS (pH 6.4)

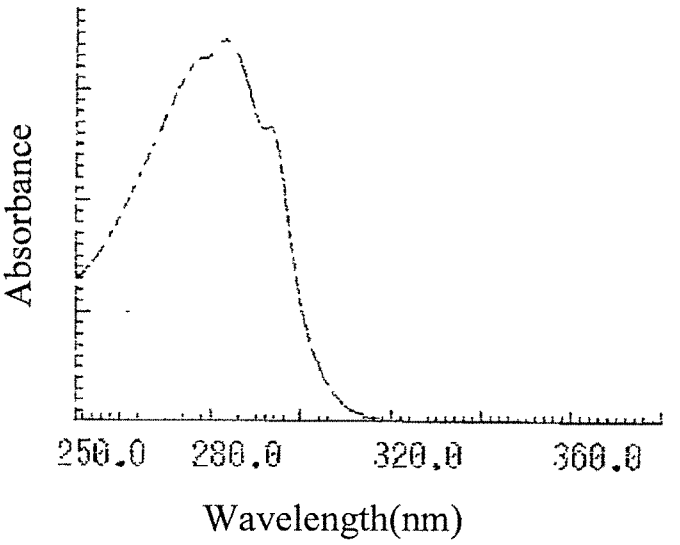

Figure 3.6 Absorptivity scan of sumatriptan succinate in PBS (pH 6.4)

Table 3.10 Mean absorbance values, regressed values and statistical data of the calibration curve for estimation of Sumatriptan succinate in Kreb's solution

Concentration (µg/ml)	Mean Absorbance ± SEM*	Regressed Values
1 25	0.027 ± 0 002	0 021
2.5	0.037 ± 0 001	0.036
5	0 069 ± 0.003	0.066
10	0 138 ± 0.003	0 126
20	0 244 ± 0 003	0 246
30	0.355 ± 0.004	0.366
40	0.472 ± 0 005	0.486
50	0.589 ± 0.004	0.606
75	0.910 ± 0.015	0.906
100	1.212 ± 0 042	1 206

Regression equation Y=0.012X + 0 0057, Correlation coefficient = 0 9994

*Mean of six values

Table 3.11 Optical Characteristics for Sumatriptan succinate in Kreb's solution

Characteristic	Value
Absorption maxima	283
Beer's law limit at 283 nm (µg/ml)	1.25-100
Apparent molar absorptivity at 283 nm(1 mol^{-1} cm^{-1})	5 72 x 10^3
Sandell's sensitivity coefficient (S) at 283 nm (µg/cm^2/0.001 abs unit)	7.23 x 10^{-2}

Table 3.12 Evaluation of accuracy and precision of the estimation method of Sumatriptan succinate in Kreb's solution

Added (µg/ml)	Found(µg/ml) ± SD[a]	Coefficient of variation (CV)	% Relative mean error	Confidence limits[b]
5	5 11 ± 0 08	1 63	2 167	5 11 ± 0 207
20	20 22 ± 0 34	1 67	1 097	20 22 ± 0 839
40	39 19 ± 1 15	2 92	2 021	39 19 ± 2.842
75	74 08 ± 1 29	1 74	1 226	74 08 ± 3 211

[a] n=3, [b] Confidence limits at P=0 95 and two degrees of freedom

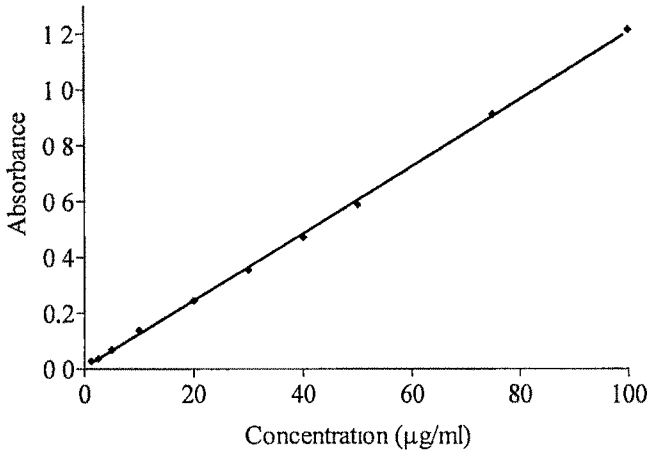

Figure 3.7 Calibration curve of sumatriptan succinate in Kreb's solution

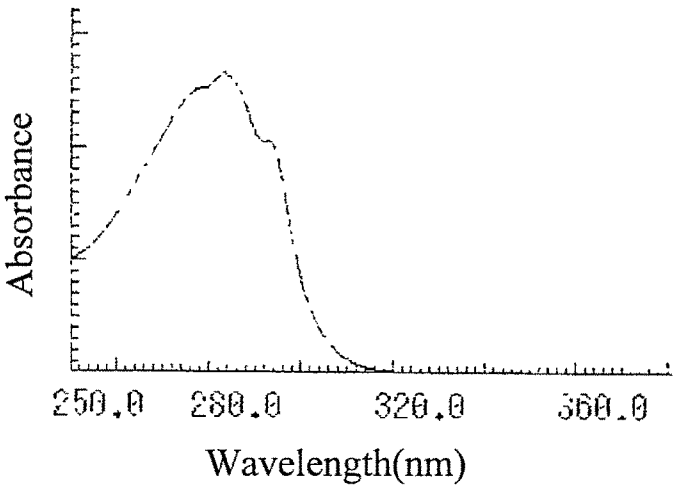

Figure 3.8 Absorptivity scan of sumatriptan succinate in Kreb's solution

Table 3.13 Mean absorbance values, regressed values and statistical data of the calibration curve for estimation of Sumatriptan succinate in Sodium free Kreb's solution

Concentration (μg/ml)	Mean Absorbance ± SEM*	Regressed Values
1.25	0.023 ± 0 001	0.017
2 5	0.038 ± 0.002	0.031
5	0.059 ± 0.002	0.059
10	0.120 ± 0.002	0.115
20	0 221 ± 0.003	0.227
30	0 324 ± 0.002	0.339
40	0 451 ± 0 002	0.451
50	0 557 ± 0.003	0.563
75	0.854 ± 0 004	0.843
100	1.122 ± 0.014	1 123

Regression equation Y=0.0112X + 0.0031, Correlation coefficient = 0.9996

*Mean of six values

Table 3.14 Optical Characteristics for Sumatriptan succinate in Sodium free Kreb's solution

Characteristic	Value
Absorption maxima	283
Beer's law limit at 283 nm (μg/ml)	1 25-100
Apparent molar absorptivity at 283 nm(1 mol^{-1} cm^{-1})	4 95 x 10^{3}
Sandell's sensitivity coefficient (S) at 283 nm (μg/cm^{2}/0 001 abs unit)	8 36 x 10^{-2}

Table 3.15 Evaluation of accuracy and precision of the estimation method of Sumatriptan succinate in Sodium free Kreb's solution

Added (µg/ml)	Found(µg/ml) ± SD[a]	Coefficient of variation (CV)	% Relative mean error	Confidence limits[b]
5	5.05 ± 0.10	2.04	1.012	5.05 ± 0.257
20	20.05 ± 0 36	1.80	0.253	20.05 ± 0.901
40	40 11 ± 0.31	0.78	0 275	40.11 ± 0 780
75	76.33 ± 0.47	0.62	1.774	76 33 ± 1.170

[a] n=3, [b] Confidence limits at P=0.95 and two degrees of freedom

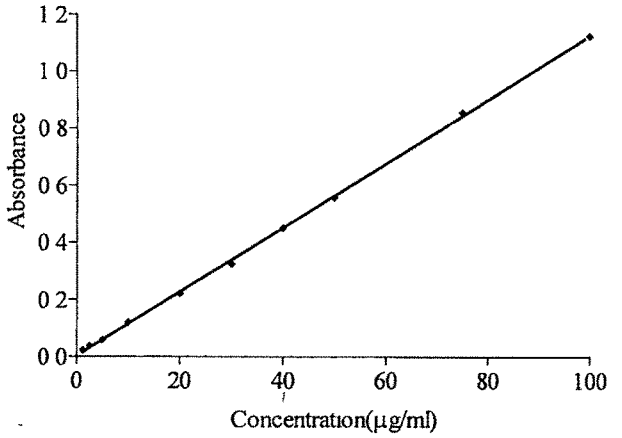

Figure 3.9 Calibration curve of sumatriptan succinate in sodium free Kreb's solution

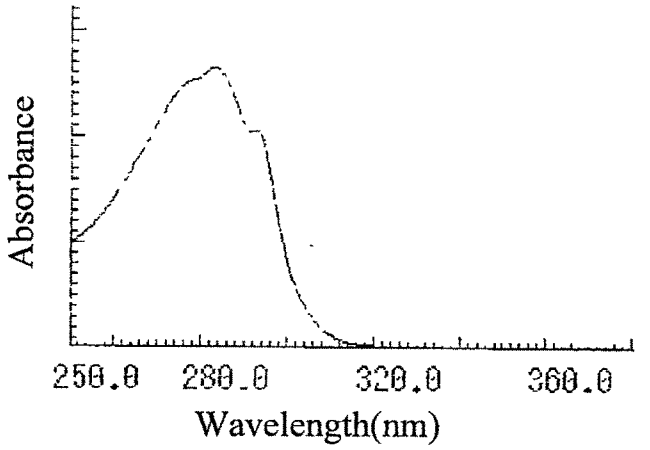

Figure 3.10 Absorptivity scan of sumatriptan succinate in sodium free Kreb's solution

Table 3.16 Mean absorbance values, regressed values and statistical data of the calibration curve for estimation of Sumatriptan succinate in Calcium free Kreb's solution

Concentration (µg/ml)	Mean Absorbance ± SEM*	Regressed Values
1.25	0.029 ± 0 002	0.020
2.5	0.042 ± 0.002	0.036
5	0.060 ± 0 002	0.067
10	0 138 ± 0.003	0.129
20	0.239 ± 0.004	0.253
30	0.364 ± 0.003	0 377
40	0 499 ± 0.004	0 501
50	0 643 ± 0.008	0.625
75	0.934 ± 0 008	0.935
100	1.250 ± 0 011	1 245

Regression equation Y=0.0124X + 0.0045, Correlation coefficient = 0 9994

* Mean of six values

Table 3.17 Optical Characteristics for Sumatriptan succinate in Calcium free Kreb's solution

Characteristic	Value
Absorption maxima	283
Beer's law limit at 283 nm (µg/ml)	1 25-100
Apparent molar absorptivity at 283 nm(1 mol^{-1} cm^{-1})	5.69×10^{3}
Sandell's sensitivity coefficient (S) at 283 nm (µg/cm^2/0.001 abs unit)	$7\ 27 \times 10^{-2}$

Table 3.18 Evaluation of accuracy and precision of the estimation method of Sumatriptan succinate in Calcium free Kreb's solution

Added (µg/ml)	Found(µg/ml) ± SD[a]	Coefficient of variation (CV)	% Relative mean error	Confidence limits[b]
5	5.07 ± 0 09	1.84	1 344	5 07 ± 0 231
20	19 61 ± 0 33	1.66	1 949	19 61 ± 0 810
40	39 26 ± 0 55	1.40	1 848	39.26 ± 1 361
75	75 52 ± 0 69	0 91	0 699	75.52 ± 1 714

[a] n=3, [b] Confidence limits at P=0.95 and two degrees of freedom

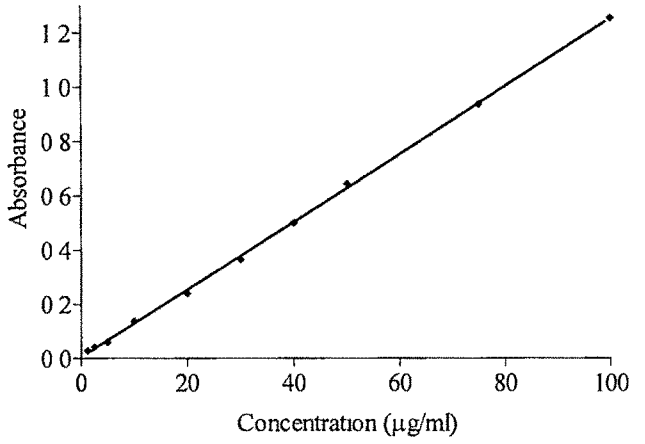

Figure 3.11 Calibration curve of sumatriptan succinate in calcium free Kreb's solution

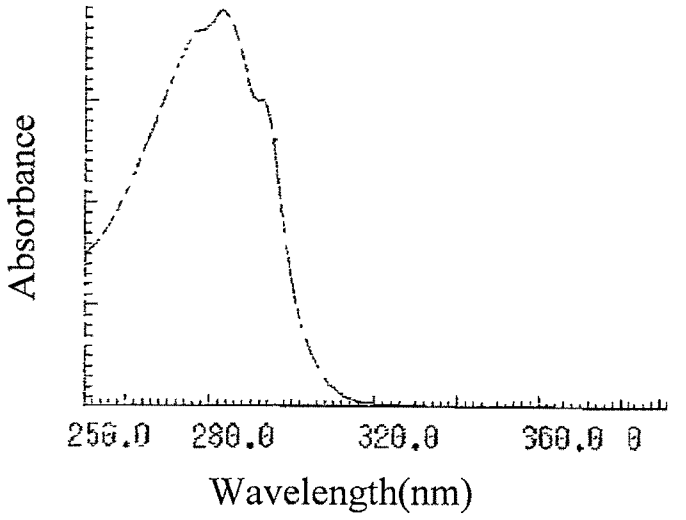

Figure 3.12 Absorptivity scan of sumatriptan succinate in calcium free Kreb's solution

3a.2. RESULTS AND DISCUSSION

Sumatriptan succinate in 0.1N HCl, distilled water, phosphate buffer saline (pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution yields characteristic curves when scanned in UV-Visible wavelength in range between 200-400 nm. The analytical wavelength 283 nm was selected because the absorptivities at 283 nm were found to be satisfactory and hence selected as the analytical wavelength and used for further studies.

The high correlation coefficients (Table 3.1, 3.4, 3.7, 3 10, 3.13 and 3.16) in the above mentioned solvents indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed in the range of 2.5-100 μg/ml (Table 3.2 and 3.5) in 0.1 N HCl and distilled water and in the range of 1.25-100μg/ml (Table 3.8, 3.11, 3.14 and 3.17) in phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution. Regression analysis of the experimental data was carried out and the experimental data along with regressed values are shown in Table 3.1, 3.4, 3.7, 3.10, 3.13 and 3.16. The slopes of the regressed lines indicate moderated sensitivity of the methods as also seen with the values of Sandell's sensitivity coefficients.

The low values of variance of the response variable, S^2y,x for sumatriptan succinate in 0.1N HCl (2.33×10^{-5}) , distilled water (5.29×10^{-5}), phosphate buffer saline(pH 6.4) (6.87×10^{-5}), Kreb's solution (1.8×10^{-4}), sodium free Kreb's solution (1.43×10^{-5}) and calcium free Kreb's solution (2.9×10^{-6}), signifies the good fit between the obtained and calculated data. This low variability of the experiments is supported by the low standard error values of mean absorbance values of the solutions used for preparing calibration curves. The variance of slope S^2b for sumatriptan succinate in 0 1N HCl (8.6×10^{-8}) , distilled water (1.88×10^{-7}), phosphate buffer saline(pH 6.4) (1.95×10^{-8}), Kreb's solution (6.37×10^{-7}), sodium free Kreb's solution (5.02×10^{-8}) and calcium free Kreb's solution (3.93×10^{-8}), indicates high sensitivity of the method which is also reflected by the high molar absorptivities of the compound and low Sandell's sensitivity coefficient values (Table 3.2 ,3.5,3.8, 3.11, 3.14 and 3.17). The variance of intercept, S^2a was calculated as 2.8×10^{-5}, 4.09×10^{-5}, 6.82×10^{-6}, 1.02×10^{-4}, 9.88×10^{-6} and 1.35×10^{-5} in 0.1N HCl , distilled water, phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution respectively. The intercept were subjected to One-way Analysis of variance (ANOVA) for the significance and was

not significantly different from zero at 6 degrees of freedom, at P=0.95, which indicates that blank does not interfere in the absorbance measurements.

The stability of drug in 0.1N HCl , distilled water, phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution was ascertained over a period of 72 hour. Analysis of variance (ANOVA) of the mean absorbance values of the solutions of different concentrations at various time intervals revealed that there was no significant difference between the readings. From this, it is concluded that sumatriptan succinate is stable over the determined period in the above solvents.

Estimation of sumatriptan succinate was carried out in presence of other constituents used in the formulation, such as chitosan glutamate, carbopol 934P, pluronic F-127 etc at appropriate levels which they were present in the final formulations. None of the materials interfered in the estimation of sumatriptan succinate using the above methods.

The results of recovery study of known amount of drug in triplicate, in in 0.1N HCl , distilled water, phosphate buffer saline(pH 6.4), Kreb's solution, sodium free Kreb's solution and calcium free Kreb's solution are summarized in Table 3.3, 3.6, 3.9, 3.12 , 3.15 and 3.18 respectively and were used to ascertain the accuracy and precision of the methods. Low relative mean error (%) values indicate low variability between each data point of analysis. Precision of the methods was ascertained from standard deviation values, the coefficient of variation (%) and confidence limits. The low coefficient of variation (%) and confidence limits indicate the precision of the methods.

3b.ESTIMATION OF SUMATRIPTAN SUCCINATE USING HPLC WITH UV-DETECTION IN BIOLOGICAL SAMPLES

3b. 1 INTRODUCTION

Several analytical methods have been developed and published for the determination of sumatriptan in plasma and serum samples including high performance liquid chromatography (HPLC) with coulometric detection (Franklin et al.,1996, Dunne et al.,1996 and Andrew et al.,1993) , HPLC with florescence detection (Ge Z et al.,2004)

and HPLC with mass spectrometric detection(Vishwanathan et al.,2000, McLoughlin et al.,1996 and Dulry et al.,1997). However no method is reported till date for determination of sumatriptan in brain samples.

Many of the above methods are sensitive, but analytical technique such as HPLC with mass spectophotometry is expensive particularly, when they are to be estimated In biological samples in routine analysis for drug monitoring. Hence, a simple, rapid and sensitive method for the determination of sumatriptan in plasma, CSF and brain homogenate using a different detection technique is highly warranted. One of the techniques possible is an HPLC with UV detection HPLC with UV detection is simple, robust and available to most analytical laboratories. Although there are several analytical methods proposed, including HPLC with UV/Vis (Shigh et al., 1997, Avadhanulu et al.,1996, Avadhanulu et al.,1996, Shirsat et al.,1998, and Nozal et al.,2002) to determine sumatriptan succinate in raw materials and pharmaceutical preparations, to our knowledge however currently no method is available for the determination of sumatriptan succinate in biological samples using a HPLC with UV detection technique. Moreover limit of detection for above mentioned analytical methods using UV detection is much higher. An analytical method using HPLC with UV detection for the estimation of sumatriptan succinate in rat plasma, CSF and brain tissue is developed and validated with relevant statistical analytical parameters. This method involves a simple liquid–liquid extraction with excellent reproducibility, which makes it suitable for pharmacokinetics studies involving sumatriptan

3b. 2 INSTRUMENTATION

All chromatographic measurements were performed on a Dionex HPLC system with a UV-visible detector (UVD170U). The separation was achieved by using 25 cm×4.6 mm

ID, 10 µm ODS Hypersil column obtained from Thermo electron corporation. Other instruments used in the study included cyclomixer, bath sonicator, water bath and cooling centrifuge (Sigma, Germany).

3b. 3 REAGENTS

(a) Ammonium phosphate monobasic (0 04 M)· Dissolve 46 gm of ammonium phosphate monobasic in sufficient distilled water to produce 1000ml.

(b) 2M sodium hydroxide solution Dissolve 80 gm of sodium hydroxide in sufficient distilled water to produce 1000ml.

(c) O-phosphoric acid (1M): Dissolve 98 gm of sodium hydroxide in sufficient distilled water to produce 1000ml

(d) PBS (7.4): Prepared as per procedure given in Indian Pharmacopoeia (1996).

3b. 4 EXPERIMENTAL

3b. 4.1 Blood collection, CSF collection and brain tissue preparation

Male wistar rats (200-250gms) were anesthetized with urethane (i.p., 1 2 g/kg) CSF (cerebro spinal fluid) samples were withdrawn by cisternal puncture as described by Dahlin et al (Dahlin et al , 2000) . Blood samples were collected from the descending aorta in tubes containing heparin as anticoagulant and centrifuged for 10 min at 8000 rpm for plasma separation. After the completion of the blood collection, the skull was opened and the brain was removed. Brain was cleaned of surrounding blood vessels, washed, weighed, and homogenized in phosphate buffer saline (pH=7.4).

3b.4.2.Preparation of calibration and validation standards

A non-zero calibration standard, ranging from 3 to 2000 ng/ml (3,25,50,75,100 1000 and 2000 ng/ml), 2 5 to 2500 ng/ml (2.5,5,25,50, 100 1000 and 2500 ng/ml) and 3 to 1000 ng/g (3,5,25,50,75,100 and 1000 ng/g)was prepared by spiking the drug free rat plasma containing heparin, drug free CSF and rat brain homogenate with an appropriate amount of sumatriptan succinate and internal standard respectively. The validation standards at four concentration levels (3, 10, 500 and 1000 ng/ml), (2.5, 10, 500 and 1000 ng/ml) and

(3, 10, 500 and 1000 ng/g) for plasma, CSF and brain samples respectively were prepared in a similar way as described above, with the lowest concentration being LOQ. Non-biological calibration curve was also performed for the determination of absolute recovery.

3b.4.3.Extraction procedure for plasma samples

A 1ml aliquot of rat plasma sample was placed in a screw cap glass tube. A 40µl of internal standard working solution (150 µg/ml ofloxacin) and 1 ml of 2M sodium hydroxide solution were added and the mixture was vortexed for 30 s. The plasma samples containing sumatriptan were then extracted with 4ml ethyl acetate. The mixture was shaken for 5 min and centrifuged for 15 min at 10°C. Extraction procedure with 4 ml ethyl acetate was repeated twice for each sample. Two extracts were mixed together in a culture tube, and then evaporated to dryness The extraction residue was reconstituted in 0 3 ml of the mobile phase and injected into the HPLC system.

3b.4.4. Preparation procedure for CSF samples

CSF samples spiked with appropriate amount of sumatriptan succinate and 40µl of internal standard working solution (150 µg/ml ofloxacin) were injected into HPLC system after centrifugation at 8000 rpm and without any further treatment.

3b.4.5.Extraction procedure for brain samples

A 40µl of internal standard working solution (150 µg/ml ofloxacin) and 1.5 ml of 2M sodium hydroxide solution were added to brain homogenate and the mixture was vortexed for 30 s. The brain samples containing sumatriptan were then extracted with 4ml ethyl acetate The mixture was shaken for 5 min and centrifuged for 15 min at 10°C Extraction procedure with 4 ml ethyl acetate was repeated thrice for each sample. All the three extracts were mixed together in a culture tube, and then evaporated to dryness. The extraction residue was reconstituted in 0.3 ml of the mobile phase and injected into the HPLC system

3b.4.6.Chromatographic conditions

The mobile phase flow rate was 1.0 ml/min and consisted mixture of ammonium phosphate monobasic (0.04 M)–acetonitrile (78:22, v/v), pH 3.7 which was adjusted with o-phosphoric acid The mobile phase was degassed and filtered (0 22μm) prior to use. The injection volume was 20μl and the detection wavelength was set at 228 nm.

3b.4.7.Method validation procedures

Analytical method was validated for specificity, robustness, absolute recovery, linearity, sensitivity, precision and accuracy.

3b.5. RESULTS AND DISCUSSION

Rapid, sensitive and novel HPLC method for determination of sumatriptan in rat plasma, CSF and brain samples was developed and validated. HPLC with UV detection has never been used in past for the determination of sumatriptan in biological samples. A liquid–liquid extraction procedure was performed for this purpose. This method is rapid, simple, selective and efficient. It is also adapted to tissue homogenate samples. Protein precipitation method was tried but analyte recovery obtained was very low.

Chromatographic conditions were studied and maximum resolution and sensitivity of the method was obtained at 228nm and mobile phase flow rate of 1ml/min. Retention time of sumatriptan and ofloxacin were 6.3 min and 12.75 min respectively. The total run time of this method was 25 min, and the system was ready for the next sample injection without the need for additional wash time

3b.5.1.Specificity

Six different lots of drug free rat plasma , drug free brain homogenate and drug free CSF(cerebro spinal fluid) were tested before spiking to ensure that there was no endogenous interference at retention times of sumatriptan and ofloxacin (internal standard). Typical chromatograms obtained after analysis of blank plasma, blank CSF blank brain homogenate and samples containing different concentrations of sumatriptan are illustrated in Fig.3.13 , Fig 3 14 and Fig 3.15 respectively.

3b.5.2.Robustness

The robustness of the HPLC method was determined by analysis of samples under a variety of conditions such as small changes in the pH (3.4 to 3.7) and in the percentage of acetonitrile (19%-22%) in mobile. The effect on retention time and peak parameters were studied. The method was found to be robust for the entire pH range and concentration of mobile phase.

3b.5.3. Absolute recovery

The absolute recovery of sumatriptan succinate at four concentration levels was determined by comparing the peak areas measured after analysis of spiked plasma samples and brain homogenate samples according to the procedure with those found after direct injection into the chromatographic system of non-biological samples at the same concentration levels. As shown in Table 3.19, the analyte recoveries were close to 100% and the extraction efficiency satisfactorily ranged from 93.4% to 104.6% for plasma samples and 89.5% to 106% for brain homogenate. Recovery of internal standard was found to be 95.2% (% R.S.D = 3.2).

Table 3.19 Absolute recovery of sumatriptan succinate

Concentration	Absolute Recovery(n=3)			
	Plasma		Brain	
(ng/ml)/(ng/g)	Mean(%)	% RSD[a]	Mean(%)	% RSD[a]
3	104.6	6.0	106.0	6.6
10	93.4	5.0	89.5	6.8
500	95.0	5.9	96.5	6.6
1000	97.5	3.6	95.3	7.7

[a] RSD (standard deviation/mean concentration)*100

3b.5.4.Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample (Hubert et al, 1999 and Hubert et al, 2003). The calibration curves were built by plotting the drug to internal standard peak area ratios versus the corresponding standard sample concentrations of the drug in ng/ml or ng/g. The concentrations for unknown samples and validation samples were obtained by using linear regression of the calibration curves. Calibration curves were established over the range of 3-2000 ng/ml for plasma samples (Figure 3.16), 2.5-2500 ng/ml for CSF samples (Figure 3.17) and 3-1000 ng/g for brain homogenate (Figure 3.18) Correlation coefficient of 0.9998 (plasma), 0.9998 and 0.9994 (brain homogenate) indicates that the system is linear over this range and there exists a strong linear relationship between the ratio of area of sumatriptan succinate and IS and its concentration The regression equation's obtained are Y=0.00066 X+0.00035 for plasma, Y=0.00086 X+0.00031 for brain and Y=0.00078 X+0.00081 for CSF. The low values of variance of the response variable, S^2y,x for sumatriptan succinate in plasma(2.95 x 10-5), brain tissue (2.24 x 10-6) and CSF (1.83 x 10-6), signifies the good fit between the obtained and calculated data. The variance of slope S^2b for sumatriptan succinate in plasma (1.41 x 10^{-9}), brain (3.42 x $10^{-10)}$ and CSF (1.44 x 10^{-11}), indicates high sensitivity of the method. The variance of intercept, S^2a was calculated as 8.54 x 10^{-6}, 1.95 x 10^{-7} and 4.47 x 10^{-7} for plasma, brain and CSF respectively. The intercept were subjected to One-way Analysis of variance (ANOVA) for the significance and was not significantly different from zero at 4 degrees of freedom, at P=0.95, which indicates that blank does not interfere in the absorbance measurements.

3b.5.5.Sensitivity

The detection limit (LOD) was 1ng/ml for all plasma, CSF and brain tissue samples and the quantification limit (LOQ) was 3ng/ml for plasma and brain tissue samples and 2 5 ng/ml for CSF samples, which were determined according to the signal/noise ratio 3:1 and 10·1 respectively.

3b.5.6.Precision

The precision of the bioanalytical method was estimated by measuring repeatability and intermediate precision at the same concentration levels as those mentioned above. Precision was also studied using plasma and brain samples spiked at four concentration levels. Each of the samples was replicated (n = 3) and analyzed on 3 consecutive days. Subsequently, the mean of each set of the concentrations and the percent deviation of the quality control samples were calculated. Relative standard deviation (R.S.D.) was calculated from the estimated concentrations (Hubert et al.,2003 and Hubert et al.,1999). The R.S.D. values for inter assay and intra assay precision are presented in Table 3.20 and Table 3.21. R.S.D. values were all lower than 9.1% which is well below the acceptance limit of 15%, illustrating the very good precision of the proposed method.

3b.5.7.Accuracy

The accuracy is expressed as %bias or % relative error (difference from added concentration) and it takes into account the total error, i.e. systematic and random errors, related to the test result. (Hubert et al, 2003 and Hubert et al, 1999). The tolerance limits for inter-assay and intra-assay samples are presented in Table 3.20 and 3.21 as a function of the introduced concentrations. As can be seen from the results, the proposed method was accurate, since the different tolerance limits of the bias were below 7.3% and did not exceed the acceptance limits (±15%)(Boulanger et al., 2003 and Guidance for industry, CDER, 2001)for all the concentration levels tested including the lowest one (3 ng/ml or ng/g).

3b.5.8.Stability

The stability of sumatriptan under different conditions was evaluated. The acceptance criteria's for all stability tests were at ±15% of the nominal value. The stability of extracted sumatriptan and the internal standard in mobile phase (processed sample stability) as well as stability of prepared CSF samples were evaluated and the results showed that processed samples are stable at 4 ± 2°C for at least 72 h. The processed sample stability was evaluated by comparing the extracted plasma samples and extracted brain tissue samples that were injected immediately (time 0) with the samples that were injected 72 h after storing in the refrigerator at 4 ± 2 °C. The CSF sample stability was

evaluated by comparing the prepared CSF samples that were injected immediately (time 0) with the samples that were injected 72 h after storing in the refrigerator at 4 ± 2 °C. Evaluation was based on back-calculated concentrations.

Table 3.20 Inter-assay and intra-assay precision and accuracy of plasma and brain validation samples (n=3)

Concentration added (ng/ml)/(ng/g)	Plasma			Brain		
	Concentration found(ng/ml)	R.E.(%)[a]	C.V.(%)[b]	Concentration found(ng/g)	R.E.(%)[a]	C.V.(%)[b]
Inter-day						
3	2 9	-1 9	2 7	2 9	-2 8	0 2
10	9 5	-5 0	5 3	9 8	-2 2	6 2
500	488 5	-2 3	6 9	497 4	-0 5	9 0
1000	1025 3	2 5	1 8	980 0	-2.0	1 9
Intra-day						
3	2 8	-6 7	4 3	3 1	2 9	7 5
10	9 4	-5 7	6 4	10 7	7 3	6 8
500	481 4	-3 7	9 1	490 0	-2 0	7 0
1000	983 4	-1 7	4 1	1005 6	0.6	5 4

[a] R.E. (mean concentration-nominal concentration)/nominal concentration x 100

[b] RSD (standard deviation/mean concentration) x 100

Table 3.21 Inter-assay and intra-assay precision and accuracy of CSF validation samples (n=3)

Concentration added (ng/ml)	CSF		
	Concentration found(ng/ml)	R.E.(%)[a]	C.V.(%)[b]
Inter-day			
2.5	2.46	-1.8	1 8
12.5	12.4	-0.8	3.7
500	495 2	-1.0	2.6
1000	980 1	-2.0	1.9
Intra-day			
2.5	2.52	0.7	1.7
12 5	12 2	-2.7	0 5
500	496 4	-0.7	1.6
1000	984.5	-1.6	2.5

[a] R.E (mean concentration-nominal concentration)/nominal concentration x 100

[b] RSD (standard deviation/mean concentration) x 100

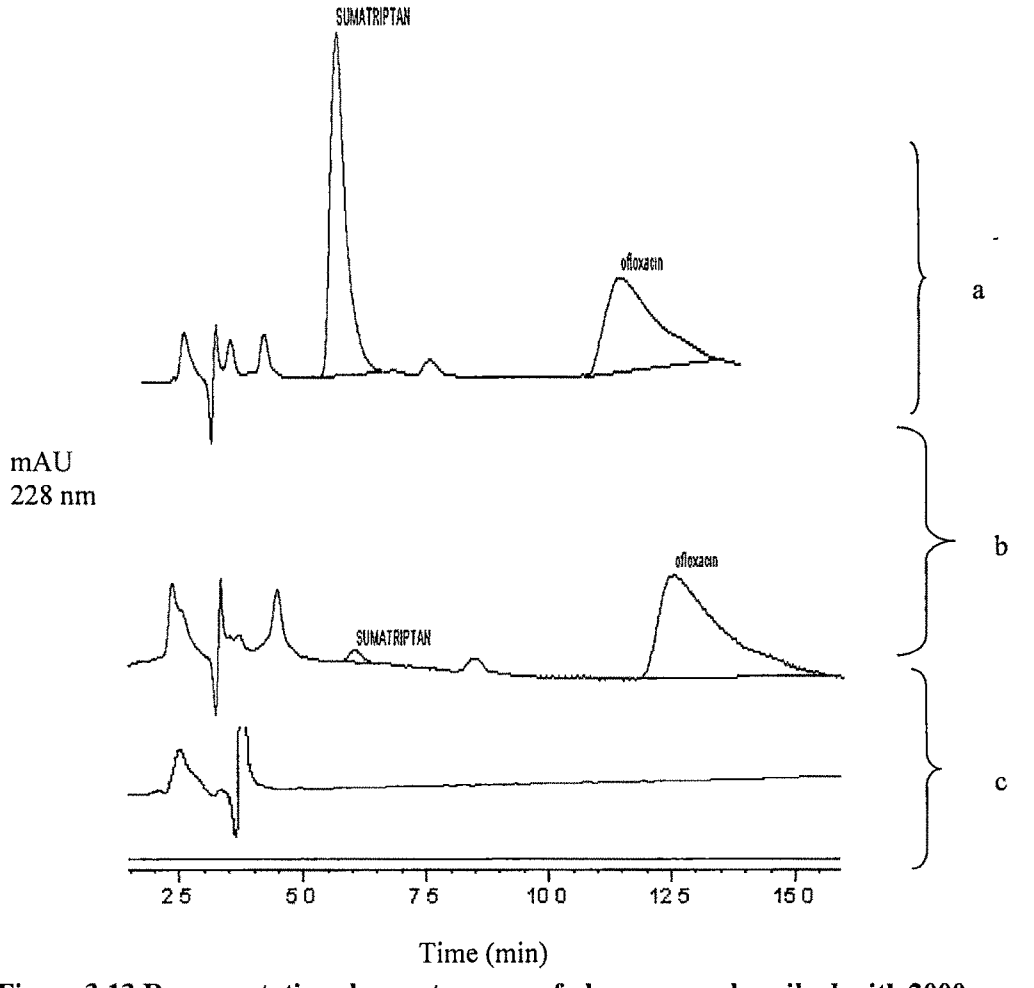

Figure 3.13 Representative chromatograms of plasma sample spiked with 2000 ng/ml sumatriptan and internal standard ofloxacin (a), plasma sample spiked with 3 ng/ml sumatriptan and internal standard ofloxacin (b) and blank plasma sample (c).

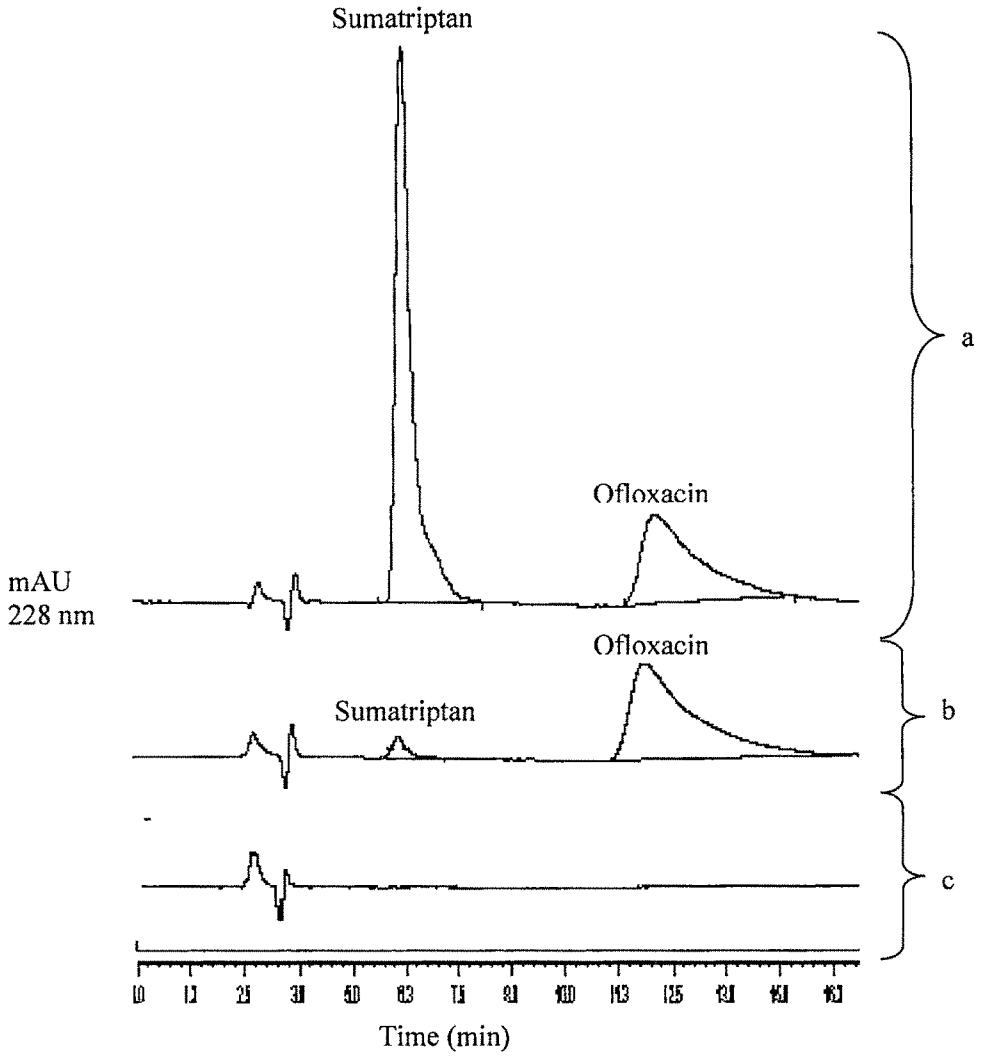

Figure 3.14 Representative chromatograms of CSF sample spiked with 2500 ng/ml sumatriptan and internal standard ofloxacin (a), CSF sample spiked with 2.5 ng/ml sumatriptan and internal standard ofloxacin (b) and blank CSF sample (c).

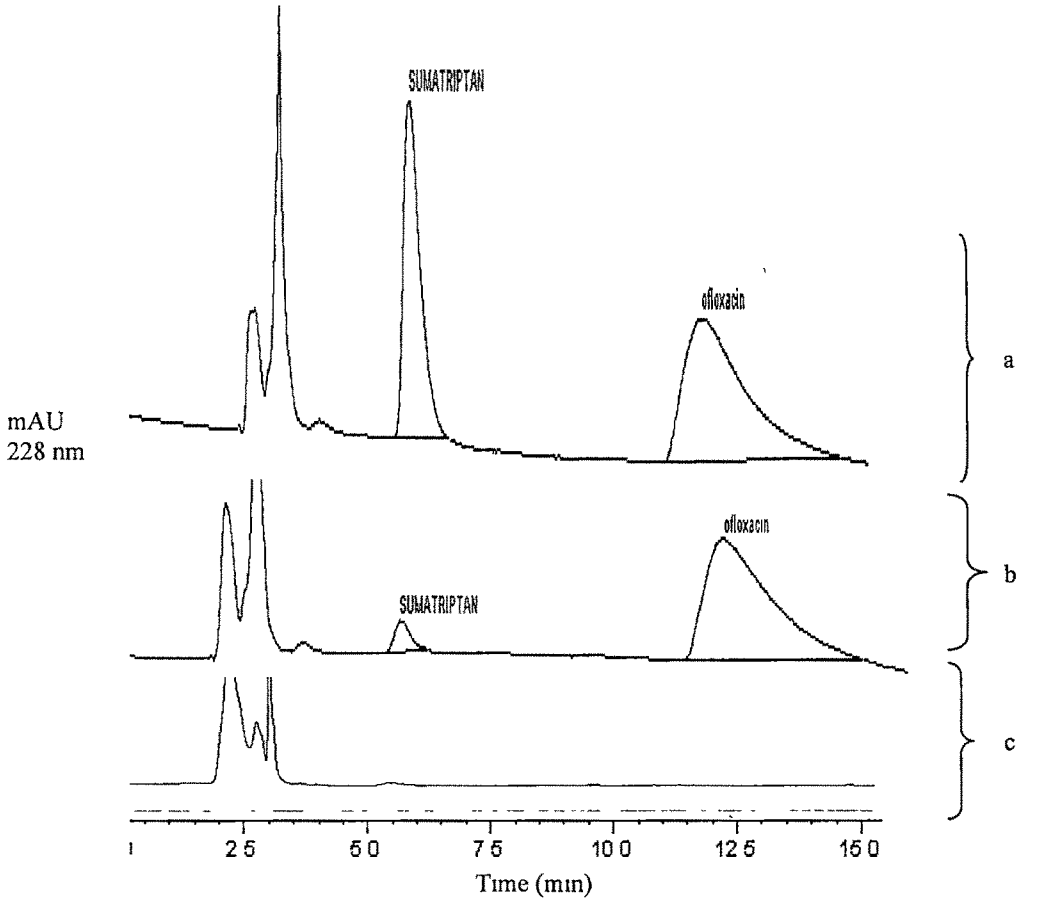

Figure 3.15 Representative chromatograms of brain homogenate sample spiked with 1000 ng/g sumatriptan and internal standard ofloxacin (a), brain homogenate sample spiked with 3 ng/g sumatriptan and internal standard ofloxacin (b) and blank brain homogenate sample

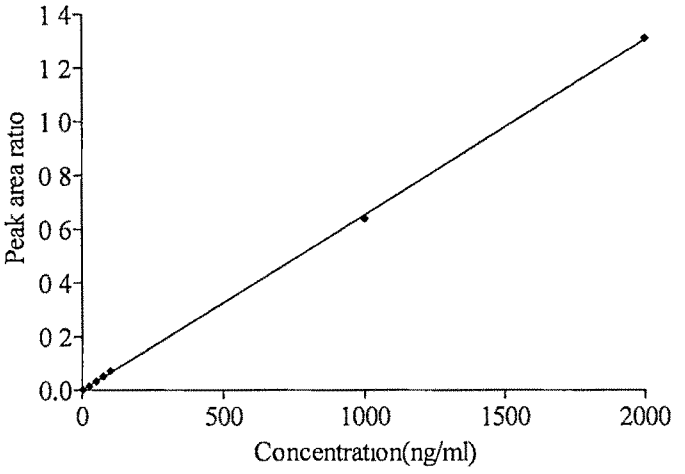

Figure 3.16 Calibration plot of sumatriptan in plasma

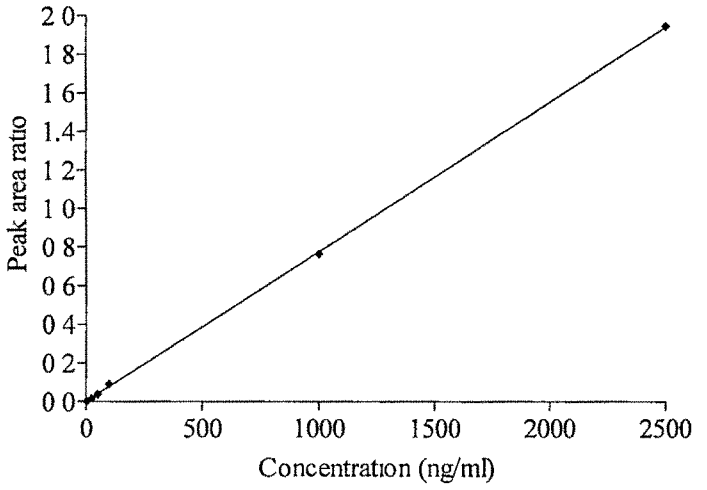

Figure 3.17 Calibration plot of sumatriptan in CSF

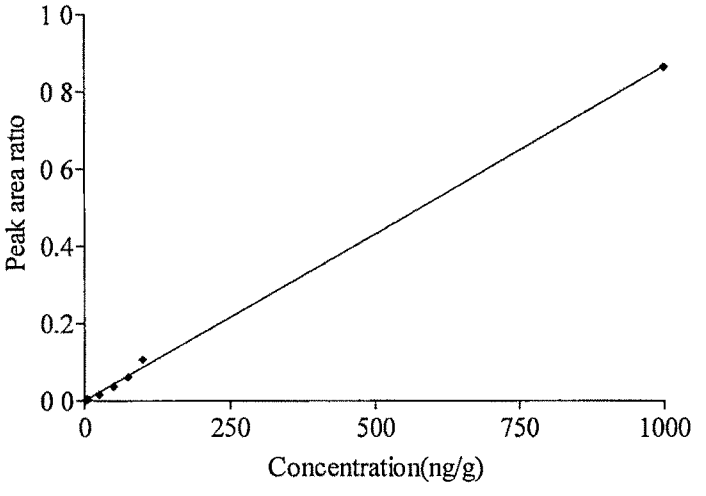

Figure 3.18 Calibration plot of sumatriptan in brain

3c. ESTIMATION OF GLUTARALDEHYDE

3c.1. EXPERIMENTAL

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.

3c.1.1.Reagents and Solution

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

(2) Hydroxylamine hydrochloride solution· Dissolve 70 mg of hydroxylamine hydrochloride in sufficient buffer to produce 100 ml 10 ml of this solution is further diluted 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 0.4ml (25%) to 100 ml with distilled water and further diluting 10 ml of this to 100 ml with distilled water Suitable dilutions were further made to give final concentration of 5,10,25,50 and 75μg/ml.

3c.1.2. 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 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 3.22. The optical characteristics of the solution prepared for estimation of glutaraldehyde is shown in table 3.23.

3c.1.3. Selectivity

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

Table 3.22 Mean absorbance values, regressed values and statistical data of the calibration curve for estimation of glutaraldehyde

Concentration (μg/ml)	Mean Absorbance ± SEM*	Regressed Values
5	0.060 ± 0.001	0.052
10	0.108 ± 0.002	0.114
25	0.317 ± 0.002	0.302
50	0.590 ± 0.002	0.614
75	0.930 ± 0.002	0.927
100	1.250 ± 0.004	1.239

Regression equation. Y=0.0125X - 0.0106, Correlation coefficient = 0.9991

* Mean of six values

Table 3.23.Optical Characteristics for Sumatriptan succinate in Calcium free Kreb's solution

Characteristic	Value
Absorption maxima	237
Beer's law limit at 283 nm (μg/ml)	5-100
Apparent molar absorptivity at 283 nm(1 mol^{-1} cm^{-1})	1.08×10^3
Sandell's sensitivity coefficient (S) at 283 nm (μg/cm^2/0.001 abs unit)	9.23×10^{-2}

3c.2.RESULTS AND DISCUSSION

Glutaraldehyde, by the method of United States Pharmacopoeia (1990) on reaction with hydroxylamine hydrochloride, gives a compound having λmax at 237 nm. The molar absorptivity of the compound 1080 1 mol^{-1} cm^{-1}. Correlation coefficient was found to be 0.9991 signifying a linear relationship between concentration and absorbance. The variance of the response variable, S^2y, x was calculated to be 1.59×10^{-6}, 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 slope S^2b was

calculated to be 2.92 x 10^{-9}. The variance of intercept, S^2a was 1.19 x 10^{-5}. To examine whether the intercept is significantly different from zero, the intercept were subjected to One-way Analysis of variance (ANOVA) for the significance and was not significantly different from zero at 6 degrees of freedom, at P=0 95, which indicates that blank does not interfere in the absorbance measurements.

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