3.0 ANALYTICAL METHODS

3.1 ESTIMATION OF CARVEDILOL BY UV SPECTROPHOTOMETRY

3.1.1 Analytical Method Development: (Ptacek et al., 2003; Ieggli et al., 2005)

3.1.1.1 Basis of development: Solvent selection for the development of analytical method was based on the solubility of the molecule in that solvent. First, isopropyl alcohol and methanol were chosen as Carvedilol is freely soluble in the above solvents. Solutions were prepared in the concentration of 10 μg/ml in both the solvents and scanned through 400 to 200nm to select the λmax for Carvedilol. Both the solutions were found to give maximum absorption at 242 nm. Therefore for the development of analytical method for Carvedilol, 242 nm was considered as λmax. When Carvedilol solutions in both isopropyl alcohol and methanol were scanned through 400 to 200nm, it was found that the absorption was more in case of methanol. Therefore, methanol was selected as the solvent and λmax -242nm for the development of UV spectrophotometric method of Carvedilol.

3.1.1.2 Stock Solution:

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

3.1.1.3 Procedure for calibration curve:

Suitable aliquots of the stock solution of Carvedilol were pipetted into 10 ml volumetric flasks and the volume was made to 10ml with methanol to give final concentrations of 4, 8, 12, 16 and 20 μg/ml. The solutions were shaken well and their absorbance was measured at 242nm using methanol as blank on Shimadzu 1601 UV-visible spectrophotometer. The above procedure was repeated six times.

3.1.1.4 Accuracy:

Accuracy of analytical method is the closeness of test results obtained by the method to the true value. For determining of Carvedilol concentration, standard solution was prepared. The above solutions were taken at 242nm and its absorbance was recorded and compared for its accuracy. The method is said to be accurate for estimation if it gives < 1.0% RSD at each concentration level.

$$RSD \approx \frac{SD}{Mean} x100$$

3.1.1.5 Precision:

The precision is agreement between the results obtained when determinations are conducted. Precision provides an indication of random errors and is generally subdivided

into two cases: repeatability and reproducibility, which were determined by calculating RSD (Relative standard deviation) of inter-day and intra-day determinations of Carvedilol. RSD should be < 2.0% for the method in both inter-day and intra-day determinations.

3.1.1.6 Linearity:

The linearity of test procedure is defined as its ability (within a given range) to produce results which are directly proportional to the concentration of analyte in the sample. Linearity of a light absorption determination should be examined to ensure that Beer's law operates over the range of interest. For evaluation of the linearity of the UV method of Carvedilol, the standard solutions were prepared at 4, 8, 12, 16, and 20 µg/ml concentrations (n=3) and absorbance were taken at 242nm in UV spectrophotometer. The method is said to be linear for estimation of Carvedilol if it is linear over 4 to 20 µg/ml range. Least square regression method was used to determine the regression coefficient, r and the equation $y = ax + b$ for the best fitting line.

3.1.2 Results and Discussion:

As shown in Table 3.1 (Calibration curve values for Carvedilol in methanol) RSD at each level is < 1.0 % which indicates that method is accurate for the analysis of Carvedilol. Inter day and intra day accuracy was calculated by comparing RSD values (Table 3.2 and 3.3). The accuracy of method was > 98% and RSD did not exceed 2%. Thus proposed method is accurate, precise and reproducible.

Table 3.1 Calibration curve values for Carvedilol in methanol.

Concentration (µg/ml)	Mean Absorbance* ± S.E.	Regressed value	RSD
4	0.250 ± 0.010	0.224	0.807
8	0.410 ± 0.012	0.424	0.823
12	0.633 ± 0.015	0.624	0.987
16	0.835 ± 0.016	0.8240	0.811
20	1.059 ± 0.019	1.0245	0.867

*** n=6**

Table 3.2 Intra-day accuracy and precision.

Intra-day accuracy and precision			
Theoretical concentration (mg)	5.0 µg	5.0 µg	5.0 µg
Observed conc. (mean) mg	5.011	5.019	4.903
S.D.	0.06	0.06	0.05
R.S.D.	1.19	1.19	1.02
n	6	6	6
Accuracy (%)	100.22	100.38	98.06

Table 3.3 Inter-day accuracy and precision.

Inter-day accuracy and precision			
Theoretical concentration (mg)	5.0 µg (Day II)	5.0 µg (Day II)	5.0 µg (Day III)
Observed conc. (mean) µg	5.011	4.980	4.951
S.D.	0.06	0.06	0.08
R.S.D.	1.19	1.21	1.62
n	6	6	6
Accuracy (%)	100.22	99.60	99.02

3.1.2.1 Linearity:

The calibration curve was found to obey Beer's law in the concentration range of 4 to 20 µg/ml. The linearity equation was $y = 0.051x + 0.0245$ with correlation coefficient (r^2) 0.9971.

3.1.2.2 Conclusion:

From the discussion above, it is clear that the developed method was simple, linear, accurate, precise and reproducible. Critical parameters are enumerated in Table 3.4.

Table 3.4 Critical parameters for developed UV spectrometric method of analysis for Carvedilol

Parameters	Results
λ_{max}	242 nm
Linearity range	4-20 µg/ml
Regression equation	y= 0.051x + 0.0245
Correlation coefficient	0.9971
% RSD	< 2.0 %
% Accuracy	> 98.00 %

3.1.3 References:

1. Ptacek, P., Macek, J., Klima, J., 2003. Liquid chromatographic determination of Carvedilol in human plasma. J. Chromatography B. 789, 405–410.
2. Ieggli, C. V., Cardoso, S. G., Belle, L. P., 2005. Validation of UV spectrophotometric and nonaqueous titration methods for the determination of Carvedilol in pharmaceutical formulations. J AOAC Int., 5, 1299-303.

3.2 ESTIMATION OF PRAVASTATIN SODIUM BY UV SPECTROPHOTOMETRY

3.2.1 Analytical Method Development: (Kawabata et al., 1998; Clarke, 2005)

3.2.1.1 Need and basis of development: As there was no analytical method reported for UV spectrophotometric analysis of Pravastatin sodium, the method was developed for the same.

Solutions were prepared in the concentration of 10 µg/ml in distilled water and phosphate buffer pH 6.8 ± 0.2 and scanned through 400 to 200nm to select the λmax for Pravastatin sodium. Both the solutions were found to give maximum absorption at 239nm. Therefore for the development of analytical method of Pravastatin sodium, 239nm was considered as λmax. When Pravastatin sodium solutions in both distilled water and phosphate buffer pH 6.8 were scanned through 400 to 200nm, it was found that the absorption was more in case of phosphate buffer pH 6.8. Therefore phosphate buffer pH 6.8 ± 0.2 was selected as the solvent and λmax was found to be 239 nm for the development of UV spectrophotometric method for analysis of Pravastatin sodium.

3.2.1.2 Estimation of Pravastatin sodium in phosphate buffer pH 6.8

3.2.1.3 Stock Solution:

Stock solution of Pravastatin sodium (100µg/ml) was prepared by dissolving 10mg of Pravastatin sodium in 100ml of phosphate buffer pH 6.8 ± 0.2. .

3.2.1.4 Calibration Curve:

Suitable aliquots of the stock solution of Pravastatin sodium were pipetted into 10 ml volumetric flasks and the volume was made up to 10ml with phosphate buffer pH 6.8 to give final concentration of 2, 4, 6, 8, 10 and 12 µg/ml. The solutions were mixed well and their absorbance measured at 239nm using phosphate buffer pH 6.8 as blank on Shimadzu 1601 UV-Visible Spectrophotometer. The above procedure was repeated six times.

The method was validated for accuracy, precision and linearity as described in UV spectrophotometric method for Carvedilol.

3.2.2 Results and Discussion:

As shown in Table 3.5 (Calibration curve values for Pravastatin sodium in phosphate buffer) RSD at each level is < 1.0 % which indicates that method is accurate for the analysis of Pravastatin sodium. Inter day and intra day accuracy was calculated by comparing RSD values (Table 3.6 and 3.7). The accuracy of method was > 98% and RSD did not exceed 2%. Therefore the proposed method is accurate, precise and reproducible.

Table 3.5 Calibration curve values for Pravastatin sodium in phosphate buffer pH 6.8

Concentration (µg/ml)	Mean Absorbance* ± S.E.	Regressed value	RSD (%)
2	0.170 ± 0.010	0.164	0.908
4	0.320 ± 0.013	0.322	0.882
6	0.493 ± 0.020	0.480	0.912
8	0.651 ± 0.011	0.638	0.835
10	0.811 ± 0.014	0.796	0.567
12	0.969 ± 0.013	0.954	0.588

*n=6

Table 3.6 Intra-day accuracy and precision.

Intra-day accuracy and precision			
Theoretical concentration (µg)	5 µg	5 µg	5 µg
Observed conc. (mean) µg	4.91	4.96	4.97
S.D.	0.06	0.07	0.05
R.S.D.	1.22	1.41	1.00
N	6	6	6
Accuracy (%)	98.20	99.20	99.40

Table 3.7 Inter-day accuracy and precision.

Inter-day accuracy and precision			
Theoretical concentration (µg)	5 µg (Day I)	5 µg (Day II)	5 µg (Day III)
Observed conc. (mean) µg	4.91	4.93	5.02
S.D.	0.06	0.08	0.07
R.S.D.	1.22	1.62	1.39
N	6	6	6
Accuracy (%)	98.20	98.60	100.40

3.2.2.1 Linearity:

The calibration curve was found to obey Beer's law in the concentration range of 2 to 12 µg/ml. The linearity equation was y= 0.080x + 0.0064 with correlation coefficient 0.9998.

3.2.2.2 Conclusion:

From the above discussion, it is clear that the developed method is linear, accurate, precise, reproducible and simple. Critical parameters are enumerated in Table 3.8.

Table 3.8 Critical parameters for developed UV spectrometric method of analysis for Pravastatin sodium.

Parameters	Results
λ_{max}	239 nm
Linearity range	2-12 µg/ml
Regression equation	y= 0.080x + 0.0064
Correlation coefficient	0.9998
% RSD	< 2.0 %
% Accuracy	> 98.00 %

3.2.3 References:

1. Clarke's analysis of drugs and poisons, 2005. Monograph-Pravastatin sodium, HMG Coenzyme reductase inhibitor, Pharmaceutical press, London.
2. Kawabata, K., Matsushima, N., Sasahara, K., 1998. An automated method for the simultaneous determination of Pravastatin and its main metabolite in human plasma by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. Biomed. Chromatogr.12, 271-275.

3.3 ESTIMATION OF CARVEDILOL IN PLASMA BY HPLC

(Hokama et al., 1999, Konishi et al., 2003, Phuong et al., 2004; Ptacek et al., 2003)

3.3.1 Stock Solution:

Stock solution of Carvedilol (100µg/ml) was prepared by dissolving 10mg of Carvedilol in 100ml of mobile phase, methanol: 50 mM KH_2PO_4, pH 2.5 (60:40, v/v).

3.3.2 Procedure for calibration curve:

The final concentrations of 20, 40, 60, 80 and 100 ng/ml of Carvedilol were prepared in mobile phase by suitably diluting stock solution with mobile phase. The solutions were mixed well and injected into HPLC system. The above procedure was repeated six times. Table 3.9 shows the calibration curve values of Carvedilol. Fig. 3.1 shows the representative chromatogram for Carvedilol.

3.3.2.1 Chromatographic conditions:

HPLC Column: C18 (4.6 x 100 mm, 3.5 µm) Waters.

Detector Wavelength: 242 nm.

Flow rate: 1ml/min.

Injection loop: 20 µl.

Retention time: 2.1 min.

Mobile phase: Methanol: 50 mM KH_2PO_4, pH 2.5 (60:40, v/v).

Internal standard: Propranolol.

Instrument: Dionex HPLC unit equipped with dionex UV-visible detector (UVD 170U) with controller.

3.3.2.2 Limit of detection and Limit of quantitation:

LoD is the lowest amount of analyte in a sample which can be detected but not quantitated as an exact value. LoQ is the lowest amount of analyte in a sample which can be quantitatively determined with defined precision and accuracy. LoD and LoQ were determined by a parameter lowest concentration (C_L) or amount (qL).

C_L (or qL) = ks_B / S

Where, k = a constant (3 for LoD and 10 for LoQ)

S_B = Standard deviation of the analytical blank signal.

S = Slope of the concentration/response graph.

3.3.3 Results and Discussion:

Table 3.9 Calibration curve for Carvedilol in Methanol: 50 mM KH_2PO_4, pH 2.5 (60:40, v/v).

Concentration (ng/ml)	mAu*	Regressed value	RSD
20	5.15	5.46	0.798
40	10.56	10.60	0.571
60	16.56	15.73	0.869
80	21.01	20.87	0.841
100	25.69	26.0	0.809

***n=6**

As shown in Table 3.9 RSD at each level is < 1.0 % which indicates that the HPLC method is accurate for the analysis of Carvedilol. Inter day and intra day accuracy was calculated by comparing RSD values (Table 3.10 and 3.11). The accuracy of method was > 98% and RSD did not exceed 2%. Thus proposed method is accurate, precise and reproducible.

Table 3.10 Intra-day accuracy and precision.

Intra-day accuracy and precision			
Theoretical concentration (ng)	50 ng	50 ng	50 ng
Observed concentration (mean, ng)	49.12	49.24	49.60
S.D.	0.97	0.90	0.80
R.S.D.	1.97	1.82	1.61
n	6	6	6
Accuracy (%)	98.24	98.48	99.20

Table 3.11 Inter-day accuracy and precision.

Intra-day accuracy and precision			
Theoretical Concentration (ng)	50 ng (Day I)	50 ng (Day II)	50 ng (Day III)
Observed conc. (mean, ng)	49.12	49.56	49.29
S.D.	0.97	0.51	0.73
R.S.D.	1.97	1.00	1.48
n	6	6	6
Accuracy (%)	98.24	99.12	98.58

3.3.3.1 Linearity:

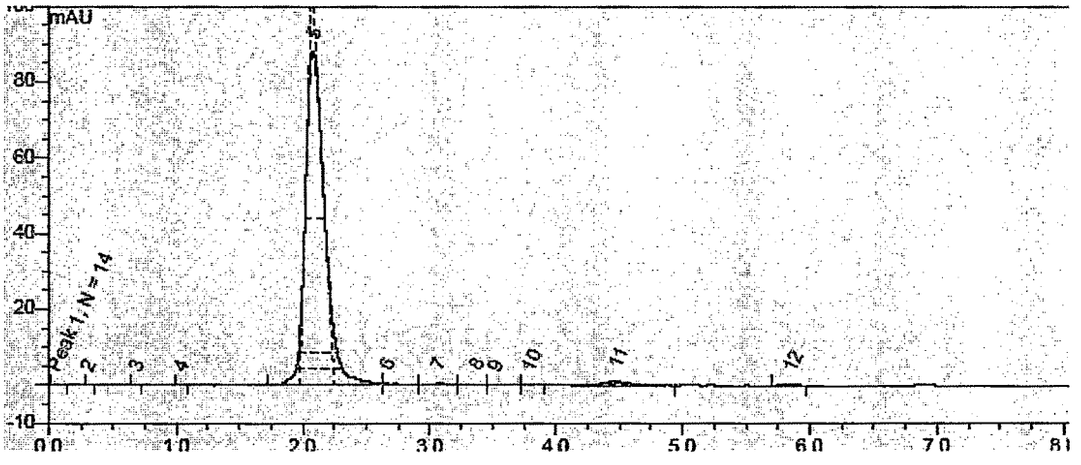
The calibration curve was found to obey Beer's law in the concentration range of 20-100 ng/ml. The linearity equation was y= 0.2577x + 0.335 with correlation coefficient 0.9967.

3.3.3.2 LoD and LoQ:

Limit of detection: 1.80 ng/ml.

Limit of quantitation: 6.00 ng/ml

Fig 3.1 Representative chromatogram of Carvedilol.



3.3.3.3 Conclusion:

From the discussion above, it is clear that the developed method is linear, accurate, precise, reproducible and simple. Critical parameters are enumerated in Table 3.12.

Table 3.12 Critical parameters for HPLC method with UV detection for Carvedilol.

Parameters	Results
λ_{max}	242 nm
Linearity range	20-100 ng/ml
Regression equation	y= 0.2577x + 0.335
Correlation coefficient	0.9967
LoD	1.80 ng/ml.
LoQ	6.00 ng/ml
% RSD	< 2.0 %
% Accuracy	> 98.00 %

3.3.4 References:

1. Konishi, H., Nishio, S., Tsutamoto, T., Minouchi, T. , Yamaji A., 2003. Serum Carvedilol concentration and its relation to change in plasma brain natriuretic peptide level in the treatment of heart failure: a preliminary study. Int. J. Clinical Pharmacology and Therapeutics 41, 578-586.
2. Ptacek, P., Macek, J., Klıma, J., 2003. Liquid chromatographic determination of Carvedilol in human plasma. J. Chromatography B. 789, 405–410.
3. Phuong, N. T., Lee, B. J., Choi, J. K., Kang, J. S., Kwon, K., 2004. Enantioselective Pharmacokinetics of Carvedilol in Human Volunteers. Arch. Pharm. Res. 27, 973-977.
4. Hokama, N., Hobara, N., Kameya, H., Ohshiro, S. ,Sakanashi, M., 1999. Rapid and simple micro-determination of Carvedilol in rat plasma by high-performance liquid chromatography. J.Chromatography B. 732, 233–238.

3.4 ESTIMATION OF PRAVASTATIN SODIUM IN PLASMA BY HPLC

(Kawabata et al., 1998; Clarke, 2005; Otter and Mignat, 1998; Zhu and Neirinck, 2003)

Instrument: Dionex HPLC unit equipped with UV-visible detector (UVD 170U) and dionex controller.

3.4.1 Solutions:

Standard stock solution of Pravastatin sodium (100µg/ml) was prepared by dissolving 10mg of Pravastatin sodium in 100ml of mobile phase, acetonitrile: ammonium acetate (0.01 M), 2:1.

3.4.2 Procedure for calibration curve:

The final concentrations of 25, 50, 75, 100 and 125 ng/ml of Pravastatin sodium were prepared in mobile phase by suitably diluting stock solution with mobile phase. The solutions were mixed well and injected into HPLC system.

3.4.2.1 Chromatographic conditions:

HPLC Column: C18 YMC packed (4.6 x 150 mm, 5 µm) Waters.

Detector Wavelength: 239 nm.

Flow rate: 1ml/min

Injection loop: 20 µl

Retention time: 7.4 min.

Mobile phase: acetonitrile: ammonium acetate (0.01 M), 2:1.

Internal standard: Lovastatin.

3.4.2.2 Limit of detection and Limit of quantitation:

LoD is the lowest amount of analyte in a sample which can be detected but not quantitated as an exact value. LoQ is the lowest amount of analyte in a sample which can be quantitatively determined with defined precision and accuracy. LoD and LoQ were determined by a parameter lowest concentration (C_L) or amount (qL).

C_L (or qL) = ks_B / S

Where, k = a constant (3 for LoD and 10 for LoQ)

S_B = Standard deviation of the analytical blank signal.

S = Slope of the concentration/response graph.

3.4.3 Results and Discussion:

As shown in Table 3.13 (Calibration curve values for Pravastatin sodium in acetonitrile: ammonium acetate (0.01 M), 2:1) RSD at each level is < 1.0 % which indicates that method

is accurate for the analysis of Pravastatin sodium. Inter day and intra day accuracy was calculated by comparing RSD values (Table 3.14 and 3.15). The accuracy of method was > 98% and RSD did not exceed 2%. Thus proposed method is accurate, precise and reproducible. Fig 3.2 showed the representative chromatogram of Pravastatin sodium.

Table 3.13 Calibration curve for Pravastatin sodium in acetonitrile: ammonium acetate (0.01 M), 2:1.

Concentration (ng/ml)	mAu	Regressed value	RSD
25	7.15	7.66	0.898
50	14.56	15.06	0.871
75	21.56	22.47	0.951
100	29.01	29.88	0.853
125	35.69	37.29	0.892

* n=6

Table 3.14 Intra-day accuracy and precision.

Intra-day accuracy and precision			
Theoretical conc. (ng)	50 ng	50 ng	50 ng
Observed conc. (mean, ng)	49.39	49.63	49.66
S.D.	0.98	0.68	0.96
R.S.D.	1.98	1.37	1.93
n	6	6	6
Accuracy (%)	98.78	99.26	99.32

Table 3.15 Inter-day accuracy and precision.

Inter-day accuracy and precision			
Theoretical conc. (ng)	50 ng (Day I)	50 ng (Day II)	50 ng (Day III)
Observed conc. (mean, ng)	49.31	49.63	49.86
S.D.	0.92	0.69	0.58
R.S.D.	1.98	1.39	1.16
n	6	6	6
Accuracy (%)	98.62	99.26	99.72

3.4.3.1 Linearity:

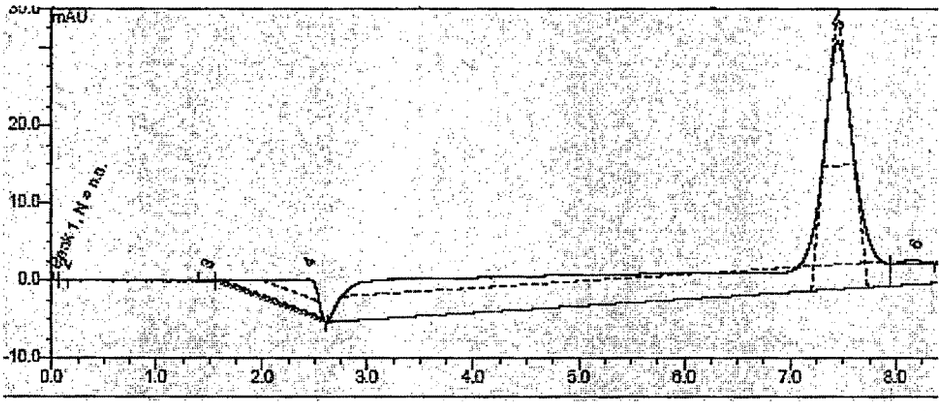
The calibration curve was found to obey Beer's law in the concentration range of 25-125 ng/ml. The linearity equation was y= 0.2861x + 0.135 with correlation coefficient 0.9979.

LoD and LoQ:

Limit of detection: 3.80 ng/ml.

Limit of quantitation: 12.66 ng/ml

Fig 3.2 Representative chromatogram of Pravastatin sodium.



3.4.3.3 Conclusion:

From the above discussion, it is clear that the developed method is linear, accurate, precise, reproducible and simple. Critical parameters are enumerated in Table 3.16.

Table 3.16 Critical parameters for HPLC method with UV detection for Pravastatin sodium.

Parameters	Results
λ_{max}	239 nm
Linearity range	25-125 ng/ml
Regression equation	y= 0.2861x + 0.135
Correlation coefficient	0.9979
LoD	3.80 ng/ml.
LoQ	12.66 ng/ml.
% RSD	< 2.0 %
% Accuracy	> 98.00 %

3.4.4 References:

1. Kawabata, K., Matsushima, N., Sasahara, K., 1998. An automated method for the simultaneous determination of Pravastatin sodium and its main metabolite in human plasma by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry, Biomed. Chromatogr. 12, 271-275.
2. Otter, K., Mignat, C., 1998. Determination of Pravastatin in human plasma by high-performance liquid chromatography with ultraviolet detection, J. Chromatography. B. 708, 235-241.
3. Clarke's analysis of drugs and poisons, 2005. Monograph-Pravastatin sodium, HMG Coenzyme reductase inhibitor, Pharmaceutical press, London.
4. Zhu, Z., Neirinck, L., 2003. High-performance liquid chromatography coupled with negative ion tandem mass spectrometry for determination of Pravastatin in human plasma. J. Chromatography B. 783,133-140.