Chapter 3
Analytical Method

3.1 Materials

Carvedilol (Torrent Research Centre, Ahmedabad, India) and Nitrendipine (USV Limited, Mumbai, India) were received as gift samples. Potassium phosphate monobasic (KH_2PO_4), Sodium hydroxide, Methanol, Hydrochloric acid and Tween 80 were procured from SD Fine Chemicals, Mumbai, India. Double distilled water (DDW) was purified by passing through 0.45µ Millipore filters (Millipore, Bangalore, India).

3.2 ESTIMATION OF CARVEDILOL BY ULTRAVIOLET SPECTROPHOTOMETRY (UV) (Ieggli et al., 2005; Yedurkar et al., 2007; Tanwar et al., 2007)

3.2.1 Calibration Plot in pH 6.2 phosphate buffer and methanol (9:1)

Standard stock solution (100 µg/ml) was prepared by dissolving 10 mg of carvedilol in 100 ml of pH 6.2 phosphate buffer and methanol (9:1). Then UV-spectrophotometric method of analysis was developed by first scanning solution of carvedilol (10 µg/ml) in the ultraviolet range between 200 and 400 nm and determining its λ_{max}.

Suitable aliquots of the stock solution of carvedilol were pipetted out into 10 ml volumetric flasks and the volume was made upto 10 ml with phosphate buffer pH 6.8 to give final concentrations ranging from 1-10 µg/ml. The solutions were mixed using vortex mixer and their absorbances measured at λmax using mixture of phosphate buffer (pH 6.2) and methanol (9:1) as blank on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted. The above procedure was repeated three times.

3.2.2 Calibration Plot in methanol and 0.1N HCl (3:2)

Standard stock solution (100 µg/ml) was prepared by dissolving 10 mg of carvedilol in 100 ml of methanol and 0.1N HCl (3:2). Then UV-spectrophotometric method of analysis was developed by first scanning solution of carvedilol (10 µg/ml) in the ultraviolet range between 200 and 400 nm and determining its λ_{max}.

Suitable aliquots of the stock solution of carvedilol were pipetted out into 10 ml volumetric flasks and the volume was made upto 10ml with methanol and 0.1N HCl (3:2) to give final concentrations ranging from 1-10 µg/ml. The solutions were mixed using vortex mixer and their absorbances measured at λ_{max} using methanol and 0.1N HCl (3:2) as blank on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted). The above procedure was repeated three times.

3.2.3 Analytical method validation

The method was validated for accuracy, precision and linearity.

3.2.3.1 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; Hubert et al., 2003). 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 1, 2, 4, 6, 8 and 10 µg/ml concentrations (n = 3) and absorbance were taken at 242 nm in UV spectrophotometer. The method is said to be linear for estimation of Carvedilol if it is linear over 1 to 10 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.

3.2.3.2 Accuracy

Accuracy refers to the closeness of an individual observation or mean of the observations to true value (Bolton, 1990). The "true" value is the result which would be observed in absence of error. Accuracy of the assay is defined as the percentage of the agreement between the measured value and the true value as follows (Merodia et al, 2000):

$$\text{Accuracy} = \frac{\text{True value} - \text{Measured value}}{\text{True value}} \times 100 \qquad \text{..... 3.1}$$

3.2.3.3 Precision

It refers to the extent of variability of a group of measurements observed under similar conditions. 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) or CV (Coefficient of variation) of inter-day and intra-day determinations. One of the common ways of expressing the variability, which takes into account its relative magnitude, is the ratio of the standard deviation (SD) to the mean, SD/Mean. This ratio, often expressed as a percentage, is called the *Coefficient of Variation* abbreviated as CV or RSD, the *relative standard deviation.* In biological data, the CV is often between 20 -50%, and one would not be surprised to see an occasional CV as high as 100% or more. The relatively large CV observed in biological experiments is due mostly to "biological variation", the lack of reproducibility in living material. On the other hand, the variability in chemical and instrumental analysis of drugs is usually relatively small (Bolton, 1990).

In order to determine precision and accuracy of the methods, solutions containing known amounts of pure drug (1-10 µg) were prepared and analyzed in three replicates. Intraday precision was determined by measuring absorbance of samples on the same day while for interday precision absorbance values were determined for three consecutive days.

3.2.4 Results and Discussion

3.2.4.1 Calibration Plot in pH 6.2 phosphate buffer and methanol (9:1)

Carvedilol in pH 6.2 phosphate buffer and methanol (9:1) yields a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The scan shows absorption maximum at 242 nm and this wavelength was chosen as the analytical wavelength. Beer's law was obeyed between 1 and 10 µg/ml (Table 3.1). Regression analysis was performed on the experimental data. Regression equation for standard curve was $y = 0.1289x - 0.0191$ (Fig. 3.1). Correlation coefficient for developed method was found to be 0.9984 signifying that a linear relationship existed between absorbance and concentration of the drug. Parameters for the developed UV spectrometric method of analysis for Carvedilol are shown in Table 3.2.

Table 3.3 and 3.4 show intraday and interday precision and accuracy for the Carvedilol assay by UV spectroscopy. The low % CV values indicate precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method (Guidance for industry, 2001; Boulanger et al., 2003).

Table 3.1 Calibration data for Carvedilol in pH 6.2 phosphate buffer and methanol (9:1)

Sr. No.	Concentration (µg/ml)	Mean Absorbance* ± SD
1	1	0.115± 0.004
2	2	0.233±0.01
3	4	0.481±0.013
4	6	0.734±0.013
5	8	0.997±0.015
6	10	1.300±0.048

*Average of 3 determinations

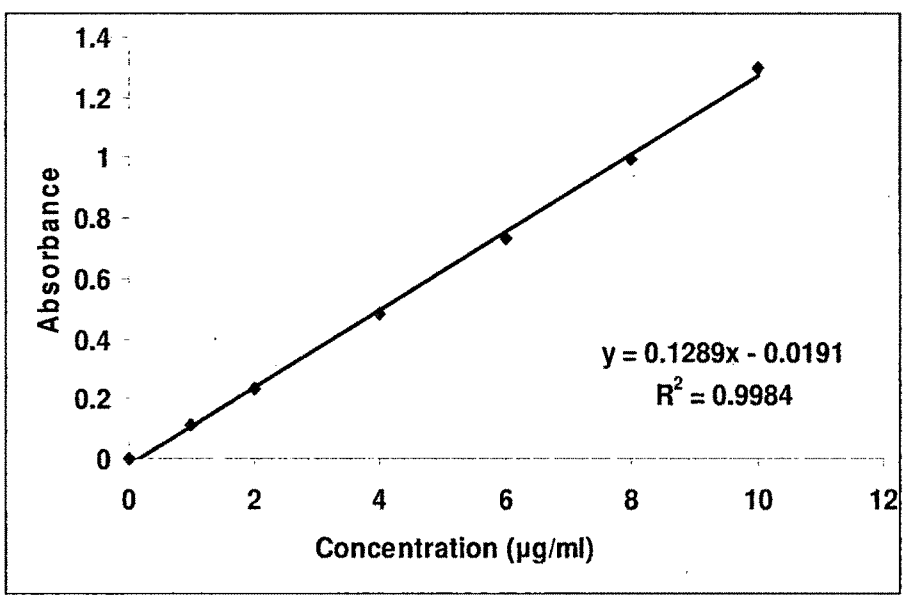

Fig. 3.1 Standard Curve of Carvedilol in pH 6.2 phosphate buffer and methanol (9:1)

Table 3.2 Parameters for UV spectrometric method of analysis for Carvedilol in pH 6.2 phosphate buffer and methanol (9:1).

Parameters	Results
λ_{max}	242 nm
Linearity range	1-10 µg/ml
Regression equation	y = 0.1289x - 0.0191
Correlation coefficient	0.9984

Table 3.3 Intraday precision and accuracy for the Carvedilol assay in pH 6.2 phosphate buffer and methanol (9:1) by UV spectroscopy.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	Observed		
1	1.02 ±0.005	0.4901	102.0
5	5.00±0.020	0.40	100.0
10	9.97±0.065	0.6519	99.7

[a] Expressed as relative standard deviation,
RSD = (standard deviation/mean concentration) x 100
[b] Expressed as (mean observed concentration/actual concentration) × 100

Table 3.4 Interday variability of Carvedilol assay in pH 6.2 phosphate buffer and methanol (9:1) by UV spectroscopy over three consecutive days.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	Observed		
1	1.00 ±0.011	1.10	100.0
5	5.01±0.020	0.399	100.2
10	9.98±0.015	0.1503	99.8

[a] Expressed as relative standard deviation,
RSD = (standard deviation/mean concentration) x 100
[b] Expressed as (mean observed concentration/actual concentration) × 100

3.2.4.2 Calibration Plot in methanol and 0.1N HCl (3:2)

Carvedilol in methanol and 0.1N HCl (3:2) yields a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The scan shows absorption maximum at 242 nm and this wavelength was chosen as the analytical wavelength. Beer's law was obeyed between 1 and 10 µg/ml (Table 3.5). Regression analysis was performed on the experimental data. Regression equation for standard curve was y = 0.1106x +0.0256 (Fig.3.2). Correlation coefficient for developed method was found to be 0.9925 signifying that a linear relationship existed between absorbance and concentration of the drug. Parameters for the developed UV spectrometric method of analysis for Carvedilol are shown in Table 3.6.

Table 3.7 and 3.8 show intraday and interday precision and accuracy for the Carvedilol assay by UV spectroscopy. The low % CV values indicate precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method (Guidance for industry, 2001; Boulangeret al., 2003).

Table 3.5 Calibration data for Carvedilol in methanol and 0.1N HCl (3:2)

Sr. No.	Concentration (µg/ml)	Mean Absorbance* ± SD
1	1	0.119±0.007
2	2	0.255±0.014
3	4	0.484±0.008
4	6	0.734±0.009
5	8	0.942±0.010
6	10	1.070±0.059

*Average of 3 determinations

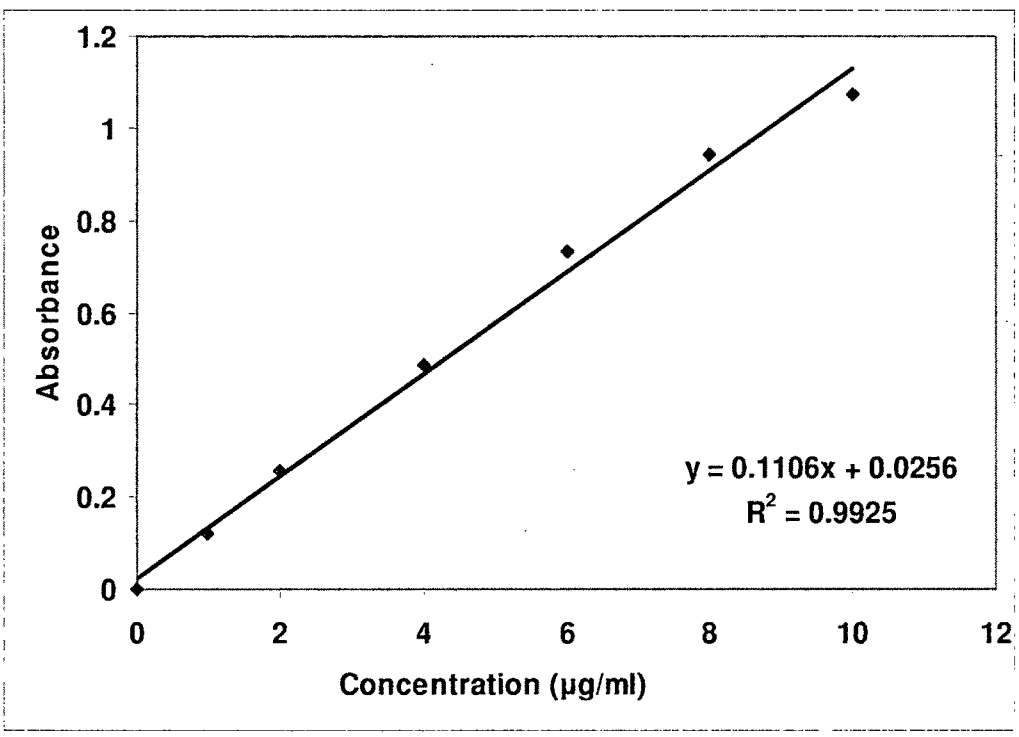

Fig. 3.2 Standard Curve of Carvedilol in methanol and 0.1N HCl (3:2)

Table 3.6 Parameters for UV spectrometric method of analysis for Carvedilol in methanol and 0.1N HCl (3:2).

Parameters	Results
λ_{max}	242 nm
Linearity range	1-10 µg/ml
Regression equation	y = 0.1106x +0.0256
Correlation coefficient	0.9925

Table 3.7 Intraday precision and accuracy for the Carvedilol assay in methanol and 0.1N HCl (3:2) by UV spectroscopy.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	Observed		
1	1.00±0.015	1.500	100.0
5	4.99±0.023	0.4609	99.8
10	9.97±0.030	0.3009	99.7

[a] Expressed as relative standard deviation,
RSD = (standard deviation/mean concentration) x 100
[b] Expressed as (mean observed concentration/actual concentration) × 100

Table 3.8 Interday variability of Carvedilol assay in methanol and 0.1N HCl (3:2) by UV spectroscopy over three consecutive days.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	Observed		
1	0.99±0.015	1.510	99.0
5	4.99±0.020	0.4008	99.8
10	9.96±0.040	0.4016	99.6

[a] Expressed as relative standard deviation,
RSD = (standard deviation/mean concentration) x 100
[b] Expressed as (mean observed concentration/actual concentration) × 100

3.3 ESTIMATION OF NITRENDIPINE BY ULTRAVIOLET SPECTROPHOTOMETRY (UV) (Yang et al., 2003; Yang et al., 2004a; Yang et al., 2004b)

3.3.1 Calibration Plot in pH 6.2 phosphate buffer containing 1% Tween 80

Standard stock solution (100 µg/ml) was prepared by dissolving 10 mg of Nitrendipine in 100 ml of pH 6.2 phosphate buffer containing 1% Tween 80. Then UV-spectrophotometric method of analysis was developed by first scanning solution of Nitrendipine (50 µg/ml) in the ultraviolet range between 200 and 400 nm and determining its λmax.

Suitable aliquots of the stock solution of Nitrendipine were pipetted out into 10 ml volumetric flasks and the volume was made upto 10ml with pH 6.2 phosphate buffer containing 1% Tween 80 to give final concentrations ranging from 5-50 µg/ml (Table 3.9). The solutions were mixed using vortex mixer and their absorbances measured at λ_{max} using pH 6.2 phosphate buffer containing 1% Tween 80 as blank on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted (Table 3.9; Fig. 3.3). The above procedure was repeated three times.

3.3.2 Calibration Plot in methanol and 0.1N HCl (3:2)

Standard stock solution (100 µg/ml) was prepared by dissolving 10 mg of Nitrendipine in 100 ml of methanol and 0.1N HCl (3:2). Then UV-spectrophotometric method of analysis was developed by first scanning solution of Nitrendipine (50 µg/ml) in the ultraviolet range between 200 and 400 nm and determining its λ_{max}.

Suitable aliquots of the stock solution of Nitrendipine were pipetted out into 10 ml volumetric flasks and the volume was made upto 10ml with methanol and 0.1N HCl (3:2) to give final concentrations ranging from 5-50 µg/ml (Table 3.10). The solutions were mixed using vortex mixer and their absorbances measured at λmax using methanol and 0.1N HCl (3:2) as blank on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted (Table 3.10; Fig 3.4). The above procedure was repeated three times.

3.3.3 Analytical method validation

The method was validated for accuracy, precision and linearity using procedure described in **Section 3.2.3** for carvedilol.

3.3.4 Results and Discussion

3.3.4.1 Calibration Plot in pH 6.2 phosphate buffer containing 1% Tween 80

Nitrendipine in pH 6.2 phosphate buffer containing 1 % Tween 80 yields a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The scan shows absorption maximum at 355 nm and this wavelength was chosen as the analytical wavelength. Beer's law was obeyed between 5 and 50 µg/ml (Table 3.9). Regression analysis was performed on the experimental data. Regression equation for standard curve was $y = 0.0166x + 0.0237$ (Fig.3.3). Correlation coefficient for developed method was found to be 0.9939 signifying that a linear relationship existed between absorbance and concentration of the drug. Parameters for the developed UV spectrometric method of analysis for Nitrendipine are shown in Table 3.10.

Table 3.11 and 3.12 show intraday and interday precision and accuracy for the Nitrendipine assay by UV spectroscopy. The low % CV values indicate precision of the method. No significant difference between the amount of drug added (actual) and

observed concentration was noticed indicating accuracy of the method (Guidance for industry, 2001; Boulangeret al., 2003).

Table 3.9 Calibration data for Nitrendipine in pH 6.2 phosphate buffer containing 1% Tween 80

Sr. No.	Concentration (µg/ml)	Mean Absorbance* ± SD
1	5	0.114± 0.014
2	10	0.187±0.012
3	20	0.363±0.007
4	30	0.543±0.010
5	40	0.716±0.006
6	50	0.814±0.015

*Average of 3 determinations

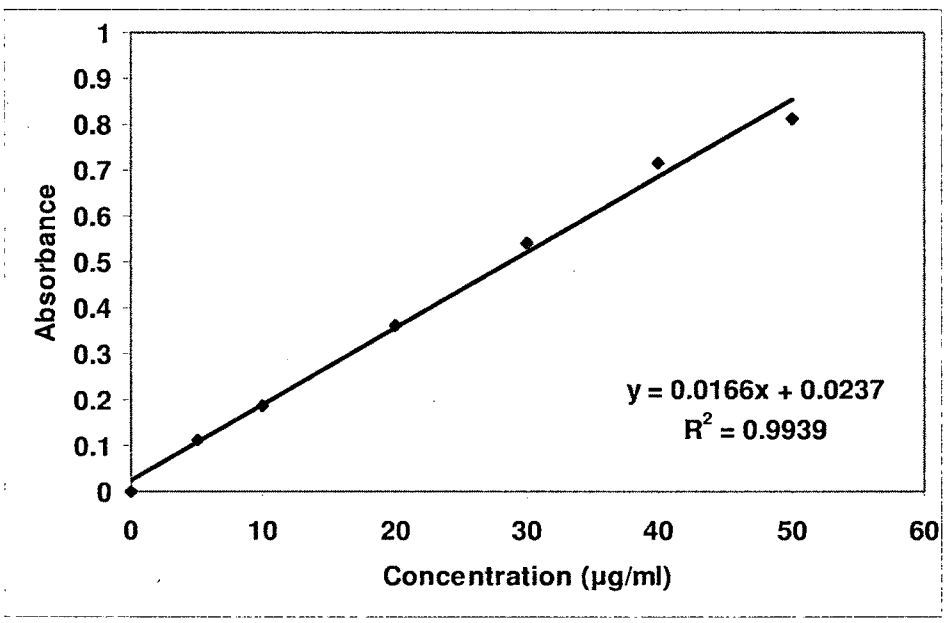

Fig. 3.3 Standard Curve of Nitrendipine in pH 6.2 phosphate buffer containing 1% Tween 80

Table 3.10 Parameters for UV spectrometric method of analysis for Nitrendipine in pH 6.2 phosphate buffer containing 1% Tween 80.

Parameters	Results
λ_{max}	355 nm
Linearity range	5-50 µg/ml
Regression equation	y = 0.0166x + 0.0237
Correlation coefficient	0.9939

Table 3.11 Intraday precision and accuracy for the Nitrendipine assay in pH 6.2 phosphate buffer containing 1% Tween 80 by UV spectroscopy.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	Observed		
10	10.04 ±0.105	1.045	100.4
20	19.95±0.162	0.8120	99.75
40	39.93±0.236	0.5910	99.82

[a] Expressed as relative standard deviation,
RSD = (standard deviation/mean concentration) x 100
[b] Expressed as (mean observed concentration/actual concentration) × 100

Table 3.12 Interday variability of Nitrendipine assay in pH 6.2 phosphate buffer containing 1% Tween 80 by UV spectroscopy over three consecutive days.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	Observed		
10	10.03±0.070	0.6979	100.3
20	19.94±0.130	0.6519	99.7
40	40.04±0.175	0.4370	100.1

[a] Expressed as relative standard deviation,
RSD = (standard deviation/mean concentration) x 100
[b] Expressed as (mean observed concentration/actual concentration) × 100

3.3.4.2 Calibration Plot in methanol and 0.1N HCl (3:2)

Nitrendipine in methanol and 0.1N HCl (3:2) yields a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The scan shows absorption maximum at 355 nm and this wavelength was chosen as the analytical wavelength. Beer's law was obeyed between 5 and 50 µg/ml (Table 3.13). Regression analysis was performed on the experimental data. Regression equation for standard curve was y = 0.0326x +0.0062 (Fig.3.4). Correlation coefficient for developed method was found to be 0.9991 signifying that a linear relationship existed between absorbance and concentration of the drug. Parameters for the developed UV spectrometric method of analysis for Nitrendipine are shown in Table 3.14.

Table 3.15 and 3.16 show intraday and interday precision and accuracy for the Nitrendipine assay by UV spectroscopy. The low % CV values indicate precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method (Guidance for industry, 2001; Boulangeret al., 2003).

Table 3.13 Calibration data for Nitrendipine in methanol and 0.1N HCl (3:2)

Sr. No.	Concentration (µg/ml)	Mean Absorbance* ± SD
1	5	0.119±0.007
2	10	0.255±0.014
3	20	0.484±0.008
4	30	0.734±0.009
5	40	0.942±0.010
6	50	1.070±0.059

*Average of 3 determinations

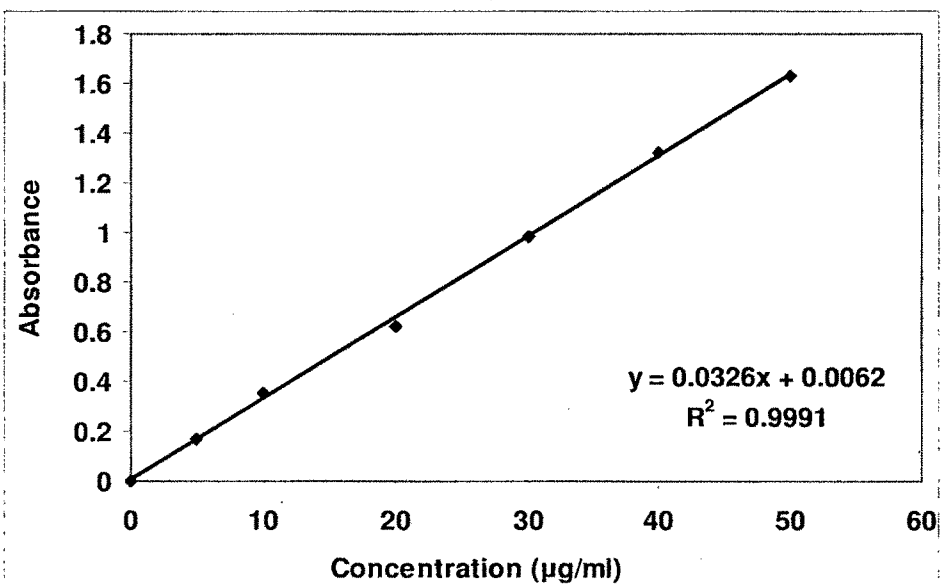

Fig. 3.4 Standard Curve of Nitrendipine in methanol and 0.1N HCl (3:2)

Table 3.14 Parameters for UV spectrometric method of analysis for Nitrendipine in methanol and 0.1N HCl (3:2).

Parameters	Results
λmax	355 nm
Linearity range	5-50 µg/ml
Regression equation	y = 0.0326x +0.0062
Correlation coefficient	0.9991

Table 3.15 Intraday precision and accuracy for the Nitrendipine assay in methanol and 0.1N HCl (3:2) by UV spectroscopy.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	Observed		
10	10.04±0.133	1.324	100.4
20	20.05±0.170	0.8478	100.25
40	40.00±0.176	0.4400	100.0

[a] Expressed as relative standard deviation,
RSD = (standard deviation/mean concentration) x 100
[b] Expressed as (mean observed concentration/actual concentration) × 100

Table 3.16 Interday variability of Nitrendipine assay in methanol and 0.1N HCl (3:2) by UV spectroscopy over three consecutive days.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	Observed		
10	9.98±0.085	0.8517	99.8
20	20.05±0.104	0.5187	100.25
40	39.96±0.185	0.4629	99.9

[a] Expressed as relative standard deviation,
RSD = (standard deviation/mean concentration) x 100
[b] Expressed as (mean observed concentration/actual concentration) × 100

3.4 Conclusion

Ultraviolet spectroscopic methods were developed and validated for the estimation of Carvedilol and Nitrendipine in different media and solvents. The methods were validated for linearity (r value closest to 0.99), precision (relative standard deviations of <2% for intra- and interday precision), and accuracy (99.6–102.0%) for Carvedilol and linearity (r value closest to 0.99), precision (relative standard deviations of <2% for intra- and interday precision), and accuracy (99.7–100.4%) for Nitrendipine.

REFERENCES

Bolton, S.; Swarbrick, J. Basic definitions and concepts In: *Pharmaceutical statistics: Practical and clinical applications*. 3rd Ed., Marcel Dekker, New York, **1990**, pp 24.

Boulanger, B.; Dewe, W.; Chiap, P.; Crommen, J.; Hubert, P. H. An analysis of the SFSTP guide on validation of bioanalytical methods: progress and limitations. *J. Pharm. Biomed. Anal.* **2003**, 32, 753-765.

Guidance for industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), May **2001**.

Hubert, P.H.; Chiap, P.; Crommen, J.; Boulanger, B.; Chapuzet, E.; Mercier, N.; Bervoas-Martin, S.; Chevalier, P.; Grandjean, D.; Lagorce, P. H.; Laparra, M. C.; Laurentie, M.; Nivet, J. C.; The SFSTP Guide on the validation of chromatographic methods for drug bioanalysis : from the Washington Conference to the laboratory. *Anal. Chim. Acta.* **1999**, 391, 135-148.

Hubert, P. H., Chiap, P., Crommen, J., Boulanger, B., Chapuzet, E., Mercier, N., Bervoas, M. S., Chevalier, P., Grandjean, D., Lagorce, P., Lallier, M., Laparra, M. C., Laurentie, M., Nivet, J. C., Validation of the quantitative analytical procedures. Harmonization of the steps. *S.T.P. Pharma practice*, **2003**, 13, 101-138.

Ieggli, C. V.; Cardoso, S. G., Belle, L. P. Validation of UV spectrophotometric and nonaqueous titration methods for the determination of Carvedilol in pharmaceutical formulations. *J.AOAC Int.* **2005**, 5, 1299-1303.

Merodia, M.; Mirshahi, T.; Mirshahi, M. Development of a sensitive method for the determination of ganciclovir by reversed phase high-performance liquid chromatography. *J. Chromatogr.*, **2000**, 870, 159-167.

Tanwar, Y. S.; Chauhan, H. S.; Sharma, A. Development and evaluation of carvedilol transdermal patches. *Acta Pharm.* **2007**, 57, 151-159.

Yang, M.; Cui, F.; You, B.; Fan, Y.; Wang, L.; Yue, P.; Yang, H. Preparation of sustained-release nitrendipine microspheres with Eudragit RS and Aerosil using quasi-emulsion solvent diffusion method. *Int. J. Pharm.* **2003**, 259, 103-113.

Yang, M.; Cui, F.; You, B.; Wang, L.; Yue, P.; Zang L.; Kawashima, Y. A novel pH-dependent gradient-release delivery system for nitrendipine I. Manufacturing, evaluation in vitro and bioavailability in healthy dogs. *J. Control. Release* **2004a,** 98, 219– 229.

Yang, M.; Cui, F.; You, B.; Wang, L.; Yue, P.; Kawashima, Y. A novel pH-dependent gradient-release delivery system for nitrendipine II. Investigations of the factors affecting the release behaviors of the system. *Int. J. Pharm.* **2004b,** 286, 99–109.

Yedurkar, P. D., Dhiman, M. K., Sawant, K. K. Mucoadhesive bilayer tablets for buccal delivery of carvedilol: in vitro and in vivo investigations. *Ars Pharm.* **2007**, 48(3), 259-274.