IJPSR (2011), Vol. 2, Issue 2 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 17 September, 2010; received in revised form 12 November, 2010; accepted 18 January, 2011

SIMULTANEOUS DETERMINATION OF NEBIVOLOL HCL AND VALSARTAN IN SOLID DOSAGE FORM BY SPECTROPHOTOMETRIC AND RP-HPLC METHOD

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Keywords:

UV-spectrophotometery,
RP-HPLC,
Nebivolol,
Valsartan,
Loratidine

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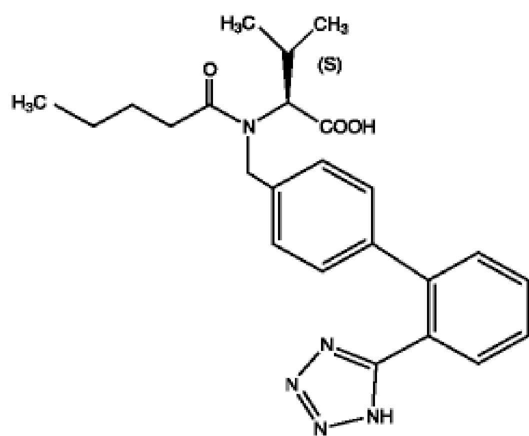
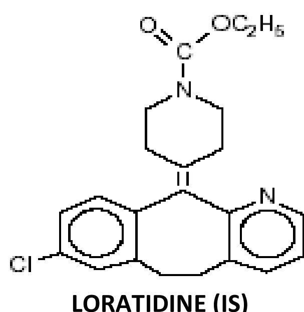
ABSTRACT

A simple high-performance liquid chromatography RP-HPLC and two reproducible ultraviolet spectrophotometric methods were developed and validated for the estimation of nebivolol HCl and valsartan simultaneously in combined pharmaceutical solid dosage form. The wavelength fixed for spectrophotometric measurement of valsartan at 246.6 nm, nebivolol HCl at 280.2 nm for the simultaneous equation and for the Q-analysis measurement the two wavelengths fixed are 246.6 nm and 275.0 nm. The linearity obtained for nebivolol HCl in the range of 0.5-2.5 µg/ml and 5-25 µg/ml for valsartan by spectrophotometric method. The nebivolol HCl and valsartan were analyzed by HPLC using reverse phase C_{18} column (25 cm x 4.6 mm i.d., 5 µm particle size), by isocratic mobile phase consisted of 50mM ammonium acetate buffer adjusted to pH 3.5 and acetonitrile (30:70 v/v), at a flow rate of 0.8 ml/min in the detection wavelength of 275 nm. The loratidine was used as an internal standard. The linearity and range of nebivolol HCl are 2-12 µg/ml and 5-30 µg/ml for valsartan by HPLC method. The correlation coefficients for all the three methods obtained were ≥ 0.998. The limit of detection and limit of quantification for nebivolol HCl and valsartan were found to be 12 ng/ml and 38 ng/ml, 32 ng/ml and 95 ng/ml respectively. The accuracy of these methods evaluated by recovery measurements and good recovery results obtained from 98.28% to 102.25% for all the methods and the relative standard deviation of below 3% were achieved.

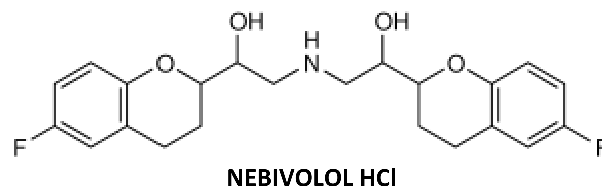
INTRODUCTION: Nebivolol hydrochloride (NEB) is (±) [2R* R* R* (S *)] œ, œ [imino bis (methylene)] bis- [6- fluoro- 3, 4 - dihydro- 2H- 1- benzopyran- 2-methanol] hydrochloride is an antihypertensive drug, It is a racemate of two enantiomers with four chiral centers. The SRRR-enantiomer (d-nebivolol) is a potent and cardio selective β_1-adrenergic blocker. The RSSS-enantiomer (nebivolol) has a favourable hemodynamic profile [1, 2]. Valsartan (VAS) (N-valeryl-N[[2-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl] valine, is an orally active, potent and specific competitive angiotensin II antagonist acting at the ATI receptor, which mediates all known effects of angiotensin II on the cardiovascular system. Valsartan is widely used in the treatment of hypertension [1, 3]. Combination of NEB and VAS is used as cardiovascular and β_1-adrenergic blocker. The chemical structures of NEB, VAS and internal standard loratidine are shown in (**Fig. 1**).



VALSARTAN



LORATIDINE (IS)



NEBIVOLOL HCl

FIG.1: CHEMICAL STRUCTURES OF VALSARTAN, LORATIDINE (IS) AND NEBIVOLOL HCl

Very few methods have been reported in the literature for the determination of valsartan in human plasma by LC-MS [4], by UV and LC [5] and nebivolol HCl in human plasma by liquid chromatography coupled with electro spray ionization tandem mass spectrometry (ESI-MS) 6 individually and estimation of valsartan and hydrochlorothiazide in tablets by first-derivative UV-spectrophotometric and LC method [7, 8].

To the best of our knowledge, no study has been reported for the simultaneous determination of NEB and VAS in pharmaceutical formulations by UV-spectrophotometer and RP-HPLC method. The significance of the developed methods is to determine the content of both drugs simultaneously in commercially available capsule dosage form and can be used in future for bioequivalence study for the same formulations. The capsule solid dosage form in combination containing nebivolol HCl (5 mg) and valsartan (80mg) is available in the market. In this paper, we reported two spectrophotometric methods and one reverse-phase HPLC method for the quantification of NEB and VAS simultaneously. The present RP-HPLC method was validated as per ICH guidelines [9, 10].

Experimental:

Equipments and conditions: A Shimadzu UV-visible Spectrophotometer-160A with data processing system was used. The sample solution was recorded in 1cm quartz cells against solvent blank over the range 200-400 nm. The optimal conditions

for recording the spectra to achieve good reproducibility included scan speed at 60nm/s, slit width at 2nm. The chromatographic separation was performed on a Waters liquid chromatographic system equipped with a Waters 1515 isocratic solvent delivery system (pump), Waters 2487 dual wavelength absorbance detector and Rheodyne 7725i injector with 50 µl loop volume. Breeze 3.3 data station was applied for data collecting and processing. A phenomenox C_{18} column (25cm x 4.6mm i.d., 5µm particle size) was used for the separation. The mobile phase consisted of a mixture of 50 mM ammonium acetate buffer pH 3.5 and acetonitrile (30:70, v/v). The mobile phase was prepared daily, filtered, sonicated before use and delivered at a flow rate of 0.8 ml/min at the detection wavelength of 275 nm.

Chemicals and reagents: The pharmaceutical grade gift reference standard of NEB obtained from Cadila pharmaceutical Ltd. Ankleshwer, VAS and loratidine (internal standard) from Hetero Labs. Hyderabad. Acetonitrile and methanol used were of HPLC grade; all analytical grade chemicals and solvents were obtained from E Merck (India) Ltd, Mumbai. Ammonium acetate and orthophosphoric acid AR grade were procured from Qualigens fine chemicals, Mumbai. Water HPLC grade was obtained from a Milli-QRO water purification system.

Standard solutions and calibration curves: Stock solutions for spectrophotometric measurement were prepared by dissolving standard NEB and VAS in methanol of the concentration of 1 mg/ml and for calibration, series of above solutions containing NEB 0.5, 1.0, 1.5, 2.0, 2.5 µg/ml and VAS 5, 10, 15, 20, 25 µg/ml were prepared by diluting the stock standard solutions of each drug with methanol in volumetric flasks (10 ml) and all dilutions were scanned in the wavelength range of 200-400 nm. The wavelength fixed for spectrophotometric measurement of valsartan at 246.6 nm and nebivolol HCl at 280.2 nm for the simultaneous equation and for the Q-analysis measurement the two wavelengths fixed are 246.6 nm and 275.0 nm as isoabsorptive point for NEB and VAS shown in (**Fig. 2**). The linearity obtained for nebivolol HCl in the range of 0.5-2.5 µg/ml and 5-25 µg/ml for valsartan by spectrophotometric method. Є (A1%, 1cm) was calculated for each standard drug by measuring absorbance of 1% solution of each at 1cm path length. Similarly, mixed standard solutions were used by simultaneous equation and Q-analysis Method.

For measurement by HPLC, standard stock solutions of 1 mg/ml of NEB and VAS were prepared separately using a mixture of water and acetonitrile (1:1, v/v). Different concentrations of standard solutions were prepared from 2 to 12 µg/ml of NEB and from 5 to 30 µg/ml of VAS by maintaining the concentration of IS at a constant level of 40µg/ml.

Sample preparation:

Simultaneous Equation method: Twenty capsules were weighed and finely powdered; an accurately weighed powder equivalent to one capsule contained 5 mg of NEB and 80 mg of VAS was transferred to a 100 ml volumetric flask, dissolved in 50ml of methanol and ultra sonicated for 20 min. and diluted to volume 100ml with the same solvent, mixed, and finally filtered through Whatmann No. 41 filter paper. The sample filtrate was further diluted with a final concentration of NEB 1µg/ml and VAS 16 µg/ml which was used for analysis. Absorbance of these solutions was measured at 280.2nm and 246.6 nm of both wavelengths as A_1 and A_2 respectively and concentrations of these two drugs in each sample were calculated using simultaneous equation. The method employs solving of simultaneous equations using Cramer's rule and matrices.

The simultaneous equations were;

$A_1 = \epsilon_{1v} \times C_1 + \epsilon_{1n} \times C_2$..........(1)

$A_2 = \epsilon_{2v} \times C_1 + \epsilon_{2n} \times C_2$..........(2)

ϵ_{1n} and ϵ_{2n} absorptivity values of NEB at 280.2 nm wavelength,

ϵ_{1v} and ϵ_{2v} absorptivity values of VAS at 246.6 nm wavelength,

C_1 and C_2 concentrations of VAS and NEB respectively in sample solution.

Q value analysis method: In this method the two wavelengths were selected in which one at 275 nm as an isoabsorptive point for NEB and VAS and second at 246.6 nm wavelength of valsartan as shown in **Fig. 2**. The dilutions of the standard and sample solutions were carried out as reported in simultaneous equation method.

The absorptivity values for both drugs at the selected wavelength were calculated and employed for Q analysis, the concentration of drugs in sample solution were determined by using the following equations.

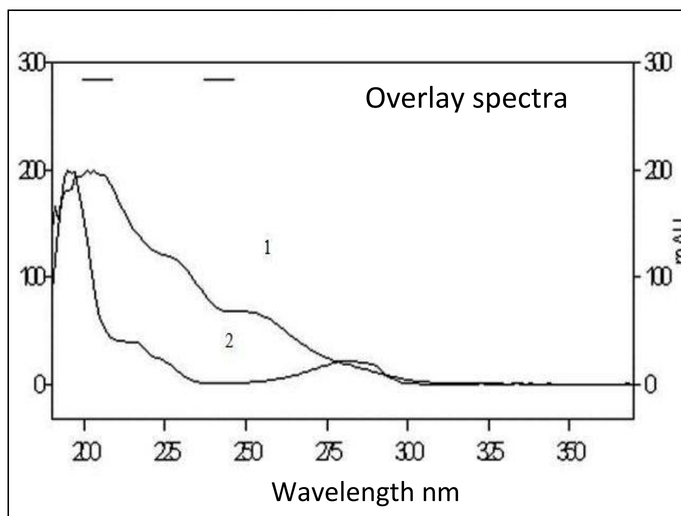


FIG. 2: TYPICAL UV-OVERLAID SPECTRUM OF VALSARTAN (1) AND NEBIVOLOL (2)

For valsartan;

$$C_1 = \frac{Q_0 - Q_2}{Q_1 - Q_2} \times \frac{A}{a_1}$$

$$Q_0 = \frac{\text{Absorbance of sample at 246.6 nm}}{\text{Absorbance of sample at 275 nm}}$$

For nebivolol hydrochloride;

$$C_2 = \frac{Q_0 - Q_1}{Q_2 - Q_1} \times \frac{A}{a_2}$$

$$Q_1 = \frac{\text{Absorbance of valsartan at 246.6nm}}{\text{Absorbance of valsartan at 275 nm}}$$

$$Q_2 = \frac{\text{Absorbance of nebivolol Hcl at 246.6 nm}}{\text{Absorbance of nebivolol Hcl at 275 nm}}$$

In the equation A was absorbance of sample at is absorptive point and a_1 and a_2 were absorptivity values of VAS and NEB respectively at isoabsorptive point.

RP-HPLC Method: Twenty capsules were weighed and finely powdered; a quantity of powder equivalent to one capsule was weighed and transferred to a sintered glass crucible. 100mg of loratidine (internal standard) was added and the drugs were extracted with three quantities, each of 20 ml of mixture of acetonitrile and water (1:1, v/v). The combined extracts were made up to 100 mL with same mixture. The required amount of solution was centrifuged and further dilutions were made with mobile phase to get a concentration of 2 µg/ml of NEB, 32 µg/ml of VAS and 40 µg/ml of internal standard loratidine. From the above mixture 50µl was injected for the estimation under the optimized chromatographic conditions, a

steady baseline was recorded; the typical chromatogram was recorded for standard as shown in **Fig. 3**.

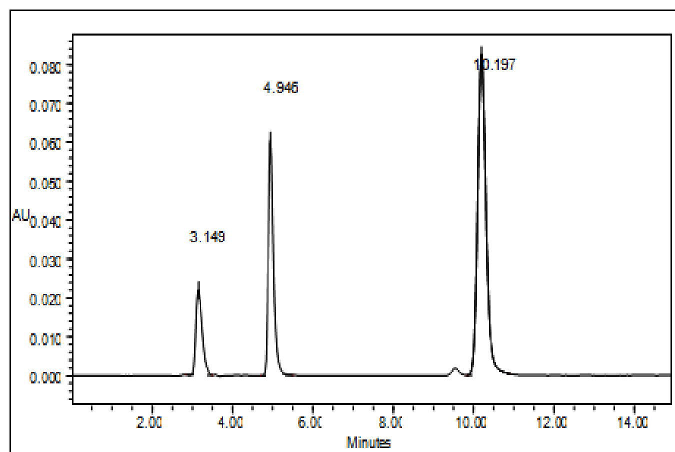


FIG. 3: TYPICAL CHROMATOGRAM OF STANDARD SOLUTION WITH IS BY HPLC (PEAK AT 3.149 MIN FOR NEBIVOLOL HCl, PEAK AT 4.946 MIN FOR VALSARTAN AND PEAK AT 10.187 MIN FOR LORATIDINE)

The retention times of standard nebivolol HCl, valsartan and internal standard were found to be 3.149, 4.946 and 10.197 min, respectively. The detection wavelength was fixed at 275 nm. The 50µL sample solution of capsule was injected; the typical sample chromatogram was recorded as shown in (**Fig. 4**).

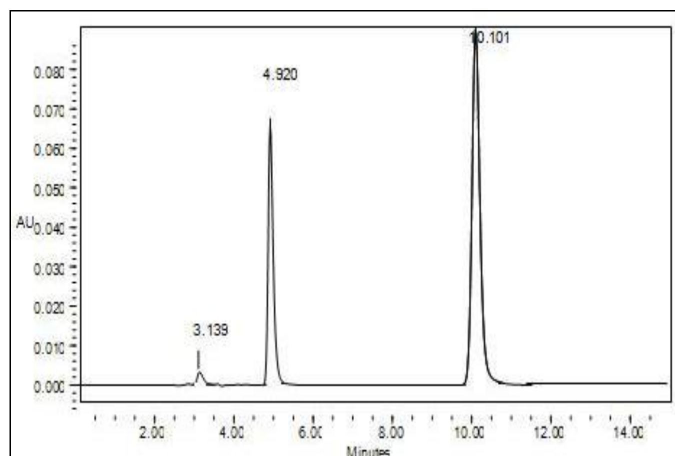


FIG. 4: TYPICAL CHROMATOGRAM OF SAMPLE SOLUTION WITH IS BY HPLC (PEAK AT 3.139 MIN FOR NEBIVOLOL HCL, PEAK AT 4.920 MIN FOR VALSARTAN AND PEAK AT 10.101 MIN FOR (IS) LORATIDINE)

The retention times of sample nebivolol HCl, valsartan and internal standard were found to be 3.139, 4.920 and 10.101 min respectively.

Method validation:

Linearity and range: A linear regression was used for quantification of both drugs. The linearity obtained for nebivolol HCl in the range of 0.5-2.5 µg/ml and 5-25 µg/ml for valsartan by spectrophotometric method. The calibration curve was constructed by plotting absorbance against concentration of drugs. The slope and intercept values of calibration curve for NEB y =0.1345X - 0.0629, (R^2=0.9994) and for VAS y = 0.3045X +0.0141, (R^2=0.9985) were obtained for calculations. The linearity and range of nebivolol HCl are 2-12 µg/ml and 5-30 µg/ml for valsartan by HPLC method. The calibration curve was constructed by plotting response factor against concentration of drugs. The slope and intercept values of calibration curve for NEB y = 0.011X-0.0025 (R^2= 0.9984) and for VAS y = 0.0132 X-0.0067 (R^2= 0.9989) where Y represents the ratio of peak area ratio of analyte to IS and X represents analyte concentration.

Accuracy and precision: The accuracy of the developed method was determined using a mixture NEB and VAS solutions containing three concentrations of drug corresponding to 80%, 100% and 120% and determining the recovery of the added drug. At each concentration, six determinations were performed. The precision and accuracy of UV and HPLC methods were obtained by analyze on the same day (intra-day accuracy) and analyze on the different days by triplicate analysis (inter-day accuracy) and expressed as relative standard deviation percentage (R.S.D. %).

LOD and LOQ: The sensitivity of NEB and VAS was estimated as limit of detection (LOD) and limit of quantification (LOQ), they were calculated by the use of the equations LOD= 3.3×N/B and LOQ=

10×N/B, where N is the standard deviation of the peak areas of the drugs (n = 3), taken as a measure of the noise, and B is the slope of the corresponding calibration plot.

RESULTS AND DISCUSSION: The aim of present work was to develop simple RP-HPLC and spectroscopic methods with ultraviolet detection for the simultaneous determination of nebivolol HCl and valsartan in solid pharmaceutical dosage forms. As the solubility of NEB and VAS was sparingly soluble in water and methanol was used as solvent for preparation of all standard and sample solutions.

Spectrophotometric conditions: The solution of each standard drug (1%,w/v) was prepared and measured absorbance at both the wavelengths of each respective content of formulations for calculating absorptivity of each drug with respect to their wavelengths ϵ_{1v}, ϵ_{2v} and ϵ_{1n} , ϵ_{2n} . The absorbances of sample solutions were also noted at the respective wavelengths for both drugs and calculated the content each of drug in formulation. In Q-analysis method absorbance was noted for standard and sample solutions and by calculating Q-values for each drug and put these values in the formula mentioned for the determination of each drug in formulation.

Chromatographic conditions for separation of analytes: Several attempts were performed in order to get satisfactory resolution of NEB and VAS in different mobile phases with various ratios of organic phase and buffers by using C_{18} column. Initially the mobile phase used was mixture of water and methanol followed by water and acetonitrile in different ratios. Other mobile phase tried was ammonium sulphate buffer pH 5.5 and acetonitrile (60:40, v/v) by isocratic elution which gave no satisfactory resolution. 50mM ammonium acetate buffer pH 3.5 and acetonitrile (30:70, v/v) mobile phase was used by isocratic elution to obtain satisfactory and good resolution with internal standard. The effect of solvent composition by changing the ratio of acetate buffer and acetonitrile in (40:60, v/v), (20:80, v/v) have not shown satisfactory resolution. The effect of change in pH of mobile phase by ±0.2 does not shown significant change in retention time of each analyte. The retention time of NEB and VAS with IS on C_{18} column was found satisfactory with above mobile phase at a flow rate of 0.8 ml/min. The resolution of standard and sample solution for nebivolol HCl, valsartan and loratidine found reproducible and satisfactory.

Selection of UV wavelength and internal standard: The detector wavelength of the present study was selected on the basis of higher sensitivity. The internal standard was selected due to its suitable retention time, recovery and lack of interference with endogenous peaks and also not much affected by the mobile phase pH. These phenomena helped their good separation with other peaks.

Linearity, accuracy and precision: The linearity and range for spectrophotometric and HPLC method was determined at six concentration levels for NEB and VAS and correlation coefficient and the data on precision and accuracy are presented in **Table 1**. The results shown that there is significant correlation exists between response factor and concentration of drugs within the concentration range indicated on Y-axis. The precision of the method was assessed by replicate analysis of pharmaceutical preparations. Precision was also studied by analysis of standard solutions containing both the drugs at concentrations covering the entire calibration range. Intra-day precision was determined by analyze the solutions three times on the same day. Inter-day precision was assessed by analyze the solutions on three different days over a period of one week. The LOD and LOQ Values were reported in **Table 1**.

TABLE 1: VALIDATION PARAMETERS OF DETERMINATION OF NEBIVOLOL HCl AND VALSARTAN BY PROPOSED METHODS

Validation parameters	Simultaneous equation		Q-analysis		RP-HPLC	
	NEB	VAS	NEB	VAS	NEB	VAS
Linearity and range (µg/mL)	0.5-2.5	5-25	0.5-2.5	5-25	2-12	5-30
Correlation coefficient	0.999	0.998	0.999	0.998	0.998	0.999
Standard deviation	0.021	0.052	0.021	0.052	0.040	0.125
LOD (µg/mL)	0.150	0.120	0.150	0.120	0.012	0.032
LOQ (µg/mL)	0.45	0.35	0.45	0.35	0.038	0.095
Accuracy (%)	98.89	100.8	99.80	101.4	100.22	99.58
Precision RSD (%)						
Inter-day	1.20	0.93	1.80	0.84	0.67	0.58
Intra-day	2.56	1.54	2.78	1.24	0.88	0.71

TABLE 2: RESULTS OF ANALYSIS OF FORMULATION AND RECOVERY STUDIES

Methods	Label claim mg/cap		Amount found mg/cap		% Recovery[a] ± SD	
	NEB	VAS	NEB	VAS	NEB	VAS
Simultaneous equation	5	80	5.10	80.2	100.21±1.48	99.82±0.72
Q- Value Analysis Method	5	80	5.12	80.5	101.20±1.24	98.80±1.02
RP-HPLC Method	5	80	5.20	79.25	102.25±0.04	99.28±0.12

[a]n=6

Recovery and stability: The accuracy of the method was determined by the method of standard addition at three different levels. The recovery studies were carried out for capsules by spiking standard of each drugs equivalent to 80%, 100%, and 120% to the original amounts present in each drug formulations. The average recoveries were as reported in Table 2.

In order to demonstrate the stability of both standard and sample solutions during analysis, both solutions were analyzed over a period of 24 h at room temperature. The retention time and peak area of NEB and VAS remained almost similar (% R.S.D. less than 2.0) and no significant degradation within the indicated period, thus indicated that both solutions were stable for at least 24 h , which was sufficient time to complete the whole analytical process.

The statistical comparison of the results of spectrophotometric, Q-analysis and RP-HPLC method was carried out and reported in **Table 3** in which there was no significant difference between all three methods because the calculated *t*- test and *F*- tests did not exceed the theoretical values at the 95% confidence level.

TABLE 3: STATISTICAL COMPARISON OF THE RESULTS OBTAINED BY PROPOSED METHODS

Methods[a]	*F*-test	*t*-test
Simultaneous equation method by UV and HPLC	1.21	0.05
Q- Analysis method by UV and HPLC	1.08	0.97

CONCLUSION: A novel HPLC method and two spectrophotometric methods were developed and validated for the simultaneous determination of nebivolol Hcl and valsartan in solid dosage form. These methods assured the satisfactory precision and accuracy and have high analytical potential. These methods were found to be simple, accurate, economical and reproducible and can be applied for routine analysis in laboratories. RP-HPLC method is suitable for the quality control of the raw materials, formulations, dissolution studies and can be employed for bioequivalence studies for the same formulation.

ACKNOWLEDGEMENT: The author's thank Hetero laboratories, Hyderabad for providing gift samples of valsartan, loratidine and M/s. Cadila Pharmaceuticals Ltd, Ankleshwer for providing a gift sample of nebivolol HCl. The authors are also thanking Mr. Supe (Drug Inspector), Miss Krishnveni N., Mr. S. Rajan Mr. S. Murlidharan (CADRAT) for their co-operation. The authors are grateful to "His Holiness Jagadguru Sri Sri Shivarathree Deshikendra Mahaswamigalavaru" of Sri Suttur Mutt, Mysore and AICTE (QIP) cell for providing facilities to carry out this work.

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