Simultaneous estimation of Nebivolol and Hydrochlorthiazide
Results and discussion

Chapter 9

9.1 Reagents and Pharmaceutical preparations

Pure Nebivolol HCl (NEB) and Hydrochlorthiazide (HCTZ) were kindly donated by M/s Micro labs limited, (Bangalore, KRN, India). The gift samples were used as standard without further purification. Acetonitrile, methanol (HPLC grade Rankem), formic acid (A.R. grade Rankem), and water of ultra pure grade of 18 MOhm resistance were obtained in-house to use in analysis.

Commercial pharmaceutical preparations Nebicard-H (Torrent Pharmaceutical Ltd, India) and Nebilong-H (Micro Caryson Pvt Ltd, India) claimed to contain 5 mg of NEB and 12.5 mg of HCTZ were used in analysis.

9.2 Procedure

Standard solutions of NEB and HCTZ

1. NEB stock solution: 1mg mL^{-1} in methanol.
1. NEB working solution: 0.1mg mL^{-1} in methanol, prepared by transferring 10.0 mL stock solution of NEB to a volumetric flask of 100 mL and completing to volume with methanol and acetonitrile: 0.03% formic acid in water, (65:35 v/v) (pH 3.3) for spectrophotometric and HPLC methods respectively.
2. HCTZ stock solution: 1mg mL^{-1} methanol.
3. HCTZ working solution: 0.04mg mL^{-1} in methanol, prepared by transferring 2.0 mL stock solution of HCTZ to a volumetric flask 50 mL and completing to volume with methanol and acetonitrile: 0.03% formic acid in water, (65:35 v/v) (pH 3.3) for spectrophotometric and HPLC methods respectively.

Preparation of mobile phase

(acetonitrile: 0.03% formic acid in water, (65:35 v/v) (pH 3.3)

A solution of 0.03% formic acid in water was prepared and the pH of the resulting solution was found to be 3.3 ± 0.14. Further alteration in pH of mobile phase was made using dilute formic acid and triethyl amine. HPLC experiments were carried out using binary pump. In one solvent reservoir acetonitrile and in another reservoir 0.03% formic acid in water were taken.

Pharmaceutical sample solution

Twenty Nebicard-H and Nebilong-H tablets were weighed accurately and powdered. An amount of the powder equivalent to 5 mg of NEB and 12.5 mg of HCTZ (content of one tablet) was dissolved separately in 60 mL of methanol. The solutions were stirred for 10 min using a magnetic stirrer and filtered into a 100 mL volumetric flask through 0.45μ nylon membrane filter. The residues were washed 3 times with 10 mL of methanol, and then the volume was completed to 100 mL with the same solvent. This solution was diluted to 1:10 with methanol. Furthermore it was demonstrated that ratio of NEB present in formulations, cannot be brought into working range by dilutions. Hence standard addition(46) of 0.01 mg mL^{-1} of NEB is made in final dilutions. The proposed spectrophotometric, chemometric based spectrophotometric and HRP-HPLC methods were applied and the concentration of each component was determined.

Spectrophotometric methods

Calibration sets for simultaneous equation, first derivative zero-crossing and ratio first derivative methods

A calibration set containing six dilutions each of HCTZ (2-16 μg mL^{-1}) and NEB (10-100 μg mL^{-1}) was prepared in methanol and UV spectra were recorded in the wavelength range between 210-350 nm versus solvent blank.

Chemometric calibration

Applying a multilevel multifactor design in which four levels concentrations of NEB and HCTZ within the stated range were introduced and 16 binary mixtures were prepared as indicated in Table 9.1 and scanned as shown in Fig. 9.6. The levels were in the calibration range of 10-100 μg mL^{-1} and 2-16 μg mL^{-1} respectively. The digitized UV absorption spectra for these samples were collected each 1 nm in the wavelength range of 231–310 nm versus solvent blank. The computations were made in R-Software Environment. CLS, PCR and PLS models were applied to the UV absorption data matrix of these mixtures.

HPLC method

The mobile phase acetonitrile: 0.03% formic acid in water (65:35 v/v) was selected because it was found ideal to resolve the peaks with retention time (R_t) 2.133 ± 0.051 min and 2.877 ± 0.073 min for NEB and HCTZ respectively and the same is shown in Figure 1. Detection wavelength 277 nm was selected by scanning both standard drugs over a wide range of wavelength 210 nm to 350 nm in spectrophotometer. The overlay UV-spectra of both the components and their binary mixture is shown in the Figure 2.

HPLC calibration

The calibration study was carried out individually for both the drugs at six different concentration levels. Aliquots of standard of NEB and HCTZ stock solution were taken in different volumetric flasks and diluted with mobile phase such that the final concentration of NEB and HCTZ were in the range of 10-100 $\mu g\ mL^{-1}$ and 2-16 $\mu g\ mL^{-1}$ respectively. All stock solutions and working solutions were filtered through the nylon membrane filter (0.45μ) and then sonicated for 5 min prior to use. Triplicate 20 μL injections were made for each concentration and chromatographed under specified condition at ambient temperature (27^0C). The peak area values obtained were plotted against corresponding concentrations. Regression analysis of the calibration data was then carried out and shown in Table 1.

Table 9.1 Concentration data for the different mixtures used in the calibration set and internal validation for the determination of NEB and HCTZ using chemometric methods

Mixture No.	Concentration ($\mu g\ mL^{-1}$)	
	NEB	HCTZ
1	10	2
2	10	8
3	10	12
4	10	16
5	30	2
6	30	8
7	30	12
8	30	16
9	60	2
10	60	8
11	60	12
12	60	16
13	100	2
14	100	8
15	100	12
16	100	16

Preparation of binary mixtures for spectrophotometric and HPLC predictions

Applying multilevel multifactorial design in which four level concentrations of HCTZ and HCTZ within the stated range were introduced and prepared 16 synthetic binary mixtures of titled ingredients and are separately used as prediction sets during the validation of the proposed methods.

9.3 Results and discussion

Spectrophotometric methods (*simultaneous equation, first derivative zero-crossing and ratio first derivative methods*)

9.3.1 Simultaneous equation method (SEM)

A calibration set of seven dilutions each of HCTZ (2-16 μg mL^{-1}) and NEB (10-100 μg mL^{-1}) in methanol was prepared and UV spectra were recorded in the wavelength range 200-350nm versus solvent blank. The overlay zero-order UV absorption spectra of standard solutions of HCTZ and NEB are shown in the Fig. 9.1 (a) and 9.1 (b). It was found that HCTZ and NEB showing λmax 271 nm and 283 nm respectively.
Binary mixture solutions for prediction of HCTZ and HCTZ were prepared in the concentration range as shown in Table 9.1. Absorbencies of binary mixture solutions were measured at 271 and 283 nm. The concentration of each component of the binary mixture was calculated by using simultaneous equation.

$$C_{HCTZ} = \frac{Am_{271} * E_{NEB283} - Am_{283} * E_{NEB271}}{E_{HCTZ271} * E_{NEB283} - E_{NEB271} * E_{HCTZ283}}$$

$$C_{NEB} = \frac{Am_{271} * E_{HCTZ283} - Am_{283} * E_{HCTZ271}}{E_{HCT283} * E_{NEB271} - E_{NEB283} * E_{HCTZ271}}$$

C_x = concentration of x,
Am_n= Absorbance of sample solution at 'n' nm,
E_{xn} = Absorptivity of x at 'n' nm,

For simultaneous determination of HCTZ and NEB in their binary mixture a simultaneous equation method was successfully developed. Standard solutions of HCTZ and NEB were prepared to determine their absorptivity values at two selected λmax 271nm and 283 nm. The absorptivity values ± SD at 271 and 283 nm for both the drugs were found to be $E_{HCTZ283}$ = 174.9444 ± 0.107212, $E_{HCTZ271}$ = 626.9722 ± 0.025181, E_{NEB283} = 69.64643 ± 0.501334 and E_{NEB271} = 38.14405 ± 0.20552.
All the optimized method parameters are summarized in Table 4.3.2

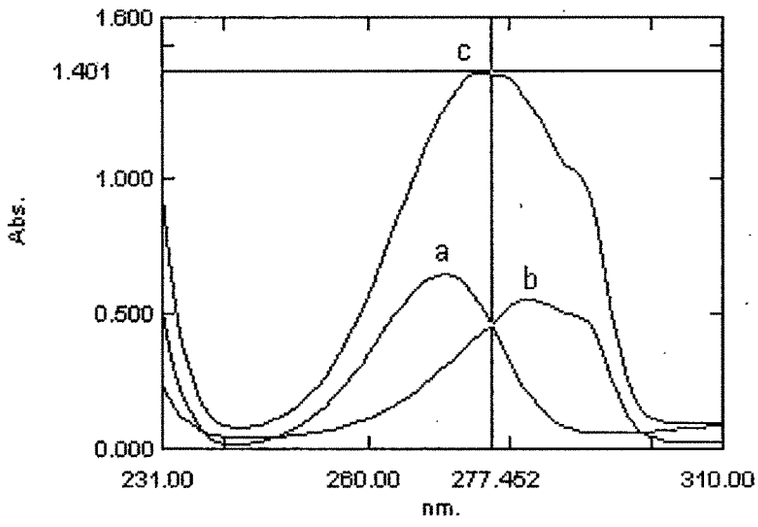


Fig. 9.1 (a) Zero-order overlay absorption spectra: a) 10 µg mL^{-1} of HCTZ, b) 60 µg mL^{-1} of NEB and c) their binary mixture in methanol

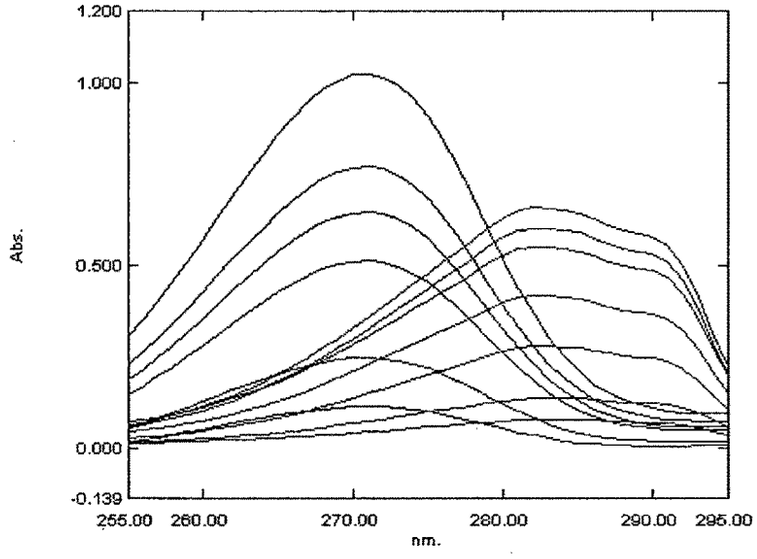


Fig. 9.1 (b) Overlay zero-order absorption spectra for standard dilutions of HCTZ and NEB

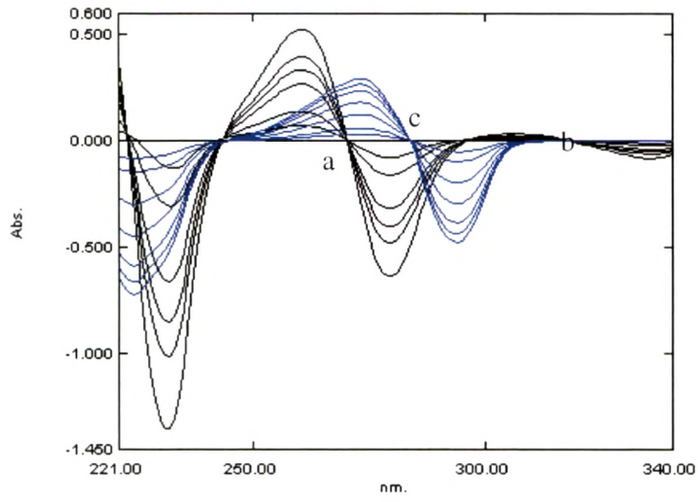


Fig. 9.2 (a) Overlay 1st derivative absorption spectra of standard dilutions of NEB and HCTZ a and b, represents zero crossing of HCTZ at 270 and 296 nm, and c, represents the zero crossing of NEB at 284 nm

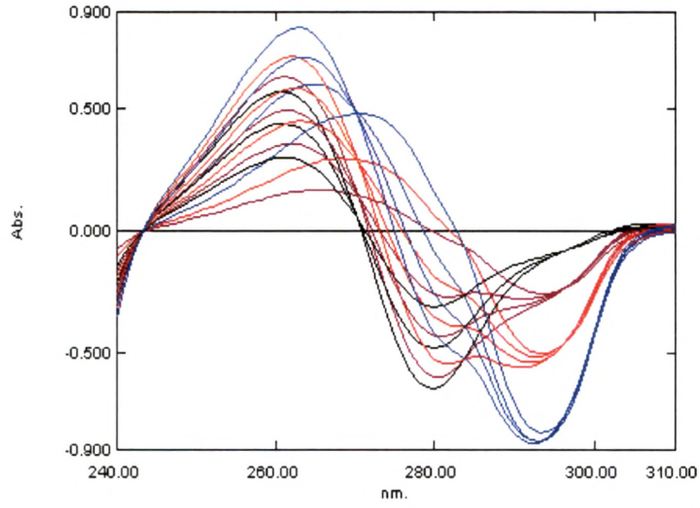


Fig. 9.2 (b) Overlay first derivative absorption spectra of binary mixture solutions of NEB and HCTZ

9.3.2 First derivative Zero-crossing method (FDZC)

A calibration set of seven dilutions each of NEB (10-100 µg mL^{-1}) and HCTZ (2 -16 µg mL^{-1}) in methanol was prepared and UV spectra were recorded and converted the

same to first derivative spectra in the wavelength range 200-350nm versus solvent blank. The first derivative overlay absorption spectra of standard solutions of NEB and HCTZ was shown in the Fig. 9.2 (a).

The absorption spectra of working standard solutions of NEB and HCTZ were recorded in the range of 221-350 nm and stored in the memory of the instrument. The first derivative of the working standard solution were traced with smoothing factor ($\Delta\lambda = 10$) and scaling the entire spectra with a constant factor 10 for determining zero cross points for both the drugs (Fig. 9.2(a)). It was found that the 1st derivative spectrum of NEB crosses at 284 nm and that of HCTZ crosses at 270 nm and 296 nm. The amplitudes at 270 nm and 296 nm were plotted against the respective concentrations of NEB. It was found that NEB shows the best linearity at 270 nm, So 270 nm was selected for the determination of NEB. The method shows good linearity in the range of 10 - 100 $\mu g\ mL^{-1}$ for NEB. Similarly the amplitudes at 284 nm were plotted against the respective concentrations of HCTZ. The method shows good linearity in the range of 2 - 16 $\mu g\ mL^{-1}$ for HCTZ. The absorption spectra of the binary mixture solutions of NEB and HCTZ were recorded in the range of 221 nm to 350 nm and stored in the memory of the instrument using smoothing factor ($\Delta\lambda = 10$) and scaling factor 10 they were converted into first derivative (Fig. 9.2(b)) and the absorbance values at 270 and 284 were used for the determination of NEB and HCTZ respectively.

Optimization and selection method parameters

All the optimized method parameters are summarized in Table 4.3.2. Methanol was selected as solvent because both NEB and HCTZ were freely soluble in this solvent. 270 nm and 296 nm were selected for determination of NEB as the first derivative spectra of HCTZ shows zero amplitude (zero cross of HCTZ) at these wavelengths. Since at 270 nm, NEB shows best linearity hence selected for the determination of NEB. Similarly 284 nm was selected for the determination of HCTZ (zero cross of NEB) (Fig 9.2).

9.3.3 Ratio spectra first derivative spectrophotometry (RFD)

Ratio derivative methods were used for analysis of mixtures with overlapped spectra. This method permits the determination of components in mixtures at wavelengths corresponding to a maximum or minimum. The values at these points permit better sensitivity and accuracy. The main instrumental parameters that affect the shape of the derivative ratio spectra are wavelength, scanning speed, the concentration of divisor spectra, smoothing ($\Delta\lambda$) and scaling factor. The effects of these parameters were studied and fast scanning speed (2800 nm min^{-1}), smoothing factor ($\Delta\lambda$ = 10) and scaling factor (=10) was selected. Divisor concentration is the main instrumental parameter; the standard spectra of 8 μg mL^{-1} of HCTZ and 10 μg mL^{-1} of NEB was considered as divisor for the determination of NEB and HCTZ in mixtures respectively.

The first derivative ratio spectra of different NEB standards at increasing concentrations in methanol were obtained by dividing each with the stored spectrum of the standard solution of 8 μg mL^{-1} of HCTZ by a computer aid as divisor spectra. These ratio spectra are shown in Fig. 9.3A. The first derivative (1DD) of these spectra traced with smoothing factor of $\Delta\lambda$ = 10 nm and scaling factor (= 10) are illustrated in Fig. 9.3B. As seen in Fig. 9.3B one maximum (283 nm) and one minimum (296 nm) and exist and we found that both were suitable for the determination of NEB in NEB and HCTZ mixtures. The wavelength of 283 nm was selected for the determination of this compound in the assay of synthetic mixtures and tablets, due to its lower RSD values and more suitable mean recovery compared with other wavelength. For the determination of HCTZ, the ratio spectra of different HCTZ standards at increasing concentrations in methanol, obtained by dividing each with stored spectrum of the standard solution of 10 μg mL^{-1} of NEB as divisor spectra by computer aid, are demonstrated in Fig. 9.4A. The first derivatives (1DD) of this spectrum traced with smoothing factor ($\Delta\lambda$ = 10) nm are illustrated in Fig. 9.4B. As seen in Fig. 9.4B, there exist one maximum (253 nm) and one minimum (274 nm) and in this also both were suitable for the determination of HCTZ in NEB and HCTZ

mixtures. The peak at wavelength 253 nm was selected because of its lower RSD and more suitable mean recoveries.

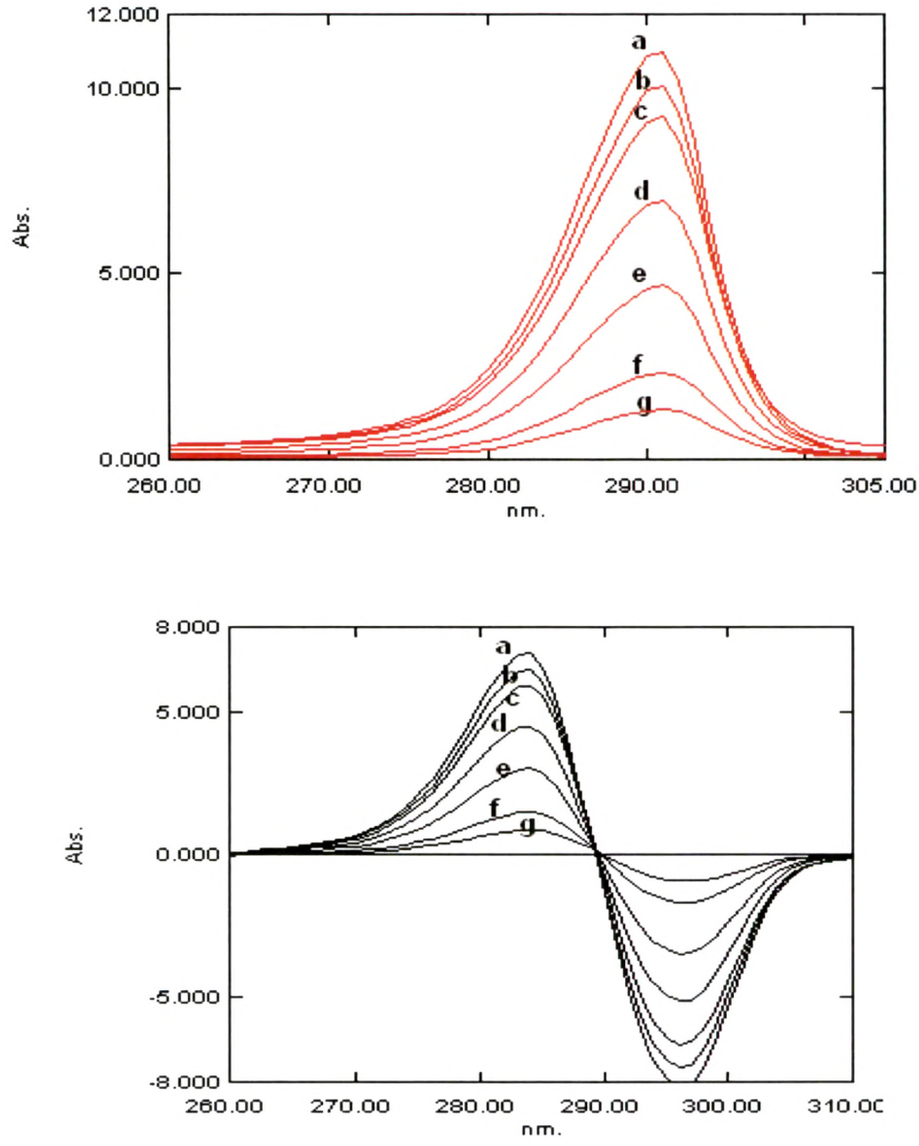
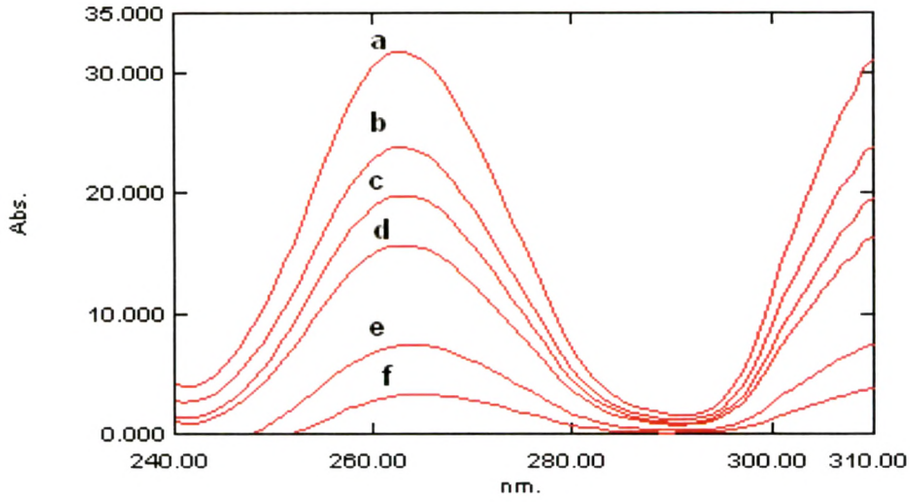


Fig. 9.3 Ratio spectra (A) and first derivative of the ratio spectra (B): a) 10, b) 20, c) 40, d) 60, e) 80, f) 90, g) 100 µg mL^{-1} solution of NEB in methanol when 8 µg mL^{-1} of HCTZ in methanol used as divisor ($\Delta\lambda$ = 10 nm); scaling factor 10



A.

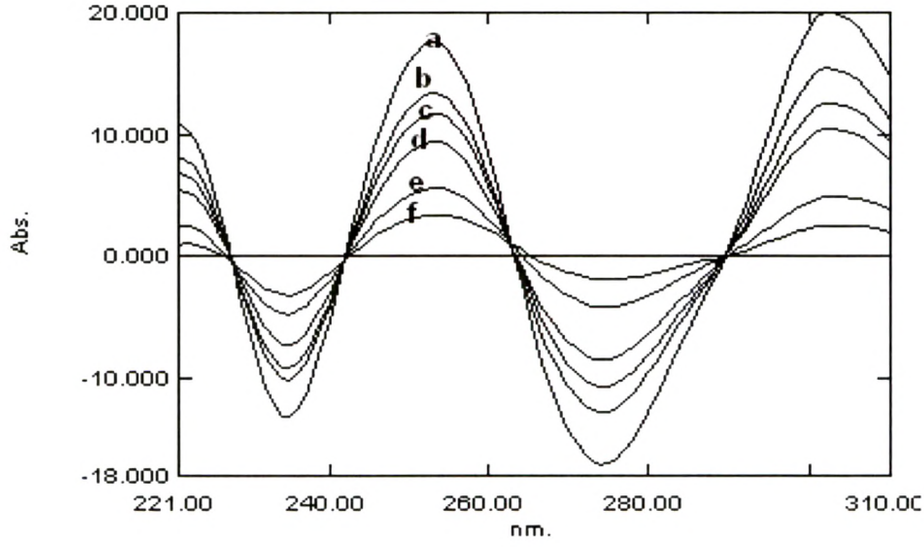


Fig. 9.4 Ratio spectra (A) and first derivative of the ratio spectra (B): a) 2, b) 4, c) 8, d) 10, e) 12, f) 16 μg mL^{-1} solution of HCTZ in methanol when 10 μg mL^{-1} of NEB in methanol used as divisor ($\Delta\lambda$ = 10 nm); scaling factor 10

Table 9.2 Analytical data of the calibration graphs for the determination of NEB and HCTZ by spectrophotometric method

Parameters	SEM		SEM		FDZC		RFD	
	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ
Wavelength (nm)	271		283		270	284	283	253
Linearity range ($\mu g\ mL^{-1}$)	10 -100	2 - 16	10 -100	2 - 16	10 -100	2 - 16	10 -100	2 - 16
Intercept (a)	0.013	0.012	0.007	0.013	0.007	0.0131	1.609	0.136
SE of Intercept[1]	0.002	0.123	0.005	0.003	0.012	0.00	0.091	0.094
Slope (b)	0.006	0.0625	0.003	0.02	0.007	0.009	0.731	0.069
SE of slope	0.002	0.003	0.001	0.21	0.014	0.016	0.006	0.008
Correl. coeff	0.9986	0.9998	0.9969	0.9994	0.9999	1	0.9991	0.9998
RSE[2]	0.134	0.164	0.002	0.004	0.0163	0.007	0.005	0.003

[1]Standard error, [2]Residual standard error

9.4 Spectrophotometric method validation

To check the validity (predictive ability) of the calibration models, the simultaneous analysis of the prediction set containing each of 16 (Table 9.3) samples in various concentrations of NEB and HCTZ (in triplicates) was carried out by proposed spectrophotometric methods. The mean recoveries, % errors and the relative standard deviations of prediction sets were computed and indicated in Table 8.4. Their numerical values were completely acceptable because of their good recoveries and hence found satisfactory for the validation.

Linearity and range

All proposed spectrophotometric method showed good linearity for NEB and HCTZ within the range of 10 - 100 $\mu g\ mL^{-1}$ and 2 – 16 $\mu g\ mL^{-1}$ respectively with lowest co-relation co-efficient, intercept and slope (Table 9.2).

Accuracy

Accuracy of the proposed methods was determined by performing recovery study in triplicates from previously analyzed formulation at five concentration levels and from laboratory prepared synthetic mixture by standard addition method at three levels of concentration so that final concentration of both the drugs lies within the stated linearity range. The method showed % recovery ± SD for both the title ingredients indicating that the developed spectrophotometric method is accurate and is free from interference of excipients (Table 9.4 and 9.5).

Precision

Inter day and intra day precision for proposed spectrophotometric methods were measured in terms of % RSD. The experiment was carried on laboratory prepared binary mixtures of title ingredients and repeated three times in a day for intra day (repeatability) and on three different days for inter-day precision (intermediate precision). The methods were found precise on intra day and inter day basis as the average %RSD value for the determination of NEB and HCTZ was as shown in Table 9.6 (a) and 9.6 (b).

Robustness

Double-beam Shimadzu (Japan) UV-vis Spectrophotometer (model UV-1700 and 1601) were used to access the robustness. The average value of % RSD of the responses for determination of NEB and HCTZ less than 2 % reveals the robustness of the method.

Limit of detection (LOD) and Limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) are calculated (Table 8.7) according to a formula given by Miller.[1]

(LOD = 3 * SD/slope of calibration curve, LOQ = 10 * SD/slope of calibration curve and SD = Standard deviation of blank determinations.)

Table 9.3 Prediction results for NEB and HCTZ in synthetic mixtures by proposed spectrophotometric techniques

Mixture added		Recovery (%)						Error%					
		SEM		FDZC		RFD		SEM		FDZC		RFD	
NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ
10	2	96.32	99.14	98.65	97.36	103.64	99.36	-3.68	-0.86	-1.35	-2.64	3.64	-0.64
10	8	96.48	96.15	102.30	98.36	102.64	94.36	-3.52	-3.85	2.30	-1.64	2.64	-5.64
10	12	96.87	98.78	104.04	97.64	104.31	97.65	-3.13	-1.22	4.04	-2.36	4.31	-2.35
10	16	97.65	100.60	96.34	95.97	99.60	97.36	-2.35	0.60	-3.66	-4.03	-0.40	-2.64
30	2	95.68	103.94	99.65	99.87	97.34	106.95	-4.32	3.94	-0.35	-0.13	-2.66	6.95
30	8	98.39	105.64	98.34	99.64	96.94	101.55	-1.61	5.64	-1.66	-0.36	-3.06	1.55
30	12	99.12	97.36	97.36	98.16	97.36	102.94	-0.88	-2.64	-2.64	-1.84	-2.64	2.94
30	16	97.85	94.36	99.98	102.65	98.65	95.95	-2.15	-5.64	-0.02	2.65	-1.35	-4.05
60	2	102.12	97.36	99.56	100.23	98.36	102.39	2.12	-2.64	-0.44	0.23	-1.64	2.39
60	8	103.56	94.87	98.34	101.34	97.61	98.95	3.56	-5.13	-1.66	1.34	-2.39	-1.05
60	12	104.38	96.87	96.49	99.34	98.67	100.20	4.38	-3.13	-3.51	-0.66	-1.33	0.20
60	16	98.45	99.01	99.78	96.87	99.99	100.80	-1.55	-0.99	-0.22	-3.13	-0.01	0.80
100	2	99.19	99.87	102.65	98.24	99.81	103.64	-0.81	-0.13	2.65	-1.76	-0.19	3.64
100	8	98.64	97.36	103.64	98.59	102.64	98.16	-1.36	-2.64	3.64	-1.41	2.64	-1.84
100	12	96.49	100.20	100.00	96.91	103.56	97.67	-3.51	0.20	0.00	-3.09	3.56	-2.33
100	16	97.36	103.00	104.96	99.99	106.97	99.19	-2.64	3.00	4.96	-0.01	6.97	-0.81
	$\bar{x}$	98.66	99.03	100.13	98.82	100.51	99.82						
	RSD:	2.01	2.18	12.66	1.78	1.02	2.17						

$\bar{x}$, mean value; RSD, Relative standard deviation

Table 9.4 Application of standard addition technique for analysis of HCTZ and NEB in Nebicard -H tablets

Serial No	Concentration in µg mL^{-1}		NEB % recovery ± SD[a]			Concentration in µg mL^{-1}		HCTZ % recovery ± SD[a]		
	Claimed	Added	SEM	FDZC	RFD	Claimed	Added	SEM	FDZC	RFD
1	5	10*	99.52 ± 1.03	98.96 ± 1.73	99.82 ± 0.98	12.5	0	98.19 ± 0.95	101.01 ± 0.90	100.12 ± 1.695
2	5	20	99.92 ± 1.38	100.82 ± 0.89	99.56 ± 1.43	12.5	1	94.95 ± 0.78	100.75 ± 0.76	98.75 ± 0.65
3	5	30	99.76 ± 0.41	100.00 ± 1.38	98.08 ± 1.21	12.5	2	99.36 ± 0.77	101.99 ± 0.63	101.52 ± 0.27
4	5	50	100.94 ± 0.37	100.17 ± 0.72	98.64 ± 0.58	12.5	3	98.12 ± 0.79	100.1 ± 0.550	99.90 ± 0.12
5	5	80	100.92 ± 1.24	99.92 ± 0.66	95. 67 ± 6.44	12.5	4	95.32 ± 0.75	101.21 ± 3.24	102.31 ± 0.99

* average of three experiments

[a] sd standard deviation

* 10 µg ml-1 of standard NEB was added to raise the level to linear calibration range

Table 9.5 Application of standard addition technique for analysis of HCTZ and NEB in certified reference material

Serial No	Concentration in µg mL^{-1}	NEB % recovery ± SD[a]			HCTZ % recovery ± SD[a]		
	Standard Addition	SEM	FDZC	RFD	SEM	FDZC	RFD
3	80	99.52 ± 1.03	98.96 ± 1.73	99.35 ± 0.73	100.94 ± 0.37	100.17 ± 0.72	100.43 ± 0.60
4	100	99.92 ± 1.38	100.82 ± 0.89	98.65 ± 1.12	100.92 ± 1.24	99.92 ± 0.66	100.00 ± 1.38
5	120	99.76 ± 0.41	100.00 ± 1.38	99.76 ± 0.41	100.14 ± 2.19	100.43 ± 1.49	100.17 ± 0.72

* average of three experiments, [a] sd standard deviation

Table 9. 6 (a) Intra day precision for determination of NEB and HCTZ

Mixture No.	% RSD					
	SEM		FDZC		RFD	
	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ
1	3.499	3.514	2.703	2.288	2.321	2.014
2	0.690	1.405	1.389	2.799	1.131	1.575
3	1.634	0.439	0.519	1.644	0.480	0.849
4	1.336	1.757	0.405	0.870	0.477	1.152
5	1.339	0	0.626	0.313	0.938	0.981
Average of % RSD	2.491	1.612	1.865	1.788	1.069	1.314

*** Average of three experiments in a day.

Table 9. 6 (b) Inter day precision for determination of NEB and HCTZ

Mixture No.	% RSD					
	SEM		FDZC		RFD	
	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ
1	2.619	1.166	1.731	1.634	2.014	2.321
2	1.7	1.994	0.143	2.045	1.575	1.131
3	0.389	0.226	0.790	1.648	0.849	0.480
4	0.887	0.915	2.712	2.440	1.152	0.477
5	0.660	0.817	1.773	0.275	0.981	0.938
Average of % RSD	1.501	1.228	1.484	1.669	1.64	1.97

*** Average of three experiments in a day.

Table 9.7 LOD and LOQ

Results observed	NEB	HCTZ
*SD	0.001633	0.000469
LOD ($\mu g\ mL^{-1}$)	0.202488	0.051543
LOQ ($\mu g\ mL^{-1}$)	0.016334	0.017181

* Standard deviation of intercepts of five calibration curves

Application of the developed method for analysis of commercial formulations

Applicability of the proposed method was tested by analyzing the commercially available tablet formulations Nebicard-H and Nebilong-H, labeled to contain 5 mg of NEB and 12.5 mg of HCTZ. Further more, it was demonstrated that in formulation NEB levels were low compared to HCTZ, ranging 1:2.5. In final dilutions NEB level remains lower than the linearity range of this compound. The levels of NEB in the prepared samples are raised by standard addition in its final concentration within the calibration linear range. The amount of standard addition was kept as low as possible to minimize error of prediction for the actual NEB levels. 0.01 mg ml^{-1} constant standard addition was chosen for NEB. The results of all methods were very close to each other as well as to the label value of commercial pharmaceutical formulation (Table 9.8 and 9.9).

Table 9.8 Results obtained for the Nebilong-H tablets by using the spectrophotometric methods

Methods	NEB (Label claim= 5 mg per tablet) Mean[a] ± SD[b]	HCTZ (Label claim= 12.5mg per tablet) Mean[a] ± SD[b]
SEM	98.19 ± 0.95	101.01 ± 0.90
FDZC	94.95 ± 0.78	100.75 ± 0.76
RFD	99.36 ± 0.77	101.99 ± 0.63

Mean[a], mean value of five determinations for each method; SD[b], Standard deviation

Table 9.9 Results obtained for the Nebicard-H tablets by using the spectrophotometric methods

Methods	NEB (Label claim= 5mg per tablet) Mean[a] ± SD[b]	HCTZ (Label claim= 12.5mg per tablet) Mean[a] ± SD[b]
SEM	98.12 ± 0.79	100.1 ± 0.550
FDZC	95.32 ± 0.75	101.21 ± 3.24
RFD	103.21 ± 0.65	105.34 ± 0.78

Mean[a], mean value of five determinations for each method; SD[b], Standard deviation

9.5 Results and discussion

9.5.1 Chromatography

In order to effect the simultaneous elution of the two component peaks with enhanced sensitivity under isocratic conditions, the mobile phase composition was optimized after several trials with methanol-water, acetonitrile-water in various combination with formic acid and ammonium acetate buffers. A satisfactory separation was obtained with a mobile phase consisting of acetonitrile: 0.03% formic acid in water (pH 3.3 ± 0.14) (65:35 v/v) at a flow rate of 1.0 ml min^{-1}. The addition of formic acid delivers the good peak symmetry for NEB and removes ghost peaks. Initial studies were performed while the effluent was monitored at 277 nm. The change in the wavelength of detection between the run was performed to achieve better detector response. Both the components show reasonably good response at 277 nm. The development of the chromatographic system was focused on short retention times in order to assure high throughput. Under the described chromatographic conditions, the analyte peak was well defined, resolved and almost free from tailing with the retention time of 2.133 ± 0.051 min and 2.877 ± 0.073 min for NEB and HCTZ respectively (Fig 9.5). This allows determination of both drugs with reasonable responses for the two well resolved peaks with reliable results. For quantitative applications linear calibration chromatograms were obtained with correlation coefficients better than 0.999.

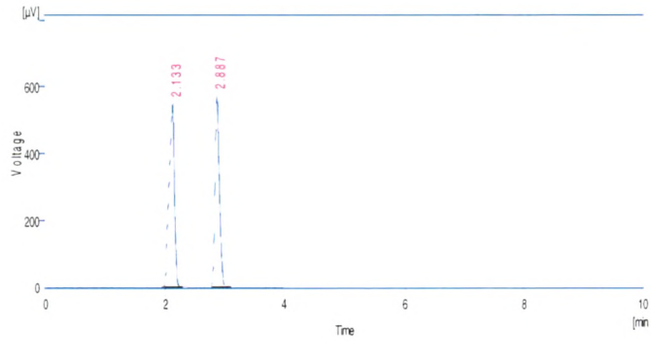


Fig. 9.5 Chromatogram showing retention time (R_t) of (a) 90 µg ml^{-1} of NEB (2.133min) and (b) 12 µg ml^{-1} of HCTZ (2.887 min) in laboratory prepared mixture

9.5.2 Chemometric methods

Fig. 9.1 shows the zero- order absorbance spectra of NEB and HCTZ as well as their corresponding binary mixture in methanol. The spectra of NEB and HCTZ were overlapped in the region of their absorption maxima. For this reason to solve overlapped spectra, two chemometric calibrations using the zero-order spectra were applied.

A calibration set consisting of binary mixtures of NEB and HCTZ was prepared in methanol in the concentration range illustrated in Table 9.1. The UV absorbance data was obtained by measuring the absorbances in the region of 231 - 310 nm. By using the correlation between calibration concentration and its absorbance data, the chemometric calibrations were calibrated within the CLS, PCR and PLS algorithms. The quality of multi-component analysis is dependent on the wavelength region and spectral mode used, calibration set chosen. All the information present in the sample target should be present in the calibration data set. It has been one of the main drawbacks in development studies of multivariate method. Chemometrics techniques are designated as full spectrum computational procedures, thus wavelength selection is seemingly unnecessary, and so all available wavelengths are often used. However, measurements from spectral wavelengths that are not informative in a model will degrade performance. Hence absorbances after 310 nm were not used because both the ingredients have no absorbance at the concentrations used in this region. Any absorbance data beyond 310 nm would have introduced a significant amount of noise, thereby decreasing the precision of estimation and predictive ability of the model. Original and reconstructed spectra of the calibration matrix were compared in order to select the range of wavelengths. The region, which is best, reconstructed also considered. This entailed using 80 experimental points per spectrum, as spectra were digitized at 1 nm intervals.

Statistical parameter

The predictive ability of a calibration model in chemometric methods can be defined in various ways. The most general expression is the standard error of calibration (SEC) and prediction (SEP), which is given by the following equation;

$$\text{SEP (SEC)} = \sqrt{\frac{\sum_{i=1}^{N}(C_i^{Added} - C_i^{Found})^2}{n}}$$

Here C_i^{Added} is the added concentration of drugs C_i^{Found} is the predicted concentration of drugs and n is the total number of the synthetic mixtures. The numerical values are quoted in Tables 9.11 and 9.12.

Selection of optimum number of factors for PCR and PLS

For PCR and PLS methods, 16 calibration spectra were used for the selection of the optimum number of factors by using the cross validation technique. This allows modelling of the system with the optimum amount of information and avoidance of overfitting or underfitting. The cross-validation procedure consisting of systematically removing one of a group of training samples in turn and using only the remaining ones for the construction of latent factors and regression applied. The predicted concentrations were then compared with the actual ones for each of the calibration samples and mean squares error of prediction (MSEP) was calculated. The MSEP was computed in the same manner each time a new factor was added to the PCR and PLS model. The selected model was that with the fewest number of factors such that its MSEP values were not significantly greater than that for the model, which yielded the lowest MSEP. A plot of MSEP values against number of components (Fig. 9.7 and 9.8) indicates that factors 3 is optimum for the estimation of principle ingredients by PCR and PLS. At the selected principal components of PCR and PLS the concentrations of each sample were then predicted and compared with known concentration and the PRESS (prediction error sum of squares) was calculated. It was given by this equation, and values are indicated in Tables 9.11.

$$\text{PRESS} = \sum_{i=1}^{n}\left(C_i^{Added} - C_i^{Found} \right)^2$$

9.6 Validation of the developed methods

To check the validity (predictive ability) of the calibration models, the simultaneous analysis of the prediction set containing each of 16 samples in various concentrations of NEB and HCTZ (in triplicates) was carried out by HPLC (Fig. 9.9) and

chemometric methods. The mean recoveries and the relative standard deviations of our proposed methods were computed and indicated in Tables 9.13. Their numerical values were completely acceptable because of their smallest values and hence found satisfactory for the validity of all calibration methods.

Linearity

The linearity of the HPLC and chemometric methods could be established for both the drugs in concentration range of 10-100 μg mL^{-1} and 2-16 μg mL^{-1} for NEB and HCTZ respectively. In this study six concentrations were chosen in triplicates. The linearity of the calibration graphs was validated by the high value of the correlation coefficient, slope and the intercept value.

Table 9.10. Characteristic parameters of the calibration equations for the proposed methods for simultaneous determination of NEB and HCTZ

Parameters	HPLC	
	NEB	HCTZ
Calibration range (μg mL^{-1})	10-100	2-16
Detection limit (μg mL^{-1})	0.0055×10^{-2}	0.027×10^{-2}
Quantitation limit (μg mL^{-1})	0.0181×10^{-2}	0.089×10^{-2}
Regression equation (Y)[a]		
Slope (b)	0.3468	2.3137
Standard deviation of the slope (S_b)	1.7×10^{-3}	1.90×10^{-2}
Relative standard deviation of the slope (%)	0.449	0.744
Intercept (a)	0.0239	0.3775
Standard deviation of the intercept (S_a)	0.00065064	0.000346
Correlation coefficient	0.9999	0.9999
Theoretical plates	2372	7818
Symmetry factor	1.050	1.389
Resolution	4.992	

[a] **$Y = a + bC$, where C is the concentration of compound in μg mL^{-1} and Y is the peak area.**

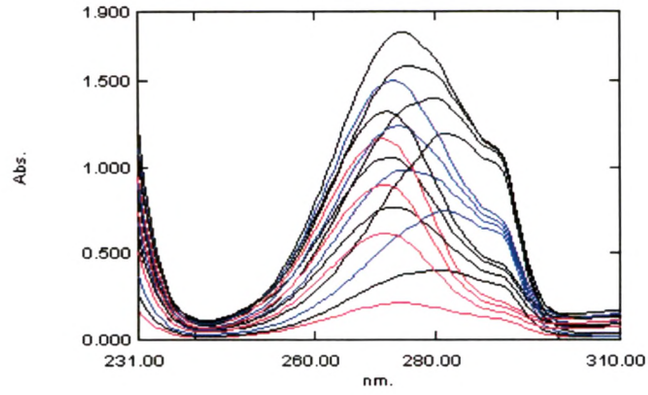


Fig. 9.6 Zero-order overlay absorption spectra of laboratory prepared NEB and HCTZ binary mixtures in methanol and used for prediction

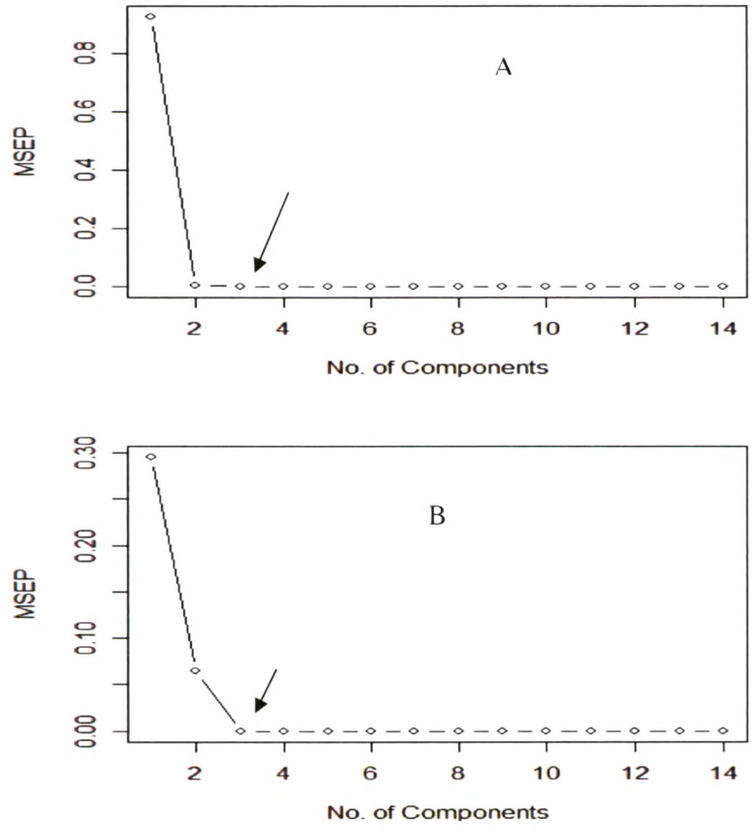


Fig. 9.7 MSEP plots of a calibration set obtained using leave-one-out (LOO) cross validation of PCR-model for A) NEB and B) HCTZ in zero-order absorption data

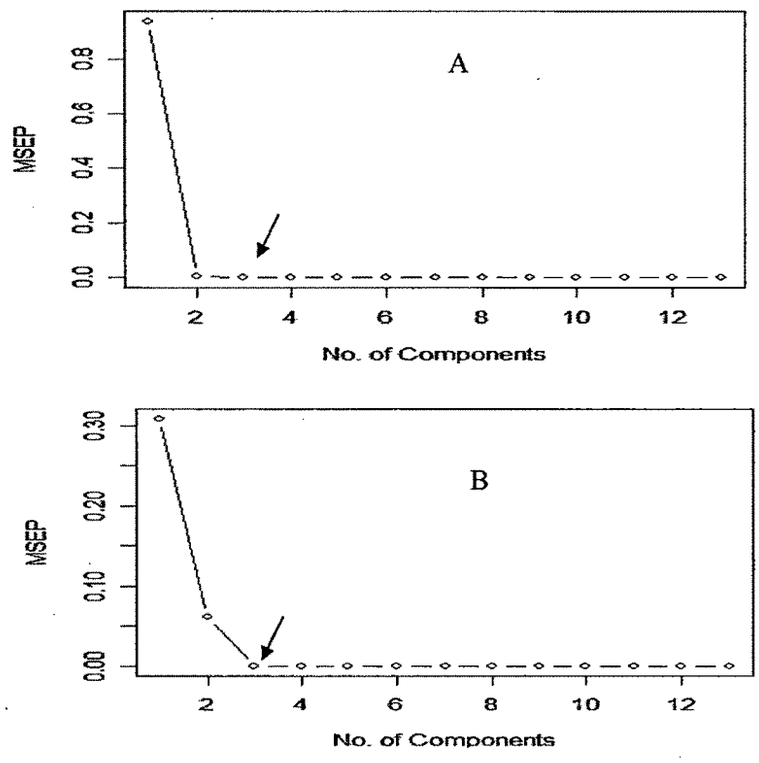


Fig. 9.8 MSEP plots of a calibration set obtained using leave-one-out (LOO) cross validation of PLS-model for A) NEB and B) HCTZ in zero-order absorption data

Accuracy

Accuracy of the proposed methods was determined by performing recovery study by standard addition method to the formulation. Nebicard-H and Nebilong-H tablet solutions were spiked with the certified reference material to reveal the accuracy. The results given in Table 9.14(a) and 9.14(b) shows excellent recoveries of the standard addition method suggested good accuracy of the proposed methods and no interference by any of the formulation excipients in their determination.

Table 9.11 Statistical parameters of chemometric methods in calibration step of zero-order spectra

Component	CLS	PCR			PLS		
	SEC	SEC	PRESS	RSE[a] (%)	SEC	PRESS	RSE (%)
NEB	0.2020	0.0253	0.0096	0.0406	0.0253	0.0096	0.0406
HCTZ	0.2438	0.0114	0.0019	0.1021	0.0114	0.0019	0.1022

[a] Relative Standard Error of calibration of single component

$$RSE^{a}(\%) = \sqrt{\frac{\sum_{i=1}^{N}\left(C_i^{Added} - C_i^{Found}\right)^2}{\sum_{i=1}^{N}\left(C_i^{Added}\right)^2}} \times 100$$

Table 9.12 Statistical parameters of chemometric methods in prediction step of zero-order spectra

Component	CLS				PCR				PLS			
	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*
NEB	0.157	0.055	1	0.999	0.016	0.083	0.997	0.999	0.015	0.083	0.997	0.9996
HCTZ	0.247	0.255	0.981	0.999	0.019	0.004	0.999	0.999	0.017	0.004	0.999	0.9999

[a] Intercept, [b] Slope, [r] Correlation coefficient

Table 9.13 Recovery results in prediction for NEB and HCTZ in synthetic mixtures by proposed HPLC and chemometric techniques

Mixture added		Recovery (%)								Error %							
		HPLC		CLS		PCR		PLS		HPLC		CLS		PCR		PLS	
NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ
10	2	100.61	99.87	98.83	109.78	99.11	102.08	102.02	102.62	0.61	-0.13	-1.17	9.78	-0.89	2.08	2.02	2.62
10	8	101.33	101.29	98.31	104.82	101.06	99.99	102.76	100.12	1.33	1.29	-1.69	4.82	1.06	-0.01	2.76	0.12
10	12	100.34	98.99	110.24	100.18	103.91	100.78	105.63	100.87	0.34	-1.01	10.24	0.18	3.91	0.78	5.63	0.87
10	16	99.84	100. 78	104.36	100.65	103.19	99.58	104.40	99.70	-0.16	0.78	4.36	0.65	3.19	-0.42	4.40	-0.30
30	2	100.86	100.56	98.37	100.76	99.29	99.21	97.56	97.69	0.86	0.56	-1.63	0.76	-0.71	-0.79	-2.44	-2.31
30	8	101.22	97.87	100.74	106.96	99.85	98.93	98.34	98.49	1.22	-2.13	0.74	6.96	-0.15	-1.07	-1.66	-1.51
30	12	101.30	100.25	101.69	103.58	99.59	99.53	99.52	99.62	1.30	0.25	1.69	3.58	-0.41	-0.47	-0.48	-0.38
30	16	101.20	100.11	101.98	100.89	99.94	100.24	99.40	100.18	1.20	0.11	1.98	0.89	-0.06	0.24	-0.60	0.18
60	2	102.12	97.59	101.78	97.81	99.67	96.68	99.72	96.34	2.12	-2.41	1.78	-2.19	-0.33	-3.32	-0.28	-3.66
60	8	101.66	99.49	100.07	99.33	99.14	99.35	99.05	99.29	1.66	-0.51	0.07	-0.67	-0.86	-0.65	-0.95	-0.71
60	12	100.41	99.42	99.94	103.44	100.26	100.12	100.17	100.03	0.41	-0.58	-0.06	3.44	0.26	0.12	0.17	0.03
60	16	100.06	98.61	99.63	100.84	99.53	100.58	99.43	100.45	0.06	-1.39	-0.37	0.84	-0.47	0.58	-0.57	0.45
100	2	99.05	98.34	98.55	98.61	98.76	99.54	99.26	100.88	-0.95	-1.66	-1.45	-1.39	-1.24	-0.46	-0.74	0.88
100	8	101.04	100.11	100.2	99.64	102.47	101.45	102.46	101.48	1.04	0.11	0.2	-0.36	2.47	1.45	2.46	1.48
100	12	100.68	99.64	100.2	98.95	99.61	100.36	100.53	100.71	0.68	-0.36	0.2	-1.05	-0.39	0.36	0.53	0.71
100	16	100.12	98.32	99.96	102.82	98.63	98.95	98.63	98.94	0.12	-1.68	-0.04	2.82	-1.37	-1.05	-1.37	-1.06
	$\bar{x}$	100.47	100.91	100.76	100.91	100.25	99.84	100.56	99.84								
	RSD:	0.76	1.05	2.5	3.2	1.59	1.21	2.26	1.50								

$\bar{x}$ mean recovery value.

[r] Relative Standard Deviation.

Table 9.14 (a) Application of standard addition technique for analysis of NEB and HCTZ in Nebicard-H tablets

Serial No	NEB						HCTZ					
	Concentration in µg ml^{-1}		% recovery ± SD[a]				Concentration in µg ml^{-1}		% recovery ± SD[a]			
	Claimed	Added	CLS	PCR	PLS	HPLC	Claimed	Added	CLS	PCR	PLS	HPLC
1	5	10[☼]	100.14 ± 2.19	100.43 ±1.49	99.35 ± 0.73	100.24 ± 1.24	12.5	0	99.09 ± 0.61	99.82± 0.98	100.75±0.13	99.78 ± 0.46
2	5	20	100.24 ± 1.24	99.78 ± .469	99.52 ± 1.03	99.35 ± 0.73	12.5	1	98.96 ± 1.7	102.31± 0.99	99.25±0.32	99.09 ± 0.61
3	5	30	100.94 ± 0.37	100.17 ± 0.729	99.92 ± 1.38	98.65 ± 1.12	12.5	2	100.82 ± 0.89	98.08± 1.21	106.25±0.35	100.43 ± 0.60
4	5	50	100.92 ± 1.24	99.92 ± 0.66	99.76 ± 0.41	99.76 ± 0.41	12.5	3	100.00 ± 1.38	100.1± 1.69	103±0.64	100.00 ± 1.38
5	5	80	100.14 ± 2.19	100.43 ± 1.49	100.94 ± 0.37	100.94 ± 0.37	12.5	4	100.17 ± 0.72	95.67± 6.44	100.5±0.01	100.17 ± 0.72

- average of three experiments, [a] sd standard deviation, [☼] 10 µg ml-1 of standard RAMP was added to raise the level to linear calibration range

Table 9.14 (b) Application of standard addition technique for analysis of of NEB and HCTZ in Nebilong-H tablets

Serial No	NEB						HCTZ					
	Concentration in µg ml^{-1}		% recovery ± SD[a]				Concentration in µg ml^{-1}		% recovery ± SD[a]			
	Claimed	Added	CLS	PCR	PLS	HPLC	Claimed	Added	CLS	PCR	PLS	HPLC
1	5	10[☼]	101.13 ± 0.48	99.35 ± 0.356	101.41 ± 0.3448	99.52 ± 1.03	12.5	0	104.1 ± 1.033	104.12 ± 0.349	102.1±0.15	98.96 ± 1.73
2	5	20	99.32 ± 1.62	101.36 ± 2.641	101.55 ± 1.5337	99.92 ± 1.38	12.5	1	102.51 ± 0.255	107.97 ± 0.798	98.25±0.34	100.82 ± 0.89
3	5	30	101.4 ± 0.3446	105.4±0.34	100.2±0.94	99.76 ± 0.41	12.5	2	104.26 ± 1.0183	104.26 ± 1.0182	100.2±0.67	100.00 ± 1.38
4	5	50	101.55 ± 1.5336	104.2±0.67	99.4±0.054	100.94 ± 0.37	12.5	3	103.61 ± 1.6635	103.61 ± 1.6638	99.79±0.011	100.17 ± 0.72
5	5	80	103.6±0.34	98.34±1.2	100.2±0.64	100.92 ± 1.24	12.5	4	100.7±0.04	99.64±0.64	102.8±0.23	99.92 ± 0.66

* Average of three experiments, [a] SD standard deviation

Precision (Repeatibility)

For evaluation of the precision estimates of the proposed methods, repeatability and intermediate precision were performed at five concentration levels (Fig. 9.10) for each compound. 3 days × 3 replicates design was performed. The percentage recoveries (± R.S.D) obtained within and between days trials are in acceptable range indicating good precision was found (Table 9.15).

Table 9.15 Precision study results of prepared binary mixture

Conc. taken in $\mu g\ mL^{-1}$		Mean % Recovery ± R.S.D					
		HPLC		PCR		PLS	
NEB	HCTZ	NEB	HCTZ	NEB	HCTZ	NEB	HCTZ
10	2	103.94 ± 0.378	104.17 ± 0.729	99.52 ± 1.038	98.96 ± 1.736	100.14 ± 2.196	99.43 ± 1.492
30	4	100.92 ± 1.248	99.92 ± .668	99.92 ± 1.385	100.82 ± 0.891	100.24 ± 1.242	101.78 ± 0.469
50	10	99.14 ± 2.196	100.43 ±1.492	99.76 ± 0.415	100.00 ± 1.389	99.99 ± 0.732	101.09 ± 0.611
70	12	97.24 ± 1.242	99.78 ± .469	98.65 ± 0.378	100.17 ± 0.729	98.96 ± 1.125	100.43 ± 0.602
90	16	98.94 ± 0.378	102.17 ± 0.729	100.92 ± 1.248	99.92 ± 0.668	101.76 ± 0.415	100.00 ± 1.389

* Average of three experiments

Limit of detection (LOD) and Limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) are calculated according to ICH(50) recommendations where the approach based on the signal-to-noise ratio. Chromatogram signals obtained with known low concentrations analytes was compared with the signals of blank samples. A signal-to-noise ratio 3:1 and 10:1 is considered for calculating LOD and LOQ respectively.

The values of LOD and LOQ are given in Table 9.10.

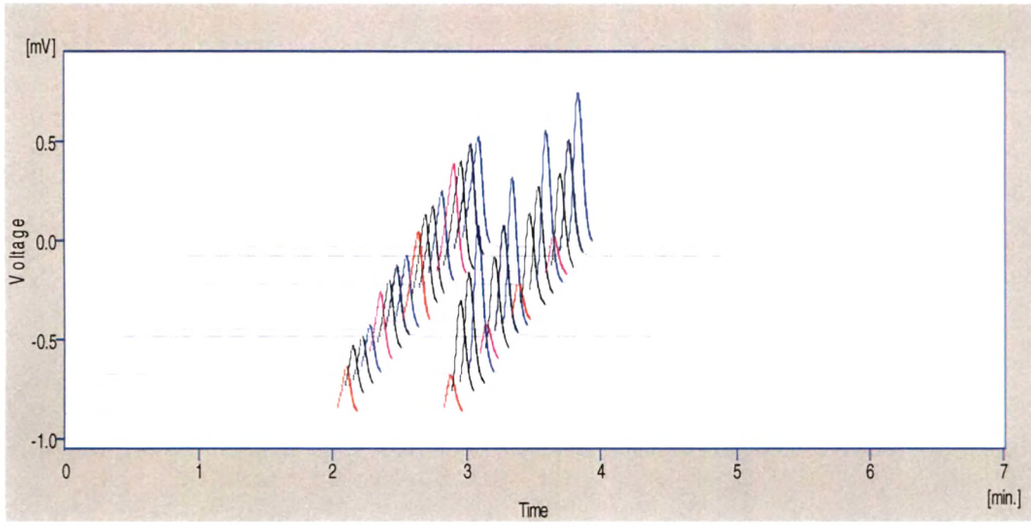


Fig. 9.9 HPLC 3-Dimensional Chromatograms set of 16 binary mixtures of NEB and HCTZ prepared by four factorial design used for prediction

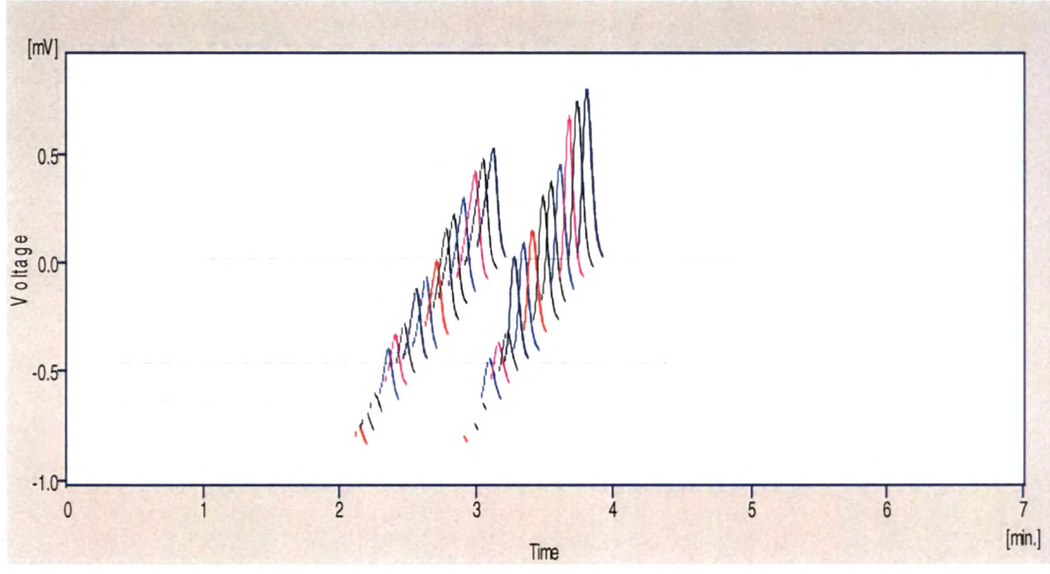


Fig 9.10 HPLC 3-Dimensional Chromatograms of NEB and HCTZ binary mixtures used for Precision analysis

Robustness

Double-beam Shimadzu (Japan) UV-vis spectrophotometer (model UV-1700 and 1601) were used in case of spectrophotometric method and the different analysts (two) carried out HPLC analysis for robustness study. Further the chromatographic condition was purposely altered and the results were evaluated. To study the effect, flow rate of mobile phase was changed by ± 0.2 units, concentration of acetonitrile by ± 3% and pH of the mobile phase varied by ± 0.3 units. The results (Table 9.16) obtained within the altered conditions did not have significant effect on the determination of active principle ingredients.

Stability

In case of an unexpected delay during analysis it is important to have information about the stability of standard and working drugs solution. The responses for the aged solutions were evaluated using a freshly prepared standard. Both the drug substances did not show evidence of significant degradation for at least 72 h when kept at normal condition.

Table 9.16 Robustness of chromatographic method

Parameter	Peak asymmetry		Resolution between NEB and HCTZ
	NEB	HCTZ	
Flow rate			
0.8	1.099	1.412	5.095
1	1.050	1.389	4.992
1.2	1.059	1.378	4.881
Acetonitrile % in mobile phase			
68	1.138	1.396	4.670
65	1.050	1.389	4.992
62	1.087	1.402	5.134
Change in pH			
3.6	1.205	1.374	4.343
3.3 (± 0.14)	1.050	1.389	4.992
3.0	1.378	1.322	4.037

Application of the developed methods for analysis of commercial tablet formulations

Applicability of the proposed method was tested by analyzing the commercially available tablet formulations Nebicard-H and Nebilong-H, labeled to contain 5 mg of NEB and 12.5 mg of HCTZ. Further more, it was demonstrated that in formulation NEB levels were low compared to HCTZ, ranging 1:2.5. In final dilutions NEB level remains lower than the linearity range of this compound. The levels of NEB in the prepared samples are raised by standard addition in its final concentration within the calibration linear range. The amount of standard addition was kept as low as possible to minimize error of prediction for the actual NEB levels. 0.01 mg ml^{-1} constant standard addition was chosen for NEB.

No published method has been reported for simultaneous determination of these binary components. So the results of the proposed PCR and PLS methods were statistically compared between results of the proposed HPLC method at the 95% confidence level with the aid Student's t-test and F-tests. The calculated t and F values never exceed the theoretical t- and F- values, at 0.05 level of significant difference. The results of all methods were very close to each other as well as to the label value of commercial pharmaceutical formulation. Therefore, these statistical tests (Table 9.17) denote no significant difference in the results achieved by the proposed methods.

Table 9.17 Results obtained for the pharmaceutical samples by using HPLC method and chemometric calibrations

Methods	HPLC	Chemometric methods		
		CLS	PCR	PLS
NEB mean[a] ± SD[b]	(5.045) ± 0.684	(4.96) ± 0.022	(5.005) ± 0.773	(5.039) ± 0.377
F		4.236	4.212	4.208
t		0.79	0.59	0.581
HCTZ mean[a] ± SD[b]	12.596 ± 0.973	±0.384	12.717 ± 1.531	12.719 ± 1.531
F		3.26	1.1545	1.1545
t		1.94	0.0689	0.0683

(Label claim: 05 mg of NEB and 12.5 mg HCTZ per tablet), a Mean recovery value of five determinations for each method

b Standard deviation, (n1 = n2 = 5), Theoretical values for *t* and F at P = 0.05 are 2.31 and 6.39 respectively