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Application of two different spectrophotometric approaches for the determination of a new antihypertensive combination: Graphical and statistical representation of the data

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RESEARCH ARTICLE

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ABSTRACT

Specific, and precise spectrophotometric methods are developed and validated for the simultaneous determination of the binary antihypertensive mixture nebivolol hydrochloride and valsartan in the zero-order spectrum. The methods applied for the determination of this antihypertensive mixture are constant center spectrophotometric resolution technique, constant center spectrum subtraction resolution technique, and advanced concentration value. Nebivolol hydrochloride was determined by its zero order spectra at 280 and 213 nm while for valsartan it determined by its zero order spectra at 250 nm. All developed methods were applied for the determination of the cited drugs in the pharmaceutical formulation and the results obtained were statistically compared with each other and with those of the reported method. The comparison showed that there is no significant difference between the proposed methods and the reported method regarding both accuracy and precision.

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1. Introduction

Nebivolol hydrochloride (NEB) (2,2'-azanediylbis(1-(6-fluorochroman-2-yl)ethanol) hydrochloride) has antihyper tensive efficacy and is a third generation β-blocker, highly selective β-adrenoceptor antagonist [1]. Valsartan (VAL) ((2*S*)-3-methyl-2-[pentanoyl-[[4-[2-(2*H*-tetrazol-5-yl)phenyl] phen yl]methyl]amino]butanoic acid) is an angiotensin II receptor blocker and it blocks the vasoconstrictor and aldosterone-secreting effects of angiotensin II [2]. Combination of antihypertensives can increase adherence, which results in improved patient outcomes [3,4]. The NEB and VAL combination is used to treat hypertension and this combination provides unique and complementary mechanisms for controlling blood pressure [5-8].

During the literature survey, many methods were found for the analysis of both NEB and VAL in its combined dosage form. Those methods include HPLC [9-17], LC-MS/MS [18], and spectrophotometry [19-21]. The aim of the study is to apply different approaches to determine the drug studied in its zero-order absorption spectrum without interference from one another. All developed methods (Constant center resolution technique (CC) [22], Constant center coupled with spectrum subtraction resolution technique (CC-SS) [22], and Advanced concentration value (ACV) [23-27] were applied for the determination of the cited drugs in pharmaceutical formulation and the results obtained were compared statistically with each other and with those of reported method.

2. Experimental

2.1. Materials and reagents

The nebivolol hydrochloride and valsartan reference standards were purchased from Sigma-Aldrich. The purity of the standards was certified to be 99.5 and 99.98%, respectively. Byvalson® tablets manufactured by Allergan labeled with 5 mg/80 mg of nebivolol/valsartan were purchased from the United States. Structures of the compounds are shown in Figure 1.

2.2. Instrumentation

UV-Vis absorption spectra were measured on a double-beam UV/Visible spectrophotometer model J-760, Jasco, Japan.

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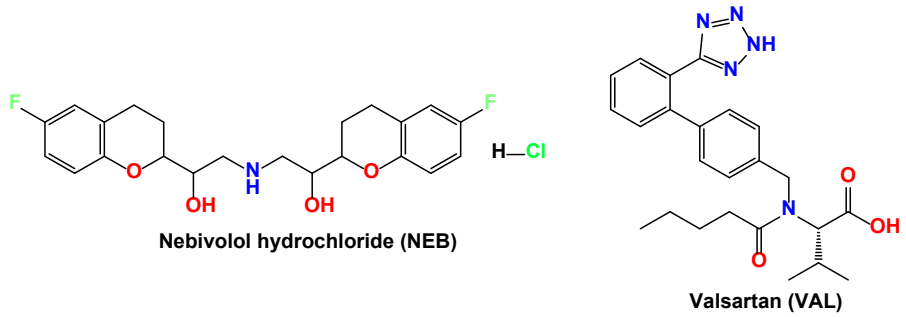

Figure 1. Chemical structure of the studied drugs nebivolol hydrochloride and valsartan.

The absorption spectra of the standard and tested solutions were recorded in 1.0 cm quartz cells in the range of 200-400 nm at room temperature using SpectraManager software.

2.3. Spectral characteristics and wavelength selection

D° absorption spectra of NEB and VAL (10 µg/mL), were scanned against blank (200-400 nm), overlaid using the Spectramanager software to detect the spectral characteristics, and predict the best methods for resolution of the mixture.

2.4. Procedures

2.4.1. Preparation of standard stock and working solutions

Primary stock solutions of standard NEB and VAL were prepared separately in 100 mL volumetric flask by dissolving 50 mg of each standard powder in ethanol. Primary stock solutions of NEB and VAL were diluted with ethanol to prepare standard working solutions (100 µg/mL).

2.4.2. Preparation of pharmaceutical dosage form

Five tablets were powdered; then, a portion equivalent to one tablet was weighed accurately and transferred to a 100-mL beaker, 50 mL of ethanol was added, stirred using a magnetic stirrer for 20 min and filtered through 0.5 µm Whatman filter paper into a 100 mL volumetric flask. The residue was washed three times each time with 10 mL of ethanol and the solution was completed to the mark with ethanol.

2.5. Methods validation

The validation of the proposed method was evaluated according to the guidelines of the International Conference on Harmonization (ICH) [28].

2.5.1. Linearity and construction of calibration curves

Accurately measured aliquots of NEB and VAL were transferred from their standard working solutions into two separate series of 10 mL volumetric flasks and the volumes were completed to the mark with ethanol. Samples were scanned from 200-400 nm and the obtained D° spectra were saved on the computer. Regression equations representing linear relationships between absorbance at λ_{max} of the D° scanned spectra of NEB 280 or 213 nm, respectively, or VAL at 250 nm, versus the corresponding concentration of NEB or VAL were obtained.

2.5.2. Determination in laboratory-prepared mixtures

From the working solutions prepared previously (100 µg/mL) of NEB and VAL, different mixtures were prepared by transferring and mixing accurate portions of both analytes into a series of 10 mL volumetric flasks. The final volume was completed by ethanol.

2.5.3. Accuracy

Three replicates of different concentrations of NEB and VAL were used to check the accuracy of the developed methods. The concentrations were obtained from the regression equation of each drug using the suggested methods.

2.5.4. Precision

2.5.4.1. Intra-day

Three different concentrations of NEB and VAL were analyzed intra-daily in triplicate. Then, relative standard deviations were calculated for each method [28].

2.5.4.2. Inter-day

The previous procedures were repeated three different days in triplicate. Then, relative standard deviations were calculated for each method [28].

2.6. Limit of quantitation (LOQ) and limit of detection (LOD)

According to ICH recommendations [28], several approaches are possible to determine the detection and quantification limits. The standard deviation of the intercept and the slope approach were used to calculate LOD and LOQ [28], Equations (1 and 2).

$$LOD = 3.3 \times (\sigma/S) \quad (1)$$

$$LOQ = 10 \times (\sigma/S) \quad (2)$$

where σ = The standard deviation of the response of the curve, S = The slope of the calibration curve (of the analyte).

2.7. Application to pharmaceutical dosage form

From the previously prepared stock solutions of pharmaceutical formulation, further dilutions were prepared in the linearity range obtained using ethanol. The stock was diluted to the concentration of 5 µg/mL NEB and 80 µg/mL VAL in a 100 mL volumetric flask and then the solutions were completed to the mark with ethanol.

3. Results and discussion

The zero-order absorption spectra of NEB with VAL were severely overlapped in the range of 200-300 nm as shown in Figure 2 to the extent that conventional spectrophotometric resolution techniques could not be used for their simultaneous determination.

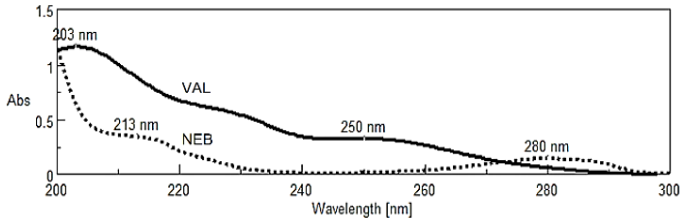

Figure 2. Zero-order absorption spectra of 10 µg/mL NEB (---) and 10 µg/mL VAL (___).

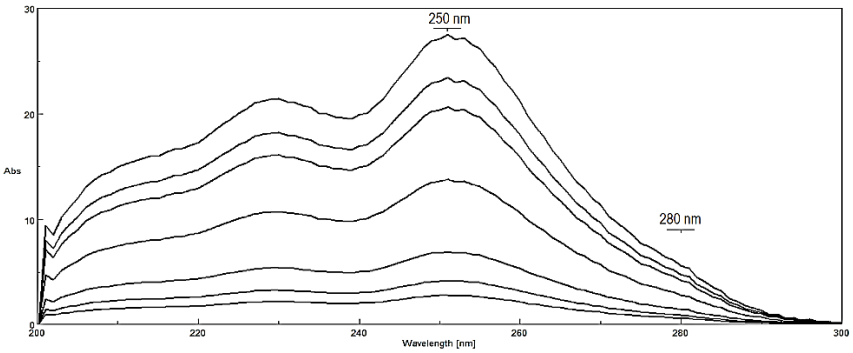

Figure 3. The difference in ratio is obtained by dividing the D° absorption spectra of VAL (2-20 µg/mL) by the D° absorption spectrum of NEB' (1 µg/mL) as a divisor showing the two selected wavelengths at 280 and 250 nm.

$$P_{250} - P_{280} = \left(\frac{a_{NEB} \times C_{NEB}}{a_{NEB} \times C_{NEB'}} + \frac{a_{VAL} \times C_{VAL}}{a_{NEB} \times C_{NEB'}}\right)\lambda_{250} - \left(\frac{a_{NEB} \times C_{NEB}}{a_{NEB} \times C_{NEB'}} + \frac{a_{VAL} \times C_{VAL}}{a_{NEB} \times C_{NEB'}}\right)\lambda_{280}$$

$$P_{250} - P_{280} = \left(\frac{a_{VAL} \times C_{VAL}}{a_{NEB} \times C_{NEB'}}\right)\lambda_{250} - \left(\frac{a_{VAL} \times C_{VAL}}{a_{NEB} \times C_{NEB'}}\right)\lambda_{280} \quad (3)$$

First; the constant center followed by spectrum subtraction (CC-SS) resolution technique was used to resolve the zero-order absorption spectrum of each drug alone, to ensure their drug profile. By substituting the absorbance for NEB or VAL in their corresponding regression equations constructed at their maximum (250 nm for VAL and 280 and 213 nm for NEB), we obtained the concentration of each drug alone [21].

Advanced Concentration Value Method (ACV) was a recently developed method that could be applied for the determination of the concentration of VAL and NEB; thus, the recovery percentage could be measured directly using this calculated concentration via difference between the recorded and calculated peak amplitudes; therefore, ACV was considered as a new simpler and easier approach depending on graphically representing of data rather statistically. Unlike the ordinarily approach, no need to use the regression equation only, we depend on the data get from the spectrum directly and the use of normalized devisor (1 µg/mL) [22].

3.1. Using NEB' normalized (1 µg/mL) as a divisor

By dividing the D° absorption spectra of VAL (2-20 µg/mL) by the D° absorption spectrum of NEB' (1 µg/mL) as a divisor at the two chosen wavelengths (250 and 280 nm), the Δ*P* was calculated by subtracting the peak amplitude at 280 nm from the peak amplitude at 250 nm applying the previous step in the mixture, resulting in canceling the contribution of the NEB response as shown in Equation (3) where, P: Peak amplitude, a_{NEB}: Absorptivity of NEB, C_{NEB}: Concentration of NEB, a_{VAL}: Absorptivity of VAL, C_{VAL}: Concentration of VAL.

From the same ratio spectra shown in Figure 3, the equation relating Δ*P* between the peak amplitudes at 250 and 280 nm to the peak amplitude at 250 nm of VAL was calculated, so upon knowing the Δ*P* of a lab mixture, the postulated peak amplitude at 250 nm of VAL present in mixture could be calculated, and by subtracting from the total recorded peak amplitude of the mixture at $P_{250\ nm}$ the amplitude of VAL at $P_{250\ nm}$, the constant representing NEB/NEB' could be obtained according to the following Equation (4),

$$\text{Recorded } P_{250} - \text{Postulated } P_{250} = \frac{NEB}{NEB'} \quad (4)$$

where the recorded P_{250} was the total peak amplitude of the ratio spectra of the mixture at 250 nm, the postulated P_{250} was the peak amplitude of VAL at 250 nm in the mixture obtained from the corresponding equation.

This constant was used in two pathways; either to directly calculate the recovery %, to obtain the concentration of NEB by graphically representing the data using the advanced concentration value (ACV), or by multiplication of this constant by the NEB (1 µg/mL) divisor curve to obtain the zero-order absorption spectrum of NEB in the mixture showing its λ_{max} at 280 or 213 nm known as the constant center method (CC).

$$\frac{NEB}{NEB'} \times NEB' = NEB \quad (5)$$

The zero-order absorption spectrum of VAL was then obtained by coupling the previously explained resolution technique CC with the SS by subtracting the obtained spectrum of NEB from the lab mixture.

$$(NEB + VAL) - NEB = VAL \quad (6)$$

Concentration of VAL was obtained by substitution in the regression equation relating the absorption of zero order absorption spectra of VAL at its λ_{max} = 250 nm.

Using the normalized divisor of VAL allows us to directly obtain the concentration of VAL through graphical representation of data without the need of regression equation, as the peak amplitude is equal to the concentration.

Table 1. Results of the validation of the assay of the proposed spectrophotometric methods for the determination of nebivolol and valsartan, dosage form analysis, and standard addition technique.

Drug	NEB			VAL	
Resolution technique	CC			CC-SS	
Method	280 nm	213 nm	ACV	250 nm	ACV
Range µg/mL	1:25	1:25	1:25	2:20	2:20
Slope	0.0123	0.0326	-	0.0351	-
Intercept	0.0138	-0.0335	-	2.00×10^{-7}	-
Correlation coefficient (r)	0.9999	0.9999	-	1	-
Accuracy (Mean±SD) [a]	101.51±1.107	100.91±1.097	100.09±1.015	101.05±0.987	100.31±1.207
Selectivity [b]	100.30±0.832	100.10±1.041	100.81±0.285	100.55±0.247	100.47±0.347
Precision					
Intraday, RSD% [c]	0.318	0.915	0.184	0.162	0.27
Interday, RSD% [d]	0.329	1.01	0.304	0.268	0.329
LOQ (µg/mL)	1	1	1	2	2
LOD (µg/mL)	0.33	0.33	0.33	0.67	0.67
Recovery of pharmaceutical preparation [e]	100.32±1.250	99.55±0.482	100.13±0.62	100.02±0.520	100.55±0.87

[a] Three concentrations of each analyte repeated three times for each concentration.
[b] Recovery±SD of five sets of laboratory prepared mixtures of NEB and VAL, each of three replicates.
[c] Intraday (n = 3), the average of three concentrations of analytes (7.5, 12.5, 15.0 µg/mL for NEB and 2.5, 5.0, 7.5 µg/mL for VAL), repeated three times within the same day.
[d] Interday (n = 3), the average of three concentrations of analytes (7.5, 12.5, 15.0 µg/mL for NEB and 2.5, 5.0, 7.5 µg/mL for VAL) repeated three times in three consecutive days.
[e] Byvalson® commercial tablet dosage form manufactured by Allergan and labeled to contain 5 mg of NEB and 80 mg of VAL.

Table 2. Statistical comparison of the results obtained by the proposed methods and the reported method for the determination of NEB and VAL in bulk powder.

Parameters	Reported method [a]		NEB			VAL	
			CC			CC-SS	
	NEB	VAL	280 nm	213 nm	ACV	250 nm	ACV
Mean	100.49	99.97	101.51	100.91	100.09	101.05	100.31
S.D.	0.652	0.585	1.107	1.097	1.015	0.987	1.207
N	3	3	3	3	3	3	3
Variance	0.425	0.342	1.225	1.203	1.030	0.974	1.457
Student's t test (2.78) [b]	-	-	1.375	0.570	0.574	1.630	0.439
F test (19.00) [b]	-	-	2.883	2.831	2.423	2.847	4.257

[a] RP-HPLC methods [8].
[b] The values in parentheses are the corresponding theoretical values of t and F at $p = 0.05$.

Table 3. One-way ANOVA testing for the different proposed methods and the reported methods used for the determination of VAL and NEB in pure powder form.

Source of variation		SS *	df **	Variance	*F* [a]	*P*-value	$F_{Critical}$ [b]
NEB	Between groups	0.061378	3	0.020459304	0.286998	0.834303	3.008787
	Within groups	1.710895	24	0.071287293			
	Total	1.772273	27				
VAL	Between groups	7.48×10^{-5}	2	3.74167×10^{-5}	0.001545	0.998456	3.554557
	Within groups	0.435963	18	0.024220146			
	Total	0.436037	20				

* Sum of squares.
** Degree of freedom between and within groups.
[a] Calculated F.
[b] Critical (tabulated) value for F at $p = 0.05$.

$$\left(\frac{VAL}{VAL'}\right) = Constant \quad (7)$$

Although the use of ACV easier and lower the manipulations steps, but in CC obtaining the zero-order absorption spectrum ensures the drug profile and working at λ_{max} ensured maximum accuracy and precision.

3.2. Validation

ICH guidelines [28] were applied in validation of the proposed methods as follows:

3.2.1. Linearity range

The linearity of the methods was evaluated by making the different calibration graphs on different days. The calibration graphs were constructed within concentration ranges that were selected based on the anticipated drug concentrations during the dosage form assay. Each concentration was repeated three times. The concentration ranges, regression equations, and other statistical parameters are listed in Table 1.

3.2.2. Accuracy

Procedures under linearity for the drugs were repeated three times for the determination of different concentrations of pure NEB and VAL. Concentrations were obtained from each method from which the mean percentage recoveries were calculated as shown in Table 1.

3.2.3. Precision

The intraday and interday precision were determined by analyzing three different concentrations of the proposed drugs, within the linearity range, three times for three pure samples of the drug on a single day and three consecutive days, the results expressed as % RSD were illustrated in Table 1.

3.2.4. Selectivity

Selectivity was ascertained by analyzing different mixtures containing the drugs in different ratios within the linearity range. Good percentage recoveries with very low standard deviation were obtained among the other methods and are listed in Table 1. The dosage form analysis results were shown in Table 1.

3.3. Statistical analysis

The proposed and reported method [8] for both NEB and VAL were statistically compared with respect to t and F values, indicating similar accuracy and precision with no significant

differences, and the results were tabulated in Table 2. The developed and reported methods were compared using one-way ANOVA and the results are shown in Table 3.

4. Conclusions

This work presented different spectrophotometric methodologies for the analysis of binary severely overlapped mixtures without prior separation and without the need for any special operations. The methods were applied by using simple steps without the need for any specific requirement in the spectrum as there is no need to have an extended part or Iso absorptive point. It also gave agreeable results regarding accuracy and recovery. The advanced concentration value method is a new approach derived from concentration value method which eliminate the use of regression equation and lets the recording of the concentration of the studied drug directly throw the spectrum with good accuracy and precision. The developed methods simple, accurate and also have the advantage of didn't require preceding treatment, complicated steps or use of organic harmful solvents like that usually used in the reported methods HPLC [9-17], LC-MS/MS [18], and spectrophotometry [19-21]. Although the use of ACV was easier and lower the manipulation steps, but in CC, obtaining the zero-order absorption spectrum ensures the drug profile and working at λ_{max} ensured maximum accuracy and precision. The proposed method used for the determination of NEB and VAL combination in several laboratory-prepared mixtures and in pharmaceutical dosage form and results show accepted accuracy and precision, consequently it could be considered as an eco-friendly alternative method for the routine analysis of this combined dosage form with least sample preparation.

Disclosure statement

Conflict of interest: The authors declare that they have no conflict of interest.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the authors.

CRediT authorship contribution statement

Conceptualization, Methodology, Investigation, Visualization: Ragaa Magdy Ismail, Validation and Formal Analysis:: Ahmed Mohamed Hemdan, Nermine Vicor Fares, Maha Farouk Abdelghany, Resources: Ahmed Mohamed Hemdan, Nermine Vicor Fares, Maha Farouk Abdelghany, Data Curation, Writing - Original Draft: Ragaa Magdy Ismail; Writing - Review and Editing: Nermine Victor Fares, Ahmed Mohamed Hemdan; Supervision: Maha Farouk Abdelghany, Ahmed Mohamed Hemdan, Nermine Victor Fares; Project Administration: Maha Farouk Abdelghany, Ahmed Mohamed Hemdan, Nermine Victor Fares.

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