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Research Article

Determination of Sacubitril and Valsartan Binary Mixture using Different Eco-friendly Spectrophotometric approaches with or without Regression Equations; greenness and whiteness assessments

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Abstract

One of the leading causes of illness and mortality worldwide is cardiovascular disease. The optimum therapy in the majority of cases is accomplished by combining several medications, each of which has a unique mechanism of action, to treat cardiovascular disease. Entresto® tablet combines Sacubitril (SAC) and Valsartan (VAL), It is proposed to lessen the risk of cardiovascular hospitalization and heart failure-related death in those with chronic heart failure and a lower ejection fraction. The aim that initiated the work was to develop Eco- friendly sustainable spectrophotometric methods that can be employed as an environmentally friendly alternative to the methods previously reported for SAC/VAL combinations. The suggested spectrophotometric methods are constant value (CV), concentration value (CNV), Absorption subtraction (AS), and Amplitude modulation (AM). Six evaluation tools were used to evaluate the greenness and whiteness of the developed methods to ensure their qualitative and quantitative safety to both humans and the environment; namely, National Environmental Methods Index, the analytical eco-scale, Green Analytical Procedure Index, complementary green analytical procedure index and the Analytical GREEnness metric, and by Whiteness tool for assessing analytical chemistry method. The suggested spectrophotometric methods were successfully applied to the studied mixture. Because the zero-order spectra were directly computed without the need for any separation stages, the established concentration value approach could identify both medications without the usage of regression equations. Additionally, depending on the iso-absorptive point, both analytes could be determined via the Absorbance Subtraction and Amplitude Modulation procedures using their same regression equation.

Keywords: *Sacubitril; Valsartan; Constant value; Concentration value; Absorbance Subtraction; Amplitude Modulation.*

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1. Introduction

The biggest leading causes of illness and mortality worldwide are the cardiovascular disease. The purpose of the therapy is accomplished by combining several medications, each of which has a unique mechanism of action, to treat cardiovascular disease **[1]**.

Valsartan, S)-3-methyl-2-(N-{[2'-(2H-1,2,3,4-tetrazol-5-yl)biphenyl-4-yl]methyl}pentanamido)butanoic acid, (VAL) blocks the vasoconstrictive and aldosterone-secreting effects of angiotensin II. This is done by preventing angiotensin II from attaching to angiotensin II type 1 receptors in various organs, such as the adrenal gland and vascular smooth muscle. Thus, its effect is independent of the angiotensin II biosynthesis routes. This reduces blood pressure and boosts the heart's blood and oxygen supply **[2]**.

The prodrug sacubitril, 4-{[(2S,4R)-1-(4-Biphenylyl)-5-ethoxy-4-methyl-5-oxo-2-pentanyl]amino}-4-oxo butanoic acid (SAC) is a neprilysin inhibitor that delays the breakdown of natriuretic peptides by acting as a neprilysin inhibitor when activated. Due to a rise in these peptide levels, sodium excretion reduces the volume of extracellular fluid and widens blood vessels **[3]**. By reducing pill load and dose frequency, antihypertensive combinations can enhance patient outcomes by increasing adherence **[4]**.

Entresto® tablet combines SAC and Valsartan VAL, It is proposed to lessen the risk of cardiovascular hospitalization and heart failure-related death in those with chronic heart failure and a lower ejection fraction **[4, 5, 6]**.

Many methods for determining both SAC and VAL in their combined dosage form were found, including HPLC **[7-12]**, LC-MS/MS **[13],** and spectrophotometry **[14-18]**. Most of these reported methods don't consider the environmental impact of the analytical procedure.

To unify the several terms for "green" or "environmentally friendly" analytical chemistry that had previously been used in isolation, the term "green analytical chemistry" (GAC) emerged at the turn of the millennium **[19]**. In the last decade, the idea of Green Analytical Chemistry has evolved into sustainable analytical chemistry **[20, 21]**, which combines GAC and quality by design concepts to argue that the technological advances made possible by GAC via the use of inexpensive, widely-available instrumentation and open-source software-should be made available to the general public.

From the viewpoint of GAC, when it comes to the utilization of instruments, the ideal capacity technique would allow direct and noninvasive measurement of samples, without the need for reagents or solvents, and can provide multiparametric information quickly and simply, all while requiring very little power. Spectroscopy is a quick-response analytical method because it allows for immediate examination with little to no sample preparation. The most environmentally friendly option to improve existing methods without adding any extra chemicals is to perform numerical treatment of signals collected using both basic instruments and even advanced ones **[22, 23]**.

Nowak *et al* **[24]** proposed the idea of White Analytical Chemistry (WAC) as a development of GAC. Green factors are important, but WAC also considers analytical (red) and practical (blue) elements that contribute to the overall quality of the approach. WAC is more in line with the concept of sustainable development because it seeks a compromise that prevents an unconditional rise in greenness at the expense of functioning.

The aim that initiated the work was to develop Eco- friendly sustainable spectrophotometric methods that can be employed as an environmentally friendly alternative to the methods previously reported for SAC/VAL combinations. Six evaluation tools were used to evaluate the greenness and whiteness of the developed methods to ensure their qualitative and quantitative safety to both humans and the environment; namely, National

Environmental Methods Index, the analytical eco-scale, Green Analytical Procedure Index, complementary green analytical procedure index and the Analytical GREEnness metric **[24-32]**, and by Whiteness tool for assessing analytical chemistry method **[33]**.

The suggested spectrophotometric methods are named, a constant value (CV), concentration value (CNV), Absorption subtraction (AS), and Amplitude modulation (AM). The validation of the methods was done according to the International Council for Harmonization (ICH) guidelines and the results were found to be appropriate and acceptable. Results obtained by the proposed methods and the reported methods were statistically compared and also the proposed methods were compared with each other's **[35]**.

Theoretical background

Constant Value **[36, 37]**, Concentration Value **[36, 38]**, Absorbance Subtraction method (AS) **[39]** Amplitude Modulation method (AM) **[39]**, and Ratio subtraction (RS) **[40]** are the method used for the determination of the studied drugs, the full theory presented in the supplementary material.

2. Experimental

2.1. Materials and reagents

Throughout the study, ethanol (HPLC grade) and distilled water were employed. Sigma-Aldrich provided the reference standards for sacubitril calcium and pure valsartan. For sacubitril calcium and valsartan, the purity was 99.10% and 99.98%, respectively. **Fig. 1** depicts the Chemical Structure of the substances under investigation.

Entresto® tablets (97/103 mg Sacubitril/Valsartan) manufactured by Novartis Pharmaceuticals, bought from the local pharmacy, in Cairo, Egypt.

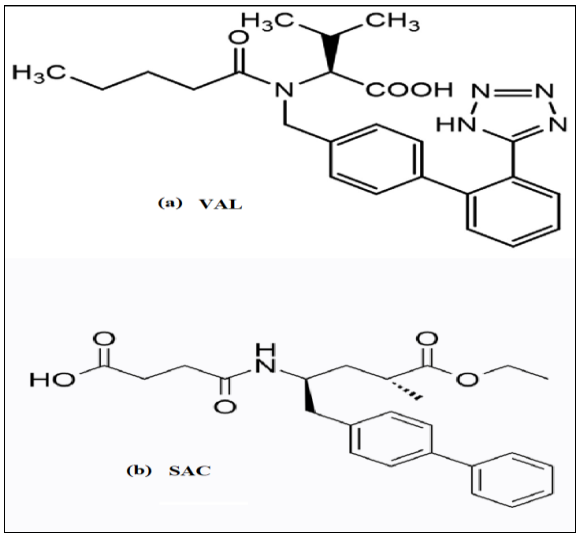


Fig.1. Chemical structures of a) valsartan and b) sacubitril.

2.2. Instruments and software

A double-beam UV/Visible spectrophotometer model J-760, Jasco, Japan was used. Using Spectra manager software, the absorption spectra of the reference solution and the test solution were captured in 1.0 cm quartz cells spanning the wavelength range of 200 nm-400 nm. Measurements were made between 200-400 nm.

2.3. Procedures

2.3.1. Standard Solutions

First, in 100-mL volumetric flasks stock solutions of the standard SAC and VAL were made by separately dissolving 20 mg of SAC and VAL in the 30 ml of ethanol, followed by adding distilled water to fill the remaining space to the mark. Then further dilution to the stock solution of both SAC and VAL was prepared using distilled water to create 100 µg/mL working solutions.

2.3.2. Spectral characteristics and wavelength selection

The Spectramanager program was used to scan against a blank spanning the range of 200

nm-400 nm and evaluate the absorption spectra of 5 µg/mL SAC and 5 µg/mL VAL individually as well as that of a lab combination having 2.5 µg/mL concentrations of each. The results are presented in **Fig. 2**.

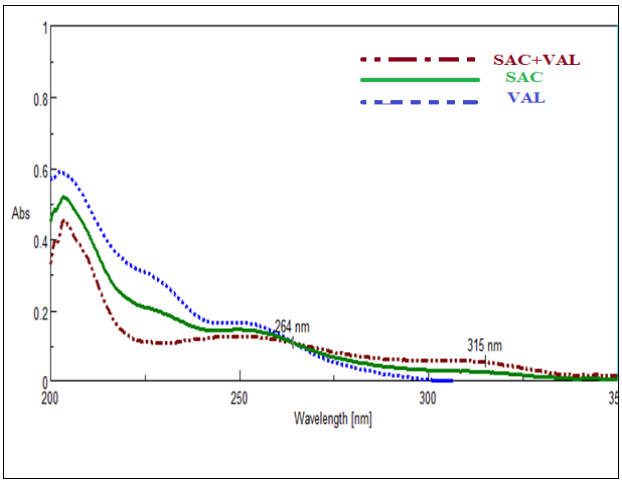


Fig. 2. Zero-order spectra of 5 µg /mL of sacubitril (____) and valsartan (-----), separately, and binary of a mixture of sacubitril and valsartan (- -- --), 2.5 µg /mL of each in ethanol and water.

2.3.3. Linearity

The calibration samples were constructed using the previously prepared stock solutions and diluted with distal water to cover a range from 1 to 30 µg/mL for SAC and VAL for all the proposed methods.

2. 4. Application to the pharmaceutical formulation

Ten Entresto® tablets were ground, and an exact weight equal to one tablet was measured out and placed in a 100-mL volumetric flask. 30 mL of ethanol was added, agitated for 15 min. using a magnetic stirrer, and then filtered. The solution reached the proper strength with water after the residue had been rinsed three times.

3. Results and discussion

In this paper, four distinct spectrophotometric techniques were used to investigate the binary mixture of SAC and VAL. CV and CNV are the two following techniques. In addition to being accurate and precise, these procedures also have the benefit of being straightforward. As will be discussed later, only one resolution step is required to resolve VAL from SAC. Time is saved, and the chance of error is decreased. When time-saving is required, the CNV approach is the best as no need for the calibration curve construction step, as CNV only depends on the graphical representation of data. For the instantaneous determination of this mixture utilizing the unified regression equation to determine both SAC and VAL, two additional progressive spectrophotometric methods (AS and AM) were created and verified. Progressive approaches rely on the iso-absorptive point (iso= 264 nm), and the calibration curve that connects the absorbance at iso to the relevant concentration yields the unified regression equation. **Fig. 2** shows that the (iso= 264 nm) is on the shoulder, which may impair the robustness of this approach. As a result, the robustness of the four generated methods was examined under the validation section, and the methods were proven to be robust. Both AS and AM methods use only a single equation to compute both drugs instantaneously from the same curve deprived of any resolution steps, and that is one of the main advantages of these methods.

3.1. Method development

3.1.1. Constant Value and Concentration Value for SAC

As seen in **Fig. 2**, SAC is stretched more than VAL. This results in a constant at the plateau region on the extended section from 300 to 350 nm; as seen in **Fig. 3** when the spectra of the mixtures of SAC and VAL are divided by the spectrum of the normalized divisor of SAC. This resulting constant is equivalent to the concentration of SAC. In the extended section, it is only connected to the concentration of SAC

according to the following equations:

VAL + SAC / SAC` =VAL/SAC` + SAC/SAC`

VAL + SAC / SAC` =VAL/SAC` + CONSTANT

The constant is equal to the concentration of SAC at this prolonged region when there is no contribution from VAL, while SAC' is a normalized divisor (1 µg/mL concentration).

To correct any errors, we build a calibration curve between the constant and the corresponding concentration, as previously mentioned, and the concentration of SAC is calculated from that calibration curve regression equation using the CV method. Using that constant, we can also easily calculate the concentration from the spectrum plateau region using the CNV method.

Both procedures have good recoveries when the outcomes of both methods are compared.

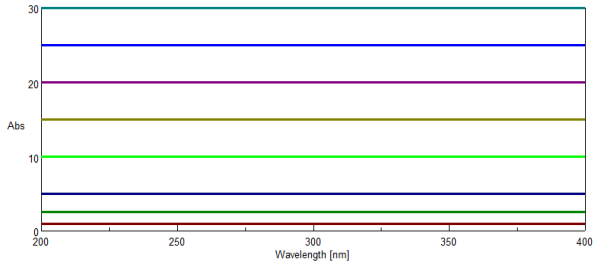


Fig. 3. The constant value obtained after the division of Zero order spectra of sacubitril (1 –30 µg /mL) by the spectrum of normalized 1 µg /mL divisor of sacubitril.

3.1.2. Ratio Subtraction Constant Value and Ratio Subtraction Concentration Value of VAL

At first, VAL was extracted by RS from SAC using the following equations:

- (VAL+SAC)/SAC` = VAL/SAC`+ SAC/SAC`, were SAC/SAC`= constant
- VAL/SAC`+ constant – constant = VAL/SAC`
- VAL/SAC`* SAC` = VAL

The resulting spectra are then divided by a normalized VAL spectrum divider. As a result, the constant found in the plateau region is connected to the concentration of VAL.as sown in **Fig. 4**.

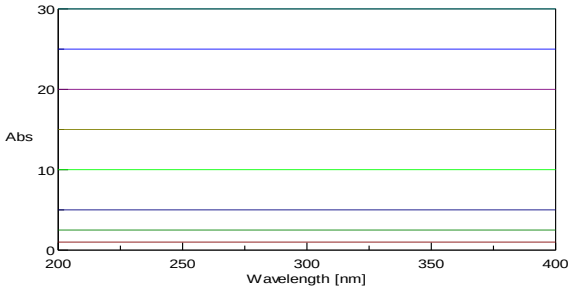


Fig. 4. The constant value obtained after the division of Zero order spectra of Valsartan (1 –30 µg /mL) by the spectrum of normalized 1 µg /mL divisor of Valsartan.

To remedy any errors, a calibration curve between a constant and its corresponding concentration is built for CV. The concentration of VAL is then determined from the calibration curve regression equation. While for CNV, that constant is used to directly extract the concentration from the spectrum plateau region. Both procedures have good recoveries when the outcomes of both methods are compared.

3.1.3. Absorption subtraction (AS) of both SAC and VAL

Absorbance subtraction is performed on the zero-order absorption spectrum, where a factor is determined by dividing SAC zero-order absorbance at 264 nm by its absorbance at 315 nm (A 264 nm / A 315 nm = 2.04), and a unified regression equation is developed at iso-absorptive point 264 nm (λ iso is shown in **Fig. 2**). The factor is multiplied by the free peak absorbance at 315 nm to determine the absorbance attributable to SAC at 264 nm. The absorbance of

VAL is calculated by subtracting SAC absorbance at 264 nm from the overall absorbance of the lab mixture; the concentration of SAC and VAL is then calculated by substituting both SAC and VAL absorbances in the unified regression equation.

3.1.4. Amplitude Modulation method (AM) of both SAC and VAL

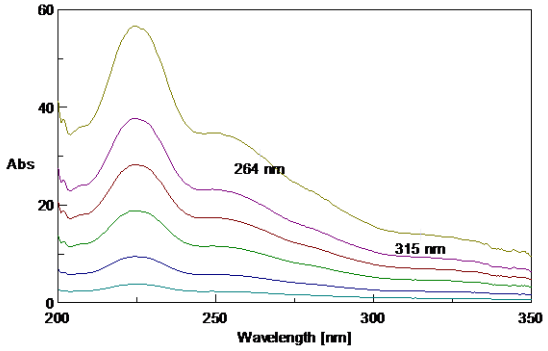


Fig. 5. Division spectra of laboratory-prepared mixtures (1-15) of sacubitril and valsartan using the normalized spectrum of sacubitril as a divisor.

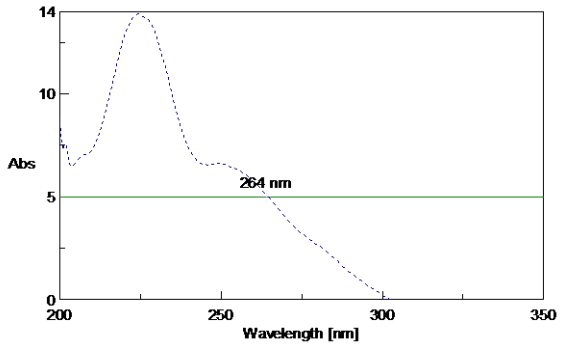


Fig. 6. Ratio spectra of 5 µg /mL of valsartan (-----) and sacubitril (____), separately using the normalized spectrum of sacubitril as a divisor.

Fig. 5 and 6 illustrate amplitude modulation on the radio spectrum following 1 µg/mL normalization of the SAC spectrum and construction of a unified regression equation at the isosbestic point 264 nm. The peak amplitude at the extended part of the division spectrum, as depicted in **Fig. 5**, **and 6**, is first substituted in the unified regression equation to obtain the concentration of the more extended SAC, and then subtracted from the amplitude at 264 nm, which is equivalent to the total laboratory prepared mixture, to obtain the peak amplitude of VAL at 264 nm, after which the concentration of VAL is determined by substitution in the unified regression equation.

3.2. Validation

According to ICH recommendations **[34]**, the proposed methodologies' validity was evaluated in terms of linearity, range, accuracy, precision, selectivity, robustness, LOQ, and LOD. **Table 1** displays each of the validation criteria.

3.2.1. Linearity, range, and calibration curves

The examination of six different concentrations covering a concentration range of 1 to 30 g/mL for SAC and VAL for CV, CNV, AM, and AS techniques, each repeated three times was used to test the linearity of the suggested method. The analysis was carried out under the previously indicated experimental conditions. **Table 1a & 1b** summarizes the linear equations.

The calibration range was chosen and created after taking into account the concentrations of SAC and VAL contained in the pharmaceutical formulations to provide accurate, precise, and linear findings, as well as the practical range required to obey Beer's law (**Table 1a & 1b**).

3.2.2. Determination in laboratory-prepared mixtures

To evaluate the methods' selectivity, different mixtures were created from the previously prepared stock solutions of SAC and VAL by accurately combining portions of the two analytes and transferring them to 10 mL volumetric flasks. The final volume is filled with water, and the results are displayed in **Tables 1a & 1b**.

Table 1a. Validation parameters of the proposed spectrophotometric methods, determination of the studied drugs in the laboratory-prepared mixtures

Drug	**sacubitril**			
Resolution technique	———————			
Method	constant value	concentration value	Absorbance subtraction	Amplitude modulation
Range µg/mL	1-30µg/mL	1-30µg/mL	1-30µg/mL	1-30µg/mL
Regression Equation	y = 0.9856x + 0.0894	-	y = 0.0213x - 0.0006	y = 0.984x + 0.1042
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9999
Accuracy (mean±SD) [a]	101.408±0.54	99.23±0.784	99.966±0.576	99.215±0.858
Intra-day Precision[b] RSD%	1.369	0.671	1.012	1.379
Inter-day precision[c] RSD%	1.369	0.671	0.731	1.379
LOQ (µg/mL)	1	1	1	1
LOD (µg/mL)	0.333	0.333	0.333	0.333
standard deviations of slope	0.0058	-	0.238	0.085
standard deviations of intercept	0.089	-	0.0839	0.005
The standard deviation of residuals, F	0.143	-	0.120	0.143
Robustness %RSD[d]	1.371	0.677	0.734	1.382
Selectivity[e]	100.02±1.414	100.21±0.176	100.25±0.883	99.98±0.471

a: three concentrations of each analyte were repeated three times for each concentration. ± SD

b: Intra-day (n=3), Average of three concentrations of the analytes (5,15, 30 µg/mL for sacubitril valsartan), repeated three times within the same day.

c: Inter-day (n=3), Average of three concentrations of the analytes (5,15, 30 µg/mL for sacubitril and valsartan), repeated three times on three different days.

d: The mean recovery ± RSD% of 5 laboratory-prepared mixtures containing different ratios (5,7,15,20, 30 µg/mL for sacubitril and valsartan).

e: RSD% of laboratory-prepared mixtures prepared using different concentrations of ethanol and to contain different ratios (5,15, 30 µg/mL for sacubitril and 5,15, 30 µg/mL valsartan).

Table 1b. Validation parameters of the proposed spectrophotometric methods, determination of the studied drugs in the laboratory-prepared mixtures

Drug	**valsartan**			
Resolution technique	RS	RS	———————	
Method	constant value	concentration value	Absorbance subtraction	Amplitude modulation
Range µg/mL	1-30µg/mL	1-30µg/mL	1-30µg/mL	1-30µg/mL
Regression Equation	y = 1.0014x + 0.0091	-	y = 0.0213x - 0.0006	y = 0.984x + 0.1042
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9999
standard deviations of slope	0.0058	-	0.238	0.085
standard deviations of intercept	0.097	-	0.0839	0.005
The standard deviation of residuals, F	0.143	-	0.120	0.143
Robustness %RSD[d]	1.071	0.804	0.552	0.920
Selectivity[e]	98.33±1.060	99.95±0.358	100.05±0.388	100±0.652

a: three concentrations of each analyte were repeated three times for each concentration. ± SD.

b: Intra-day (n=3), Average of three concentrations of the analytes (5,15, 30 µg/mL for sacubitril valsartan), repeated three times within the same day.

c: Inter-day (n=3), Average of three concentrations of the analytes (5,15, 30 µg/mL for sacubitril and valsartan), repeated three times on three different days.

d: The mean recovery ± RSD% of 5 laboratory-prepared mixtures containing different ratios of (5,7,15,20, 30 µg/mL for sacubitril and valsartan).

e: RSD% of laboratory-prepared mixtures prepared using different concentrations of ethanol and to contain different ratios of(5,15, 30 µg/mL for sacubitril and 5,15, 30 µg/mL valsartan).

3.2.3. Accuracy

For accuracy testing of the devised procedures, three replicates of SAC and VAL samples at various concentrations were used. Except for CNV, where the concentration was derived directly from the graph without the requirement for a calibration curve, the concentrations were determined from the respective regression equations for each method. As indicated in **Tables 1a & 1b**, the percentage recoveries demonstrate the suggested approaches' commendable accuracy.

3.2.4. Intermediate precision

The proposed approaches were used to examine three inter-dailies on three different days. For the examination of the three selected concentrations of both SAC and VAL (5, 15, 30 µg/mL). Calculations were made for the relative standard deviations. **Table 1a & 1b** shows the outcome.

3.2.5. Repeatability

The proposed approaches were used to examine three intra-daily concentrations of SAC and VAL (5, 15, 30 µg/mL). Calculations were made for the relative standard deviations. **Table 1a & 1b** shows the outcome.

3.2.6. Limit of quantitation and limit of detection:

The quantitation and detection limits can be determined in many ways, per ICH recommendations. LOD and LOQ were calculated using the sloping approach and intercept standard deviation, where:

LOD = 3.3 x-intercept standard deviation/slope coefficient, LOQ = 10 x SD of the intercept/slope coefficient.

3.2.7. Robustness

As we mentioned before, for the SAC and VAL methods, the (λ_{iso}= 264 nm) is on the shoulder, so it may affect the robustness of these methods, for this reason, the robustness of the developed four methods was tested.

Three concentrations of each analyte (5, 15, 30 µg/mL) were prepared using different concentrations of ethanol (80%, 60%, 40%, and 20%) and analyzed three times using the proposed methods. The percent relative standard deviations were found to be below 2.0% as shown in **Table 1** and the methods proved to be robust.

3.3. Application

The methods were successfully used for the determination of SAC and VAL in Entresto® tablet dosage form and results were presented in **Table 2**.

Table 2. Determination of Sacubitril and Valsartan in pharmaceutical dosage form by the proposed methods

Drug	**sacubitril**			
Resolution technique	———————			
Method	constant value	concentration value	Absorbance subtraction	Amplitude modulation
Recovery of Pharmaceutical Dosage form [f]	99.98±1.35	100.01.085	99.85±0.383	100.12±0.751
Drug			valsartan	
Resolution technique	RS	RS	———————	
Method	constant value	concentration value	Absorbance subtraction	Amplitude modulation
Recovery of Pharmaceutical Dosage form[f]	99.53±1.02	99.89±1.021	100.04±0.88	100.12±0.652

f: Entresto® commercial tablet (97/103 mg Sacubitril/Valsartan) from Novartis Pharmaceuticals

3.4. Greenness and whiteness Assessment

The evaluation of analytical methodologies of the proposed methods was done from the perspective of green, white, and sustainable chemistry using six different environmental green and white evaluation tools **[24-34]** without compromising the validation criteria and requirements. Different evaluation tools and techniques have been used to ensure the greenness of the whole protocol and method qualitatively and quantitatively starting from sampling ending to the way we handle the waste,

The first one is qualitative methods National Environmental Methods Index (NEMI), the second method is Analytical Eco-scale is a method considered to give a semi-quantitative data by calculating penalty points of all aspect of methodology then subtracted from a base of 100. Another evaluation tool named Green Analytical Procedure Index (GAPI) was developed by Potka-Wasylka in 2018 **[27]**. That method is capable to deliver a complete evaluation of the entire analytical process, early from the sample gathering to the end of the study. It consists of pictograms and the color of the pictogram parts may be green, yellow, or red; green color means a safe procedure while red refers to non-green. The results show that the methods are considered to be green as shown in **Tables 3a, 3b, and 3c**.

Table 3a. green assessments comparison and results of the proposed methods using ECO-SCALE, and NEMI

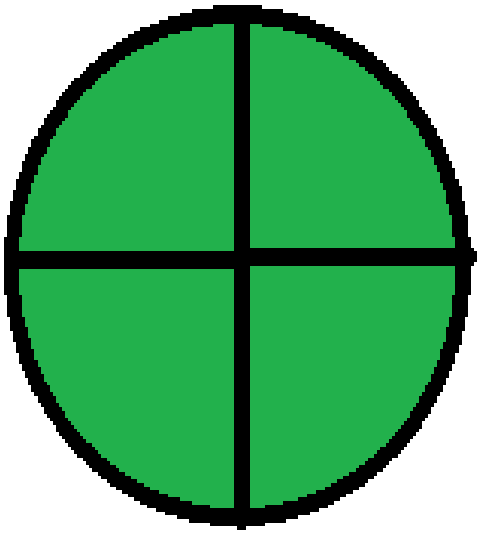
National Environmental Methods Index	***Analytical Eco-scale***	
	Reagents	PPs
	Ethanol	
	Amount <10	1
	hazardous	2
	Instruments	
	spectro-photometric	(Energy and Occupational Hazard) 0
	Waste	1
	Total PPs	4
	Score	96
the analytical methods meet all the requirements, they were fully green analytical methods	the score over 75 so the methods were excellent green analytical methods	

Table 3b. Green assessments comparison and results of the proposed methods using GAPI, and AGREE

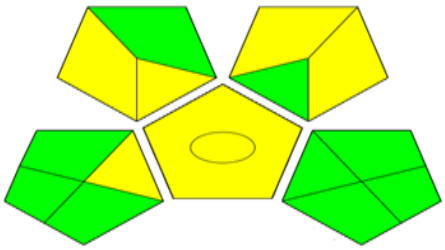
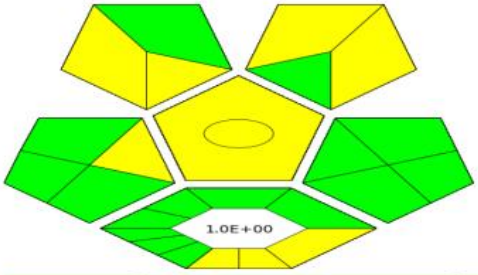
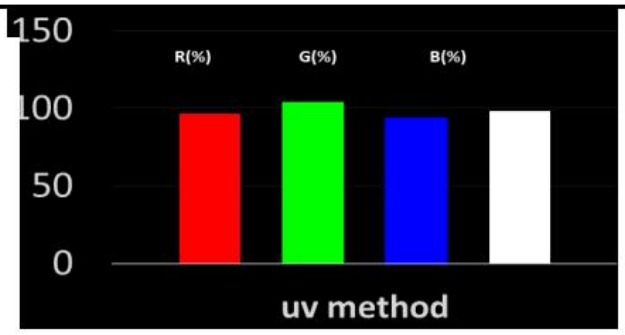
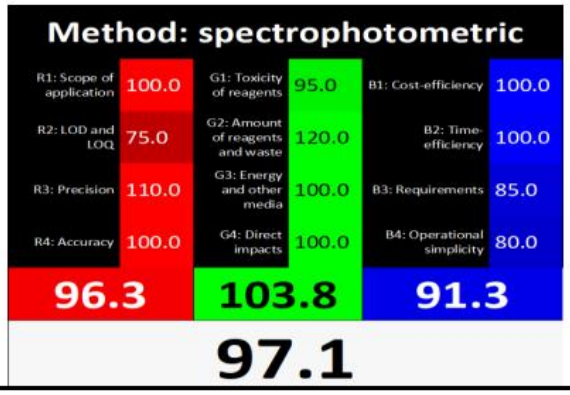
Green Analytical Procedure Index (GAPI)	***Analytical GREEnness metric (AGREE)***
	
1:collection of samples: online	1: sample treatment in(on-line analysis).
2:preservation: N/A 3:transport:N/A	2: Minimal Sample Size and Minimal Number of Samples (1mL) 3:In Situ Measurements (ON-LINE)
4:storage:N/A	4:Integration of Analytical Processes and Operations Save Energy and Reduce the Use of Reagents (only one step required)
5:type of method: simple procedures require like filtration	5:Automated and Miniaturized Methods Should Be Selected.(automatic, miniaturized)
6:scale of extraction: Nano extraction	6:Derivatization (no Derivatization needed)
7: solvent and reagent: ethanol green solvent	7:Volume and management of Analytical Waste: (1mL)
8: additional treatment (not required)	8: Multianalyte or Multiparameter Methods Are Preferred versus Methods Using One Analyte at a Time
9:amount of reagent :1 ml	9:The Use of Energy Should Be Minimized (<0.1 kWh per sample UV−vis spectrophotometry)
10:health hazard: Moderately toxic; ; NFPA = 2	10:Reagents Obtained from Renewable Source Should Be Preferred (ethanol is the only used reagent)
11: safety hazard: Highest NFPA flammability	11:Toxic Reagents Should Be Eliminated or Replaced (no toxic reagent used)
energy:<0.1 kWh per	12:The Safety of the Operator Should Be Increased
13:occupational hazard: No vapors	
14:waste: 1ml reused	
15: waste treatment: reused	the score of 0.88 indicates that the spectrophotometric methods were green

Table 3c. Green assessments comparison and results of the proposed methods using Complementary green analytical procedure index & Whiteness Assessment (RGB12 Model)

Complementary green analytical procedure index	Whiteness Assessment (RGB12 Model)
 	 

Another two smart and simple methods Analytical GREEnness metric (AGREE) developed In June 2020, as a new technique has been proposed using innovative, smart greenness assessment software named AGREE, And Complementary green analytical procedure index expands on the well-known green analytical procedure index by adding additional fields about the processes performed before the analytical procedure itself. Each field of the hexagon that was added to the GAPI pictogram corresponds to a different aspect of the described process and is colored green if certain requirements are met also to facilitate the use of this tool; freeware software has been created for generating the Complex GAPI pictogram hat considered an advantage over GAPI.

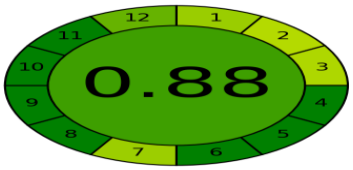
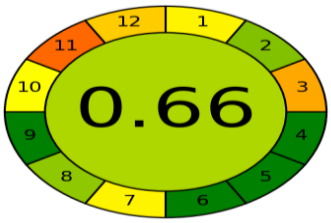
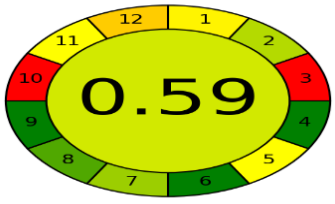
The last method named Whiteness Assessment (RGB12 Model) **[24]** is an evaluation tool of analytical methods provided via the recently introduced Red-Green-Blue (RGB) model which is a quantitative evaluation tool that provides sustainability by calculating the whiteness of the analytical method. Red is assigned to analytical efficiency as expressed by validation criteria such as accuracy, precision, LOD, sensitivity, and others), and green stands for compliance with GAC principles related to environment safety such as toxicity of the reagents, number and amount of reagents used, and waste generated during the complete process, energy used and the total impact on the environment, while blue represents productivity and practice with economic efficiency including

the cost, time, least practical requirements, and operational simplicity.

Table 2 shows an illustration and comparison between the six evaluation tools used for the assessment of the environmental impact of the developed methods and also shows the results of the assessments.

Also, a comparison between the proposed spectrophotometric method and reported methods using AGREE assessment show that the developed method is the highest in the score of greenness assessment as presented in **Table 4**.

Table 4. Comparison between the greenness profile of the developed and reported methods using AGREE

method	Analytical GREEnness metric (AGREE)
PROPOSED spectrophotometric methods	
Reported method (200)	
Reported method (188)	
Reported method (189)	
Reported method (95)	

Statistical comparison

The developed spectrophotometric methods were statistically compared with the reported one to ensure that there was no significant difference in the determination of both SAC and VAL **[14]**, results were presented in **Table 5** Also, the results of the developed methods were compared using One-way ANOVA and the results were presented in **Table 6**.

Table 5. Statistical comparison of the results obtained by the proposed methods and the reported method [14] for the analysis of sacubitril and valsartan in bulk powder

Drug	Method	Mean	S.D	N	Variance	Student's t-test(2.23)[a]	F test (7.15)[a]
sacubitril	constant value	100.408	0.540	6	0.292	1.271	1.226
	concentration value	99.230	0.784	6	0.615	2.049	1.719
	Absorbance subtraction	99.966	0.576	6	0.332	0.071	1.078
	Amplitude modulation	99.215	0.858	6	0.736	1.815	0.486
valsartan	RS-constant value	99.496	1.020	6	1.040	0.881	1.020
	concentration value	100.117	1.020	6	1.040	0.179	1.020
	Absorbance subtraction	98.870	0.912	6	0.832	2.056	1.226
	Amplitude modulation	99.466	0.998	6	0.996	0.942	1.024
sacubitril	Reported method[b]	99.990	0.598	6	0.358	———	———
valsartan	Reported method[b]	100.012	1.010	6	1.020	———	———

[a] The values in parenthesis are the corresponding theoretical values of t and F at p=0.05.

[b] Method **[14]**

Table 6. Results of ANOVA (single factor) for comparison of the proposed methods for the determination of sacubitril and valsartan in pure powder form

Source of Variation		SS *	df **	Variance	F b	P-value	F crita
SAC	Between Groups	2.437	3	0.8123	0.7657	0.5266	3.0984
	Within Groups	21.220	20	1.061			
	Total	23.657	23				
VAL	Between Groups	5.128	3	1.7092	1.998	0.1467	3.0984
	Within Groups	17.109	20	0.8555			
	Total	22.237	23				

[*] Sum of squares

[**] degree of freedom between and within groups

[a] Critical (tabulated) value for F at p=0.05

[b] Calculated F

Conclusions

This work presented different spectrophotometric methodologies for the analysis of binary severely overlapped mixtures without prior separation and the need for any special operations. The work involves a novel smart method that determines both SAC and VAL for the first time using simple steps and a unified equation with the need for only one specific requirement in the spectrum (the presence of an iso-absorptive point) as there is no need to have an extended part like most methods. The developed methods are simple, and accurate without requiring preceding treatment nor complicated steps or the use of organic harmful solvents like that usually used in the reported methods. Six evaluation tools were used for the first time to confirm the safety, sustainability, eco-friendliness, and cost-effectiveness of the approaches, indicating that the methods are regarded as green and sustainable.

To determine the mixture of SAC and VAL, the developed spectrophotometric methods were effectively applied for the determination of the studied drugs with accepted validation parameters. Because the zero-order spectra were directly computed without the need for any separation stages, the established CNV approach can identify the binary medications without the usage of regression equations. Additionally, depending on the iso-absorptive point, both analytes could be determined via the Absorbance Subtraction and Amplitude Modulation procedures using their same regression equation. The suggested methods' minimal steps reduced analysis errors and enabled them to be applied to routine analysis of the combination in quality control laboratories.

Declarations

Consent to publish

All authors have read and agreed to the published version of the manuscript

Ethics approval and consent to participate

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article in the main manuscript.

Competing interests

No competing interests were declared by the authors

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Authors' contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by [raga magdy], [ahmed hemdan], and [nermine v. fares]. The first draft of the manuscript was written by [raga magdy] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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