Analytical Chemistry Letters

ISSN: 2229-7928 (Print) 2230-7532 (Online) Journal homepage: http://www.tandfonline.com/loi/tacl20

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To cite this article: Hayam M. Lotfy, Yasmin Mohammed Fayez, Eman M. Morgan, Engy Shokry & M. Abdelkawy (2016) Comparative Study of the Spectral Resolution Efficiency of the Recently Developed and Conventional Spectrophotometric Methods in the Analysis of Severely Overlapped Zero-Order Absorption Spectra with the Same Geometrical Features, Analytical Chemistry Letters, 6:5, 540-560, DOI: 10.1080/22297928.2016.1232176

To link to this article: http://dx.doi.org/10.1080/22297928.2016.1232176

Published online: 28 Oct 2016.

Taylor & Francis
Taylor & Francis Group

ISSN Print: 2229-7928
ISSN Online: 2230-7532

Comparative Study of the Spectral Resolution Efficiency of the Recently Developed and Conventional Spectrophotometric Methods in the Analysis of Severely Overlapped Zero-Order Absorption Spectra with the Same Geometrical Features

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Received 09 June 2016; accepted in revised form 01 September 2016

Abstract: Simple, cost-effective, safe, accurate, precise and environmentally friendly spectrophotometric methods were developed and validated for the quantitative determination of valaciclovir (VAL) in the presence of its related impurity in bulk powder and in its pharmaceutical preparation. This related impurity namely guanine (GUA) is the potential and synthesis impurity of VAL. The spectra of VAL and GUA show the same geometrical features with different absorptivities, so their resolution is very challengeable. A Comparative study was conducted for the results of the conventional methods namely, dual wavelength (DW), first derivative of ratio spectra (^{1}DD) and mean centering of ratio spectra (MCR) versus the recently developed methods namely, induced dual wavelength (IDW), ratio difference (RD) and constant center (CC). The optimized methods allow the estimation of VAL in the concentration range 5-50 µg/mL. The methods were validated as per ICH guidelines and the specificity was assessed by analyzing synthetic mixtures containing different percentages of the related impurity with the drug. The obtained results were compared with that of the official HPLC method by using one-way analysis of variance (ANOVA) and proved to be suitable for quality control laboratories.

Keywords: Valaciclovir; Guanine; Induced dual wavelength; Ratio difference; Constant center.

Introduction

Chemically, Valaciclovir (VAL) known as 2-(2-Amino-6-oxo-1,6-dihydro-9H-purine-9-yl) methoxyethyl L-valinate hydrochloride [1] (Figure 1) is an antiviral drug used in the treatment of herpes zoster (shingles) and herpes simplex infections of the skin and mucous membranes, including genital herpes [2]. VAL is a prodrug, an esterified version of aciclovir which has the potent antiviral activity. Aciclovir, the active metabolite of VAL is slowly and incompletely absorbed from the human gastrointestinal tract (GIT). VAL is absorbed rapidly and extensively from the human GIT therefore, has a greater oral bioavailability (about 55 %) than aciclovir (10-20 %) [3].

Valaciclovir (VAL) is an official drug in the British Pharmacopoeia [1]. Few analytical methods have been reported for the quantitative estimation of VAL in pharmaceutical formulations and

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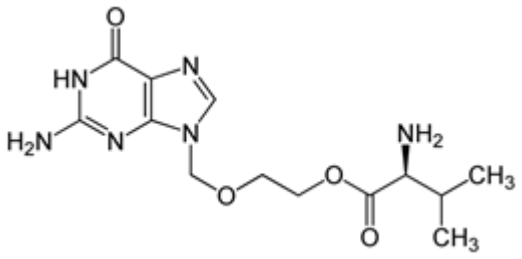


Fig. 1. Chemical structure of Valaciclovir

in body fluids. These methods include colorimetric methods using 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone [4], wool fast blue dye [5], diazotization coupled with resorcinol [6], MBTH (3-methyl-2-benzothiozolinone hydrazone) with ferric chloride Fe(III), MBTH with sodium periodate ($NaIO_4$) [7], para dimethyl amino benzaldehyde (PDAB) in acidic condition [8], 1,2-napthaquinone-4-sulfonic acid sodium (NQS) in alkaline media [9], phenyl hydrazine hydrochloride (PHH) in the presence of hexacyano ferrate in acidic condition and 1,10-phenanthroline [10], para dimethyl amino benzaldehyde (PDAB) and vanillin [11], high performance liquid chromatographic methods [12-21] , electrochemical method [22] and electro-spray ionization mass spectrometry [23].

Related impurity is the simplest unwanted constituent in a pharmaceutical agent; it may be formed by the degradation of the pharmaceutical agent itself or through an interaction or reaction of the active ingredient in a formulation with one of the other constituents in a dosage form, it can also be brought into the drug product through the formulation process. Hence, it has generally closely related structure to the drug molecule [24].

The most common degradation pathways encountered involve the oxidation or hydrolysis with/without thermal stress to the drug molecule. Generally, the level of a degradation product will increase with time. Forced degradation study (Stress testing) is a well-established method to identify the possible degradation impurities. Stress testing is necessary to identify the actual degradation impurities in an active pharmaceutical ingredient, which is considered as potential impurities, while synthesis impurity is the one that can originate during its synthetic process from raw materials. The presence of these unwanted chemicals, even in small amounts, may influence the efficacy and safety of the pharmaceutical products [25].

Valaciclovir (VAL) was reported to give aciclovir upon alkaline hydrolysis which subsequently undergoes further degradation to give guanine (GUA) [26] while upon acidic degradation it gives guanine (GUA) therefore, GUA is the acid induced degradation product obtained due to stress condition and is considered as a potential impurity of VAL [21, 27-31]. In addition, guanine is considered as a synthesis impurity of VAL [32, 33].

Literature survey revealed that no spectrophotometric methods were reported for the determination of VAL in the presence of its related impurity. Therefore, this work is focused on the development of spectrophotometric methods for the determination of VAL in presence of GUA as related impurity either potential or synthesis impurity of VAL which is reported to be pharmacologically inactive in order to complete the impurity profile of VAL, which is now receiving a critical attention from the regulatory authorities.

The structure similarity of VAL and GUA leads to that their spectra are very close irrespective to their absorptivities. Therefore, their resolution becomes challengeable. The severely overlapped spectra hinders the spectrophotometric determination of VAL by most of the conventional methods and even the applicable ones show poor recovery results upon analysis of synthetic mixtures containing more than 70 % of the related impurity.

Several analytical approaches were applied for resolving mixtures in order to obtain accurate, precise and safe results. Among the previous years, manipulating ratio spectra techniques attracted the attention of analysts who are interested in the quantitative spectrophotometric resolution of complex mixtures having overlapped spectra, such as: ratio difference (RD) method [34-37], ratio subtraction and extended ratio subtraction (EXRS) method [34, 38-40], constant center (CC) method [39, 41, 42], successive spectrophotometric resolution technique (SSRT) [43, 44], amplitude modulation (AM) [45, 46] and mean centering of ratio spectra (MCR) method [47-49].

Derivative spectrophotometry has been applied as the manipulating tool for ratio spectra in dif-

ferent forms. Salinas *et al*. [50] introduced the derivative of ratio spectra for binary mixtures [51-53]. Then Berzas Nevado *et al*. [54] introduced the derivative of ratio spectra using zero-crossing point (DR-ZC) for ternary mixtures [55, 56]. In addition, Dinc and Onur [57] developed the derivative of ratio spectra using double-divisor (DR-DD) applied for ternary mixtures [56, 58, 59]. Then Afkhami and Bahram [60] developed the successive derivative of ratio spectra (SDR) for the analysis of ternary mixtures [61]. Merging between derivative of ratio spectra area under curve (AUC) method has been generated for the analysis of ternary mixtures using Cramer's rule [62].

The ultimate goal of this study is to develop and conduct simple, rapid, selective, low cost and less time-consuming spectrophotometric methods to obtain results with more and more precision and accuracy and at increasingly lower concentration levels of the substances being determined. The novelty of the present work is to conduct a comparative study that compares the resolution efficiency of the recently developed spectrophotometric methods namely induced dual wavelength (IDW), ratio difference (RD) and constant center (CC) versus the conventional methods namely dual wavelength (DW), first derivative of ratio spectra (^{1}DD) and mean centering of ratio spectra (MCR).

Theory

Induced dual wavelength (IDW) method

This method can be applied for a drug and its related impurity of X and Y with complete overlapped zero order absorption spectra at two wavelengths λ_1 and λ_2, where the absorbance of the related impurity between these two wavelengths were not equal (absorbance difference does not equal zero), or by applying the conventional dual wavelength method the recorded absorbance values at the two selected wavelengths were two small, or they were two close to each other; thus leads to very small difference and therefore, leads to high error. By applying this method, we chose the two wavelengths, which have high absorbance values and large difference between them to get maximum accuracy and sensitivity. The concentration of X was calculated using the regression equation (obtained by plotting the absorbance difference values of the zero order spectra of X at the two chosen wavelengths ($\Delta A = A_1 - F_Y A_2$) against their corresponding concentrations X). The concentration of Y was calculated using the same procedure, which can be done to calculate the concentration of Y equality factor of pure X at the two chosen wavelengths (F_X).

Experimental

Apparatus and software

- A double beam UV / VIS spectrophotometer (Shimadzu, Japan) UV / VIS model UV-1800 PC with quartz cell of 1cm path length. The spectral band width was 2 nm and the wavelength scanning speed was 2800 nm/min.
- An IR spectrophotometer (Shimadzu 435, Kyoto, Japan) sampling was undertaken as potassium bromide discs.
- A mass spectrophotometer: MS-QB 1000 EX, Finnigan Nat (USA).
- TLC-plates (20 cm × 20 cm) coated with silica gel 60 F254 (Merck, Germany).
- Software: All computations regarding MCR method were performed in Matlab (Natick, MA) for Windows™ Version 6.5 [63].

Materials and solvents

Pure sample

Valaciclovir (VAL) pure sample was kindly supplied from the National Organization for Drug Control and Research (NODCAR), Egypt. Its purity was found to be 99.92 ± 0.66 according to the official HPLC method [1]. Guanine (GUA) was kindly supplied from oxford laboratory, Egypt. Batch No. 4510. Its purity was certified to be 98.00%.

Market sample

VALTREX® tablets were labeled to contain 500 mg valaciclovir manufactured by GlaxoSmith-Kline (GSK), Spain. Batch No. 2121A.

Solvents

All chemicals used throughout this work were of analytical grade

- Concentrated hydrochloric acid solution (Adwic-Egypt) used to prepare 0.1M, 0.2M & 2M HCl in distilled water.
- Sodium hydroxide pellets (Adwic-Egypt) used

to prepare 2M NaOH in distilled water.
- Isopropanol (SDFCL-India).
- Concentrated ammonia solution (Teba-Egypt).
- De-ionized water (Egypt Otsuka Pharmaceutical Co., S. A. E. - Egypt).

Standard solutions

Stock standard solutions

Stock standard solution of VAL and GUA (1 mg/mL) was prepared in 0.1M HCl.

Working standard solution

The solutions were freshly prepared by dilution from the stock solutions with the same solvent mixture to obtain a concentration (100 µg/mL).

Procedures

Preparation of GUA by acid induced degradation method

Ten milligrams of VAL powder were heated with 10 mL 2M HCl solution in a thermostatic water bath at 80°C for eight hours (Figure 2). The solution was neutralized with 2M NaOH solution (pH=7.0). Complete degradation was confirmed by TLC through the disappearance of the drug spot using isopropanol: ammonia: water (14:2:4 by volumes) as a developing system and the R_f of the related impurity was compared with that of standard guanine. The related impurity was separated as a band on TLC plates using the same developing system and the band was scratched and dissolved in methanol. The solution was stirred, filtered and then the solvent was allowed to evaporate. The separated related impurity was subjected to IR and mass spectral analysis for subsequent identification.

Spectrophotometric methods

Spectral characteristics

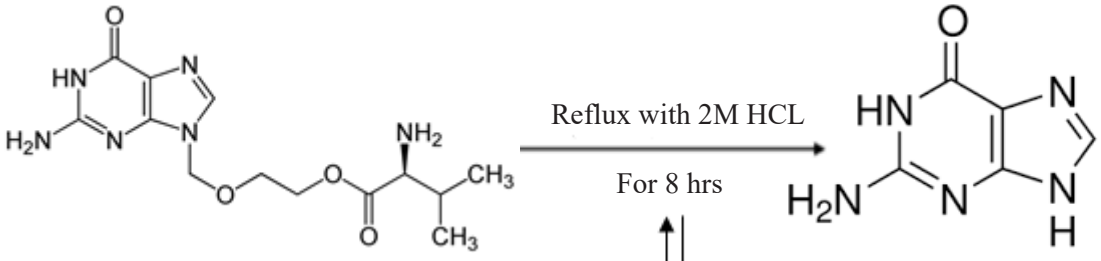
The absorption spectra of VAL and GUA in 0.1M HCl were recorded over the range 200-400 nm using 0.1M HCl as a blank as shown in **(**Figure 3).

Construction of calibration graphs

Accurately measured aliquots of working standard solution containing 50-500 µg VAL were transferred into a series of 10 mL measuring flasks and the volume was completed using 0.1M HCl. The absorption spectra of the resulting solutions were scanned and stored in the computer.

Dual wavelength (DW) method

The absorbance of the stored spectra were measured at 240 nm and 256.8 nm. Calibration graph was constructed by plotting the difference between the recorded absorbance versus the corre-



Valaciclovir (VAL)
Mol.wt = 324.336 g/mol

Guanine (GUA
Mol.wt = 151.13 g/mol
Keto Form

Guanine (GUA)
Mol.wt = 151.13 g/mol
Enol Form

Fig. 2. Proposed scheme for preparing VAL related impurity, GUA.

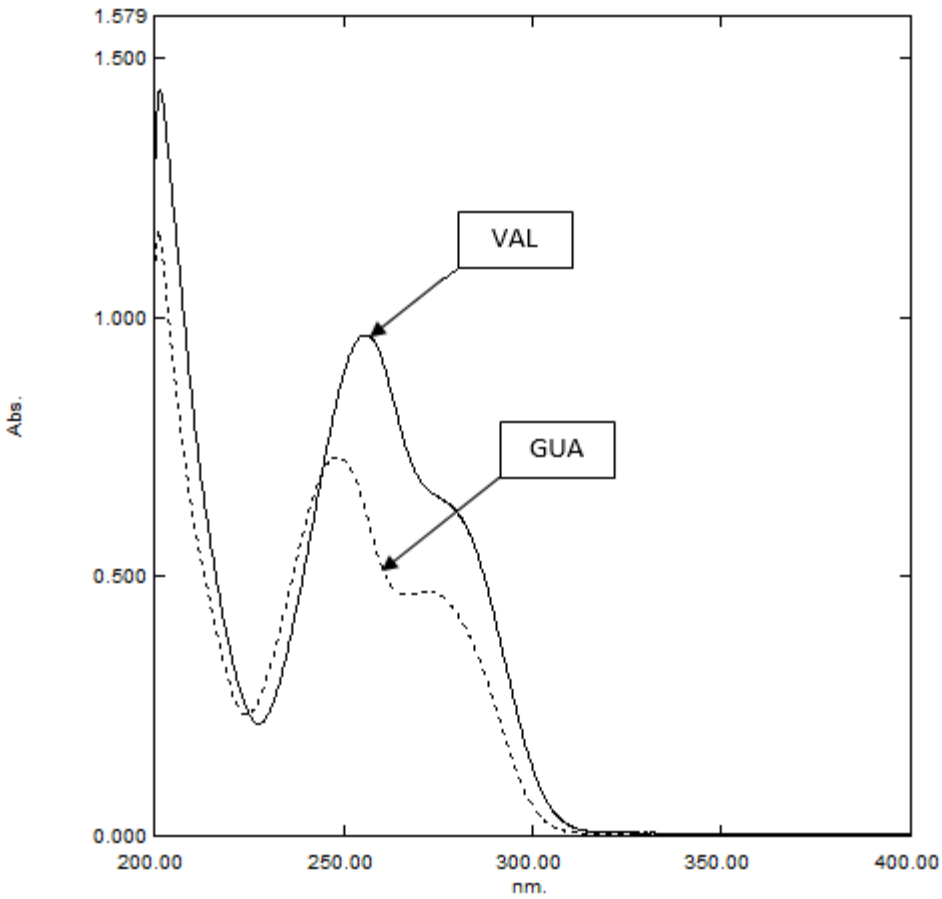


Fig. 3. Zero order absorption spectra of 30 µg/mL VAL (–) and 10 µg/mL of its related impurity GUA (….) in 0.1M HCl.

sponding concentrations and the regression equation was computed.

Induced dual wavelength (IDW) method

The equality factor (F) was calculated which, is the average of the ratio between the recorded absorbance of different concentration of GUA at 227 nm and 256 nm. The absorbance of the stored spectra was measured at 227 nm and 256 nm and calibration graph was constructed by plotting the difference between the recorded absorbance after multiplication by the equality factor (F) (A_{256} × F) - A_{227} versus the corresponding concentrations and the regression equation was computed.

First derivative of ratio spectra (^{1}DD), Mean centering of ratio spectra (MCR), Ratio difference (RD) and Constant center (CC) methods

The stored absorption spectra were measured and divided by the absorption spectrum of 10 µg/mL of GUA (as a divisor) to obtain ratio spectra.

For ^{1}DD method: The amplitude of the first derivative values of the ratio spectra were calculated using peak to peak graphical measurement (P_{max} - P_{min}) at 256 nm - 266.4 nm using Δλ=4.00, scale factor =10. Calibration graph representing the relationship between the measured amplitudes (P_{max}-P_{min}) and the corresponding concentrations of VAL was constructed and the regression equation was computed.

For MCR method: The obtained ratio spectra were mean centered using matalab. The mean-centered values at 252 nm for VAL were plotted versus the corresponding concentrations and the regression equation was computed.

For RD method: Calibration graph was constructed by plotting the difference between the amplitudes of the obtained ratio spectra at 241.4 nm and 261.4 nm versus the corresponding con-

centrations and the regression equation was computed.

For CC method: Calibration graph was constructed by plotting the difference between the amplitudes of the obtained ratio spectra at (241.4 nm and 261.4 nm) versus the amplitudes at 261.4 nm and the regression equation was computed. Another calibration graph was constructed by plotting the absorbance at 256 nm versus the corresponding concentrations and the regression equation was computed.

Assay of synthetic mixtures

Accurate aliquots of working solution (100 µg/mL) of VAL and its related impurity (GUA) were transferred into a series of 10 ml volumetric flasks, completed to volume with 0.1M HCl and mixed well in order to obtain different mixtures containing different ratios. The absorption spectra of different synthetic mixtures (10 - 90 %) were scanned then proceed as detailed in each method. The concentrations of VAL in the mixtures were calculated from the corresponding regression equations.

Assay of pharmaceutical formulation

Twenty tablets of VALTREX® tablet were powdered and mixed well; an accurately weighed amount of the powder equivalent to 0.01 g of VAL was transferred into 100 mL beaker, 50 mL of distilled water was added, mixed well, centrifuged for 30 minutes, filtered and quantitatively transferred into 100 mL measuring flask then completed to the mark to get a final concentration of 100 µg/mL. Make appropriate dilution using 0.2M HCl to get a final concentration of 50 µg/mL. Proceed as mentioned in each method. The concentrations of VAL were calculated from the corresponding regression equations.

Results and discussion

This work introduced an application of a comparative study of different spectrophotometric methods for the determination of VAL in the presence of its related impurity (GUA) in its authentic powder and in pharmaceutical dosage form which can be a stability indicating methods.

Different solvents were tried such as De-ionized water, methanol, ethanol, 0.1M HCl and 0.1M NaOH. It was found that 0.1M HCl was the solvent of choice which could be used for the determination of VAL in the presence of GUA taking in to account the poor solubility of GUA in organic solvents and the poor stability of VAL in higher alkaline medium (1M NaOH) and in higher acidic medium (2M HCl). The stability of VAL in 0.1M HCl was monitored and it was found that the stock solution was stable up to 5 days at 5°C.

Valaciclovir undergoes hydrolysis under acidic conditions according to the previously reported study [27] without any structural elucidation of the products. This work was extended to confirm such investigation where complete acid induced degradation was conducted upon refluxing of pure VAL with 2M HCl for eight hours at 80°C. The potential degradation product was isolated and showed one new spot at R_f = 0.580, which is different from that of the intact drug R_f = 0.654 and is the same as that of standard guanine. The IR spectrum of intact VAL reveals a stretching band at 1750 cm^{-1} which disappears in the IR spectrum of the potential impurity, thus confirming the cleavage at ester linkage and appearance of carbonyl group of acid at 1650 cm^{-1} also, there is a broad band appears at 3000 cm^{-1} which indicates the tautomeric form of GUA (enol form) [64] (Figure 4). In the MS chart, the parent peak of isolated potential impurity was identified at 151 *m*/*z* (molecular weight of guanine) while that of the intact VAL was identified at 324 *m*/*z* (molecular weight of VAL) (Figure 5).

The International Conference on Harmonization (ICH) guideline entitled "stability testing of new drug substances and products" requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance [65]. An ideal stability indicating method is the one that quantifies the standard drug alone and resolves its degradation product.

Spectrophotometric methods

Dual wavelength (DW) method

The pre requisite parameter for applying dual wavelength method is the selection of two wavelengths where the interfering component shows same absorbance whereas the component of interest shows significant difference in absorbance with concentration, which is directly proportional to the concentration of the component of interest

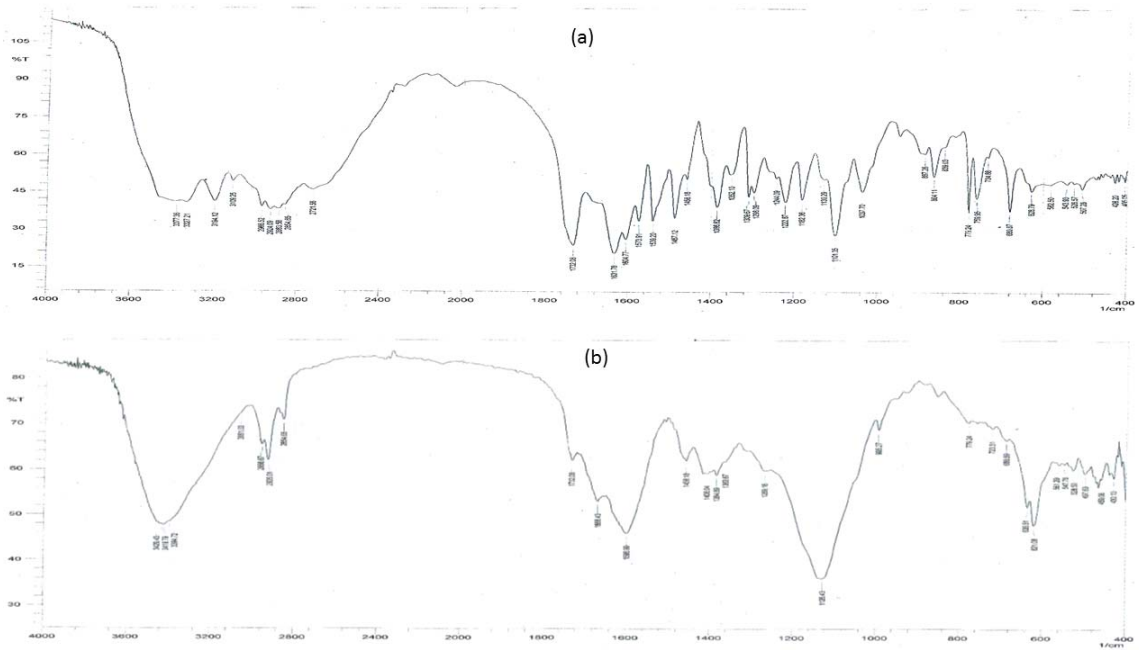


Fig. 4. IR spectra of intact drug VAL and its prepared related impurity, GUA in (a) and (b) respectively

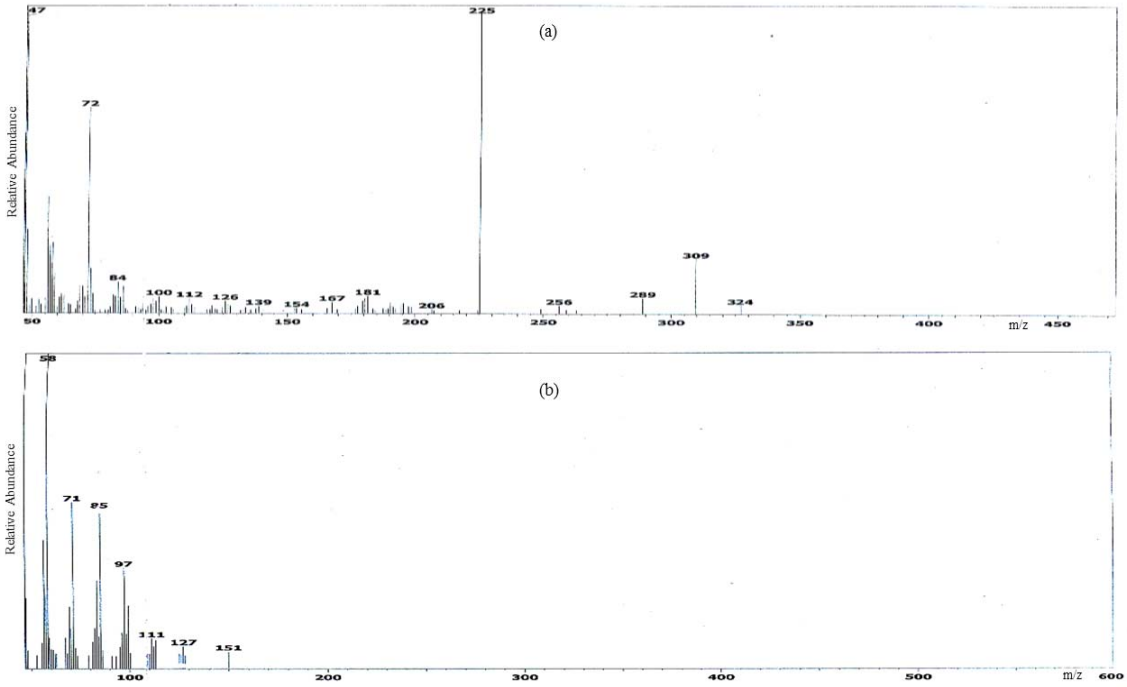


Fig. 5. Mass charts of intact drug VAL and its prepared related impurity, GUA in (a) and (b) respectively.

and independent to that of the interfering components [37, 66].

Selection of suitable wavelengths plays an important role with respect to selectivity and sensitivity; hence, two wavelengths 240 nm and 256.8 nm were chosen for GUA and at these wavelengths the absorbance difference was zero while there was a considerable absorbance difference in case of VAL (Figure 6).

The regression equation for absorbance differ-

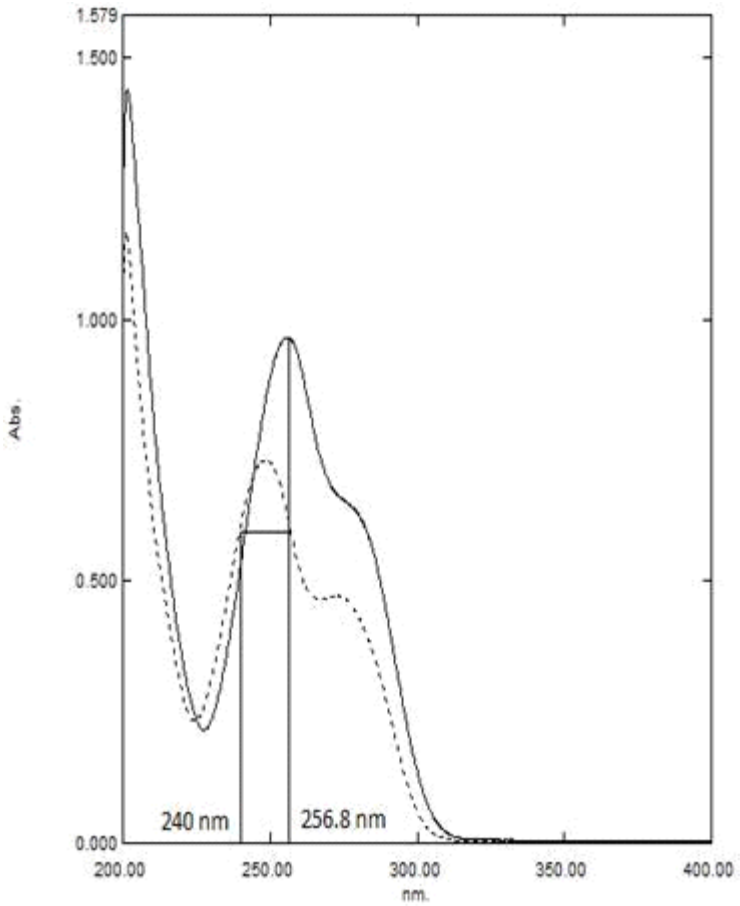


Fig. 6. Zero order absorption spectra of 30 µg/mL VAL (–) and 10 µg/mL of GUA (….) showing DW method

ence (256.8 nm - 240 nm) versus concentrations of VAL was calculated as shown in table 1.

First derivative of ratio spectra (^{1}DD) method

The absorption spectra of VAL and GUA in 0.1M hydrochloric acid solution are strongly overlapped. This spectral overlapping was sufficient enough to demonstrate the resolving power of the derivative ratio technique. In order to improve the selectivity of the analysis of VAL in the presence of its related impurity GUA, first derivative of ratio spectra method [50] was applied. In this method, the spectra of the mixture were divided by 10 µg/mL of GUA as a divisor and first derivative spectra of these ratio spectra were generated. The main advantage of the ratio-spectra of derivative spectrophotometry is the chance of doing easy measurements in correspondence of peaks, so it allows the use of the wavelength of highest value of analytical signals (a maximum or a minimum). Moreover, the presence of lot of maxima and minima is another advantage by the fact that these wavelengths give an opportunity for the determination of active compounds in the presence of other compounds and excipients which possibly interferes the assay as well as that the whole spectrum of the interfering substance is cancelled. Accordingly, the choice of the wavelength selected for calibration is not as critical as in the ^{1}D method

In order to optimize ^{1}DD method, several concentrations of the divisor were tried as 5, 10, 15 and 20 µg/mL where, the best results were obtained when using 10 µg/mL of GUA as a divisor. Different smoothing and scaling factors were tested where a smoothing factor $\Delta\lambda = 4$ and a scaling factor = 10 were suitable to enlarge the signal of VAL to facilitate its measurement and to diminish error in reading the signal.

Dividing the previously stored absorption spec-

Table 1. Regression parameters and results of determination of pure samples of VAL by the proposed methods

Parameter	DW	[1]DD (P_{max}-P_{min})	MCR	IDW	RD	CC
Linearity (µg/mL)	5-50	5-50	5-50	5-50	5-50	5-50
Slope	0.0146	0.0348	0.015	0.0066	0.0289	0.0318
Intercept	0.0014	0.0079	0.0047	0.0016	0.0059	0.0007
Correlation coefficient (r)	0.9999	0.9999	0.9996	0.9999	0.9999	0.9999
Mean	99.73	100.19	100.06	100.2	100.09	100.24
± SD	± 0.53	± 0.79	± 1.44	±1.09	± 0.60	± 0.65
Accuracy	100.2	99.81	100.94	100.8	98.97	99.32
± SD	± 0.52	± 0.33	± 1.50	± 0.99	± 0.47	± 0.54
*RSD % [a]	0.545	0.542	0.466	0.479	0.594	0.441
**RSD % [b]	0.536	0.512	0.112	0.363	0.534	0.453

*RSD % [a] & **RSD % [b]: the intra-day & inter-day respectively relative standard deviation of concentrations (10, 25, 30, 50 µg/mL)

tra of VAL in the range of 5-50 µg/mL by the absorption spectrum of 10 µg/mL of GUA (as a divisor) then different graphical measurements were tried either Peak to zero (P-0) or peak to peak (P-P): 224.8 nm (P-0), 256 nm (P-0), 266.4 nm (P-0) and 256 nm - 266.4 nm (P_{max}-P_{min}). It was found that P_{256}-$P_{266.4}$ (Figure 7) showed good linearity and reproducibility and it has the advantage of producing higher slope and therefore higher sensitivity. Linear regression equation was computed as shown in Table 1.

First order derivative of ratio spectra method enable the quantitation of pure VAL with good accuracy and precision, either in synthetic mixtures or in pharmaceutical product. The procedure is fast and specific and works without solving equations or separation steps. As a further advantage of the ratio spectra method proposed over the zero-crossing derivative method, is the possibility of performing measurements in correspondence of peaks hence a potentially greater sensitivity and accuracy. Disadvantages of the zero-crossing method are the risk of small drifts of the cross over points and the fact that the working wavelengths do not coincide with the peaks. This may be particularly dangerous when the slope of the spectra is very high, with consequent loss of precision and accuracy. This procedure seems to be time and labor consuming but gives good results.

Mean centering of ratio spectra (MCR) method

The absorption spectra of VAL and GUA in 0.1M HCl were overlapped in the wavelength range of 210 - 290 nm. Therefore, the absorption spectra of the standard solutions of VAL with different concentrations were recorded in the wavelength range of 210 - 290 nm and divided by the spectrum of 10 µg/mL of GUA then the ratio spectra were obtained. Mean centering of the ratio spectra [47-49] were obtained in the wavelength range of 210 - 290 nm (Figure 8). The concentration of VAL was determined by measuring the amplitude at 252 nm corresponding to a maximum wavelength.

The effect of using divisor concentration on the analytical parameters such as slope, intercept and correlation coefficient of the calibration graphs was also tested. Different concentrations of divisor were tried but it was observed that changing the concentration made no significant effect in their linear calibration range and the calculated analytical parameters. Therefore, a spectrum of 10 µg/mL of GUA was used as divisor spectrum in the proposed method. The regression equation for mean centering of ratio spectra versus con-

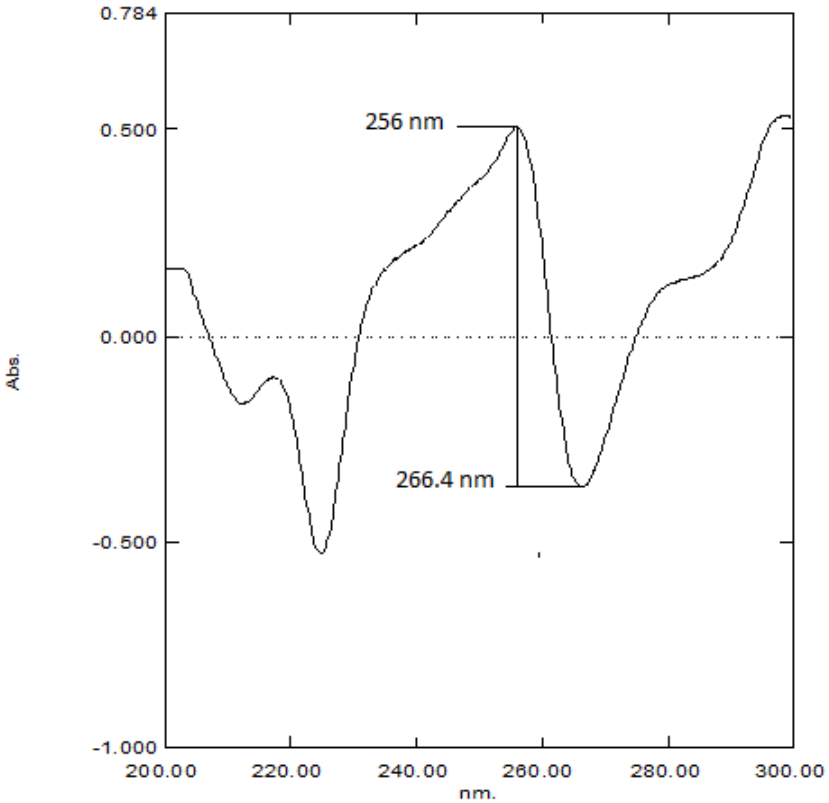


Fig. 7. First Derivative ratio spectra of 30 µg/mLVAL (–) and 10 µg/mL GUA (….) as a divisor.

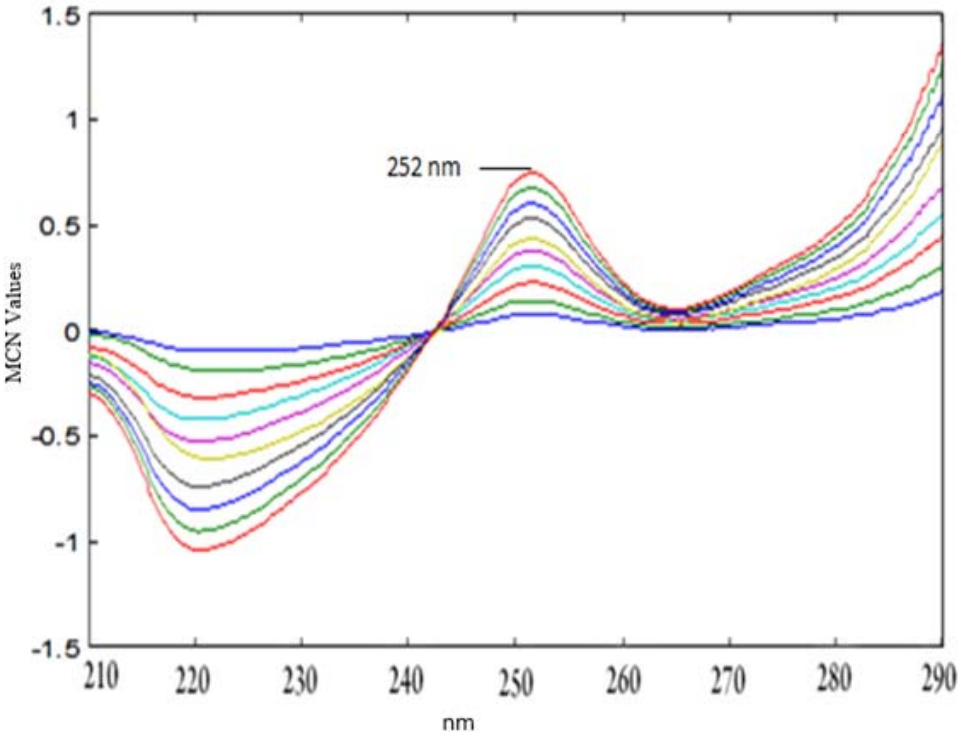


Fig. 8. Mean centered ratio spectra of VAL in the concentration range of 5-50 µg/mL using 10 µg/mL GUA as a divisor.

centrations of VAL was calculated as shown in Table 1.

This method has the advantages of high sensitivity, good selectivity, rapid analysis and inexpensive instruments. The disadvantage is the need for using a special software (Matlab).

Induced dual wavelength (IDW) method

This method is based on eliminating the absorbance of the interfering substance between two selected wavelengths irrespective to the absorbance difference of interfering substance in order to increase the sensitivity of the method [67], where at the chosen wavelengths the absorbance difference (ΔA) of the interfering substance is not equals to zero so highest absorbance values were obtained. By utilizing the λ_{max} of the component of interest together with the equality factor, the difference in the absorbance values of the related impurity is induced to be zero, whereas the component of interest shows significant difference in absorbance values dependent on concentration at the selected wavelengths. Induced dual wavelength method was applied by calculating the equality factor for pure GUA at the two selected wavelengths ($F = A_{227}/ A_{256} = 0.4081$). The difference in the absorbance values of GUA is induced to be zero, whereas VAL shows significant difference in absorbance, which is directly proportional to the concentration of the component of interest and independent to that of the interfering components Figure 9. The concentration of VAL was calculated using the regression equation as shown in Table 1.

Induced dual wavelength (IDW) method is found to be superior than the conventional DW method. DW method has poor robustness and any shift in the wavelengths will led to high variation in the absorbance difference and subsequently alter the accuracy and precision of the

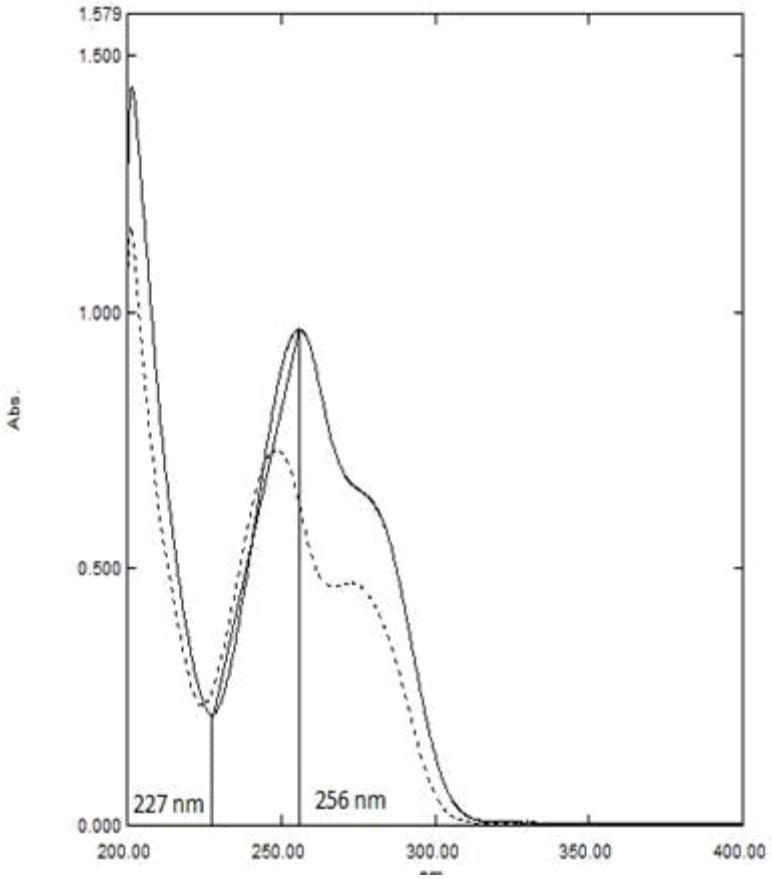


Fig. 9. Zero order absorption spectra of 30 µg/mL VAL (–) and 10 µg/mL of GUA (….) showing IDW method.

method, while IDW method shows high reproducibility since the two chosen wavelengths were maxima and minima which gave a maximum robustness therefore, enhance accuracy and sensitivity. The advantages of the IDW method over the other proposed methods is the application of minimum manipulation steps using the zero order absorption ^{0}D spectra, it requires no divisor unlike derivative ratio, ratio difference etc. Moreover, The IDW method shows maximum resolution of the drug of interest without any limitation.

Ratio difference (RD) method

The most striking feature of the ratio difference method is its simplicity, rapidity and accuracy. This is a newly developed method having the ability for resolving severely overlapped spectra without prior separation; meanwhile it does not require any sophisticated apparatus or computer programs.

The utility of ratio difference method is to calculate the unknown concentration of a component of interest present in a mixture containing both component of interest and an unwanted related impurity.

For the determination of the concentration of VAL by the ratio difference method, The only requirement for the selection of the two wavelengths λ_1 & λ_2 is the contribution of the two spectra at the chosen wavelengths where the ratio spectrum of the related impurity shows the same amplitudes (constant), while the component of interest shows significant difference [34-37].

The overlapped spectra of VAL and its related impurity suggest that a ratio difference spectrophotometric method is a suitable method for determination of VAL in the presence of its related impurity GUA. In ratio difference method, the amplitudes at 241.4 nm and 261.4 nm were selected for determination of VAL using ratio spectrum of VAL and 10 µg/mL of GUA as a divisor **(Figure 10).** The regression equation for amplitude difference (241.4 nm - 261.4 nm) versus concentrations of VAL is calculated as shown in Table 1.

The advantages of RD method is the minimum manipulation steps of the spectra and it is considered to be sensitive as it has a higher slope than that of DW and IDW and very close to those of other methods, in addition there is no critical measurement at two selected wavelengths, so it has good robustness. The main drawback is the use of divisor and subsequently, the method is divisor concentration dependent.

Constant center (CC) method

By applying the proposed method, the original spectra of VAL will be obtained. In constant center method [39, 41, 42] the absorption spectrum of VAL was scanned and divided by the absorption spectrum of a known concentration of its related impurity (GUA) as a divisor. The selected divisor should compromise between minimal noise and maximum sensitivity. The divisor concentration of 10 µg/mL of GUA gave the best results regarding average recovery percent when used for the analysis of VAL concentrations.

Ratio difference at two selected wavelengths was applied to the ratio spectra of the cited drug, where the interfering substance was cancelled and subsequently showed no interference. The only requirement for the selection of these two wavelengths was the contribution of the two components at these two selected wavelengths λ_1 & λ_2, where the ratio spectrum of the related impurity shows the same value (constant) whereas the component of interest shows significant difference in these two ratio values at these two selected wavelengths with concentrations. The two selected wavelengths were (241.4 nm and 261.4 nm) as shown in (Figure10).

For the determination of VAL in the synthetic mixture, the zero order absorption spectra of the mixtures were scanned and the ratio spectra of the mixtures were obtained by using 10 µg/mL of GUA as a divisor where recorded amplitude at 261.4 nm were measured for each synthetic mixture, while the postulated amplitude value at 261.4 nm can be calculated by using the equation representing the linear relationship between the ratio difference of ratio spectra at 241.4 nm and 261.4 nm (related impurity was cancelled) versus the corresponding ratio amplitudes at 261.4 nm **(**Figure 11).

P_1-P_2= 0.4928 P_1 - 0.0009 r = 1.0000

Where P_1 and P_2 are the postulated amplitudes

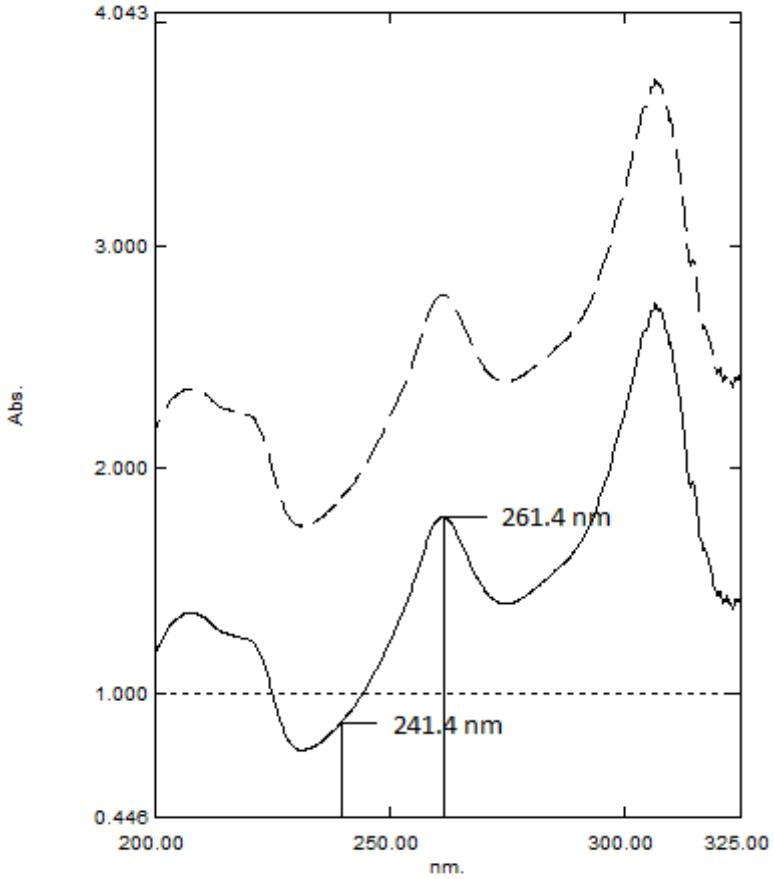


Fig. 10. Ratio spectra of 30 µg/mL VAL (–), 10 µg/mL GUA (….) as a divisor and their synthetic mixture (- - -)

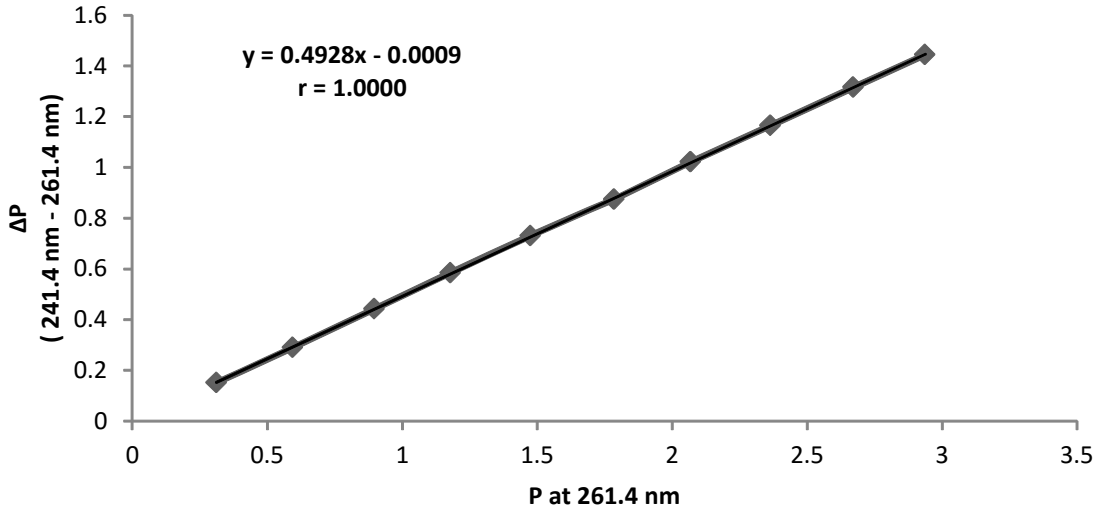


Fig. 11. Linear relationship between ΔP (241.4 nm and 261.4 nm) on y-axis and P (261.4 nm) on x-axis

at 241.4 nm and 261.4 nm respectively and r is the correlation coefficient. The constant value was calculated by monitoring the effect on the amplitude of the ratio spectrum of VAL at 261.4 nm ($\Delta P_{recorded - postulated}$), so the constant value was calculated by measuring the difference between the

recorded amplitude and postulated amplitude at this wavelength.

$C.V = (P_{recorded}) - (P_{postulated})$

P (GUA/ GUAï) = P (VAL/GUAï+ GUA/ GUAï) -P (VAL/ GUAï)

Where C.V is the constant value, $P_{recorded}$ is the recorded amplitude of the ratio spectrum of the synthetic mixture using 10 µg/mL of GUA as a divisor at 261.4 nm and $P_{postulated}$ is the calculated amplitude using the specified regression equation. The obtained constant values were multiplied by the absorption spectrum of 10 µg/mL of GUA as a divisor to obtain the zero order absorption spectrum of GUA then be subtracted from the zero order absorption spectrum of the mixture to get the zero order absorption spectrum of VAL. The concentration of VAL in the mixture could be calculated by substitution in the corresponding regression equation obtained by plotting the absorbance at its λ_{max} (256 nm) and its corresponding concentration as shown in **Table 1.**

Constant center method provides many alluring features such as maximum resolution since zero order absorption spectrum of the drug of interest is obtained which acts as a defined fingerprint and provides enhancement of sensitivity and specificity in mixtures analysis.

Selectivity indication

To assess the selectivity-indicating efficiency of the proposed methods, the related impurity of VAL was mixed with its intact sample in different ratios and analyzed by the proposed methods. **Table 2** showed good selectivity for the determination of VAL in the presence of up to 90 % of its related impurity for induced dual wavelength, ratio difference, constant center and mean centering of ratio spectra methods and up to 70 % of its related impurity for dual wavelength and derivative ratio methods. The suggested methods were valid and applicable for the analysis of VAL in its dosage form. The validity of the proposed methods was assessed by applying standard addition technique, which showed accurate results and there was no interference from excipients as shown in Table 2.

From the obtained results, it was clear that the

Table 2. Determination of VAL in the synthetic mixtures and dosage form by the proposed methods

Sample method	Recovery% ± SD					
	DW	$^{1}DD (P_{max}-P_{min})$	MCR	IDW	RD	CC
Synthetic mixtures	98.82±1.09*	98.87±0.79*	99.70±1.81**	100.36±0.71**	99.19±0.71**	99.21±0.61**
Valtrex® Tablet *** Batch No. 2121A	91.51±0.69	93.80±0.72	93.46±0.97	93.47±0.73	92.77±0.69	94.69±0.91
Standard addition	101.33±0.69	101.17±0.56	101.78±1.06	100.93±1.17	100.24±0.88	101.86±0.76

* The results of the analysis of synthetic mixtures were up to 70%
** The results of the analysis of synthetic mixtures were up to 90%
***Accepted recovery% of USP for Valtrex tablets is 90% - 110%

recently developed methods namely, induced dual wavelength, ratio difference and constant center methods have higher resolution efficiency than the conventional methods namely dual wavelength and derivative ratio since satisfactory results were obtained upon analysis of synthetic mixtures with higher percentage of related impurity up to 90 %, while mean centering method showed the same efficiency but with high relative standard deviation (1.815) in addition of using special software (Matlab) for its application.

Method validation

Method validation was performed according to ICH recommendation [68] for all the proposed methods as follows:

Range and linearity: The linearity of the proposed methods was evaluated by processing 10 point calibration curves on 3 different days. The calibration graphs of which were constructed within concentration ranges that were selected on the basis of the anticipated drug concentration during the assay of the dosage form. A linear least-squares regression analysis was conducted to determine slope, intercept and coefficient of determination to demonstrate linearity of the method. The goodness of fit in all cases was found to be ≥0.9996 indicating a functional linear relationship. The relevant slope values were statistically different from zero ($P < 0.05$); although intercepts of the calibration curves were significantly different from zero, they did not affect the accuracy of the method. The linear regression analysis data were summarized in Table 1.

Accuracy: The accuracy of the proposed methods was tested by analyzing triplicate samples of standard VAL solutions. The recovery percentages were listed in Table 1 and the results revealed the high accuracy of the proposed methods. Furthermore, the methods accuracy was assessed as recovery obtained when spiking the sample solution with known concentrations of the intact drug (standard addition technique) as illustrated in Table 2.

Precision: The precision of the methods was checked by analyzing three different concentrations of VAL in triplicate during the same day (intraday precision). Precision study was determined by performing the same procedure on three consecutive days (interday precision). The average recovery percentages were around 100 % and the low percentage relative standard deviations (RSD %) indicated the good precision of the proposed methods as were illustrated in Table 1.

Statistical analysis

The proposed analytical methods were compared with the official HPLC method [1] using statistical analysis. The Student's t-test and F test were applied and revealed that there was no significant difference between the official HPLC method and the experimental values which were obtained in the pure sample analysis by the proposed methods as shown in Table 3.

Analysis of variance (ANOVA) was also used to verify the validity of the methods. The p-value (0.862) is greater than 0.05 and the F calculated (0.4216) is lower than the F tabulated (2.2596) at $p = 0.05$, indicating that there is no significant difference between the proposed methods and the official method as shown in Table 3 and 4.

Conclusion

The present work was concerned for the resolution of pure VAL from its related impurity (potential and synthesis impurity, GUA) with zero order absorption spectra of the same geometrical features. In this paper, simple, sensitive and rapid methods were described for the determination of VAL in pure form, in synthetic mixtures with its related impurity and in pharmaceutical dosage form.

The methods used in this study were more versatile and easy to apply than the HPLC, polarographic, and voltammetric methods. In addition, these methods did not require any sophisticated instrumentation, such as HPLC, which requires organic solvents and consumes time or advanced methodologies like chemometric methods. To sum up, the spectrophotometric methods offer cost effective and time saving alternative to other methods.

From the obtained results, we concluded that the recently developed methods showed higher resolution efficiency and specificity since they determine pure VAL in the presence of up to 90

Table 3. Statistical analysis of the proposed methods and the official method of VAL in its pure form

Parameter	VAL						
	DW	[1]DD (P_{max}-P_{min})	MCR	IDW	RD	CC	Official method **
Mean ± SD	99.73±0.53	100.19±0.79	100.06±1.44	100.2±1.09	100.09±0.60	100.24±0.65	99.92±0.66
n	10	10	10	10	10	10	5
Variance	0.2809	0.6241	2.0736	1.1881	0.36	0.4225	0.4356
t-test	0.368 (2.100)*	0.501 (2.100)*	0.802 (2.100)*	0.548 (2.100)*	0.645 (2.100)*	0.392 (2.100)*	
F	1.551 (3.822)*	1.433 (5.120)*	4.760 (5.120)*	2.728 (5.120)*	1.210 (3.822)*	1.031 (3.822)*	

* The figures in parenthesis are the corresponding theoretical values at P = 0.05.

** The official method used is HPLC method using a mobile phase consisting of water containing trifluoroacetic acid (2:1000 V/V) and methanol containing trifluoroacetic acid (2:1000 V/V)

Table 4. One way ANOVA testing the different proposed methods used for the determination of VAL

Source of variation	SS	df	MS	F	P-value	F crit
Between groups	2.006965	6	0.334494	0.421615	0.861669	2.259605
Within groups	46.01506	58	0.793363			
Total	48.02202	64				

At the 0.05 level

The population means are not significantly different

% of its related impurity (GUA); therefore, they are superior to be used as selectivity indicating methods. Moreover, these methods were simple and inexpensive allowing their application in quality control laboratories without any preliminary separation steps or using any special software program.

Finally, it was revealed that the constant center (CC) method is superior over all of the proposed methods since it has good resolution efficiency of the synthetic mixtures up to 90 % of the related impurity (GUA) with the smallest relative standard deviation (0.615). Moreover, the resolved zero order absorption spectrum of pure VAL acts as spectral profile of the drug that could be extracted from its synthetic mixture after few manipulation steps in the spectrophotometer software with no need of sophisticated instrument like derivatization performed in ^{1}DD or special software (Matlab) as that used in MCR. This method enables the quantitation of mixtures of VAL with good accuracy and precision, either in synthetic mixtures or in pharmaceutical products. In addition, the procedure is fast and specific and works without solving equations or separation steps.

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