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DETERMINATION OF SIMVASTATIN AND GEFITINIB IN BINARY MIXTURE USING SPECTROPHOTOMETRIC, CHEMOMETRIC - ASSISTED SPECTROPHOTOMETRIC AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS

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ABSTRACT

Four methods were developed for simultaneous determination of simvastatin and gefitinib without previous separation. In the first method both drugs were determined using first derivative UV spectrophotometry, with zero crossing measurement at 237.2 and 348 nm for simvastatin and gefitinib, respectively. The second method depends on first derivative of the ratios spectra by measurements of the amplitudes at 240.6nm for simvastatin and 235.8 nm for gefitinib. Calibration graphs are established in the range of 1-3.5 µg/ml and 6-21 µg/ml for simvastatin and gefitinib, respectively. The third method describes the use of multivariate spectrophotometric calibration for the simultaneous determination of the analyzed binary mixture where the resolution is accomplished by using partial least squares (PLS), Classical least square (CLS), Inverse least square (ILS), Principal component regression (PCR) regression analysis.. In the fourth method (HPLC), a reversed-phase column and a mobile phase of Acetonitrile: Methanol: Water (60: 30: 10 v/v/v) at 1 ml/min flow rate is used to separate both drugs and UV detection at 245 nm. Good linearities are obtained in concentration range of 0.1-15 µg/ml for simvastatin and 0.6-90 µg/ml for gefitinib. All the proposed methods are extensively validated. They have the advantage of being economic and time saving. The results obtained by adopting the proposed methods are statistically analyzed and compared with those obtained by reported methods.

Keywords: Simvastatin; Gefitinib; First derivative Spectrophotometry; Ratio derivative Spectrophotometry; Multivariate; HPLC.

INTRODUCTION

Simvastatin (SIM) is a selective and competitive inhibitor of 3-hydroxy-3- methylglutaryl -coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl coenzyme A to Mevalonate, a precursor of cholesterol [1]. SIM is used to treat hypercholesterolemia and related conditions and to prevent cardiovascular disease. The most recent methods for determination of Simvastatin include Chromatographic [2-6], Mass spectrometry[7-10], micellar electrokinetic [11] and spectrophotometric [12-16] methods.

Gefitinib (GEF) (Martindale, 2005), [Iressa] is a synthetic anilinoquinazoline compound. Gefitinib is used to treat several types of lung cancer. Gefitinib inhibits the epidermal growth factor receptor (EGFR) tyrosine kinase by binding to the adenosine triphosphate (ATP)-binding site of the enzyme[17]. The most recent methods for determination of Gefitinib include Chromatographic[18-20], Mass spectrometry[21-22], Voltametric[23] and Spectrophotometric methods[24].

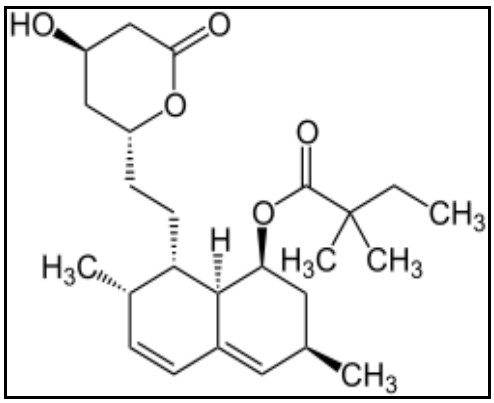

Fig.1:structure of simvastatin(SIM)

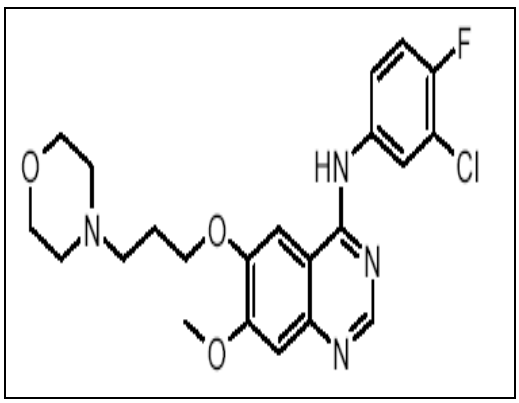

Fig.2:structure of gefitinib (GEF)

The randomized, open-label, phase II study of gefitinib and simvastatin versus gefitinib alone was conducted at an institution (National Cancer Center) from May 2006 to September 2008[25]. The impact of statins on cholesterol and isoprenoid synthesis may have anticancer effects through at least 2 primary mechanisms, which include impairment of protein prenylation and interference with the formation of cholesterol-rich lipid microdomains called lipid rafts within the cell membrane[26]. The impact of statins on epidermal growth factor

receptor (EGFR) function and signaling and in vivo activity against tumor cells has generated interest in studying statins as a potential EGFR-targeted therapeutic intervention. However, GEF and SIM improved the response rate and Progression free survival compared with GEF in the exploratory subgroup analysis of the patients with wild type EGFR nonadenocarcinomas. This finding suggests that inhibiting the mevalonate pathway using Simvastatin may enhance the efficacy of Gefitinib in this relatively Gefitinib resistant subpopulation.

Recently, derivative spectrophotometry has been found to be a useful method in the determination of mixtures with two or more components having overlapping spectra and in eliminating interference from formulation matrix by using the zero-crossing techniques [27-29]. Furthermore, ratio-spectra derivative spectrophotometric method has also been found to be useful in the estimation of drugs in their mixtures[30-34]. This method permits the determination of a component in their mixture at the wavelengths corresponding to a maximum or minimum and also the use of the peak-to-peak between consecutive maximum and minimum. The main advantage of derivative of the ratio-spectra method may be the chance of easy measurements in correspondence of peaks so it permits the use of the wavelength of highest value of analytical signals (maximum or minimum). Moreover, the presence of a 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 active compounds and excipients which possibly interfere the analysis. Multivariate calibration methods applied to spectral data are being increasingly used for pharmaceutical analysis. The greatest difficulties with UV-Vis multi determination methods arise when the analytes to be determined give partly or fully overlapped spectra. Multivariate calibration is a useful tool in the analysis of multicomponent mixtures because it allows rapid and simultaneous determination of each component in the mixture with reasonable accuracy and precision and without the need of lengthy separation procedures. With the aid of modern instrumentation to acquire and digitize spectral information and powerful computers to process large amounts of data, chemometric methods such as classical least squares (CLS), inverse least squares (ILS), principle component regression (PCR) and partial least squares (PLS) regression are finding increasing use in quantitative analysis of complex mixtures, offering an interesting alternative to chromatographic techniques. Classical least squares (CLS) and Principal components regression (PCR) analysis are the most simplest multivariate methods that can be performed with easily accessible statistical software[35-39]. each frequency

(wavelengths) are proportional to component concentration units. Model errors are assumed to derive from the measurement of spectral absorbance. So CLS requires that all interfering chemical components be known and included in the calibration data set. CLS has the advantage of improved precision when using many frequencies, due to signal averaging. Calibration is realized by recording the spectra at *n* wavelengths of *m* standard mixtures, of known composition of *c* components. The spectra (absorbance or emission) are arranged into the columns of matrix *Y* (dimensions *n* x *m*), with the composition of each mixture forming the columns of concentration matrix *X* (*c* x *m)*

$Y = K \,.\, X$ ---------- (1)

With a prior knowledge of *X* and by recording data for *Y*, then the matrix of sensitivities, *K*, can be calculated, but after the rearrangement of equation 1 to the following equation by multiplying the equation components by Xt value as:

$Y \,.\, X^t = K \,.\, X \,.\, X$

$K = (X \,.\, X^t)\text{-}1 \,.\, Y \,.\, X^t$---------- (2)

To avoid being under-determined, there must be measurements at more wavelengths than there are components (i.e. $n \geq c$). If $n > c$ then the component concentrations in an unknown mixture are obtained from its spectrum by,

X unknown $= (K^t \,.K\,)^1\text{-} \,.Kt \;\; y_{unknown}$

This CLS method is intuitively appealing since it is based on some generally assumed relationship, e.g. Beer's law, and it can be used for moderately complex composition of the calibration mixtures, i.e. the concentration of each absorbing species. PCR is a two step procedure, in the first step, one estimating the number of principal components by one or more of the following criteria, the percentage of explained variance, eigen value-one criterion, the Scree-test and Cross validation. They can be considered as new variables that summarize in an optimal way the variation present in the spectra, in the second step, CLS is applied to the newly obtained latent variables. When co-linearity between original variables occurs, principal component plots often allow better interpretation of the variations observed in the data set than plots of original variables selected by CLS. As modeling method, it is less performant than CLS when performing prediction within the calibration domain and when the model is indeed linear. It is more reliable if extrapolation may be required. It is a linear method, but it is able to perform quite well for moderately nonlinear data. As CLS, it is a global method.

HPLC methods are useful in the determination of drugs in pharmaceutical dosage forms and biological sample. Owing to the widespread use of HPLC in routine analysis. The purpose of

the present study was to investigate the utility of derivative, derivative ratio spectrophotometry, multivariate and HPLC techniques in the assay of simvastatin and gefitinib in pharmaceutical preparations without the necessity of sample pretreatment.

MATERIALS AND METHOD

1.1 chemicals and reagents

SIM is obtained as a gratis sample from Dr. Reddy's Lab, Hydrabad and GEF as a gratis sample from Natco Pharma Pvt. Ltd., Hydrabad. Methanol (AR grade) was used as a solvent for UV spectrophotometric method. HPLC grade methanol and acetonitrile (Merk, Darmstadt, Germany) and doubly distilled water were used for preparing mobile phase solutions.

1.2 instrumentation

UV Visible Double beam Spectrophotometer with Pair Matched Cuvette (Analytical Spectro 2080), HPLC (Shimadzu LC-20 AT) system used consisted of pump (model Perkin Elmer; Reciprocating Series-200) with universal loop injector (Hamilton Syring Perkin Elmer) of injection capacity 20µL. Detector consists of Ultraviolet detector SPD 20 A, the column used was Enable C_{18} (5 µm, 25cm X 4.6 mm i.d.), EIE Ultra-sonic Cleaner and Electronic Digital Balance, Shimadzu.

1.3 preparation of standard solutions and calibration

Stock standard solutions containing 1.0 and 6.0 mg simvastatin and gefitinib respectively, were dissolved in 10 ml methanol. Standard solutions of both drugs were prepared individually by dilution of the stock solutions with methanol for spectrophotometric methods to obtain concentration range of 1-20 µg/ml for simvastatin and 1-20 µg/ml for gefitinib and in mobile phase preparation for chromatographic method to reach concentration range of 0.1-15 and 0.6-90 µg/ml for simvastatin and gefitinib, respectively.

For derivative spectrophotometric method (D1): The values of the D1 amplitudes were measured at 237.2 nm (zero-crossing of gefitinib) and 348 nm (zero-crossing of simvastatin) for the determination of simvastatin and gefitinib, respectively.

For first derivative of the ratio spectrophotometric method (DD1): According to the theory of the ratio spectra derivative method. The stored UV absorption spectra of standard solutions of simvastatin were divided wavelength-by-wavelength by a standard spectrum of

gefitinib (12 µg/ml). The first derivative was calculated for the obtained spectra with $\Delta\lambda = 10$ nm. The amplitudes at 240.6 nm were measured and found to be linear to the concentrations of simvastatin. For gefitinib, the stored UV absorption spectra of standard solutions of gefitinib were divided wavelength-bywavelength by a standard spectrum of simvastatin (2 µg/ml). The first derivative was calculated for the obtained spectra with $\Delta\lambda = 10$ nm. The amplitudes at 235.8 nm were measured and found to be linear to the concentration of gefitinib.

For multivariate method: In order to obtain the calibration matrix for applying CLS, ILS, PCR and PCR analysis, twenty solutions of each of the pure components (simvastatin and gefitinib) were prepared in a concentration range of 51-20 µg/ml for simvastatin and gefitinib. These ranges were previously verified to obey Beer's law for each of the studied drugs in the selected solvent. The absorption data in the range of 217-257 nm (digitized every 2.0 nm) were subjected to least squares analysis in order to obtain the calibration K matrix. Laboratory prepared mixtures were prepared by mixing known amounts of simvastatin with gefitinib in different varied proportions in order to verify the precision of the method for analysis of such mixture.

For high-performance liquid chromatographic method: Standard solutions were prepared separately with mobile phase by varying concentrations of simvastatin and gefitinib in the range 0.1-15 and 0.6-90 µg/ml, respectively. Triplicate 20 µl injections were made for each concentration and peak area ratio of each concentration to the internal standard was plotted against the corresponding concentration to obtain the calibration graph.

Application to synthetic binary mixture: For all methods, accurately weighed 2.5 mg SIM and 15.0 mg of GEF were transferred to 10 ml volumetric flask, dissolved in sufficient amount of methanol and diluted up to mark with methanol to get concentration of 1000 µg/ml SIM and 6000 µg/ml GEF. This solution was further diluted with methanol to get 10 µg/ml of SIM and 60 µg/ml of GEF. 2 ml of combined working solution is taken in 10 ml volumetric flask and diluted up to 10 ml with Methanol to get concentration of 2.0 µg/ml and 12.0 µg/ml of SIM and GEF respectively.

Accuracy study: To study the accuracy of the proposed methods, and to check the interference from excipients present in the dosage form, recovery experiments were carried out by the standard addition method. This study was performed by addition of different

amounts of simvastatin and gefitinib to a known concentration of the binary mixtures. The resulting mixtures were analyzed as described under Section 2.3.

RESULTS AND DISCUSSION

D1 method: As shown in Fig. 3, the zero-order spectra of standard drugs were found to be overlapped making their simultaneous determination difficult. In contrast, the D1 spectrum of each pure drug was found to show zero-crossing points (Fig. 4) and assisted in their simultaneous estimation. In practice, the wavelength selected is that which exhibits the best linear response, giving a zero or near zero intercept on the coordinate of the calibration graph, and not affected by the presence of any other component. The shape of the first derivative spectra is adequate for determining simvastatin in the presence of gefitinib and vice versa. Simvastatin was determined by measuring of its D1 amplitude at the zero-crossing point of gefitinib (at 237.2 nm). While gefitinib was determined by measuring of its D1 at the zero-crossing point of simvastatin (at 348 nm). Linear relationships between derivative amplitude and drug concentration were obtained over the concentration range 1-3.5 µg/ml for simvastatin and 6-21 µg/ml for gefitinib. The linear regression equations together with correlation coefficients slope and intercept, R.S.D. of slope and intercept repeatability (within day) and reproducibility (between-day) obtained for each drug are collected in Table 1 and 2.

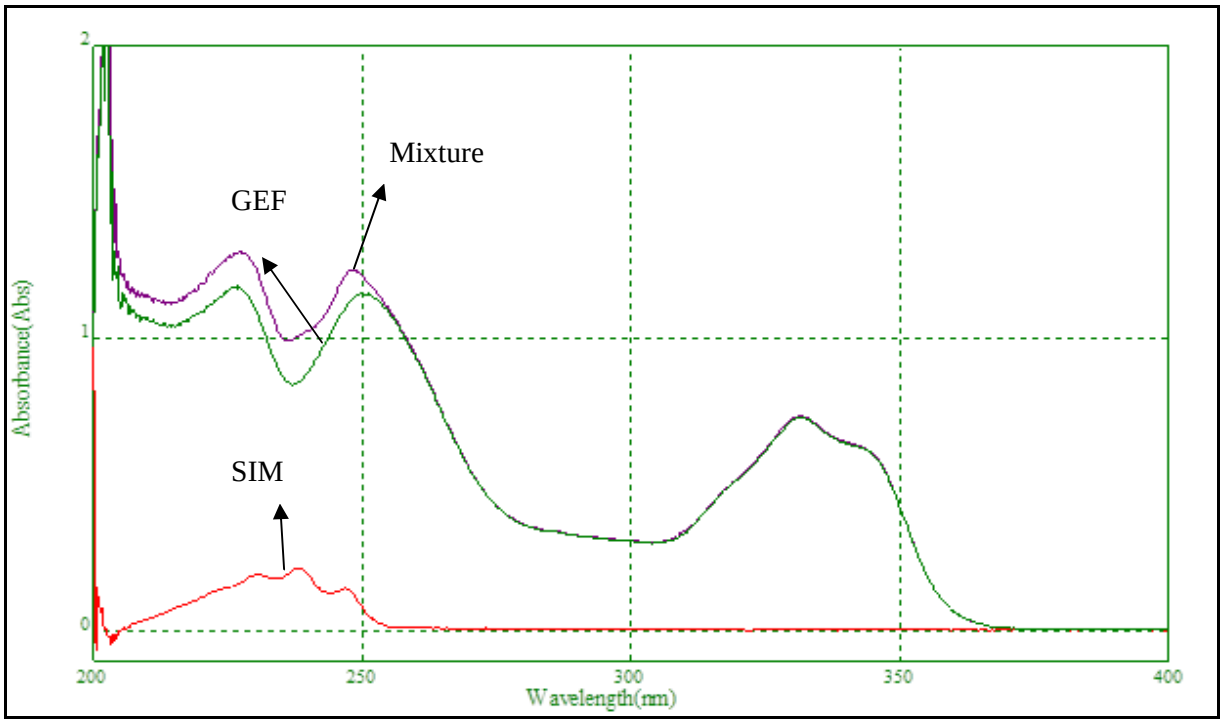

Fig. 3: overlay zero order uv spectra of simvastatin and gefitinib and their binary mixture

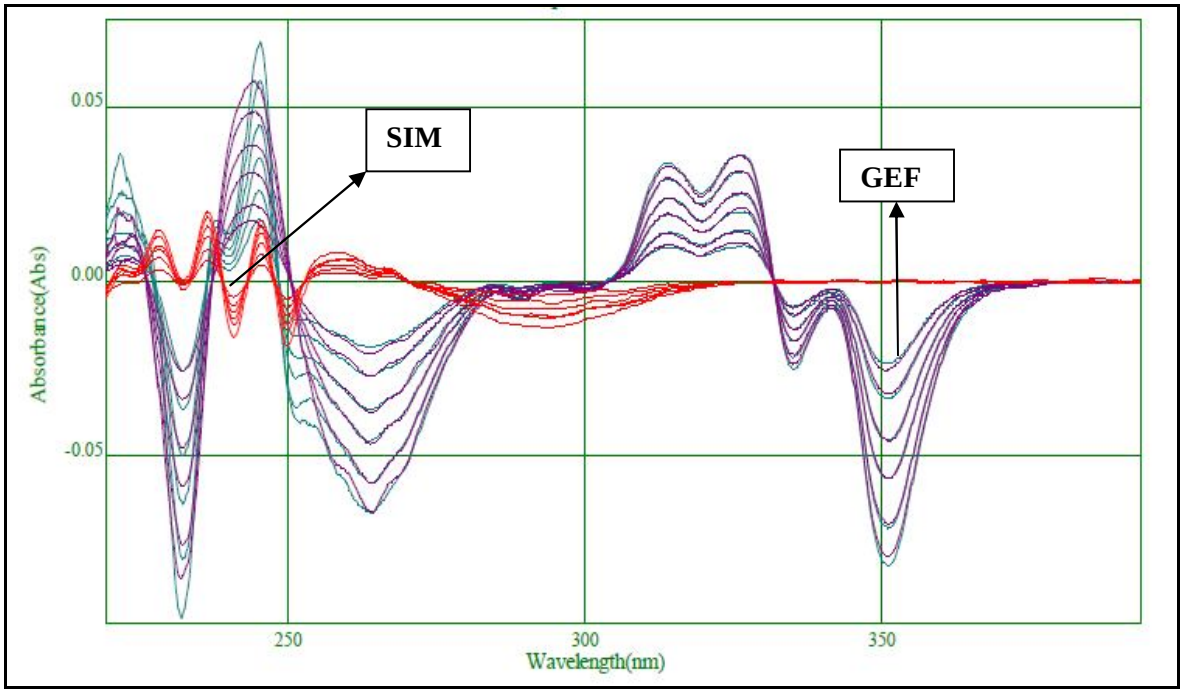

Fig. 4:overlay uv first derivative spectra of SIM, GEF and their mixture

DD1 method: The influence of Δλ for obtaining the first derivative of the ratio spectra as well as, the effect of divisor concentration on the calibration graphs for the proposed mixture was studied in order to select the best factors affecting the determination. Results indicated that Δλ = 10 nm was most suiTable one, while the divisor concentration has no significant effect on the assay results for the studied mixtures. For determination of simvastatin, the absorption spectra of simvastatin were divided by that of standard solutions of gefitinib (12 µg/ml) and the absorption spectra of gefitinib were divided by that of standard solutions of simvastatin (2 µg/ml), fig. 5 and 6, respectively. The first derivative of the developed ratio spectra were calculated with Δλ = 10 nm. Fig. 7 and 8 show that, simvastatin can be determined by measuring the amplitude at many wavelengths where gefitinib has no contribution, but it was found that the amplitude at 240.6 nm give the most accurate and sensitive results (Fig. 7). Fig. 8, shows that gefitinib can be determined by measuring the amplitude at many wavelengths where simvastatin have no contribution, but it was found that the amplitude at 235.8 nm give the most accurate and sensitive results. The proposed method is applicable over the range 1-3.5 µg/ml for simvastatin and 6-21 µg/ml for gefitinib. The characteristic parameters and necessary statistical data of the regression equations, LOD and LOQ values, respectively and reproducibility data are collected in Table 1 and 2. Repeatability and reproducibility variables were characterized by R.S.D.(%) and by the difference between theoretical and measured concentrations. There was no significant

difference for the assay, which was tested within-day (repeatability) and between-days (reproducibility). In order to demonstrate the validity and applicability of the proposed DD1 method, recovery studies were performed by analyzing laboratory prepared mixtures of simvastatin and gefitinib with different composition ratio (Table 3).

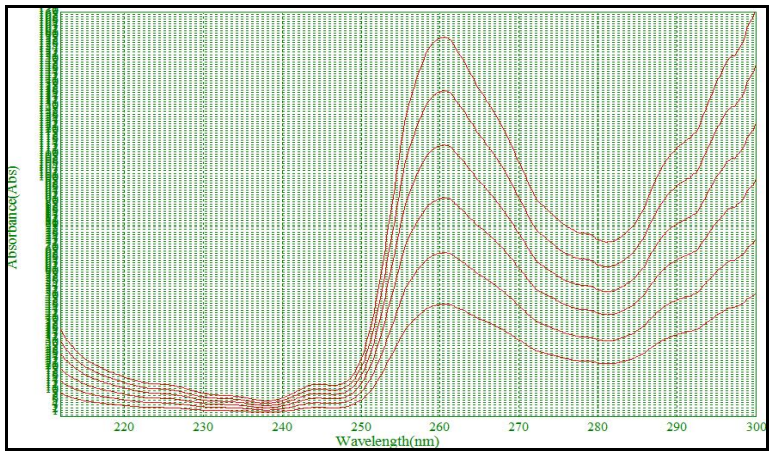

Fig. 5: ratio spectra of simvastatin (1-3.5 µg/ml) divisor is gefitinib (12 µg/ml)

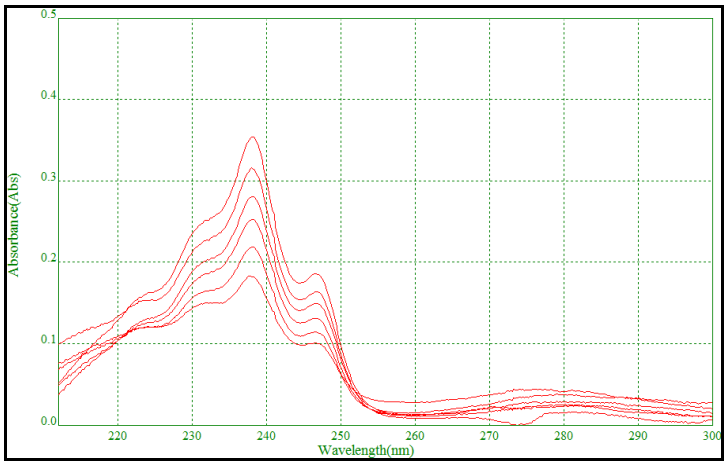

Fig. 6: ratio spectra of gefitinib (6-21 µg/ml) divisor is simvastatin (2 µg/ml)

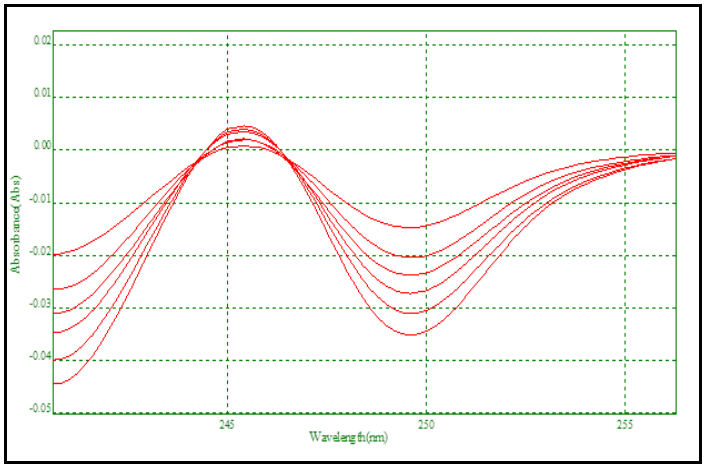

Fig. 7: first derivative of ratio spectra of simvastatin (1-3.5 µg/ml) divisor is gefitinib (12 µg/ml)

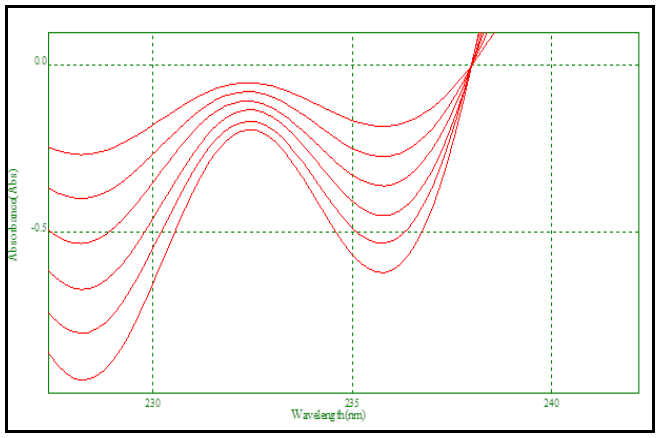

Fig. 8: first derivative of ratio spectra of gefitinib (6-21 µg/ml) divisor is simvastatin (2 µg/ml)

Multivariate method: The absorption spectra of the studied drugs are shown in Figure 3. As could be seen, a considerable degree of spectral overlapping occurs in the region from 217 to 257 nm for simvastatin and gefitinib. The degree of spectral overlapping was given by (Di)0.5. Several laboratory prepared mixtures were subjected to the CLS, ILS, PLS and PCR analysis in order to confirm the suitability of the calibration model for determination of the studied drugs in the pharmaceutical sample solutions. The results of laboratory prepared mixtures analysis with comparable concentrations were found closely matched. This indicated that, excipients and additives did not interfere with the determinations. Moreover, the results of binary mixture analysis were compared with those obtained by applying reported methods. As shown in Table 3, the results are in good agreement with those of the reported procedures. RMSEP values are shown in Table 4.

High-performance liquid chromatographic method: Drug concentration is monitored during various phases of pharmaceutical development, such as formulation and stability studies, quality control and pharmacological testing in animals and humans. All these investigations require reliable and validated analytical methods in order to measure drugs in pharmaceutical formulations and biological samples. In order to perform the simultaneous elution of simvastatin and gefitinib peaks under isocratic conditions, the mixtures of methanol, acetonitrile and water in different combinations at various flow rates were assayed. The optimum wavelength for detection was 245 nm at which much better detector responses for both drugs were obtained. The mixture of acetonitrile: methanol: water (60:30:10 (v/v/v)) at 1 ml/min flow rate, proved to be better than the other mixtures and flow rates for separation, since the chromatographic peaks were better defined, resolved and free from

tailing. The retention times were 7.75 min for simvastatin, 5.23 min for gefitinib Resolution and selectivity factors for this system were found 4.26 and 2.11 for simvastatin and gefitinib, respectively. Tailing and capacity factors were obtained as 0.917 and 1.60 for simvastatin and 1.387 and 0.76 for gefitinib. The variation in retention times among six replicate injections of simvastatin and gefitinib standard solutions was very low, rendering a R.S.D of 1.33 and 0.28%, respectively. The results obtained from system suitability tests are in agreement with the USP requirements. Peak area ratios (Asample\AIS) were plotted against corresponding concentrations in the range of 0.1-15 µg/ml for simvastatin and 0.6-90 µg/ml for gefitinib (fig. 9). Linear regression parameters of the peak area ratios versus concentrations of simvastatin and gefitinib are presented in Table 1 and 2. The results showed highly reproducible calibration curves with correlation coefficients > 0.999. Statistical data of the regression equations are shown in Table 1 and 2. The LOD and LOQ values were calculated as described in Section 3.1. In order to demonstrate the validity and applicability of the proposed HPLC method, recovery tests were carried out by analyzing laboratory prepared mixtures of simvastatin and gefitinib, with different ratios (Table 3).

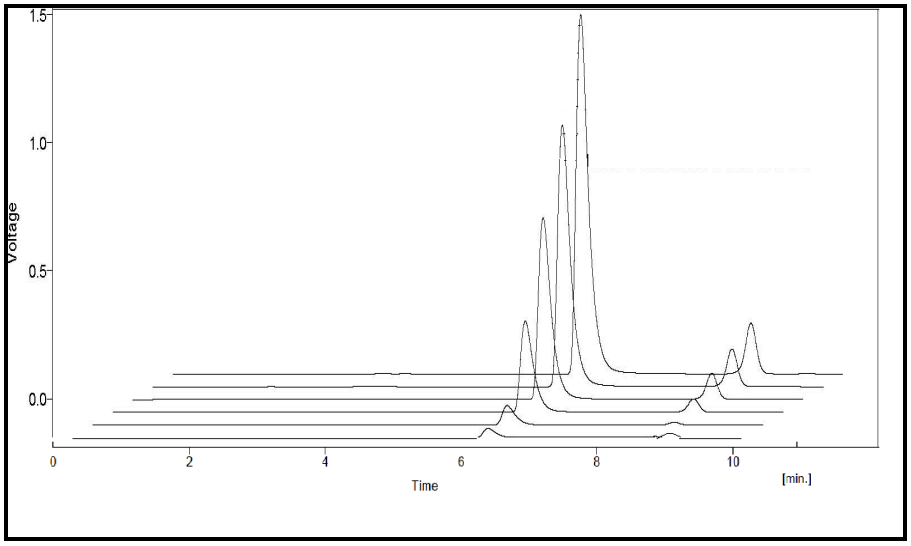

Fig. 9:overlay chromatogram for calibration curve of SIM (0.1- 15 µg/ml) and GEF (0.6 - 90 µg/ml)

Validity of the proposed methods: Statistical analysis of the results obtained for the proposed methods (Table 3), shows that all the suggested measurements are equally precise and accurate to the reported methods [40].

TABLES

Table 1: statistical data for the calibration graphs of simvastatin by D1, DD1, CLS, ILS, PLS, PCR and HPLC methods

	D1	DD1	CLS	ILS	PCR	PLS	HPLC
Linearity range (µg/ml)	1-3.5	1-3.5	3-18	3-18	3-18	3-18	0.1-15
Slope	0.0039	0.0096	1.0111	0.9858	1.0406	1.048	70.29
Intercept	-0.0002	0.0042	0.1646	0.1827	-0.3002	-0.3107	9.1491
Correlation coefficient	0.9998	0.9984	0.9903	0.9917	0.9931	0.9959	0.9998
LOD	0.17	0.38	0.17	0.61	0.95	0.98	0.005
LOQ	0.51	0.97	0.51	1.85	2.88	2.96	0.176
Repeatibility (RSD %)	1.39	0.70	1.25	1.17	0.98	1.04	0.71
Reproducibility (RSD %)	1.84	1.09	1.63	1.43	1.11	0.58	0.81

Table 2: statistical data for the calibration graphs of gefitinib by D1, DD1, CLS, ILS, PLS, PCR and HPLC methods

	D1	DD1	CLS	ILS	PCR	PLS	HPLC
Linearity range (µg/ml)	6-21	6-21	3-18	3-18	3-18	3-18	0.6-90
Slope	0.0033	0.0291	0.9326	0.9789	0.9716	0.9622	96.322
Intercept	-0.0009	0.0111	0.5392	0.0239	0.233	0.2746	78.301
Correlation coefficient	0.9999	0.9999	0.9956	0.9956	0.9968	0.9975	0.9996
LOD	0.90	1.26	1.91	0.08	0.79	0.09	0.03
LOQ	2.72	3.81	5.78	0.24	2.40	0.26	0.36
Repeatibility (RSD %)	0.86	0.61	0.54	0.73	0.64	0.89	0.72
Reproducibility (RSD %)	0.48	0.71	0.78	0.89	0.76	0.56	0.78

Table 3: recovery data of simvastatin and gefitinib by d1, dd1, cls, ils, pls, pcr and hplc methods

Percentage Accuracy (%)							
	D1	**DD1**	**CLS**	**ILS**	**PCR**	**PLS**	**HPLC**
SIM	101.65 ± 0.94	100.24 ± 0.80	103.27 ± 5.55	100.68 ±3.68	101.39 ± 2.95	101.99 ± 2.59	101.13 ± 0.48
GEF	100.29± 0.76	100.21 ± 1.40	102.45 ± 7.35	98.27 ± 5.23	101.00 ± 5.02	101.28 ± 5.68	100.63 ± 0.45

Table 4: RMSEP values of simvastatin and gefitinib

Drug	RMSEP			
	CLS	ILS	PCR	PLS
SIM	0.5315	0.4486	0.4728	0.4788
GEF	0.5575	0.4714	0.3748	0.3734

CONCLUSION

The D1and DD1, multivariate (CLS, ILS, PLS and PCR) and HPLC methods enable the quantitation of simvastatin and gefitinib binary mixture with good accuracy and precision, in laboratory prepared samples. By comparing the results of the four proposed methods, no significant difference is obtained; HPLC method has the superiority over spectrophotometric methods in analyzing the binary mixture of studied drugs. All of the proposed procedures have the advantage of being rapid, precise and direct. DD1 method has two advantages over the D1 method, the first is the high sensitivity and accuracy, the second is the easy measurements at any peak signal giving higher values. CLS, ILS, PLS and PCR multivariate calibration analysis were developed for the analysis of the laboratory prepared mixtures. The good recoveries obtained in all cases as well as the reliable agreement with the reported procedures proved that, the proposed method could be applied efficiently for determination of simvastatin and gefitinib binary mixture with quite satisfactory precision and could be easily used in a quality control laboratory for their analysis.

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