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Simultaneous determination of salbutamol sulphate, bromhexine hydrochloride and etofylline in pharmaceutical formulations with the use of four rapid derivative spectrophotometric methods

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Abstract

Four simple, rapid, accurate, precise, reliable and economical spectrophotometric methods have been proposed for simultaneous determination of salbutamol sulphate (SS), bromhexine hydrochloride (BH) and etofylline (ET) in pure and commercial formulations without any prior separation or purification. They were first derivative zero crossing spectrophotometry (method 1), simultaneous equation method (method 2), derivative ratio spectra zero crossing method (method 3) and double divisor ratio spectra derivative method (method 4). The ranges for SS, BH and ET were found to be 1–35 $\mu g\,mL^{-1}$, 4–40 $\mu g\,mL^{-1}$ and 5–80 $\mu g\,mL^{-1}$. For methods 1 and 2, the values of limit of detection (LOD) were 0.2314 $\mu g\,mL^{-1}$, 0.4865 $\mu g\,mL^{-1}$ and 0.2766 $\mu g\,mL^{-1}$ and the values of limit of quantitation (LOQ) were 0.7712 $\mu g\,mL^{-1}$, 1.6217 $\mu g\,mL^{-1}$ and 0.9221 $\mu g\,mL^{-1}$ for SS, BH and ET, respectively. For method 3, LOD values were 0.3297 $\mu g\,mL^{-1}$, 0.2784 $\mu g\,mL^{-1}$ and 0.7906 $\mu g\,mL^{-1}$ and LOQ values were 0.9325 $\mu g\,mL^{-1}$, 0.9282 $\mu g\,mL^{-1}$ and 2.6352 $\mu g\,mL^{-1}$ for SS, BH and ET, respectively. For method 4, LOD values were 0.3161 $\mu g\,mL^{-1}$, 0.2495 $\mu g\,mL^{-1}$ and 0.2064 $\mu g\,mL^{-1}$ and LOQ values were 0.9869 $\mu g\,mL^{-1}$, 0.8317 $\mu g\,mL^{-1}$ and 0.6879 $\mu g\,mL^{-1}$ for SS, BH and ET. The precision values were less then 2% R.S.D. for all four methods. The common excipients and additives did not interfere in their determinations. The results obtained by the proposed methods have been statistically compared by means of Student *t*-test and by the variance ratio *F*-test.

Keywords: First derivative zero crossing spectrophotometry; Simultaneous equation method; Derivative ratio spectra zero crossing spectrophotometry; Double divisor ratio spectra derivative spectrophotometry; Salbutamol sulphate; Bromhexine hydrochloride; Etofylline

1. Introduction

Salbutamol sulphate (SS), chemically known as bis [(1RS)-2-[(1, 1-dimethylethyl) amino]-1-[4-hydroxy-3-(hydroxymethyl) phenyl] ethanol] sulphate, is beta-adenocepter agonist used as an anti-asthmatic drug. Bromhexine hydrochloride (BH), *N*-(2-amino-3,5-dibromobenzyl)-*N*-methyl cychlohexanamine hydrochloride, is an expectorant use in the treatment of various respiratory disorders. Etofylline (ET), 7-(2-hydroxyethyl)-1,3-dimethyl-3,7-dihydro-1,4-purine-2,6-dione, is a xanthine bronchodilator used for the treatment of respiratory diseases and asthma in combination with SS. SS [1], BH [2] and ET [3] are official in BP. The official methods involve determination of SS [1], BH [2] and ET [3] using potentiometry.

Some procedures have been described for the assay of either SS or BH or ET in single dosage forms [4–7]. A spectrophotometric method has been reported for determination of SS and BH in combine dosage forms [8]. The SS, BH and ET mixture is not yet official in any pharmacopoeia. As per literature, no analytical method could be traced for the analysis of SS, BH and ET combination in pharmaceutical dosage forms. Therefore, simple, rapid and reliable methods for simultaneous estimation of these drugs in mixture seemed to be necessary.

Spectrophotometric methods of analysis are more economic and simpler, compared to methods such as chromatography and electrophoresis. Under computer-controlled instrumentation, derivative spectrophotemetry is playing a very important role in the multicomponent analysis of mixtures by ultraviolet–vis molecular absorption spectrophotometry [9]. Ternary mixtures

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can be easily resolved by means of a spectrophotometric method, which is based on the simultaneous use of "zero crossing" and "ratio spectra derivative" methods [10–12]. The aim of this work was to investigate the utility of derivative spectrophotometry and to develop reliable spectrophotometric procedures for the simultaneous determination of SS, BH and ET either in laboratory samples, or in commercial dosage forms without any prior separation of individual drugs. SS, BH and ET have closely overlapping spectra, which prevents the use of zero-order UV–vis spectrophotometry for their determination. Derivative spectrophotometry is a very useful tool for overcoming this problem. This technique has been successfully applied in pharmaceutical and environmental analyses for the determination of drugs in multicomponent systems [13–18].

In this work, various orders of derivative and different kinds of measurements were assayed, i.e., zero crossing first derivative [19], simultaneous equation method [20], ratio-spectra first derivative zero crossing [21–22] and double divisor ratio spectra derivative method [23–24]. Four methods were successfully developed for the above combination and satisfactory results obtained. A brief comparison between the usefulness of different procedures was attempted.

2. Experimental

2.1. Instruments

Spectrophotometric measurements were made on a Shimadzu 1700 double beam UV–vis spectrophotometer with a fix slit width of 1 nm coupled HP7540 computer loaded with Shimadzu UV PC software of version 2.0 and EPSON-300 printer.

2.2. Reagents

All chemicals used were of analytical grade and double distilled water was used throughout. Pure SS and BH were obtained from Dial Pharmaceuticals Pvt. Ltd., India and ET was obtained from Cadila Healthcare Pvt. Ltd., India. Various pharmaceutical formulations of SS, BH and ET in their combined dosage forms were obtained commercially.

2.3. Solutions

Stock solutions, 0.1 mg mL^{-1} in methanol, of pure samples of SS, BH and ET were freshly prepared individually. Commercial formulations containing tablets of Air-Vent (Dial Pharmaceuticals Ltd., India, Mfg. Lic. No.: G/1648), Butabrom (Rekvina Pharmaceutical Pvt. Ltd., India, Mfg. Lic. No.: 26/UA/LL/of2005) and Saans (Kosha Laboratories, India, Mfg. Lic. No.: G/964), which were labeled to contain 4 mg of SS, 16 mg of BH and 200 mg of ET par tablets were use for the study.

2.4. Procedure

All reagents were tested for stability in solution and during the actual analysis. The behavior of the analytes remained unchanged up to about 24 h from their preparation at the room temperature. All the three drugs were found to be stable during each kind of experimental measurements. Each measurement was done at room temperature.

While for the commercial formulation analysis in all the four methods, 20 tablets were weighed and ground to a fine powder. From the resultant powder, an accurately weighed powder equivalent to one tablet was taken to a calibrated volumetric flask containing methanol. Solution was filtered through Whatman filter paper number 41 and absorbance of the derivative spectra was measured at 273 nm, 323 nm and 279 nm for SS, BH and ET determination, respectively. All the three brands of tablets were tested according to the procedure described above.

2.4.1. First derivative zero crossing spectrophotometry (method 1)

The absorption spectra of the samples were recorded between 220 nm and 350 nm against a reagent blank (the same of the samples without the compounds to be determined) using a 1.0 cm quartz cell. The zero-order spectra of pure drugs were stored individually within the above concentration ranges and were derivatized in first order using delta lambda 4 and scaling factor 10 for all three drugs. The first derivative amplitudes were recorded at 273 nm, 323 nm and 279 nm for determination of SS, BH and ET, respectively. Standard laboratory mixtures of SS, BH and ET in 1:4:50 ratios were prepared and absorbance was measured at 273 nm, 323 nm and 279 nm for SS, BH and ET, respectively. Commercial formulation was analysed as described above.

2.4.2. Simultaneous equation method (method 2)

This method is based on first derivative and the wavelengths selected for estimation of SS, BH and ET were 273 nm, 323 nm and 279 nm, respectively. However, in contrast to first method, this method utilized simultaneous equations (Vierdot's method) on derivative spectra to overcome spectral interference at selected wavelength. The first derivative absorptivity coefficients were determined at the selected wavelengths. A set of three equations framed using these coefficient values are listed below:

$$C_{SS} = 0.001561 DA_{SS} \quad (1)$$

$$C_{BH} = 0.037803 DA_{BH} \quad (2)$$

$$C_{ET} = 0.006791 DA_{ET} \quad (3)$$

where C_{SS}, C_{BH} and C_{ET} are the concentration of SS, BH and ET, respectively; DA_{SS}, DA_{BH} and DA_{ET} are the first derivative amplitudes of mixture at 273 nm, 323 nm and 279 nm. These equations were directly utilized for the simultaneous estimation of SS, BH and ET in standard laboratory mixture. While the equation was used for the commercial formulation after applying the procedure described before.

2.4.3. Derivative ratio spectra zero crossing spectrophotometry (method 3)

The absorption spectra of pure drugs and their ternary mixtures were recorded between 220 nm and 300 nm. The absorption spectra of pure SS and their ternary mixture were divided by a standard spectrum of 10 μg mL^{-1} of ET, the absorption spectra of pure BH and their ternary mixture were divided by a standard spectrum of 10 μg mL^{-1} of ET and the absorption spectra of pure ET and their ternary mixture were divided by a standard spectrum of 10 μg mL^{-1} of BH and first derivative of the ratio spectra were plotted using delta lambda 8 nm and scaling factor 1. In the ternary mixture, the concentration of SS, BH and ET were proportional to the first derivative ratio signals at 276.8 nm (zero crossing point for BH where 10 μg mL^{-1} of ET was used as divisor), 248.6 nm (zero crossing point for SS where 10 μg mL^{-1} of ET was used as divisor) and 247.8 nm (zero crossing point for SS where 10 μg mL^{-1} of BH was used as divisor), respectively. Calibration graphs were obtained by measuring the derivative ratio amplitudes against the increasing concentration of pure SS, pure BH and pure ET using respective divisors. The content of SS, BH and ET in standard laboratory mixture was determined by use of above-mentioned procedure.

While for the commercial formulation analysis, after applying following procedure above-mentioned procedure was applied, 20 tablets were weighed and ground to a fine powder. From the resultant powder, an accurately weighed powder equivalent to one tablet was taken to a calibrated volumetric flask containing methanol. Solution was filtered through Whatman filter paper number 41 and absorbance of the ratio derivative spectra was measured at 276.8 nm, 248.6 nm and 247.8 nm for SS, BH and ET determination, respectively. All the three brands of tablets were tested according to the procedure described above.

2.4.4. Double divisor ratio spectra derivative method (method 4)

The absorption spectra of the pure drugs and their ternary mixtures were recorded between 220 nm and 300 nm. The absorption spectra of SS and their ternary mixture were divided by a standard spectrum obtained by the addition of stored spectrum of 10 μg mL^{-1} of BH and 10 μg mL^{-1} of ET and first derivative of the ratio spectra was plotted using delta lambda 8 nm and scaling factor 1. In the ternary mixture, the concentration of SS was proportional to the first derivative ratio signals at 235.6 nm. Calibration graph was obtained by measuring the derivative ratio amplitudes against the increasing concentration of pure SS by using same divisor described above. The content of SS was determined by use of above-mentioned calibration graph. Similarly, for determination of BH, a standard spectrum obtained by the addition of stored spectrum of 10 μg mL^{-1} of SS and 10 μg mL^{-1} of ET was used as divisor and for ET determination, a standard spectrum obtained by the addition of stored spectrum of 10 μg mL^{-1} of SS and 10 μg mL^{-1} of BH used as divisor. First derivative of the ratio spectra plotted using delta lambda were 8 nm and scaling facture 1. In the ternary mixture, the concentration of BH and ET were proportional to the first derivative ratio signals at 239.8 nm and 263.2 nm, respectively. Calibration graphs were obtained by measuring the derivative ratio amplitudes against the increasing concentration of pure BH and ET by using same respective divisors described above. While for the commercial formulation analysis, the same procedure was applied as in other methods and followed by above-mentioned procedure.

2.5. Validation parameters

2.5.1. Accuracy

For studying the accuracy of the proposed methods, and for checking the interference from excipients used in the dosage forms, recovery experiments were carried out by the standard addition method. This study was performed by addition of known amounts of salbutamol sulphate, bromhexine hydrochloride and etofylline to a known concentration of the commercial tablets. The amounts of standard recovered were calculated in terms of mean recovery with the upper and lower limits of percent relative standard deviation.

2.5.2. Precision

Intra-day precision and inter-day precision for the developed methods were measured in terms of % R.S.D. The experiments were repeated five times a day for intra-day precision and on 5 different days for inter-day precision. The concentration values for both intra-day precision and inter-day precision were calculated five times separately and percent relative standard deviation were calculated. Finally, the mean of % R.S.D. (% R.S.D. = [S/X] 100, where S is standard deviation and X is mean of the sample analysed) were taken for conclusion.

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

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to the 3s/m and 10s/m criterions, respectively, where s is the standard deviation of the absorbance ($n = 10$) of the sample and m is the slope of the corresponding calibration curve.

2.5.4. Reproducibility

The reproducibility of the method was determined by the use of different instruments: Shimadzu UV 1700 and Shimadzu UV 1601. The average value of % R.S.D. (% R.S.D. = [S/X] 100, where S is standard deviation and X is mean of the sample analysed) of the responses for the determination of SS, BH and ET were found as mentioned below which reveals the reproducibility of the method.

3. Results and discussion

The absorption spectra of the three compounds, SS, BH and ET overlapped closely shown in Fig. 1. For this reason, the determination of the above compounds was not possible by direct measurements of absorbance in zero-order spectra. On the other hand, derivative spectroscopy shows more resolution and makes it possible to analyse each drug in presence of one another as well as in presence of other excipients without any pretreatment.

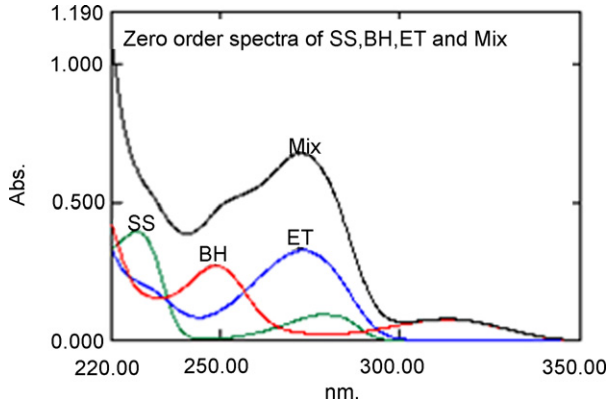

Fig. 1. Zero-order overlain spectra of SS (10 μg mL^{-1}), BH (10 μg mL^{-1}), ET (10 μg mL^{-1}) and their ternary mixture.

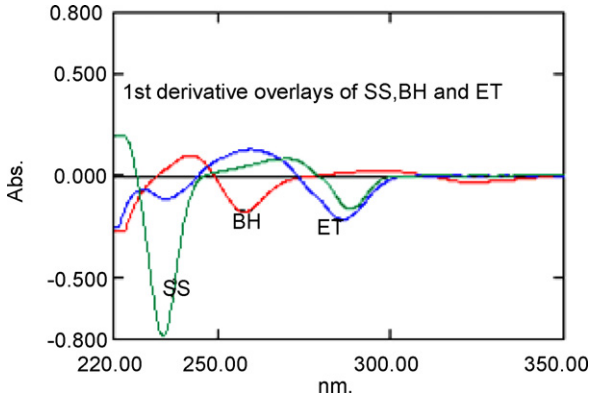

Fig. 2. First derivative overlain spectra of SS, BH and ET.

3.1. First derivative zero crossing spectrophotometry

In contrast to zero-order spectra, first derivative spectra show more resolution in terms of zero crossing points shown in Fig. 2. The first derivative wavelengths were considering 273 nm for SS determination, 323 nm for BH determination and 279 nm for ET determination. At 273 nm there is no contribution of BH and ET, SS was determined at this wavelength in the presence of other two drugs represented in Fig. 3a. At 323 nm, both SS and ET show zero absorbance; therefore BH was determined at this wavelength without any interference of other two represented in Fig. 3b. At 279 nm, ET was determined because it was zero crossing point for both SS and BH shown in Fig. 3c. The developed method was validated accurately and results of accuracy are shown in Table 1, summary of various validation parameters are listed in Table 2a, results of commercial formulation analysis are listed in Table 3a.

3.2. Simultaneous equation method

A set of three equations framed using these coefficient values are listed below:

$$C_{SS} = 0.001561 DA_{SS} \quad (1)$$

$$C_{BH} = 0.037803 DA_{BH} \quad (2)$$

$$C_{ET} = 0.006791 DA_{ET} \quad (3)$$

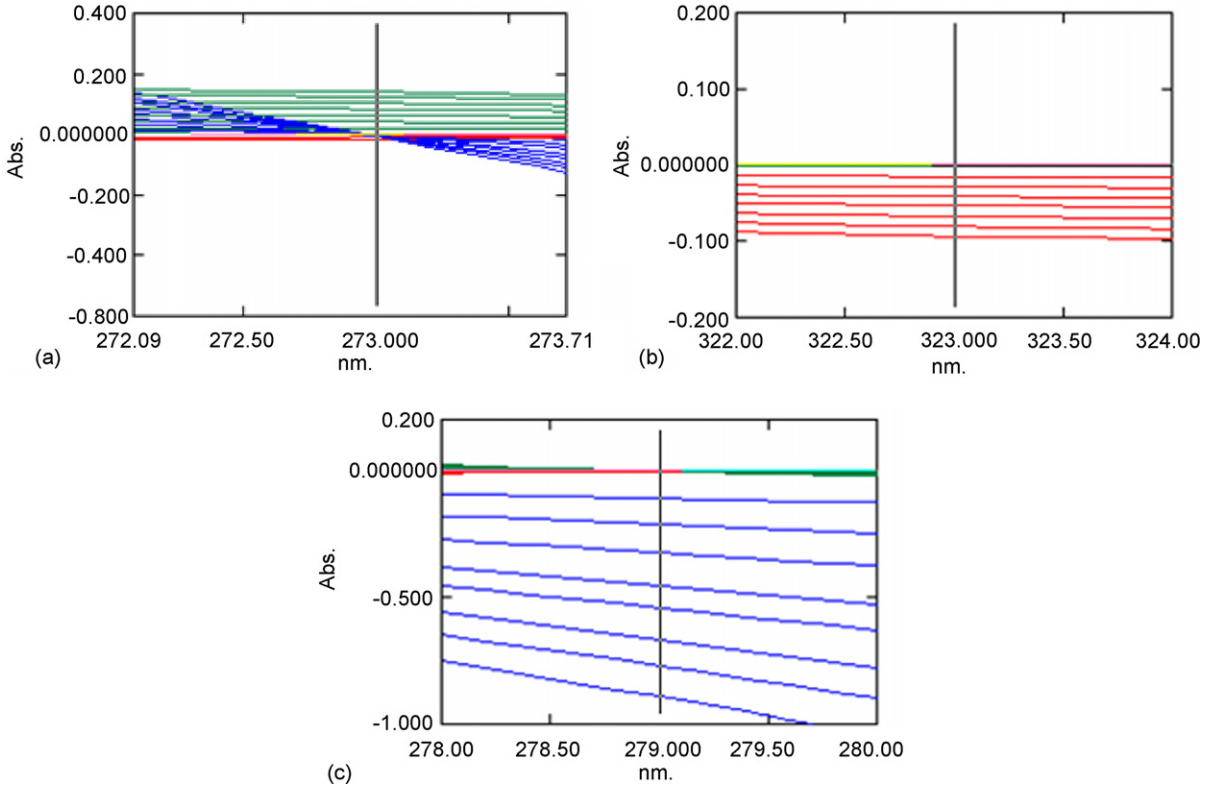

Fig. 3. (a) Determination of SS at 273 nm by method 1 in the presence of BH and ET. (b) Determination of SS at 273 nm by method 1 in the presence of BH and ET determination of BH at 323 nm by method 1 in the presence of SS and ET. (c) Determination of ET at 279 nm by method 1 in the presence of SS and BH.

Table 1
Results of recovery study of SS, BH and ET by all four methods

Amount added ($\mu g\,mL^{-1}$)			% Recovery[a] (method 1)[b]			% Recovery[a] (method 2)[c]			% Recovery[a] (method 3)[d]			% Recovery[a] (method 4)[e]		
SS	BH	ET	SS	BH	ET	SS	BH	ET	SS	BH	ET	SS	BH	ET
1.04	4.16	52	101.92	101.9	99.2	102	101.7	99.2	97.68	98.49	99.73	97.96	101.42	100.96
1.3	5.2	65	98.58	99.885	98.88	98.5	99.38	98.68	98.76	99.82	100.91	98.86	100.82	100.21
1.56	6.24	78	98.67	99.98	99.61	98.61	99.18	99.51	99.82	101.4	100.82	99.12	99.2	99.96
Mean recovery			99.72	100.58	99.33	99.7	100.08	99.62	98.66	99.91	100.42	98.64	100.48	100.37
S.D.			1.907	1.129	0.468	1.89	1.141	0.344	1.095	1.468	0.652	0.617	1.143	0.518

[a] Mean and standard deviation for 10 determinations.
[b] Method 1: First derivative zero crossing spectrophotometry.
[c] Method 2: Simultaneous equation method.
[d] Method 3: Derivative ratio spectra zero crossing spectrophotometry.
[e] Method 4: Double divisor ratio spectra derivative method.

where C_{SS}, C_{BH} and C_{ET} are the concentration of SS, BH and ET respectively; DA_{SS}, DA_{BH} and DA_{ET} are the first derivative amplitudes of mixture at 273 nm, 323 nm and 279 nm. These equations were directly utilized for the simultaneous estimation of SS, BH and ET in standard laboratory mixture as well as the commercial formulations. The developed method was validated accurately and results of accuracy are shown in Table 1, summary of various validation parameters are listed in Table 2a, results of commercial formulation analysis are listed in Table 3b.

3.3. Derivative ratio spectra zero crossing spectrophotometry

An accurate choice of either standard divisors or working wavelengths is fundamental for this method. In particular, by increasing or decreasing the concentration of divisor, the resulting derivative values and, hence, the slope of lines of regression are proportionately decreased or increased, with consequent variation of both sensitivity and linearity range. Several tests were made in a preliminary investigation by using standard divi-

Table 2
(a) Results of validation parameters obtained by method 1 and method 2 and (b) results of validation parameters of methods 3 and 4

Parameters	Method 1[a]			Method 2[b]		
	SS	BH	ET	SS	BH	ET
Part A						
Range ($\mu g\,mL^{-1}$)	1–35	4–40	5–80	1–35	4–40	5–80
Slope	0.0041	0.0026	0.0112	0.0041	0.0026	0.0112
Intercept	0.0008	0.0025	−0.007	0.0008	0.0025	−0.007
Correlation-coefficient (R^2)	0.9991	0.9997	0.9995	0.9991	0.9997	0.9995
Accuracy	99.72 ± 1.907	100.59 ± 1.13	99.33 ± 0.468	99.7 ± 1.89	100.08 ± 1.14	99.62 ± 0.344
Precision (% R.S.D.)	1.323	1.165	1.2012	1.14	0.9892	1.187
LOD ($\mu g\,mL^{-1}$)	0.2314	0.4865	0.2766	0.2314	0.4865	0.2766
LOQ ($\mu g\,mL^{-1}$)	0.7712	1.6217	0.9221	0.7712	1.6217	0.9221
Reproducibility (% R.S.D.)	1.112	0.987	1.254	1.01	0.9972	1.452
Parameters	Method 3[c]			Method 4[d]		
	SS	BH	ET	SS	BH	ET
Part B						
Range ($\mu g\,mL^{-1}$)	1–35	4–40	5–80	1–35	4–40	5–80
Slope	0.0013	0.0099	0.0016	0.0091	0.02	0.0343
Intercept	−0.0004	0.0085	−0.0011	0.0018	0.0169	−0.021
Correlation-coefficient (R^2)	0.9997	0.9997	0.9995	0.9991	0.9997	0.9995
Accuracy	98.66 ± 1.095	99.91 ± 1.468	100.48 ± 0.65	98.6 ± 0.61	100.48 ± 1.14	100.37 ± 0.52
Precision (% R.S.D.)	1.085	1.0615	1.045	1.45	1.5004	1.378
LOD ($\mu g\,mL^{-1}$)	0.3297	0.2784	0.7906	0.3161	0.2495	0.2064
LOQ ($\mu g\,mL^{-1}$)	0.9325	0.9282	2.6352	0.9869	0.8317	0.6879
Reproducibility (% R.S.D.)	1.652	1.152	1.225	1.04	1.456	1.687

[a] Method 1: First derivative zero crossing spectrophotometry.
[b] Method 2: Simultaneous equation method.
[c] Method 3: Derivative ratio spectra zero crossing spectrophotometry.
[d] Method 4: Double divisor ratio spectra derivative method.

Table 3
(a) Assay results of SS, BH and ET in combined commercial formulations by First derivative zero crossing spectrophotometry (method 1); (b) assay results of SS, BH and ET in combined commercial formulations by Simultaneous equation method (method 2); (c) assay results of SS, BH and ET in combined commercial formulations by derivative ratio spectra zero crossing spectrophotometry (method 3); (d) assay results of SS, BH and ET in combined commercial formulations by double divisor ratio spectra derivative method (method 4)

Formulation	% Labeled claim obtained for SS[a]	% Labeled claim obtained for BH[a]	% Labeled claim obtained for ET[a]
Part A			
Air vent[b]	99.94 ± 0.0356	100.42 ± 0.512	100.75 ± 0.282
Butabrom[c]	98.72 ± 0.0421	98.82 ± 0.426	99.86 ± 0.356
Saans[d]	98.46 ± 0.0254	99.75 ± 0.653	100.05 ± 0.269
Part B			
Air vent[b]	99.94 ± 0.356	100.42 ± 0.512	100.75 ± 0.282
Butabrom[c]	98.72 ± 0.421	98.82 ± 0.426	99.86 ± 0.356
Saans[d]	98.46 ± 0.254	99.75 ± 0.653	100.05 ± 0.269
Part C			
Air vent[b]	101.684 ± 0.569	100.928 ± 0.269	100.689 ± 0.785
Butabrom[c]	99.497 ± 0.945	98.928 ± 0.958	99.869 ± 1.122
Saans[d]	101.212 ± 0.456	100.694 ± 0.648	99.625 ± 0.647
Part D			
Air vent[b]	98.627 ± 0.429	99.686 ± 0.686	101.22 ± 1.147
Butabrom[c]	100.121 ± 0.986	99.845 ± 0.845	100.926 ± 0.465
Saans[d]	99.686 ± 0.784	100.687 ± 0.486	99.829 ± 0.869

[a] Mean and standard deviation for 10 determinations. Here ± sign indicates the upper and lower limits of standard deviation of 10 determinations.
[b] Brand A tablets.
[c] Brand B tablets.
[d] Brand C tablets.

sors in the concentration range from 5 to 40 μg mL^{-1}. The best results in terms of signal-to-noise ratio, sensitivity, repeatability, and range of validity of Beer's law were found by using 10 μg mL^{-1} of ET as divisor for SS and BH determination and 10 μg mL^{-1} of BH as divisor for ET determination. The first derivative wavelengths were considering 276.8 nm for SS determination, 248.6 nm for BH determination and 247.8 nm for ET determination. 276.8 nm was used for SS determination because

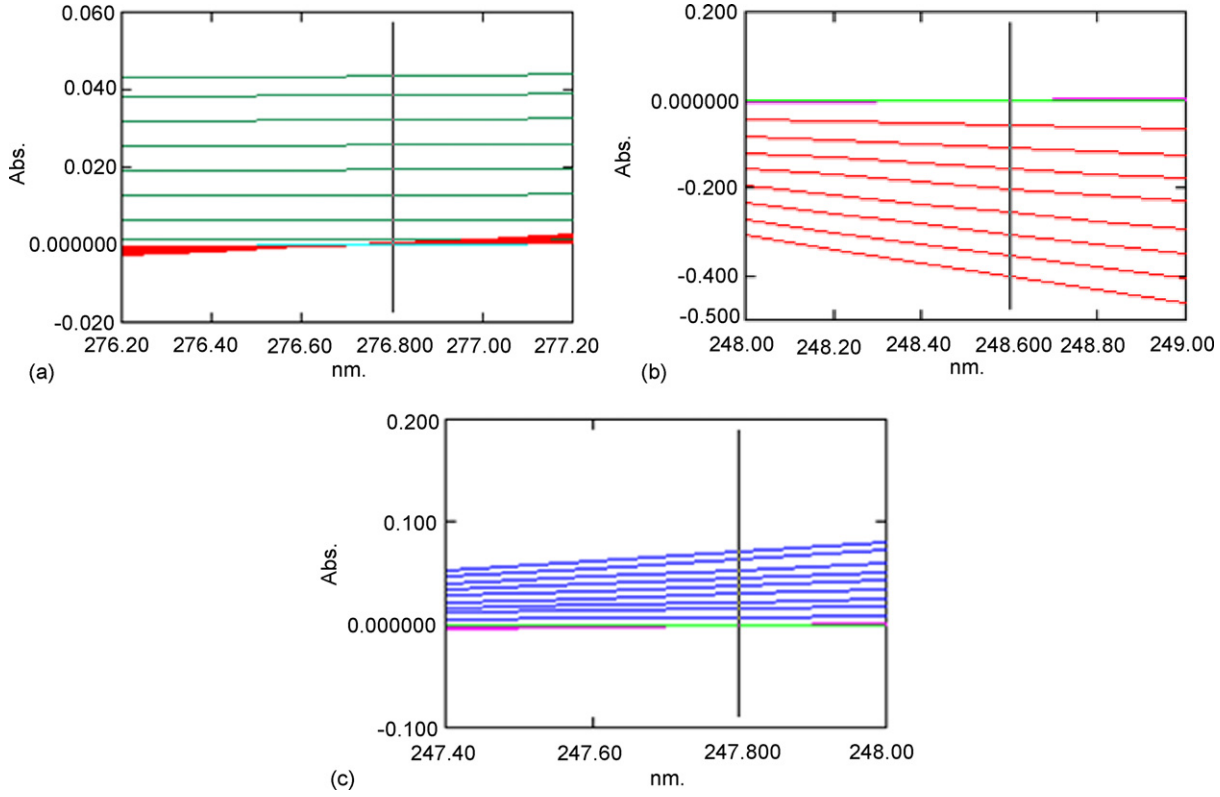

Fig. 4. (a) Determination of SS at 276.8 nm by method 3 in the presence of BH using ET (10 μg mL^{-1}) as divisor. (b) Determination of BH at 248.6 nm by method 3 in the presence of SS using ET (10 μg mL^{-1}) as divisor. (c) Determination of ET at 247.8 nm by method 3 in the presence of SS using BH (10 μg mL^{-1}) as divisor.

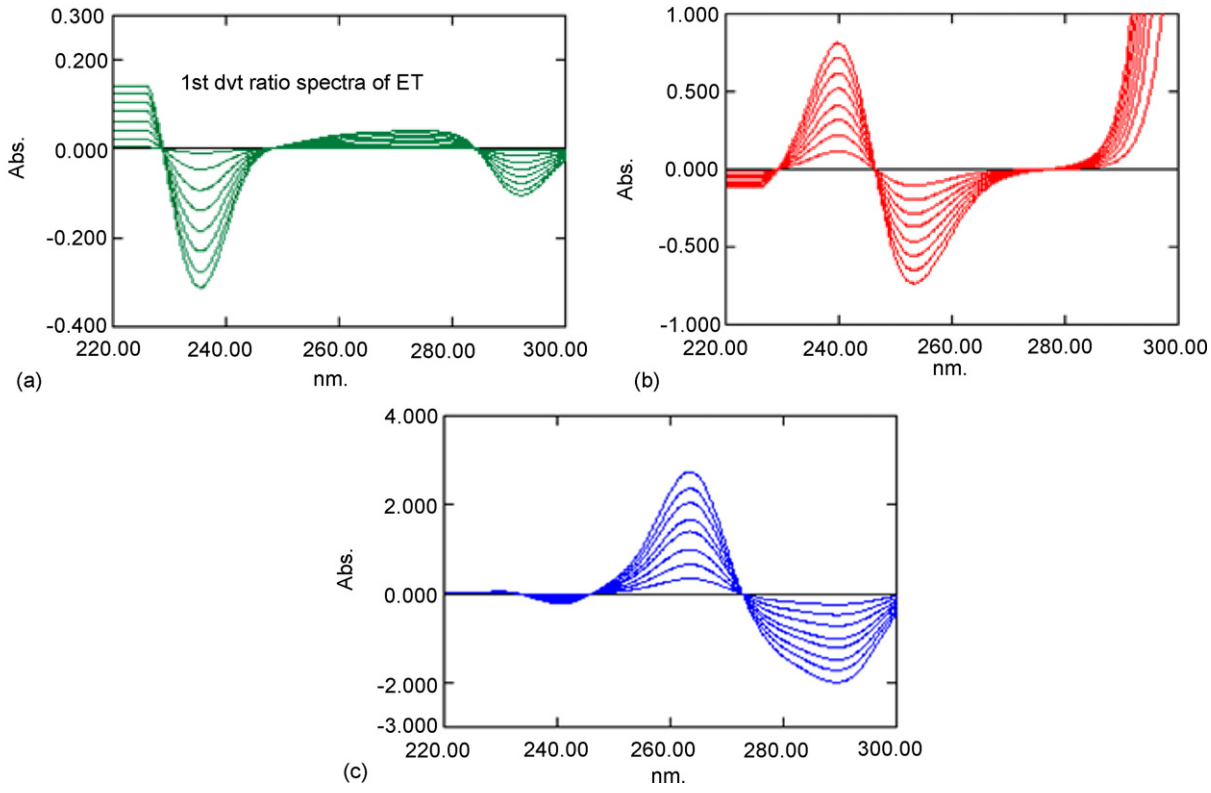

Fig. 5. (a) Determination of SS at 235.6 nm by method 4. (b) Determination of BH at 239.8 nm by method 4. (c) Determination of ET at 263.2 nm by method 4.

there is no contribution of BH at this wavelength (10 μg mL^{-1} ET as divisor) shown in Fig. 4a. Similarly, 248.6 nm was used for BH determination because it was zero crossing point for SS (10 μg mL^{-1} ET as divisor) shown in Fig. 4b. 247.8 nm was used for ET determination because at this wavelength SS had zero absorbance (10 μg mL^{-1} BH as divisor) shown in Fig. 4c. The developed method was validated accurately and results of accuracy are shown in Table 1, summary of various validation parameters are listed in Table 2b, results of commercial formulation analysis are listed in Table 3c.

3.4. Double divisor ratio spectra derivative method

In order to obtain the best spectra recoveries for SS, BH and ET it is necessary to study and optimize parameters such as standard divisor concentration, scaling factor, delta lambda,

Table 4
Statistical comparison of the results obtained by the developed four methods

Drugs	[a]Method 1	[b]Method 2	[c]Method 3	[d]Method 4
SS (mean ± S.D.)	99.72 ± 1.907	99.7 ± 1.89	98.66 ± 1.095	98.6 ± 0.61
$t_{calculated}$	0.864	1.248	1.235	0.956
$t_{theoretical}$	2.26	2.26	2.26	2.26
$F_{calculated}$	0.425	1.045	1.123	0.689
$F_{theoretical}$	3.18	3.18	3.18	3.18
BH (mean ± S.D.)	100.59 ± 1.13	100.08 ± 1.14	99.91 ± 1.468	100.48 ± 1.14
$t_{calculated}$	0.768	0.954	1.425	0.681
$t_{theoretical}$	2.26	2.26	2.26	2.26
$F_{calculated}$	0.245	0.478	1.289	0.525
$F_{theoretical}$	3.18	3.18	3.18	3.18
ET (mean ± S.D.)	99.33 ± 0.468	99.62 ± 0.344	100.48 ± 0.65	100.37 ± 0.52
$t_{calculated}$	1.054	0.879	1.845	0.652
$t_{theoretical}$	2.26	2.26	2.26	2.26
$F_{calculated}$	0.975	0.648	1.524	0.254
$F_{theoretical}$	3.18	3.18	3.18	3.18

Results obtained are average of 10 experiments for each; S.D., standard deviation.
[a] Method 1: First derivative zero crossing spectrophotometry.
[b] Method 2: Simultaneous equation method.
[c] Method 3: Derivative ratio spectra zero crossing spectrophotometry.
[d] Method 4: Double divisor ratio spectra derivative method.

etc. The best results in terms of signal-to-noise ratio, sensitivity, repeatability, and range of validity of Beer's law were found by using a standard spectrum obtained by the addition of stored spectrum of 10 μg mL^{-1} of BH and 10 μg mL^{-1} of ET as divisor for SS determination. For BH determination, best results were obtained using a standard spectrum obtained by the addition of stored spectrum of 10 μg mL^{-1} of SS and 10 μg mL^{-1} of ET as divisor. Similarly, for ET determination best results were obtained when a standard spectrum obtained by the addition of stored spectrum of 10 μg mL^{-1} of SS and 10 μg mL^{-1} of BH is used as divisor. The first derivative wavelengths were considering 235.6 nm for SS determination, 239.8 nm for BH determination and 263.2 nm for ET determination. The wavelengths were selected on the basis of maximum amplitudes and best linearity represented in Fig. 5a–c. The developed method was validated accurately and results of accuracy are shown in Table 1, summary of various validation parameters are listed in Table 2b, results of commercial formulation analysis are listed in Table 3d.

3.5. Statistical comparison of the results of the developed four methods

The proposed methods were successfully applied to the analysis of SS, BH and ET in combine pharmaceutical formulations without any interference of excipients and pretreatments. The results obtained were compared statistically by Student *t*-test and by the variance ratio *F*-test with those obtained by each method. The calculated values of the Student *t*-values at 95% confidence level and the variance ratio *F*-values did not exceed the theoretical values indicating that there were no significant differences among the results of the developed four methods represented in Table 4.

4. Conclusion

All the newly developed spectrophotometric methods for the simultaneous estimation of SS, BH and ET are simple, specific, accurate, precise, rapid and economical which indicates its adequacy for routine pharmaceutical analysis. It is concluded that derivative spectrophotometry is successfully utilized for the simultaneous estimation of SS, BH and ET in the combine dosage forms without any prior separation of individual drugs. In the absence of official monograph they can be used for their determination.

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