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# Simultaneous Determination of Salmeterol Xinafoate and Fluticasone Propionate in Bulk Powder and Seritide® Diskus using High Performance Liquid Chromatographic and Spectrophotometric Method

**Ahmed Samir[1], Hesham Salem[1]* and Mohammad Abdelkawy[2]**

[1]*Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA University), October 6 City, Egypt*
[2]*Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Egypt*

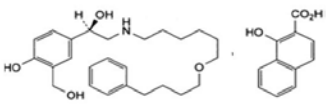
## Abstract

Five methods were developed for simultaneous determination of Salmeterol xinafoate (SAM) and Fluticasone propionate (FLU) without previous separation. In the first method (HPLC), a reversed-phase column and a mobile phase of acetonitrile: methanol (80:20 v/v) at 0.5 mLmin$^{-1}$ flow rate was used to separate both drugs and UV detection at 220 nm. Linearity was obtained in concentration ranges of 50-500 µgmL$^{-1}$ for Salmeterol xinafoate Fluticasone propionate. In the second method both drugs were determined using first derivative UV spectrophotometry, with zero crossing measurement at 352 and 269.5 nm for Salmeterol xinafoate and Fluticasone propionate, respectively. The third method depends on first derivative of the ratios spectra by measurements of the amplitudes at 334 and 337.5 nm for Salmeterol xinafoate and at 225 and 231.5 nm for Fluticasone propionate. Calibration graphs were established in the range of 4-28 µgmL-1 for both Salmeterol xinafoate and Fluticasone propionate. The fourth one depend on isosbestic point at 237.5 nm, while the content of Salmeterol xinafoate was determined by measuring the absolute value of the ultraviolet curves at 343 nm, without interference from Fluticasone propionate. All the proposed methods were extensively validated. They have the advantage of being economic and time saving. All the described methods can be readily utilized for the analysis of pharmaceutical formulations. The results obtained by adopting the proposed methods were statistically analyzed and compared with those obtained by official methods.

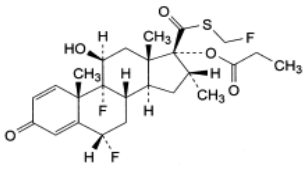
**Keywords:** Salmeterol xinafoate; Fluticasone propionate; HPLC; First derivative spectrophotometry; Ratio derivative spectrophotometry; Isosbestic point

## Introduction

Salmeterol xinafoate (SAM) and Fluticasone propionate (FLU) are formulated together in inhalation known as Seritide® Diskus inhalation, used to treat asthma. SAM is in a class of medications called $\beta_2$ adrenergic receptors agonists. FLU is in a class of medications called corticosteroids. The combination of SAM and FLU works by reversing the bronchoconstriction occurs in asthma.



Salmeterol xinafoate (SAM)



Fluticasone propionate (FLU)

Salmeterol xinafoate was determined by several methods including spectrophotometry [1-7], HPTLC [8], HPLC [9-11] and electrophoresis [12].

Fluticasone propionate was determined by spectrophotometric methods [13,14] and by HPLC [15-17].

HPLC is successful for the determination of drugs in pharmaceutical dosage forms and biological samples. Owing the widespread use of HPLC in routine analysis, it is thoroughly validated [18-20].

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 [21-23].

Ratio-spectra derivative spectrophotometric method has also been found to be useful in the estimation of drugs in their mixtures. 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 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 interferes the analysis [24-26].

The isosbestic point technique was extended for the analysis of two drugs in their binary formulation. Where, the spectra of two drugs are crossed at certain points, these points are so-called "isosbestic points" at which the two drugs have the same absorptivity, so the mixture of the two drugs act as single component that condemns the absorbance at isosbestic points as good measurements of the total concentration of both drugs in combination, if the concentration of either two drugs

***Corresponding author:** Hesham Salem, Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA University), October 6 City, Egypt, Tel: +2-100-60-500-29; E-mail: h_salem_eg@yahoo.com

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could be determined, separately, the concentration of the second one could be calculated by subtraction [27,28].

The aim of this paper was to demonstrate the capability of HPLC, first derivative spectrophotometry, derivative ratio spectrophotometry, isosbestic points for simultaneous analysis of both drugs in mixture form without the need of preliminary separation steps.

## Experimental

### Apparatus

HPLC, Agilent 1200 series, vacuum degasser, thermostatted column compartment G1316A/G1316B, diode array and multiple wavelength detector, quaternary pump (Germany). Chromatographic column; Zorbax ODS (4.6 cm × 250 mm, 5 µm). P.N. 880952-702. S.N. USF 0060920.

UV-Visible Spectrophotometer, Shimadzu UV-1800. connected to an IBM compatible computer. The software was UVPC personal spectroscopy software version 3.7 (Shimadzu). The absorption spectra of the reference and test solutions were carried out in a 1 cm quartz cells.

### Chemicals and pharmaceutical preparation

- All solvents were of HPLC grade and reagents were analytical grade.

- Salmeterol xinafoate pure sample was kindly provided by ADCO Co., Cairo, Egypt while Fluticasone propionate pure sample was kindly provided by Galaxo wellcome., Cairo, Egypt. Their percentage purity was found to be 99.87 ± 0.342 and 100.31 ± 0.52 according official B.P. methods 2007 [1], respectively.

-Seritide® diskus inhaler batch No R467369 (GalaxoSmithKline group) purchased from Egyptian market labeled to contain 50 µg Salmeterol xinafoate and 100 µg Fluticasone propionate / dose.

### Standard stock and working solutions

-For HPLC method, stock standard solutions of SAM and FLU were prepared separately by dissolving 50 mg of SAM or 50 mg of FLU in 100 mL mobile phase (80 mL acetonitrile and 20 mL methanol). Working standard solutions were prepared individually by diluting the stock solutions with the same mobile phase to obtain a concentration range of 50-500 µgmL$^{-1}$ for SAM or FLU.

-For spectrophotometric methods, stock standard solutions of SAM and FLU were prepared separately by dissolving 40 mg of each drug in methanol and completing the volume to 100 mL using the same solvent. Working stock solutions were prepared by diluting 10 mL of stock solution to 100 ml in a volumetric flask using the same solvent to get working solutions contain 40 µg/mL of SAM or FLU. Series of concentration of each drug is prepared by diluting this working solution to obtain a concentration range of 4-28 µg/mL for SAM and FLU.

### Methods of analysis

**HPLC method:** Triplicate 20 µL injections were made for each working standard solution. The peak area for each concentration was recorded and then plotted against the corresponding concentration to obtain the calibration graph for the determination of SAM and FLU (Tables 2 and 3), respectively.

**First derivative spectrophotometric method:** The values of the D1 amplitudes were measured at 352 nm (zero-crossing of FLU) and 269.5 nm (zero-crossing of SAM) for the determination of SAM and FLU (Tables 2 and 3), respectively.

**First derivative of the ratio spectrophotometric method:** According to the theory of the ratio-spectra derivative method. The stored UV absorption spectra of standard solutions of SAM were divided wavelength-by-wavelength by a standard spectrum of FLU (24 µgmL$^{-1}$). The first derivative was calculated for the obtained spectra with Δλ=4 nm. The amplitudes at 334 and 337.5 nm were measured and found to be linear to the concentrations of SAM. For FLU, the stored UV absorption spectra of standard solutions of FLU were divided wavelength-by-wavelength by a standard spectrum of SAM (12 µgmL$^{-1}$). The first derivative was calculated for the obtained spectra with Δλ=4 nm. The amplitudes at 225 and 231.5 nm were measured and found to be linear to the concentration of FLU (Tables 2 and 3).

**Isosbestic method:** The zero order absorption spectra of 24 µgmL$^{-1}$ of each SAM and FLU were recorded, and the spectrum of 12 µgmL$^{-1}$ of SAM/FLU in a mixture was recorded. Construct calibration curve relating the absorbance of the zero order spectra of SAM at wavelength 237.5 nm (isosbestic point) to the corresponding concentrations in µgmL$^{-1}$ of SAM. Construct a calibration curve relating the absorbance of the zero order spectra of SAM at λ=343 nm to the corresponding concentrations in µgmL$^{-1}$ of SAM (Tables 2 and 3).

| | | HPLC | | | D1 | | | DD1 | | | | iso | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | official method | taken | found | recovery | taken | found | recovery | found | recovery at 337.5 | found | recovery at 334 | found | recovery at 343 |
| | | 50 | 48.74 | 97.49 | 4 | 4.01 | 100.27 | 4.04 | 101.1 | 3.99 | 99.79 | 3.99 | 99.76 |
| | | 100 | 99.62 | 99.62 | 8 | 7.98 | 99.72 | 7.91 | 98.88 | 8.02 | 100.22 | 8.09 | 101.18 |
| | | 200 | 200.4 | 100.2 | 12 | 11.94 | 99.54 | 12.19 | 101.59 | 12.04 | 100.37 | 12.20 | 101.65 |
| | | 300 | 306.69 | 102.23 | 16 | 16.22 | 101.36 | 15.92 | 99.49 | 16.07 | 100.44 | 16.04 | 100.23 |
| | | 400 | 399.63 | 99.91 | 20 | 19.88 | 99.39 | 19.79 | 98.93 | 19.73 | 98.65 | 19.74 | 98.72 |
| | | 500 | 496.48 | 99.3 | 24 | 23.85 | 99.36 | 24.07 | 100.27 | 24.12 | 100.51 | 23.98 | 99.92 |
| | | | | | 28 | 28.12 | 100.42 | 28.07 | 100.25 | 28.03 | 100.09 | 28.08 | 100.30 |
| X- | 100.29 | | | 99.79 | | | 100.01 | | 100.07 | | 100.01 | | 100.25 |
| ±SD | 1.226 | | | 1.53 | | | 0.725 | | 1.041 | | 0.647 | | 0.959 |
| %RSD | 1.222 | | | 1.533 | | | 0.725 | | 1.04 | | 0.647 | | 0.957 |
| N | | | | 6 | | | 7 | | 7 | | 7 | | 7 |
| t | | | | 0.246 | | | 0.194 | | 0.219 | | 0.473 | | 0.549 |
| F | | | | 1.80 | | | 2.057 | | 1.878 | | 1.794 | | 1.052 |

**Table 1:** determination and statistical analysis of the results obtained for assay of authentic SAM using, HPLC, D1, DD1, isosbestic point methods compared with official method [1].

## Analysis of laboratory prepared mixtures

**HPLC method:** In volumetric flasks 10 mL, aliquot volumes of SAM and FLU from their corresponding stock solutions were transferred accurately to prepare mixtures containing different ratios of the two drugs (Table 4).

**First derivative spectrophotometric method:** Aliquots equivalent to 80-200 µg of SAM from its working standard solution were transferred into a series of 10-mL volumetric flasks. Different aliquots equivalent to 80-200 µg of FLU from their stock standard solutions were added to the same flasks with different ratios and the volume is completed to 10 with methanol (Table 4).

**First derivative of the ratio spectrophotometric method:** The spectra of the prepared solutions were recorded and stored, then divided by the spectrum of 24 $\mu gmL^{-1}$. FLU then from the peak amplitude at ($DD1_{334\ nm\ and\ 337\ nm}$), the concentration of SAM in the mixtures was obtained by substituting in regression equation. To determine FLU in mixtures, the stored spectra were divided by the spectrum of 12 $\mu gmL^{-1}$ SAM, then the peak amplitude at ($DD1_{225\ nm\ and\ 231.5nm}$) were obtained and the concentration of FLU was calculated by substituting in regression equation. Results obtained are shown in Table 4.

**Isosbestic method:** In volumetric flask 10 mL, transfer accurately aliquots of SAM and FLU from their corresponding working solutions, to prepare mixtures containing different ratios of each two drug in their binary mixtures. For analysis of SAM/FLU binary mixture, measure the absorbance of the resulting solutions at 237.5 nm (the isosbestic point) corresponding to the total content of SAM and FLU in SAM/FLU binary mixture and at 343 nm is corresponding to the content of SAM. Calculate the total concentrations of SAM and FLU, then subtract the corresponding concentration of SAM from the result

| | | HPLC | | | D1 | | | DD1 | | | | iso | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | offecial method | taken | found | recovery | taken | found | recovery | found | recovery at 225 | found | recovery at 231.5 | found | recovery at 237.5 |
| | | 50 | 51.27 | 102.54 | 4 | 4.04 | 101.04 | 4.01 | 100.25 | 4.03 | 100.66 | 4.11 | 102.63 |
| | | 100 | 99.22 | 99.22 | 8 | 7.79 | 97.43 | 8.12 | 101.55 | 8.04 | 100.47 | 8.04 | 100.56 |
| | | 200 | 202.48 | 101.24 | 12 | 12.12 | 101.04 | 12.06 | 100.52 | 11.97 | 99.77 | 11.90 | 99.20 |
| | | 300 | 296.09 | 98.7 | 16 | 16.17 | 101.04 | 16.08 | 100.5 | 15.91 | 99.42 | 15.87 | 99.19 |
| | | 400 | 404.24 | 101.06 | 20 | 19.92 | 99.6 | 20.14 | 100.7 | 19.99 | 99.97 | 19.92 | 99.59 |
| | | 500 | 500.7 | 100.14 | 24 | 23.96 | 99.84 | 24.27 | 101.1 | 24.08 | 100.34 | 24.10 | 100.42 |
| | | | | | 28 | 28 | 100 | 28.18 | 100.63 | 27.99 | 99.98 | 28.07 | 100.24 |
| X- | 100.58 | | | 100.48 | | | 99.99 | | 100.48 | | 100.09 | | 100.26 |
| ±SD | 0.805 | | | 1.421 | | | 1.293 | | 0.437 | | 0.430 | | 1.187 |
| %RSD | 0.800 | | | 1.414 | | | 1.239 | | 0.435 | | 0.428 | | 1.184 |
| N | | | | 6 | | | 7 | | 7 | | 7 | | 7 |
| t | | | | 1.172 | | | 0.592 | | 0.011 | | 0.0235 | | 0.244 |
| F | | | | 2.494 | | | 3.172 | | 1.741 | | 4.395 | | 3.268 |

**Table 2:** Determination and statistical analysis of the results obtained for assay of authentic Fluticasone propionate using, HPLC, D1, DD1 and isosbestic points methods compared with official method.

| | | HPLC | | | D1 | | | DD1 | | | | iso | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | offecial method | taken | found | recovery | taken | found | recovery | found | recovery at 225 | found | recovery at 231.5 | found | recovery at 237.5 |
| | | | | | | | | | | | | | |
| | | 50 | 51.27 | 102.54 | 4 | 4.04 | 101.04 | 4.01 | 100.25 | 4.03 | 100.66 | 4.11 | 102.63 |
| | | 100 | 99.22 | 99.22 | 8 | 7.79 | 97.43 | 8.12 | 101.55 | 8.04 | 100.47 | 8.04 | 100.56 |
| | | 200 | 202.48 | 101.24 | 12 | 12.12 | 101.04 | 12.06 | 100.52 | 11.97 | 99.77 | 11.90 | 99.20 |
| | | 300 | 296.09 | 98.7 | 16 | 16.17 | 101.04 | 16.08 | 100.5 | 15.91 | 99.42 | 15.87 | 99.19 |
| | | 400 | 404.24 | 101.06 | 20 | 19.92 | 99.6 | 20.14 | 100.7 | 19.99 | 99.97 | 19.92 | 99.59 |
| | | 500 | 500.7 | 100.14 | 24 | 23.96 | 99.84 | 24.27 | 101.1 | 24.08 | 100.34 | 24.10 | 100.42 |
| | | | | | 28 | 28 | 100 | 28.18 | 100.63 | 27.99 | 99.98 | 28.07 | 100.24 |
| X- | 100.58 | | | 100.48 | | | 99.99 | | 100.48 | | 100.09 | | 100.26 |
| ±SD | 0.805 | | | 1.421 | | | 1.293 | | 0.437 | | 0.430 | | 1.187 |
| %RSD | 0.800 | | | 1.414 | | | 1.239 | | 0.435 | | 0.428 | | 1.184 |
| N | | | | 6 | | | 7 | | 7 | | 7 | | 7 |
| t | | | | 1.172 | | | 0.592 | | 0.011 | | 0.0235 | | 0.244 |
| F | | | | 2.494 | | | 3.172 | | 1.741 | | 4.395 | | 3.268 |

**Table 3:** Determination of SAM and Fluticasone propionate in Seritide® Diskus inhaler by the proposed procedures.

| | SAM | | | | | FLU | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HPLC | D1 | DD1 | | iso | HPLC | D1 | DD1 | | Iso |
| | | | 334 | 337.5 | 343 | | | 225 | 231.5 | 237.5 |
| X- | 100.47 | 100.48 | 100.132 | 100.62 | 100.73 | 100.81 | 101.04 | 100.65 | 100.46 | 100.48 |
| ±SD | 0.788 | 1.182 | 0.924 | 0.957 | 1.298 | 0.498 | 1.311 | 0.971 | 1.543 | 1.330 |
| %RSD | 0.784 | 1.176 | 0.923 | 0.951 | 1.289 | 0.494 | 1.298 | 0.965 | 1.536 | 1.324 |
| N | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

**Table 4:** Determination of SAM and Fluticasone propionate in laboratory prepared mixtures by the proposed methods.

(corresponding to the sum of SAM and FLU concentration), to get the concentration of FLU. The results are shown in table 4.

## Results and Discussion

### HPLC method

The developed RP-HPLC method has been applied for the simultaneous determination of SAM and FLU. To optimize the RP-HPLC assay parameters, the mobile phase composition was studied. A satisfactory separation was obtained with a mobile phase consisting of acetonitrile-methanol (80:20, v/v). Aqueous mobile phase like buffer was avoided to prevent the probability of salmeterol xinafoate ionization to give two different peaks one for salmeterol base and the other for xinafoic acid, the ratio of acetonitrile/methanol was chosen to give complete separation with short analysis time. The optimum wavelength for detection was 220 nm at which much better detector responses for the two drugs were obtained. The mobile phase was found to be suitable to improve the sharpness of SAM and FLU peaks. The retention times for the investigated drugs were found to be 2.610 min (SAM), and 3.390 min (FLU). The specificity of the RP-HPLC method is illustrated in Figure 1 where complete separation of the studied compounds was noticed.

### First derivative spectrophotometric method

The zero-order absorption spectra of SAM and FLU in methanol are shown in Figure 2. The spectra display overlapping in the region of 200-300 nm. This makes the determination of FLU in the presence of SAM by conventional UV spectrophotometry difficult, but the determination of SAM from 300 to 350 nm might be possible without interference from FLU. The derivative spectrophotometry technique was, however, chosen for the determination of both the drugs since it could remove broadband contributions from excipients and might also overcome the interference from peak overlapping. The experiments showed that the first derivative spectra of SAM and FLU were simple and gave results with suitable precision at Δλ=6 nm. In this first derivative spectrum the signals at 352 nm (FLU reads zero) are proportional to the SAM concentration and the signals at 269.5 nm (zero crossing point of SAM) are proportional to the FLU concentration (Figure 3).

### First derivative of the ratio spectrophotometric method

Figure 2 shows the UV absorption spectral overlap of SAM and FLU at their nominal concentrations. However, solving this spectral overlap was sufficiently enough to demonstrate the advantage of the derivative ratio method. To optimize the simultaneous determination of the two drugs by using DD1 method, it is necessary to test the influence of the divisor standard concentration, the Δλ and smoothing function (Figures 4 and 5). Δλ=4 nm was selected as the optimum value. From several tests for correct choice of the divisor standard concentration, the best results in terms of signal to noise ratio, sensitivity and repeatability followed using normalized spectra as divisor (obtained by dividing the spectra of several standards of different concentrations by their corresponding concentrations and subsequently averaging them, in order to obtain a spectrum of unit concentration). The first derivative of the ratio peak amplitudes at the specified wavelength 334 and 337.5 nm were found to be proportional to the concentration of SAM and at 225 and 231.5 nm were found to be proportional to the concentration of FLU (Figures 6 and 7).

### Isosbestic method

In the isosbestic method, total concentration of both drugs in the mixture (T) was determined at the previously chosen isosbestic points. This theory could be confirmed experimentally by recording

| | | HPLC | | | | Spectro | | recovery | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | taken | | recovery | | taken | | D1 | | DD1 | | | iso | |
| mix | SAM : FLU ratio | SAM | FLU | SAM | FLU | SAM | FLU | SAM | FLU | SAM at 334 | SAM at 337.5 | FLU at 231.5 | SAM at 343 | FLU 237.5 |
| 1 | 1::1 | 100 | 100 | 101.53 | 100.52 | 12 | 12 | 99.54 | 101.04 | 98.14 | 100.37 | 101.05 | 99.44 | 101.84 |
| 2 | 1::3 | 100 | 300 | 100.93 | 100.58 | | | | | | | | | |
| 3 | 1::2 | 100 | 200 | 101.19 | 99.61 | 8 | 16 | 99.54 | 102.48 | 101.98 | 102.07 | 100.85 | 101.18 | 100.20 |
| 4 | 3::2 | 300 | 200 | 100.79 | 101.58 | 12 | 8 | 102.10 | 101.04 | 98.93 | 99.87 | 100.23 | 101.65 | 99.53 |
| 5 | 2::3 | 200 | 300 | 101.25 | 99.85 | 8 | 12 | 99.72 | 101.04 | 100.60 | 98.17 | 99.61 | 100.23 | 100.24 |
| 6 | 2::5 | | | | | 8 | 20 | 99.72 | 101.04 | 102.33 | 98.70 | 100.26 | 99.52 | 100.66 |
| 7 | 4::3 | | | | | 16 | 12 | 101.30 | 101.04 | 98.14 | 100.37 | 101.23 | 100.26 | 100.24 |
| 8 | 4::5 | | | | | 8 | 20 | 101.43 | 101.48 | 102.08 | 101.97 | 100.26 | 99.40 | 101.01 |
| x- | | | | 100.82 | 100.74 | | | 100.48 | 101.31 | 100.31 | 100.22 | 100.49 | 100.24 | 100.53 |
| ±SD | | | | 0.738 | 0.665 | | | 1.089 | 0.542 | 1.889 | 1.481 | 0.568 | 0.889 | 0.735 |
| %RSD | | | | 0.732 | 0.660 | | | 1.084 | 0.535 | 1.883 | 1.478 | 0.565 | 0.887 | 0.731 |

**Table 5:** Determination of SAM and Fluticasone propionate in laboratory prepared mixtures by the proposed methods.

| HPLC | | | | | | | | | | %Recovery | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| drug taken (µg\mL) | | standard added(µg\mL) | | %Recovery | | drug taken (µg\mL) | | standard added (µg\mL) | | D1 | | DD1 | | | | ISO | |
| SAM | FLU | SAM | FLU | SAM | FLU | SAM | FLU | SAM | FLU | SAM | FLU | SAM at 334 | SAM at 337.5 | FLU 225 | FLU at 231.5 | SAM at 343 | FLU at 237.5 |
| 100 | 100 | 50 | 50 | 99.00 | 101.92 | 10 | 10 | 4 | 4 | 101.25 | 99.25 | 100.25 | 99.75 | 100.50 | 99.75 | 100.50 | 101.75 |
| 100 | 100 | 100 | 100 | 101.36 | 100.96 | 10 | 10 | 6 | 6 | 98.50 | 98.17 | 102.00 | 100.33 | 101.83 | 101.00 | 99.67 | 101.50 |
| 100 | 100 | 150 | 150 | 99.53 | 100.66 | 10 | 10 | 8 | 8 | 101.50 | 100.62 | 99.25 | 99.25 | 98.63 | 99.00 | 99.88 | 98.52 |
| x- | | | | 99.96 | 101.09 | | | | | 100.42 | 99.35 | 100.50 | 99.77 | 100.32 | 99.92 | 100.01 | 100.50 |
| ± SD | | | | 1.238 | 0.719 | | | | | 1.665 | 1.230 | 1.392 | 0.542 | 1.612 | 1.010 | 0.438 | 1.952 |
| %RSD | | | | 1.238 | 0.711 | | | | | 1.658 | 1.238 | 1.385 | 0.543 | 1.606 | 1.011 | 0.438 | 1.942 |

**Table 6:** Results of the application of the standard addition technique for the determination of Seritide® Diskus inhaler by the proposed procedures.

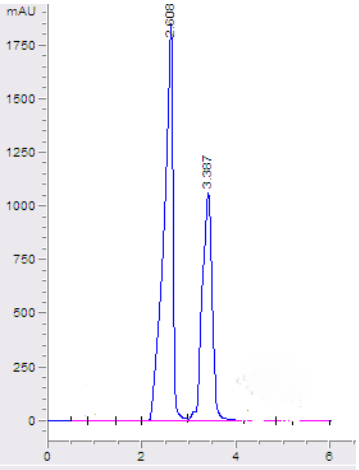


**Figure 1:** Chromatogram of the separation of SAM (retention time=2.6 min) and FLU(retention time=3.39 min) using RP-HPLC method.

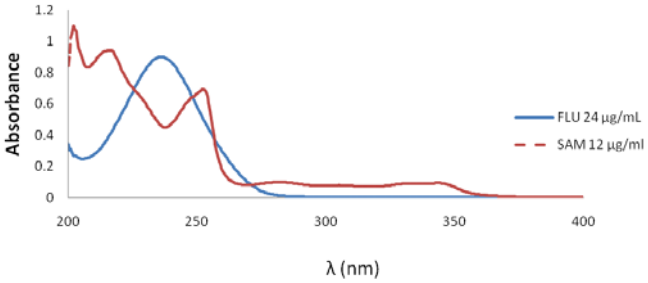


**Figure 2:** Zero order absorption spectra of SAM, 12 µg/mL and FLU 24 µg/mL using methanol as solvent.

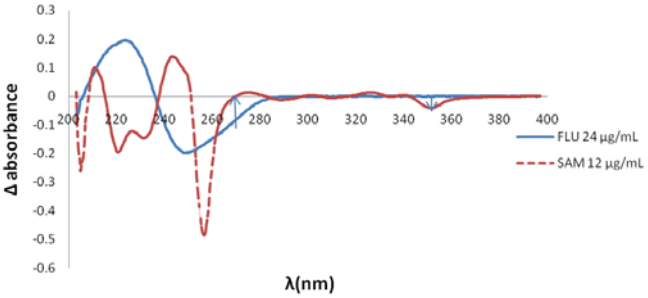


**Figure 3:** First derivative of absorption spectra of SAM 12 µg/mL and FLU 24 µg/mL in methanol showing zero crossing points, Δλ = 6 nm.

the absorbance spectra of 16 µgmL$^{-1}$ of SAM and FLU separately and a mixture containing same total concentration (8 µgmL$^{-1}$ of each) as shown in Figure 8. As shown in Fig.2, SAM has maxima at 343 nm while FLU has no absorbance, so the concentration of SAM (B) could be determined without any interference from FLU. The concentration of FLU could be calculated by subtraction (T-B). In the isosbestic method, the linearity between the zero order absorption of its spectra of SAM at 237.5 and 343 nm and the corresponding concentrations of the drug were studied.

## Analysis of pharmaceutical products

The proposed HPLC, D1, DD1, Isosbestic and Absorptivity factor

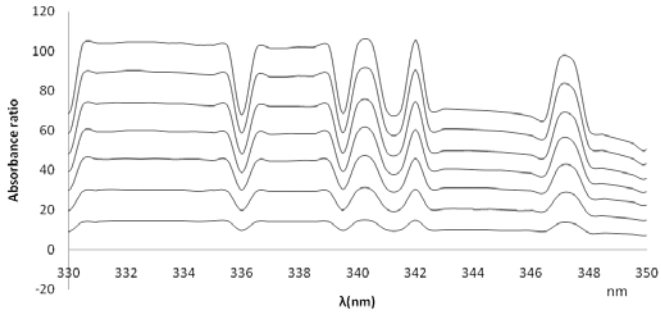


**Figure 4:** Ratio spectra of SAM (8-28 µg/mL), using the spectrum of FLU (24 µg/mL) as a divisor.

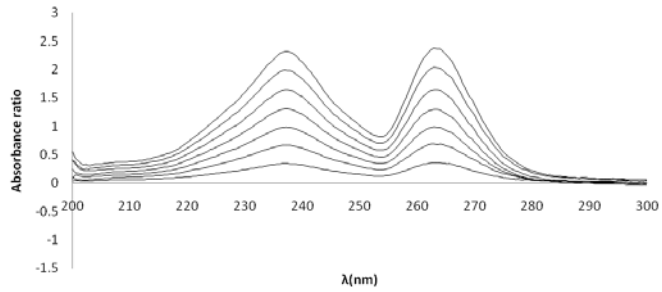


**Figure 5:** Ratio spectra of FLU (4-28 µg/mL) using the spectrum of SAL 12 µg/mL as a divisor.

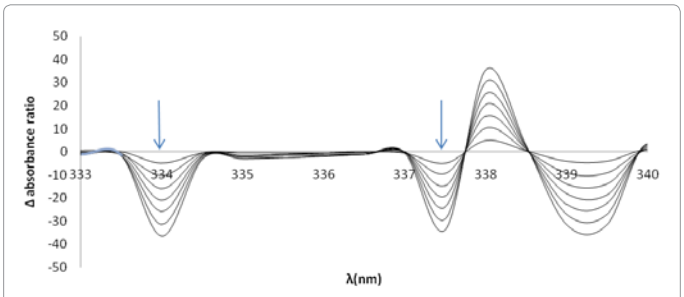


**Figure 6:** First derivative of Ratio spectra of SAM (8-28 µg/mL), using the spectrum of FLU 24 µg/mL as a divisor, Δλ=4nm.

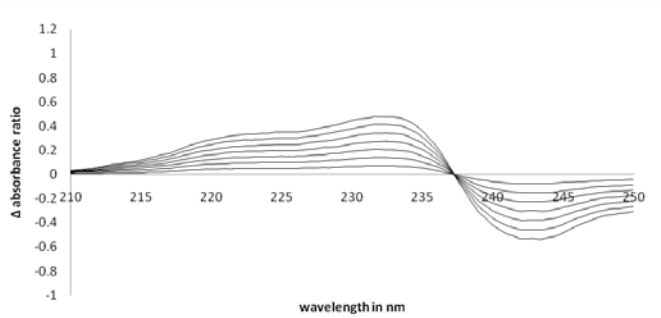


**Figure 7:** First derivative of Ratio spectra of FLU (4-28 µg/mL) using the spectrum of SAL 12 µg/mL as a divisor.

methods were applied for the simultaneous determination of SAM and FLU in Seritide® Diskus inhaler**.** The performance of the HPLC, D1, DD1, isosbestic and Absorptivity factor methods were statistically compared with that of a reported method by Student`s t-test and f-values at 95% confidence level. The calculated t and F-values did not exceed the theoretical values, indicating that there was no significant difference between HPLC, D1, DD1, isosbestic and Absorptivity factor methods and the reported method with regard to accuracy and precision (Table 5). Further, to check the validity of the proposed methods, the standard addition method was applied by adding different amounts of SAM and FLU to the previously analyzed inhaler (Table 6). The results of analysis of the commercial inhaler and the recovery study suggested that there is no interference from any excipients present in the inhaler.

## Validation of the methods

**Linearity:** The linearity of the HPLC, D1, DD1, isosbestic and Absorptivity factor methods for determination of SAM and FLU was evaluated by analyzing a series of different concentrations of each drug. In this study six concentrations ranging between 50-500 $\mu gmL^{-1}$ for SAM and FLU, respectively, for HPLC method and seven concentrations ranging between 4-28 $\mu gmL^{-1}$ for SAM and FLU, respectively, for the spectrophotometric methods. Each concentration was repeated three times; in order to provide information on the variation peak area and spectrophotometric values between samples of same concentration. The linearity of the calculated graphs was validated by the high value of the correlation coefficient and the intercept value (Table 1). Characteristic parameters for regression equations of the studied methods obtained by least squares treatment of the results are given in Table 1.

**Range:** The calibration range was established through consideration of the practical range necessary, according to each compound concentration present in the pharmaceutical product, to give accurate, precise and linear results. The calibration range of the proposed methods is given in Table 1.

**Detection and quantitation limits:** According to the International Conference on Harmonization (ICH) recommendations, the approach based on the standard deviation (SD) of the response and the slope was used for determining the detection and quantitation limits. The theoretical values for the proposed methods were assessed practically and given in Table 1.

**Precision:** The precision of the methods was evaluated by calculating the relative standard deviation of the assay results. The mean relative standard deviations are presented in tables 2-5 and can be considered to be satisfactory.

**Selectivity:** Methods selectivity was achieved by preparing several laboratory-prepared mixtures of the studied compounds at various concentrations within the linearity range. The laboratory-prepared mixtures were analyzed according to the previous procedures described under the proposed methods. Satisfactory results were obtained. Table 4 indicating the high selectivity of the proposed methods for simultaneous determination of SAM and FLU.

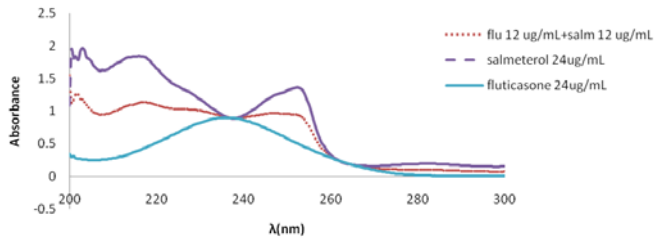


**Figure 8:** Spectra of SAM 24 µg/mL , FLU 24 µg/mL and a mixture contains 12 µg/mL of each showing isosbestic point at 237.5 nm.

**Accuracy:** The interference of excipients in the pharmaceutical formulations was studied in detail by HPLC, D1, DD1, isosbestic and absorpativity factor methods. For this reason, standard addition method was applied to the pharmaceutical formulation containing these compounds. In application of standard addition method to the pharmaceutical formulation, the mean percentage recoveries and their standard deviation for the proposed methods for nine replicate were calculated (Table 6). According to the obtained results a good precision and accuracy was observed for these methods. Consequently, the excipients in pharmaceutical formulation do not interfere in the analysis of these compounds in the pharmaceutical formulation.

**Analytical solution stability:** The analytical solutions of the studied compounds in mobile phase or spectrophotometric solvent exhibited no chromatographic or absorbance changes for 6 h when kept at room temperature and for 1 day when stored refrigerated at 5°C.

## Conclusion

For routine analytical purposes, it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with due accuracy and precision. Spectrophotometric techniques can generate large amounts of data within a short period of analysis, In this paper also a comparative study of the use of HPLC and spectrophotometric methods for the resolution of SAM and FLU in their multicomponent mixtures have been accomplished achieving short time of analysis and comparative law flaw rate, avoiding usage of buffer eliminate the risk of error due to the PH range, showing that D1, DD1 and isosbestic methods provide, a clear example of the high resolving power and low cost of this technique. Although the HPLC method is more specific, it needs expensive equipment and materials.

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