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Development and validation of spectroscopic simultaneous equation method for simultaneous estimation of betamethasone and luliconazole in synthetic mixture

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Abstract

A simple, specific, accurate and precise spectrophotometric method was developed and validated for simultaneous estimation of Betamethasone and Luliconazole in Synthetic Mixture. The wavelength of estimation for Betamethasone was 243.20 nm and for Luliconazole was 225.00 nm. Beer's law is obeyed in the concentration range of 10-50µg/ml and 20-100µg/ml and correlation coefficient of 0.999 and 0.999 for Betamethasone and Luliconazole respectively. The % recovery for Betamethasone and Luliconazole were found to be 100.49 % & - 100.68 % respectively. Intraday precision of Betamethasone and Luliconazole were found to be 0.10 %– 0.28 % and 0.11 % – 0.24 % RSD and Interday precision were found to be 0.27 %– 0.37 % and 0.23 %– 0.35 % RSD respectively. The proposed method was also evaluated by the Assay of Synthetic mixture containing Betamethasone and Luliconazole. The % Assay was found to be 100.60 % for Luliconazole and 100.52 % for Betamethasone. Validation of proposed methods was carried out according to ICH Q_2R_1Guidelines. The proposed methods were found accurate and reproducible for routine analysis of both the drugs in synthetic mixture.

Keywords: Betamethasone; Luliconazole; Simultaneous equation method; UV Spectroscopy

1 Introduction

Fungi are eukaryotic, heterotrophic organisms that live as parasites.

Complex organisms in comparison to bacteria. Have nucleus and well-defined nuclear membrane, and chromosomes. Have rigid cell wall composed of chitin (bacterial cell wall is composed of peptidoglycan) Fungal cell membrane contains ergosterol, human cell membrane is composed of cholesterol Antibacterial agents are not effective against fungi. Fungal infections are also called as mycoses.

Systemic fungal infections are a major cause of death in patients whose immune system is compromised, cancer or its chemotherapy, organ transplantation, HIV-1 infection.

The development of antifungal agents has lagged behind that of antibacterial agents. This is a predictable consequence of the cellular structure of the organisms involved.

Bacteria are prokaryotic and hence offer numerous structural and metabolic targets that differ from those of the human host. Fungi, in contrast, are eukaryotes, and consequently most agents toxic to fungi are also toxic to the host. Furthermore, because fungi generally grow slowly and often in multicellular forms, they are more difficult to quantify

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than bacteria. This difficulty complicates experiments designed to evaluate the in vitro or in vivo properties of a potential antifungal agent.

Patent No. 46. WO2017/203456A1, Luliconazole stable topical compositions, 2017.Hence, there is a scope to develop analytical methods for Betamethasone and Luliconazole in combination.

Literature review reveals that, various analytical methods have been reported for the estimation of Betamethasone & Luliconazole in pharmaceutical formulation and bulk drug include UV spectrophotometric method, High-performance liquid chromatography method (HPLC), NMR Spectroscopy and GC-FID Methods, Stability indicating RP-HPLC method, HPTLC method, TLC method, Capillary electrophoresis and UPLC method in individual and/or in combination of other drug.

Literature review shows that, there is no reported method available for simultaneous estimation of both the drugs in combination. Therefore, it is thought of interest to developed simple, accurate, precise and rapid methods for simultaneous estimation of Betamethasone and Luliconazole in combination.

2 Material and methods

2.1 Instrument and apparatus

Table 1 Lists of Instrument and Apparatus

Component	Model/Software	Manufacturer
Double Beam UV-Visible Spectrophotometer	Shimadzu-2450, UV Probe 2.34	Shimadzu
Analytical Balance	Sartorius (CD2250)	Wensar
Sonicator	-	Trans-o-Sonic
Volumetric Flask	-	Borosil
Pipettes	-	Borosil
Beaker	-	Borosil

2.2 Reagents and material

All the Reagents and Solvents used were of AR or HPLC grades.

Table 2 Working Standard API

Standard	Purpose	Source
Betamethasone	Analysis	Apex Healthcare Ltd.
Luliconazole	Analysis	Luxica Pharma Inc.

2.3 Preparation of standard solutions

2.3.1 Preparation of stock solution of LUL

Accurately weighed quantity of Luliconazole 10mg was transferred to 100 ml volumetric flask, dissolved and diluted up to 25 ml with methanol to give a stock solution having strength of 100 µg/ml.

2.3.2 Standard solution of Betamethasone (BTN)

Preparation of stock solution of BTN

Accurately weighed quantity of Betamethasone 10 mg was transferred to 100 ml volumetric flask, dissolved and diluted up to 25ml with methanol to give a stock solution having strength of 100µg/ml.

2.3.3 Preparation of standard mixture solution (LUL + BTN)

From the stock solution of LUL take 1ml and from stock solution of BTN take 2ml and transferred in to 10ml volumetric flask and diluted up to 25ml with methanol to give a solution having strength of LUL was 10 µg/ml and BTN was 20 µg/ml.

2.3.4 Preparation of test solution

The preparation of synthetic mixture was used which containing Luliconazole (1%w/v) and Betamethasone (2%w/v) (content 15 ml). From which 10ml transferred in 100 ml volumetric flask and made up to 25ml with the Methanol. Final solution contained 100µg/mL LUL and 200µg/mL BTN. From that flask pipette out 1ml and transferred in 10ml volumetric flask and make up the volume with methanol and Concentration of LUL was 10µg/ml and BTN were 20µg/ml.

2.4 Simultaneous equation method

To determine wavelength for measurement, standard spectra of Betamethasone and Luliconazole was scanned between 200-400nm in Methanol. The method was based on the measurement of Betamethasone absorbance of at 243.20 nm and Luliconazole at 225.00 nm and in both wavelengths..

$$Cx = A_2ay_1 - A_1ay_2 / ay_1ax_2 - ay_2ax_1$$

$$Cy = A_1ax_2 - A_2ax_1 / ay_1ax_2 - ay_2ax_1$$

Where,
C_x = Concentration of Luliconazole
C_y = Concentration of Betamethasone
A_1 = Absorbance of test at λ_1 (λ_{max} of LUL)
A_2 = Absorbance of test at λ_2 (λ_{max} of BTN)
ax_1 = Absorptivity of x drug (LUL) at λ_1
ax_2= Absorptivity of x drug (LUL) at λ_2
ay_1 = Absorptivity of y drug (BTN) at λ_1
ay_2= Absorptivity of y drug (BTN) at λ_2

2.5 Spectrophotometric condition

Table 3 Spectrophotometric conditions for Spectroscopic Method

Mode	**Spectrum**
Scan Speed	Fast
Wavelength Range	400-200 nm
Slit width	1 nm

2.6 Preparation of calibration curve

2.6.1 Calibration curves for Luliconazole

This series consisted of five concentrations of standard LUL solution ranging from 10-50µg/ml. The solutions were prepared by pipetting out Standard LUL stock solution (1ml, 2ml, 3ml, 4ml, 5ml) was transferred into a series of 10 ml volumetric flask and volume was adjusted up to mark with methanol. A zero-order derivative spectrum of the resulting solution was recorded and convert in ratio second order derivative and, measure the absorbance at 225nm against a reagent blank solution (Methanol). Calibration curve was prepared by plotting absorbance versus respective concentration of LUL.

2.6.2 Calibration curve for Betamethasone

This series consisted of five concentrations of standard BTN solution ranging from 20-100µg/ml. The solutions were prepared by pipetting out Standard BTN stock solution (2ml, 4ml, 6ml, 8ml, 10ml) was transferred into a series of 10 ml volumetric flask and volume was adjusted up to mark with Methanol. A zero-order derivative spectrum of the resulting solution was recorded and convert in ratio second order derivative and, measure the absorbance at 243.20nm

against a reagent blank solution (Methanol). Calibration curve was prepared by plotting absorbance versus respective concentration of BTN.

3 Validation of proposed method

3.1 Linearity and range

The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of 10-50µg/ml and 20-100µg/ml for LUL and BTN respectively (n=6).

3.2 Precision

3.2.1 Intraday Precision

The precision of the developed method was assessed by analyzing combined standard solution containing three different concentrations 20, 30, 40 µg/ml for LUL and 40,60,80 µg/ml for BTN. Three replicate (n=3) each on same day. For ratio second order derivative spectra absorbance was measured at 225 nm for LUL and 243.20 nm for BTN. The % RSD value of the results corresponding to the absorbance was expressed for intra-day precision.

3.2.2 Interday Precision

The precision of the developed method was assessed by analyzing combined standard solution containing three different concentrations 20,30,40 µg/ml for LUL and 40,60,80 µg/ml BTN triplicate (n=3) per day for consecutive 3 days for inter-day precision. For ratio second order derivative spectra absorbance was measured at 225 nm for LUL and 243.20 nm for BTN. The % RSD value of the results corresponding to the absorbance was expressed for interday precision.

3.3 Accuracy

The developed UV spectroscopic method was checked for the accuracy. It was determined by calculating the recovery of LUL and BTN from synthetic mixture by standard addition method in the combined mixture solution. The spiking was done at three levels 80 %, 100 % and 120 %. Each solution was scanned between 200 nm to 400 nm methanol as a blank. The spectrum of each was obtained. The amount of LUL and BTN was calculated at each level and % recoveries were computed.

- Solution-A (assay preparation): 10µg/ml + 20µg/ml (1ml)
- Solution-B (LUL): 100µg/ml
- Solution-C (BTN): 200µg/ml

Table 4 Solutions for accuracy study

Concentration of Formulation (µg/ml)		**Concentration of API in spiking solution (µg/ml)**		**Total concentration of (µg/ml)**	
LUL	BTN	LUL	BTN	**LUL**	**BTN**
10	20	8	16	18	36
10	20	10	20	20	40
10	20	12	24	22	44

3.4 LOD and LOQ

The Limit of detection and Limit of Quantification of the developed method was assessed by analyzing ten replicates of standard solutions containing concentrations 10µg/ml for LUL and 20µg/ml for BTN

3.4.1 LOD

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD was calculated out by using following formula:

$$DL = 3.3\ \sigma / S$$

Where,
σ = the standard deviation of the response
S = the slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte.

3.4.2 LOQ

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated out by using following formula:

$$DL = 10\ \sigma / S$$

3.5 Robustness & ruggedness

- Robustness and Ruggedness of the method was determined by subjecting the method to slight change in the method condition, individually, the:
- Change in wavelength (225.00nm and 243.20 to 225±0.2 and 243.20±0.2nm)
- Change in instrument (UV-Vis Spectrophotometer model 1800 and 2450)
- Three replicates were made for the concentration (20,30,40µg/ml of LUL and 40,60,80 µg/ml of BTN) with different stock solution preparation.
- % RSD was calculated

3.6 Assay by UV spectrophotometric method

The synthetic mixture was used for assay which containing Luliconazole (1%w/v) and Betamethasone(2%w/v) (content 15 ml). From, which 10ml transferred in 100 ml volumetric flask and made up to the mark with the Methanol. Final solution contained 100µg/mL LUL and 200µg/mL BTN and From, that pipette out within the linearity.

4 Results and discussion

The methods were validated with respect ICH Q_2R_1 guidelines.

- The combination of Luliconazole and Betamethasone is present in 1:2 ratios, respectively. Ratio Second Order Derivative spectra of both drugs in methanol were shows satisfactory absorbance with respect to selected wavelength so Simultaneous Equation method was developed.

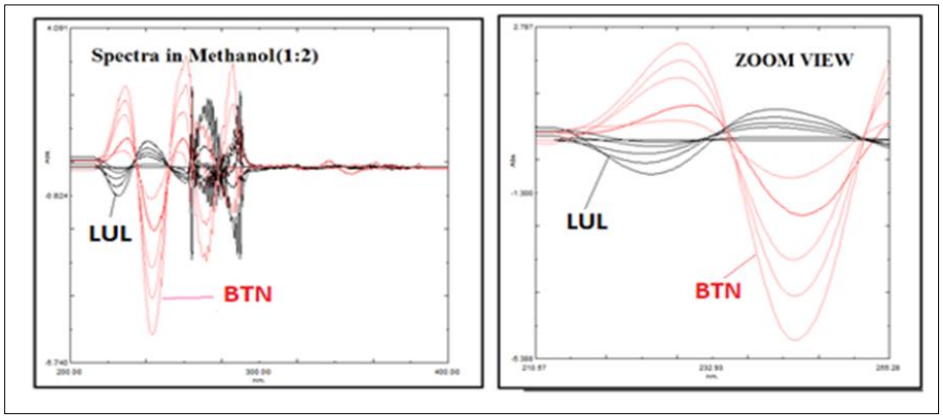

Figure 1 Overlain ratio second order spectra of mixture (LUL +BTN) in methanol (1:2)

4.1 Validation of Proposed Spectrophotometric Method for Simultaneous Method

4.1.1 Linearity and range

Different concentrations of Luliconazole (10-50µg/ml) and Betamethasone (20-100µg/ml) were prepared from respective stock solutions. The absorbances were noted at 225.00 and 243.20 nm. It was noted that at the wavelengths 225.00 and 243.20 nm good linearity was observed and hence these wavelengths were fixed for their simultaneous estimation.

Table 5 Calibration data for LUL and BTN at 225.00nm and 243.20nm, respectively. *(n=6)

LUL*			**BTN***		
Conc. µg/ml	**Mean Abs.* At 225.00nm**	**Mean Abs.* At 243.20nm**	**Conc. µg/ml**	**Mean Abs.* At 225.00nm**	**Mean Abs.* At 243.20nm**
10	-0.048±0.0005	0.045±0.0005	20	0.455±0.0006	-0.959±0.0006
20	-0.201±0.0006	0.268±0.0007	40	0.998±0.0007	-2.025±0.0006
30	-0.365±0.0006	0.370±0.0004	60	1.273±0.0005	-2.945± 0.0010
40	-0.545±0.0004	0.505±0.0005	80	1.580±0.0005	-3.844± 0.0007
50	0.702±0.0006	0.694±0.0005	100	1.818±0.0006	-4.912± 0.0006

Table 6 Average of absorptivity at 225.00and 243.20nm

at 225.00nm		**at 243.20nm**	
ax1	-0.0111	ax2	0.0116
ay1	0.021	ay2	-0.048

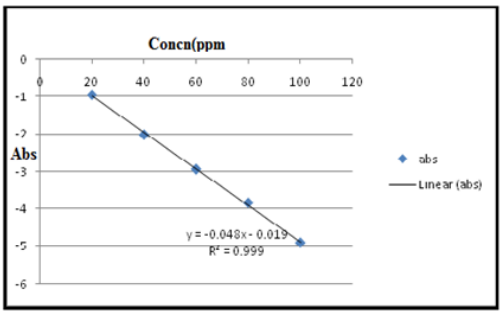

Figure 2 Calibration graph of Luliconazole at 225.00 nm

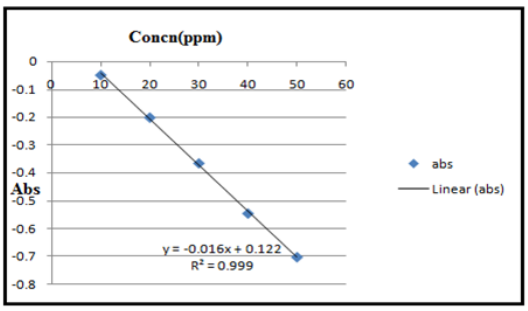

Figure 3 Calibration graph of Betamethasone at 243.20 nm

Table 7 Absorbance of Mixture at 225.00nm and at 243.20nm

Conc. of LUL and BTN (µg/ml)	**At 225.00nm**	**At 243.20nm**
10+20	-0.048	-0.949
20+40	-0.203	-2.024
30+60	-0.366	-2.932
40+800	-0.543	-3.845
50+100	-0.703	-4.913

4.2 Intraday precision

Mixed solutions of LUL and BTN containing 20, 30 and 40 µg/ml and 40, 60 and 80 µg/ml respectively series were analyzed three times on the same day using developed spectroscopic method and %RSD was calculated. The % R.S.D was found to be 0.10-0.28% for LUL and 0.11-0.24% for BTN. These %RSD value was found to be less than ±2.0 indicated that the method is precise.

Table 8 Intraday precision data for estimation of LUL and BTN *(n=3)

Conc. (µg/ml)		Abs.* (LUL) Avg. ± SD(225.0nm)	% RSD	Abs.* (BTN) Avg.± SD(243.20nm)	% RSD
LUL	BTN				
20	40	-0.201±0.0005	0.28	-2.026±0.0023	0.11
30	60	-0.364±0.0005	0.15	-2.945±0.0040	0.13
40	80	-0.545±0.0005	0.10	-3.848±0.0094	0.24

4.3 Interday precision

Mixed solutions of LUL and BTN containing 20, 30 and 40 µg/ml and 40, 60 and 80 µg/ml respectively series were analyzed three times on the different day using developed spectroscopic method and %RSD was calculated. The % R.S.D was found to be 0.27-0.37% for LUL and 0.23-0.35% for BTN. These %RSD value was found to be less than ±2.0 indicated that the method is precise.

Table 9 Interday precision data for estimation of LUL and BTN *(n=3)

Conc. (µg/ml)		Abs.* (LUL) Avg. ± SD (225.0nm)	% RSD	Abs.* (BTN) Avg. ±SD (243.20nm)	% RSD
LUL	BTN				
20	40	-0.203 ± 0.0007	0.37	-2.026±0.0049	0.24
30	60	-0.367 ± 0.001	0.27	-2.948 ± 0.007	0.23
40	80	-0.547 ± 0.002	0.36	-3.846 ±0.0136	0.35

4.4 Accuracy

The developed UV spectroscopic method was checked for the accuracy. It was determined by calculating the recovery of LUL and BTN from synthetic mixture by standard addition method in the combined mixture solution. The spiking was done at three levels 80 %, 100 % and 120 %.

Percentage recovery for LUL and BTNS by this method was found in the range of 100.07-101.33 %and 100.18-101.61%, respectively.

Table 10 Recovery data of LUL *(n=3)

Conc. of LUL from formulation (µg/ml)	Amount of Std. LUL added (µg/ml)	Total amount of LUL (µg/ml)	Total amount of LUL found (µg/ml) * Mean ± SD	% Recovery (n=3)	% RSD LUL
10	0	10	10.02 ± 0.0057	100.20	0.05
10	8	18	18.12 ± 0.001	101.33	0.01
10	10	20	20.01 ± 0.005	100.09	0.02
10	12	22	22.01 ± 0.005	100.07	0.02

Table 11 Recovery data of BTN *(n=3)

Conc. of BTN from formulation (µg/ml)	Amount of Std. BTN added (µg/ml)	Total amount of BTN(µg/ml)	Total amount of BTN found (µg/ml) * Mean ± SD	% Recovery (n=3)	% RSD BTN
20	0	20	20.06 ± 0.0051	100.03	0.02
20	16	36	36.28 ± 0.011	101.61	0.03
20	20	40	40.04 ±0.005	100.18	0.01
20	24	44	44.07±0.005	100.26	0.01

4.5 LOD and LOQ

The LOD for LUL and BTN was conformed to be 0.0837µg/ml and 0.2498µg/ml, respectively. The LOQ for LUL and BTN was conformed to be 0.2537µg/ml and 0.7570µg/ml, respectively.

Table 12 LOD and LOQ data of LUL and BTN *(n=10)

Conc. (µg/ml)		Avg. ± SD (225nm) * LUL	% RSD	Avg. ± (243.20nm)* BTN	% RSD
LUL	BTN				
10	20	-0.048 ± 0.0004	0.83	-2.047 ± 0.003	0.17
LOD (µg/ml)		0.083		0.249	
LOQ (µg/ml)		0.253		0.757	

4.6 Robustness & ruggedness

Table 13 Robustness and Ruggedness data of LUL and BTN *(n=3)

Condition	Concentration (µg/ml)	Change in Wavelength 225.00±0.2nm and 243.20±0.2nm		Change in Instrument	
		224.8nm	225.2nm	UV 1800	UV 2450
LUL Mean (n=3) ± % RSD	20	-0.203 ±0.28	-0.206 ±0.48	-0.201 ±0.28	-0.202 ± 0.37
	30	-0.366 ±0.31	-0.368 ±0.27	-0.363 ±0.57	-0.366± 0.41
	40	-0.547 ±0.38	-0.548 0.18	-0.545± 0.10	-0.542± 0.28
		243.00nm	243.4nm		
BTN Mean (n=3) ± % RSD	40	-2.034 ±0.69	-2.036 ±0.35	-2.029 ±0.41	-2.027 ± 0.51
	60	-2.951±0.25	-2.941 ±0.34	-2.952 ±0.37	-2.944 ± 0.12
	80	-3.850±0.19	-3.866 ±0.29	-3.859 ±0.68	-3.862 ± 0.83

4.7 Assay by UV spectrophotometric method for simultaneous method

Table 14 Analysis data of commercial formulation *(n=3)

Sr. No.	Formulation		Amount found* LUL	% Assay LUL ± SD	Amount found* BTN	% Assay BTN±SD
	LUL	BTN				
1	20	40	20.12	100.60 ± 0.05	40.22	100.52 ± 0.02
2			20.11		40.21	
3			20.13		40.20	

Table 15 Summary of validation parameter

Sr No.	Parameters	LUL	BTN
1.	Concentration Range (µg/ml)	10-50µg/ml	20-100µg/ml
2.	Regression Equation	y = -0.016x+0.122	y = -0.048x -0.019
3.	Correlation Coefficient (r^2)	0.999	0.999
4.	Accuracy (%Recovery)	100.49%	100.68%
5.	Intraday Precision (%RSD)	0.10-0.28%	0.11-0.24%
6.	Interday Precision (%RSD)	0.27-0.37%	0.23-0.35%
7.	Robustness(%RSD)	0.10- 0.57%	0.12 – 0.83%
8.	LOD (µg/ml)	0.083µg/ml	0.249µg/ml
9.	LOQ (µg/ml)	0.253µg/ ml	0.757µg/ml
10.	Assay	100.60 %	100.52 %

5 Conclusion

All the parameters for two substances met the criteria of the ICH guidelines for the method validation and found to be suitable for routine quantitative analysis in pharmaceutical dosage forms. The result of linearity, accuracy, precision proved to be within limits with lower limits of detection and quantification. Ruggedness and Robustness of method was confirmed as no significant were observed on analysis by subjecting the method to slight change in the method condition. Assay results obtained by proposed method are in fair agreement. Validation of proposed methods was carried out according to ICH Q_2R_1Guidelines.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest.

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