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RESEARCH ARTICLE

Development and Validation of Reversed-phase High-performance Liquid Chromatography Method for the Simultaneous Estimation of Benzoyl Peroxide and Resveratrol

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ABSTRACT

A new, reliable, and sensitive reversed-phase high-performance liquid chromatography method has been developed and validated for simultaneous assay of benzoyl peroxide (BPO) and resveratrol. An isocratic separation of BPO and resveratrol was achieved on C18, 250 mm × 4.6 mm I.d., 5 µm particle size columns with a flow rate of 1.2 ml/min and using a UV detector to monitor the elute at 245 nm. The mobile phase consisted of an ammonium acetate (pH 4) and ethanol. Response was a linear function of drug concentration in the range of 10–100 mg/mL range with an R^2 of 0.993 for BPO and 10–100 µg/mL range with an R^2 of 0.995 for resveratrol, accuracy with percent relative standard deviation of 100.65 ± 0.23 (benzoic peroxide) and 100.48 ± 0.45 (resveratrol) and with a limit of detection and quantification for BPO and resveratrol, respectively. The result of analysis has been validated statistically and by recovery study. The accuracy ranged between 99.65 and 101.91%. The method was found to be precise, reproducible, and rapid.

Keywords: Benzoyl peroxide, resveratrol, reversed-phase high-performance liquid chromatography.

INTRODUCTION

Chemically, resveratrol is 3,5,4'-trihydroxy-trans-stilbene, a type of natural phenol and a phytoalexin produced naturally by several plants in response to injury or when the plant is under attack by pathogens such as bacteria or fungi.

It is a powerful antioxidant produced by some plants to protect them against environmental stresses.

A derivative used topically for the treatment of acne vulgaris.[1] It is white colored amorphous powder, relatively insoluble in water and freely soluble in methanol[2] as benzoyl peroxide (BPO) is a potent antibacterial agent which is the first-line drug in the treatment of acne vulgaris.[3] The chemical structure of resveratrol and BPO is shown in Figures 1 and 2. BPO is an organic compound in the peroxide family. It consists of two benzoyl groups bridged by a peroxide link. Its structural formula is $[C_6H_5C(O)]_2O_2$. It is one of the most important organic peroxides in terms of applications and the scale of its production. BPO is used as an acne treatment.

However, for the combination therapy of two drugs, if they are administered in the form of a single formulation, a simultaneous estimation of the two would be required. Literature survey revealed that there was no validated method for the estimation of resveratrol and BPO simultaneously by high-performance liquid chromatography (HPLC). The present study is aimed at to develop selective, precise, accurate, and reliable HPLC method for the determination of BPO and resveratrol in a mixture. However, the method validation study was not developed for this combination pharmaceutical dosage formulation. The purpose of this study was to validate a simultaneous estimation method by reverse-phase HPLC for the quantitative analysis of resveratrol and BPO according to the guideline of ICH and USP.[4,5]

MATERIALS AND METHODS

Resveratrol and BPO standard powder was kindly supplied as a gift sample from having purities of

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98.99% and 70.00%, respectively. Ammonium acetate (RCI Lab Scan), ethanol (RCI Lab Scan), and water (Milli-Q) used were of HPLC grade to prepare the mobile phase and diluents.

Instrument and chromatographic condition

The instrument used: LC-2010, Shimadzu, Japan,equipped with an UV-Visible detector and a photodiode array detector (an auto-sampler, Luna®)
Stationary Phase: C18, 250 × 4.6 mm, 5µ particle size
Elution mode: Linear gradient elution [A:B (20:80)v/v]
Mobile phase: A: Ammonium acetate pH 4.0 (20.0 mM of Ammonium acetate was prepared in distilled water for 1000 ml volume and pH was maintained up to 4.0 with glacial acetic acid)
B: Ethanol
Absorption maxima: 245 nm
Column Temperature: 40 °C
Flow rate: 1.2 ml/min.
Injection volume: 10 µl
Diluent: Ethanol

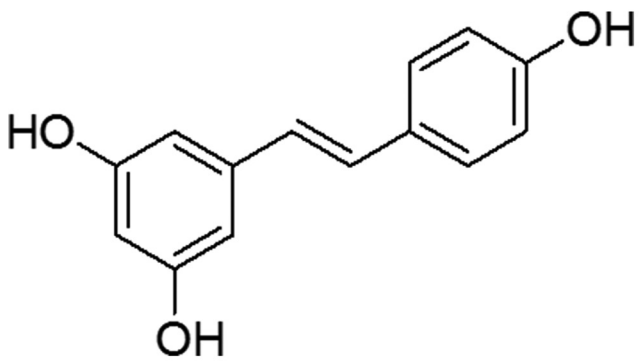

Figure 1: Chemical structure of resveratrol

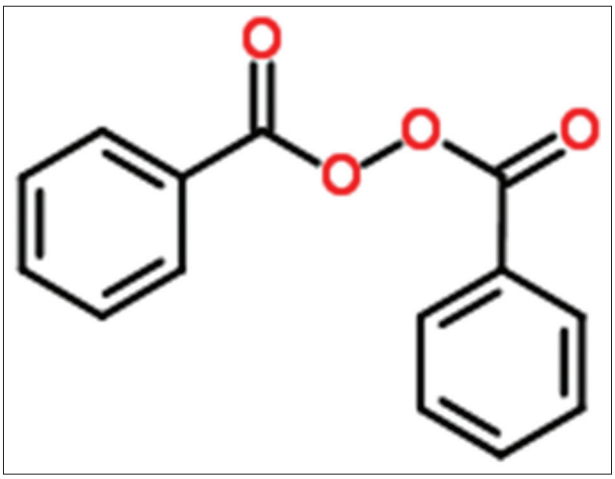

Figure 2: Structure of benzoyl peroxide

EXPERIMENTAL WORK

Standard stock solution preparation

Standard solution preparation

About 100 mg of drug was dissolved in 100 ml of diluents (as mentioned above) to obtain a solution of 1000 µg/ml as stock.

Standards mixture preparation

Standard solution of both the drugs was mixed in the ratio of 1:1 to prepare a mixture solution.

HPLC chromatogram of mixture sample

On HPLC analysis of mixture of standards, chromatogram was optimized in which retention time of drugs [Tables 1-9].

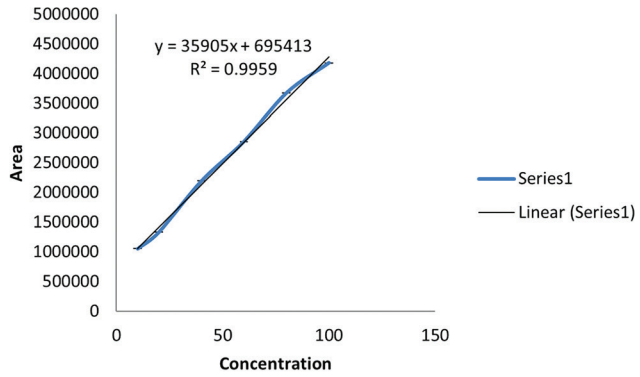

Figure 3: A linear curve was obtained in the range of 10–100 µg/ml with an equation of y=35905.x +69541 and R2=0.995

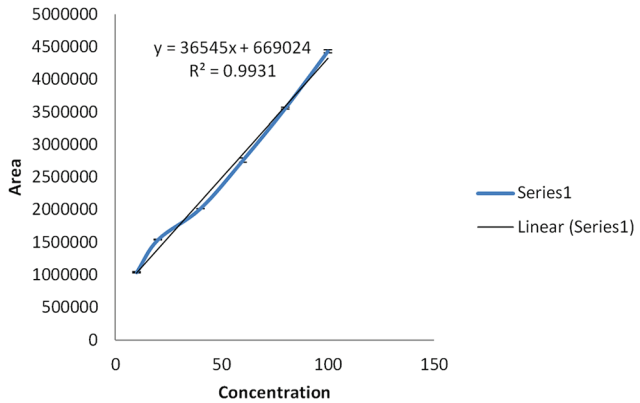

Figure 4: A linear curve was obtained in the range of 10–100 µg/ml with an equation of y=36545.x+66902 and R2=0.993

Table 1: Retention time of drugs (resveratrol and benzoyl peroxide)

Name of drug	Retention time (min)
Resveratrol	2.361
Benzoyl peroxide	4.486

Table 2: Resveratrol linearity

Concentration (μg/ml)	Retention time	Mean ± SD	Area	Mean ± SD
10	2.361	2.360667 ± 0.000577	1056164	1054246 ± 6367.468
	2.361		1059434	
	2.36		1047140	
20	2.36	2.358667 ± 0.004163	1336893	1334957 ± 1682.97
	2.354		1334135	
	2.362		1333843	
40	2.361	2.363333 ± 0.002082	2198612	2194148 ± 5446.838
	2.364		2195753	
	2.365		2188079	
60	2.359	2.362333 ± 0.004933	2853813	2859021 ± 6842.789
	2.368		2866771	
	2.36		2856479	
80	2.358	2.36 ± 0.002	3651879	3676891 ± 22593.12
	2.362		3695820	
	2.36		3682973	
100	2.36	2.361 ± 0.001732	4178500	4183833 ± 12757.26
	2.36		4174607	
	2.363		4198391	

SD: Standard deviation

Table 3: Linearity of benzoyl peroxide

Concentration (μg/ml)	Retention time	Mean ± SD	Area	Mean ± SD
10	4.698	4.585667 ± 0.106566	1036331	1039717 ± 7506.454
	4.486		1048320	
	4.573		1034500	
20	4.547	4.546333 ± 0.001155	1534173	1540111 ± 5823.588
	4.547		1540347	
	4.545		1545813	
40	4.549	4.545667 ± 0.003512	2017205	2016893 ± 461.9679
	4.542		2017111	
	4.546		2016362	
60	4.545	4.542 ± 0.003	2774099	2758076 ± 32867.71
	4.539		2720270	
	4.542		2779860	
80	4.543	4.542667 ± 0.005508	3570584	3557502 ± 11400.87
	4.537		3549688	
	4.548		3552233	
100	4.541	4.538 ± 0.003606	4430887	4460296 ± 25675.68
	4.534		4478254	
	4.539		4471746	

SD: Standard deviation

Validation of HPLC method[6,7]

Linearity and range

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy, and linearity. Linearity range for resveratrol and benzoyl peroxide was found to be 10-100μg/ml. All the dilutions were filtered through 0.22 μ filter and injected.

Accuracy

Accuracy of the method was determined in terms of percentage recovery of standard. Recovery studies were carried out by addition of standard

drug solution at the three concentration levels 50%, 75%, and 100% in pre-analyzed sample. In this method, the known concentration of standard drug was added to the assay sample.

Precision

The intraday and interday variation for the determination of all the three drugs were carried out with concentrations over three levels in the same day and 3 consecutive days where repeatability was determined with lower concentration and injected 6 times and percentage relative standard deviation (RSD) was calculated.

Repeatability

a. Intraday (Day-1)
b. Interday (Day-2)
c. Interday (Day-3).

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of developed method were studied as per ICH guidelines. Several approaches for determining the LOD and LOQ are possible, depending on the procedure, i.e., a non-instrumental or instrumental. Among them, here, employed method was as follows:

$LOD=3.3\sigma/S$ and

$LOQ=10\sigma/S$

Table 4: Data of accuracy study of resveratrol and benzoyl peroxide

% Concentration	Concentration (resveratrol and benzoyl peroxide)	Resveratrol (area)	Corresponding conc.	% Amount recovered	Mean ± SD	%RSD
50%	200 (100 + 100)	3658744	99.963877	99.9638769	99.69056 ± 0.987024	0.990088
	200 (100 + 100)	3678429	100.51213	100.5121292		
	200 (100 + 100)	3609619	98.595683	98.59568305		
75%	300 (150 + 150)	5601450	154.07071	102.7138096	102.5966 ± 1.645966	1.604308
	300 (150 + 150)	5503505	151.34282	100.8952142		
	300 (150 + 150)	5680463	156.27133	104.1808847		
100%	400 (200 + 200)	7495021	206.80908	103.4045398	105.0732 ± 1.460639	1.390116
	400 (200 + 200)	7690034	212.24044	106.12022		
	400 (200 + 200)	7659477	211.38939	105.6946943		

SD: Standard deviation, RSD: Relative standard deviation

Table 5: Data of accuracy study of resveratrol and benzoyl peroxide

% Concentration	Concentration (resveratrol and benzoyl peroxide)	Resveratrol (area)	Corresponding conc.	% Amount recovered	Mean±SD	%RSD
50%	200 (100+100)	3658744	99.963877	99.9638769	99.69056±0.987024	0.990088
	200 (100+100)	3678429	100.51213	100.5121292		
	200 (100+100)	3609619	98.595683	98.59568305		
75%	300 (150+150)	5601450	154.07071	102.7138096	102.5966±1.645966	1.604308
	300 (150+150)	5503505	151.34282	100.8952142		
% Concentration	**Concentration (resveratrol and benzoyl peroxide)**	**Benzoyl peroxide (area)**	**Corresponding conc.**	**Amount recovered**	**Mean±SD**	**%RSD**
50%	200 (100+100)	3889227	104.5923	104.5923	102.316±2.469858	2.413952
	200 (100+100)	3710068	99.68986	99.68986		
	200 (100+100)	3818822	102.6658	102.6658		
75%	300 (150+150)	6436533	174.2956	116.197	114.5104±1.74565	1.524447
	300 (150+150)	6245447	169.0668	112.7112		
	300 (150+150)	6350241	171.9343	114.6229		
100%	400 (200+200)	8618838	234.0111	117.0056	116.4403±0.516023	0.443166
	400 (200+200)	8544935	231.9889	115.9944		
	400 (200+200)	8568799	232.6419	116.3209		

SD: Standard deviation, RSD: Relative standard deviation

Table 6: Repeatability of resveratrol and benzoyl peroxide

Level (%)	Concentration (resveratrol+benzoyl peroxide) µg/ml	Resveratrol (area)	Mean±SD	%RSD
50%	200 (100+100)	2164878	2164163±12139.3	0.560924
	200 (100+100)	2175929		
	200 (100+100)	2151682		
75%	300 (150+150)	3811606	3841656.67±26149.27	0.680677
	301 (150+150)	3854132		
	302 (150+150)	3859232		
100%	400 (200+200)	4674307	4667711.33±8482.116	0.181719
	401 (200+200)	4658143		
	402 (200+200)	4670684		
Level (%)	**Concentration (resveratrol+benzoyl peroxide) µg/ml**	**Benzoyl peroxide (area)**	**Mean±SD**	**%RSD**
50%	200 (100+100)	1435391	1441013±4891.813	0.33947
	200 (100+100)	1444298		
	200 (100+100)	1443350		
75%	300 (150+150)	3094714	3133094.333±35106.18	1.120495
	300 (150+150)	3163583		
	300 (150+150)	3140986		
100%	400 (200+200)	3484898	3521029.333±46980.71	1.334289
	400 (200+200)	3574139		
	400 (200+200)	3504051		
Intraday (Day-1)				
Level (%)	**Concentration (resveratrol+benzoyl peroxide) µg/ml**	**Resveratrol (area)**	**Mean±SD**	**%RSD**
50%	200 (100+100)	1424069	1422765.67±1339.885	0.094175
	200 (100+100)	1422836		
	200 (100+100)	1421392		
75%	300 (150+150)	2769772	2751407±20761.28	0.754569
	300 (150+150)	2755569		
	300 (150+150)	2728880		
100%	400 (200+200)	3675649	3660626±17332.19	0.473476
	400 (200+200)	3664566		
	400 (200+200)	3641663		
Level (%)	**Concentration (resveratrol+benzoyl peroxide) µg/ml**	**Benzoyl peroxide (area)**	**Mean±SD**	**%RSD**
50%	200 (100+100)	1542407	1568090±23364.79	1.490016
	200 (100+100)	1588087		
	200 (100+100)	1573776		
75%	300 (150+150)	1497280	1516066.667±16315.11	1.076147
	300 (150+150)	1526676		
	300 (150+150)	1524244		
100%	400 (200+200)	2631543	2662991.333±40850.18	1.533996
	400 (200+200)	2648269		
	400 (200+200)	2709162		
Interday (Day-2)				
Level (%)	**Concentration (resveratrol+benzoyl peroxide) µg/ml**	**Resveratrol (area)**	**Mean±SD**	**%RSD**
50%	200 (100+100)	1863824	1864840±9605.385	0.515078
	200 (100+100)	1874913		
	200 (100+100)	1855783		
75%	300 (150+150)	2831537	2808808±20694.65	0.736777
	300 (150+150)	2803832		

(Contd...)

Table 6: *(Continued)*

Level (%)	Concentration (resveratrol+benzoyl peroxide) µg/ml	Resveratrol (area)	Mean±SD	%RSD
	300 (150+150)	2791055		
100%	400 (200+200)	3960252	3933132.33±56655.86	1.440477
	400 (200+200)	3868014		
	400 (200+200)	3971131		
Level (%)	**Concentration (resveratrol+benzoyl peroxide) µg/ml**	**Benzoyl peroxide (area)**	**Mean±SD**	**%RSD**
50%	200 (100+100)	2196213	2182203±12389.11	0.567734
	200 (100+100)	2177704		
	200 (100+100)	2172692		
75%	300 (150+150)	3260804	3251008.333±39763.96	1.223127
	300 (150+150)	3207262		
	300 (150+150)	3284959		
100%	400 (200+200)	4565591	4493090±71131.53	1.583132
	400 (200+200)	4423412		
	400 (200+200)	4490267		
Interday (Day-3)				
Level (%)	**Concentration (resveratrol+benzoyl peroxide) µg/ml**	**Resveratrol (area)**	**Mean±SD**	**%RSD**
50%	200 (100+100)	2016975	2029919.67±22972.07	1.131674
	200 (100+100)	2016341		
	200 (100+100)	2056443		
75%	300 (150+150)	3109663	3090264±19739.38	0.63876
	300 (150+150)	3090928		
	300 (150+150)	3070201		
100%	400 (200+200)	4023902	4032820.33±23179.12	0.574762
	400 (200+200)	4015425		
	400 (200+200)	4059134		
Level (%)	**Concentration (resveratrol+benzoyl peroxide) µg/ml**	**Benzoyl peroxide (area)**	**Mean±SD**	**%RSD**
50%	200 (100+100)	1303259	1310352±23746.74	1.812241
	200 (100+100)	1290960		
	200 (100+100)	1336837		
75%	300 (150+150)	3332588	3281068±50522.45	1.539817
	300 (150+150)	3279010		
	300 (150+150)	3231606		
100%	400 (200+200)	4354741	4408520.667±46776.38	1.061045
	400 (200+200)	4439751		
	400 (200+200)	4431070		

SD: Standard deviation, RSD: Relative standard deviation

Table 7: LOD and LOQ

Sample name	LOD	LOQ
Resveratrol	1.1724 (µg/ml)	3.5529 (µg/ml)
Benzoyl peroxide	2.3184 (µg/ml)	7.0255 (µg/ml)

LOD: Limit of detection, LOQ: Limit of quantification

Where, σ=The standard deviation of response
S=The slope of calibration curve.

Robustness

The robustness was studied by analyzing the sample of lower concentration with deliberate variation in the method parameters. The change in the responses of drugs was noted in terms of percentage RSD. Robustness of the method was studied by change in flow rate or change in mobile phase ratio.

Ruggedness

The ruggedness was studied by analyzing the same samples of three drugs by changing analyst. The change in the responses of drugs was noted in terms of percentage RSD.

The percentage RSD should not be more than 2. The percentage RSD obtained for change of

Table 8: Robustness data of resveratrol a and b

a. Robustness data of resveratrol and benzoyl peroxide with deliberate change in flow rate				
Flow rate	**Concentration (resveratrol + benzoyl peroxide) μg/ml**	**Resveratrol (area)**	**Mean±SD**	**%RSD**
1	100 (50 + 50)	1953117	1947541 ± 14368.74807	0.737789
	100 (50 + 50)	1931220		
	100 (50 + 50)	1958286		
1.2	100 (50 + 50)	1819267	1815792.3 ± 3009.788254	0.165756
	100 (50 + 50)	1813993		
	100 (50 + 50)	1814117		
1.4	100 (50 + 50)	685362	673528.67 ± 10253.87426	1.522411
	100 (50 + 50)	667264		
	100 (50 + 50)	667960		
Flow rate	**Concentration (resveratrol + benzoyl peroxide) μg/ml**	**Benzoyl peroxide (area)**	**Mean±SD**	**%RSD**
1	100 (50 + 50)	1630083	1618465 ± 15330.09	0.947199
	100 (50 + 50)	1624223		
	100 (50 + 50)	1601090		
1.2	100 (50 + 50)	1635828	1625210 ± 9948.474	0.612135
	100 (50 + 50)	1616104		
	100 (50 + 50)	1623699		
1.4	100 (50 + 50)	679452	672858 ± 8730.604	1.29754
	100 (50 + 50)	662957		
	100 (50 + 50)	676165		
b. Robustness data of resveratrol and BPO with deliberate change in mobile phase ratio				
Mobile phase ratio (ethanol: buffer)	**Concentration (resveratrol + benzoyl peroxide) μg/ml**	**Resveratrol (area)**	**Mean±SD**	**%RSD**
78:22	100 (50 + 50)	1108670	1126952.3 ± 19597.20991	1.738956
	100 (50 + 50)	1124545		
	100 (50 + 50)	1147642		
80:20	100 (50 + 50)	772479	778712 ± 7062.016638	0.906884
	100 (50 + 50)	777275		
	100 (50 + 50)	786382		
82:18	100 (50 + 50)	1221815	1208931.3 ± 16808.75553	1.390381
	100 (50 + 50)	1215061		
	100 (50 + 50)	1189918		
Mobile phase ratio (ethanol: buffer)	**Concentration (resveratrol + benzoyl peroxide) μg/ml**	**Benzoyl peroxide (area)**	**Mean±SD**	**%RSD**
78:22	100 (50 + 50)	871441	864326 ± 9006.983	1.042082
	100 (50 + 50)	867338		
	100 (50 + 50)	854199		
80:20	100 (50 + 50)	883716	868564.3 ± 16300.26	1.876689
	100 (50 + 50)	870659		
	100 (50 + 50)	851318		
82:18	100 (50 + 50)	1319203	1324616 ± 5054.562	0.381587
	100 (50 + 50)	1325431		
	100 (50 + 50)	1329213		

SD: Standard deviation, RSD: Relative standard deviation

analyst was found to be <2, which was within the acceptance criteria. Hence, the method was rugged.

Specificity

Specificity of the methods was achieved by the analysis of different laboratory prepared

Table 9: Analyst

Analyst-I						
Concentration (resveratrol + benzoyl peroxide) μg/ml	**Resveratrol (area)**	**Mean ± SD**	**%RSD**	**Benzoyl peroxide (area)**	**Mean ± SD**	**%RSD**
100 (50 + 50)	1569155	1561248 ± 8852.24	0.566998	934788	932131.3 ± 15019.23	1.611278
100 (50 + 50)	1547757			947062		
100 (50 + 50)	1568798			945648		
100 (50 + 50)	1567420			935229		
100 (50 + 50)	1560266			922800		
100 (50 + 50)	1554091			907261		
Analyst-II						
Concentration (resveratrol + benzoyl peroxide) μg/ml	**Resveratrol (area)**	**Mean ± SD**	**%RSD**	**Benzoyl peroxide (area)**	**Mean ± SD**	**%RSD**
100 (50 + 50)	781047	788059.2 ± 5310.945	0.673927	906716	920431.2 ± 17438.57	1.894609
100 (50 + 50)	784247			900704		
100 (50 + 50)	787597			941010		
100 (50 + 50)	787056			936630		
100 (50 + 50)	794747			929984		
100 (50 + 50)	793661			907543		

mixtures of resveratrol and BPO within the linearity range.

RESULTS AND DISCUSSION

The results indicate that the recoveries are well within the acceptance range of 99–116%, indicating a good degree of sensitivity of the method toward detection of analytes in sample. Therefore, method is accurate, and it can be used for the estimation of both drugs.

Precision

The method was found to be precise due to low values of the percentage RSD.

LOD and LOQ

The LOD and LOQ of the method were calculated based on standard deviation of the response and the slope (s) of the calibration curve at approximate levels of the LOD and LOQ. The results obtained were within the limit.

Robustness

The percentage RSD should not be more than 2. The percentage RSD obtained for change of flow rate, change in mobile phase ratio was found to be <2, which was within the acceptance criteria. Hence, the method was robust.

Ruggedness

The ruggedness was studied by analyzing the same samples of three drugs by changing analyst. The change in the responses of drugs was noted in terms of percentage RSD.

The percentage RSD should not be more than 2. The percentage RSD obtained for change of analyst was found to be <2, which was within the acceptance criteria. Hence, the method was rugged.

CONCLUSION

The HPLC method was developed and validated for the simultaneous analysis of BPO and RES. The results together established that the method is simple, accurate, precise, reproducible, rapid, and sensitive. The method could be applied successfully and economically for the simultaneous estimation of BPO and RES in laboratory samples for efficient data generation and for combination formulations of these two drugs in the future.

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