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Research Article

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DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR ESTIMATION OF GRISEOFULVIN AND PRESERVATIVES IN ORAL SUSPENSION

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Abstract A simple, fast, accurate and precise reverse phase high performance liquid chromatographic method has been developed for the estimation of Griseofulvin in oral suspension. The chromatographic separation was achieved on Zorbax eclipse plus C8 (250 x 4.6) mm; 5µm particle size column with a gradient mixture of mobile phase containing acetonitrile: ortho-phosphoric acid buffer: methanol (10:50:40 v/v). The injection volume was kept at 20 µl with mobile phase at a flow rate of 1.5 ml/min. The wavelength of detection was kept at 254 nm with column temperature at 25 ºC. The selected chromatographic conditions were found effectively to separate Griseofulvin at 12.84 min with its excipients methylhydroxy benzoate at 4.192 min and propylhydroxy benzoate at 11.021 min. The linearity was obtained in range of 125-1250 µg/ml for Griseofulvin, 7.5-75.01 µg/ml for methylhydroxy benzoate and 0.5-5.01 µg/ml for propylhydroxy benzoate with correlation coefficient 0.999, 0.999 and 0.999 for GRS, methylhydroxy benzoate and propylhydroxy benzoate, respectively. The proposed methods were validated as per ICH guidelines and successfully applied for the determination of investigated drugs in oral suspension.

Keywords Griseofulvin, oral suspension, RP-HPLC, methylhydroxy benzoate, propylhydroxy benzoate

Introduction

GRS (Griseofulvin)[1-3] is an antifungal drug which acts by inhibiting microtubules and blocking fungal mitosis, therefore a fungistatic. It is poorly insoluble in water when administered orally in a microcrystalline form (improved when taken with fatty foods). It is systemically used for dermatophytosis (e.g. skin and especially nail infections) or diseases requiring extended treatments. Methylhydroxy benzoate (MHB) [4] and propylhydroxy benzoate (PHB) [5] are generally use as preservatives. Both are generally considered safe for food and cosmetic preservation. They are readily metabolized by common soil bacteria, making it completely biodegradable. They are hydrolyzed to *p*-hydroxybenzoic acid and rapidly excreted in urine without accumulating in the body.

Rationale

From the literature review, it is revealed many that spectrometric and chromatographic methods have been reported for the estimation of GRS alone [6-10] and GRS in combination with other drugs [11-14]. But not a single spectrophotometric method has been reported for estimation of GRS with MHB and PHB as preservatives. Therefore, we have tried to develop an accurate, precise analytical method for estimation of GRS with MHB and PHB and carry out its validation according to ICH guidelines.

Material and Methods

Materials

The formulation GRS in Oral Suspension (label claim: Griseofulvin 2.50gm/100ml, methylhydroxy benzoate 0.15gm/100ml and propylhydroxy benzoate 0.01gm/100ml), manufactured by Thames laboratory ltd. was procured from analytical research laboratory, Ahmadabad. All the chemicals used were of analytical grade and were purchased from MERCK Chem. Ltd., Mumbai.

Instruments

Following instruments with given specification were used for estimation of GRS from oral Suspension, HPLC from Agilent technology (1260 infinite) USA Prominence Liquid Chromatograph comprising Prominence PDA detector and ZORBAX ECLIPSE PLUS C8, (250x4.6) mm; 5µm. The HPLC system was equipped with "EZ chrome" data acquisition software. The mobile phase consists of a mixture of Acetonitrile: orthophosphoric acid buffer: methanol (pH adjusted to 3.0 using o-phosphoric acid) in ratio of 10:50:40 v/v. The mobile phase was set at a flow rate of 1.5 ml/min and an injection volume of 20µl. In addition, an Electronic balance {Libror AEO-210 (capacity=200.00 gm)}, digital pH meter (Systronic model 361), a sonicator (systronic), gallenkamp (model 5A 6797, electronic) melting point instrument were used in this study.

Method Development

Preparation of standard solution for GRS

Accurately weighed 250 mg of GRS was transferred to a 50 ml clean, dry volumetric flask. Mobile phase was added to it and then sonicated to dissolve the compound. The volume was made up to the mark with mobile phase. From this solution 15.6 ml was withdrawn and dissolved in 50 ml of mobile phase (Acetonitrile: Ortho-Phosphoric acid Buffer: Methanol (10:50:40 %v/v). From this solution, again 4 ml was taken and dissolved in 10 ml of mobile phase to prepare the stock solution of GRS with concentration of 625 µg/ml. Five working standard solutions for calibration were prepared by adding defined volumes of the stock standard solution and finally diluting.

Preparation of standard solution for methylhydroxy benzoate (MHB)

Accurately weighed 100 mg of MHB was transferred to a 50 ml clean, dry volumetric flask. To this mobile phase was added and then sonicated to dissolve the compound. The volume was made up to the mark with mobile phase. From this solution 2.3 ml was withdrawn and dissolved in 50 ml of mobile phase and finally 4 ml of this solution was dissolved in 10 ml of mobile phase to obtain the stock solution. This is stock preparation of MHB with concentration of 37.5 µg/ml. Five working standard solutions were prepared for calibration curve by adding defined volumes of the stock standard solution and finally diluting.

Preparation of standard solution for phenylhydroxy benzoate (PHB)

Accurately weighed 50 mg of PHB was transferred to a 50 ml clean, dry volumetric flask. To this mobile phase was added and then sonicated to dissolve the compound. The volume was made up to the mark with mobile phase. From this solution 0.3 ml was withdrawn dissolved in 50 ml of mobile phase and finally 4 ml of this solution was dissolved in 10 ml of mobile phase to obtain the stock solution. This is stock preparation of PHB with concentration of 2.5 µg/ml. Five working standard solutions were prepared for calibration by adding defined volumes of the stock standard solution and finally diluting.

Preparation of sample solution

Accurately weighed 12.5 gm of sample was transferred to a 50 ml volumetric flask. To this 20 ml of mobile phase was added and then placed in an ultra-sonication bath until dissolution is complete. The resulting solution was filtered using 0.2 µm filter and degassed by sonication.

Selection of Detection Wavelength

The UV spectrum of diluted solutions of various concentrations of GRS, MHB and PHB in methanol was recorded using UV spectrophotometer. The sharp and separated peaks for GRS, MHB and PHB were obtained at 254 nm.

Method Optimization

Selection of Mobile phase

Several trials were taken for the optimization of RP HPLC method by changing the ratio of the mobile phase. The optimized parameters are shown in Table 1 and the optimized chromatogram with its parameters is shown in Figure 1. Separation was achieved on a ZORBAX ECLIPSE PLUS C8 (250 x 4.6) mm ; 5 µm column as a stationary phase with mobile phase acetonitrile: orthophosphoric acid buffer: methanol (HPLC grade) in the ratio of 10:50:40 v/v.

Table 1: Optimized Conditions

SAMPLE NAME	GRS ORAL SUSPENSION (125mg/5mL)
SAMPLE PPM	625 ppm
COLUMN	ZORBAX ECLIPSE PLUS C8, (250x4.6)mm ; 5µm
FLOW RATE	1.5mL/min
COLUMN TEMPRATURE	25˚C
WAVELENGTH	254nm
INJECTION VOLUME	20µL
RUN TIME	22min
DILUENT	Methanol : Water (85:15)
MODE	GRADIENT
MOBILE PHASE	Acetonitrile: ortho-Phosphoric acid buffer: methanol

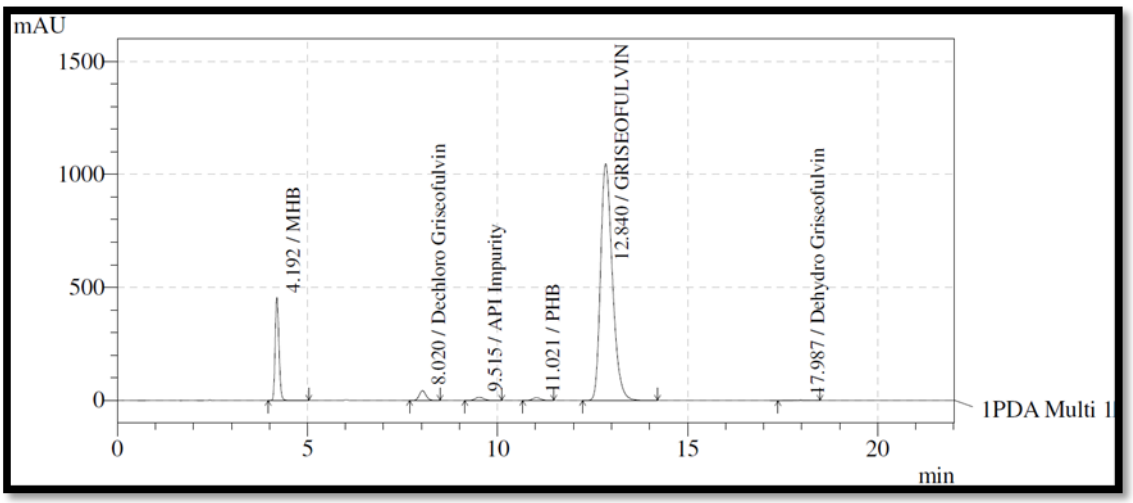

Figure 1: Chromatogram with separated peaks and retention time (optimized condition)

Calibration curve

Calibration curves were constructed for GRS, MHB and PHB and were linear over the concentration range of 125-1250 µg/ml for GRS, 7.5-75.01 µg/ml for MHB and 0.5-5.01 µg/ml for PHB with correlation coefficient 0.999,

0.999 and 0.999 for GRS, MHB and PHB, respectively. Calibration curves were prepared using ratio of analyte peak area to internal standard peak versus concentration of analytes. The calibration curves are shown in Figure 2, 3 and 4.

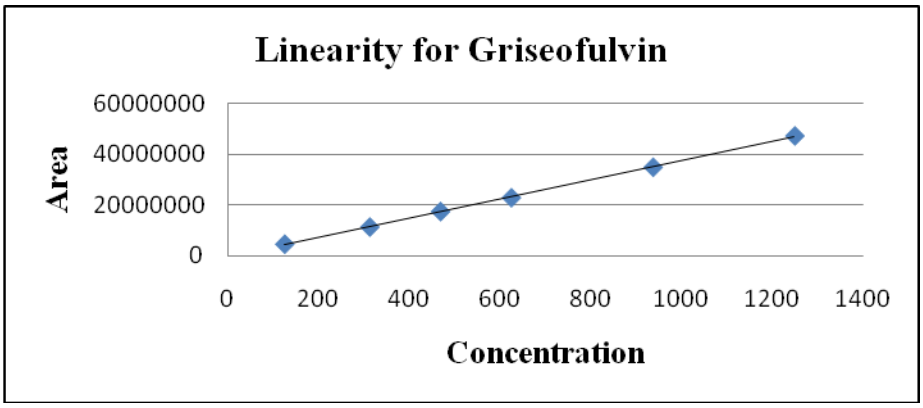

Figure 2: Linearity graph for GRS

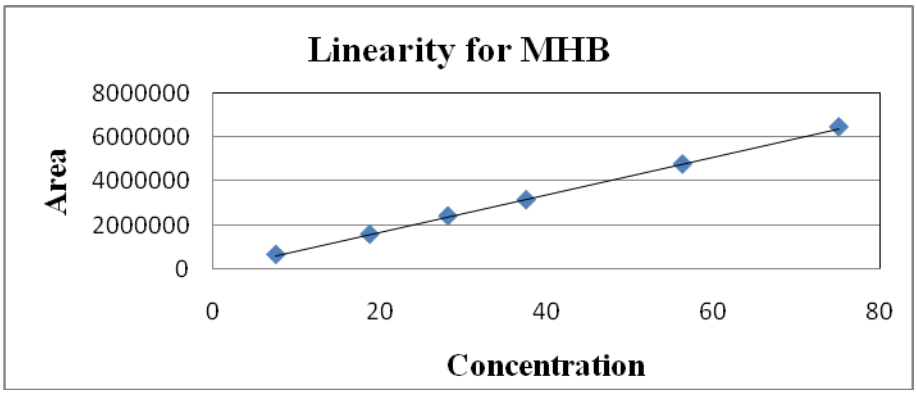

Figure 3: Linearity graph for MHB

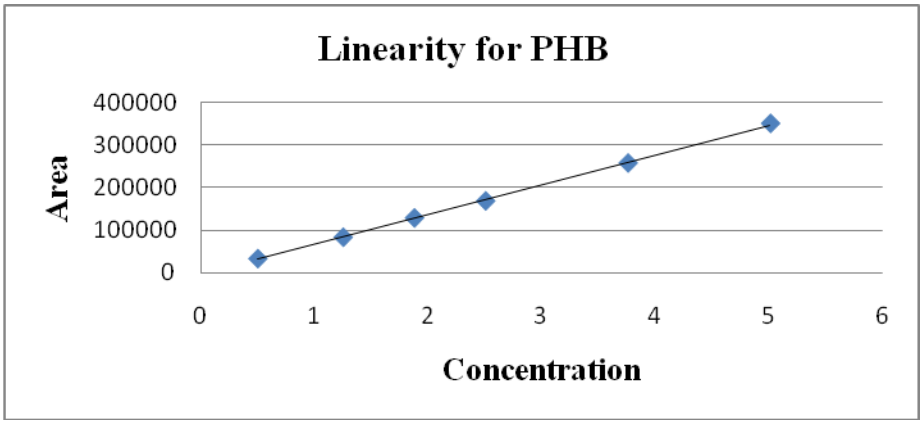

Figure 4: Linearity graph for PHB

Method Validation

The proposed method was validated in accordance with ICH guidelines for accuracy, precision, LOD, LOQ, linearity and percentage recovery.

Linearity

Linearity studies were carried out for GRS, MHB and PHB at different concentration levels. Calibration curves constructed were linear over the range of 125-1250 µg/ml, 7.5-75 µg/ml and 0.5-5.0 µg/ml for GRS, MHB and PHB, respectively. The correlation coefficients were found to be 0.9998, 0.9998 and 0.9997 for GRS, MHB and PHB, respectively. The evaluation was performed by UV detector at 254.

Limit of Detection and Limit of Quantification

The LOD for GRS, MHB and PHB were found to be 26.653 µg/ml, 1.715 µg/ml and 0.124 µg/ml, respectively and LOQ for GRS, MHB and PHB were found to be 80.768 µg/ml, 5.197 µg/ml and 0.377 µg/ml, respectively.

Accuracy

The accuracy was assessed by recovery studies of GRS, MHB and PHB in combined dosage form at three different concentration levels. A fixed amount of pre analyzed sample was taken and standard drug was added at 80%, 100% and 120% levels. Each level was repeated for three times. The results are shown in Table 2.

Table 2: Accuracy data

Drug	Spiked level	Amount taken (µg/ml)	Amount found (µg/ml)	Mean % recovery ± SD
GRS	80%	500	507.13	101.4 ± 0.48
	100%	625	627.86	100.5 ± 0.25
	120%	750	733.53	97.8 ± 0.20
MHB	80%	30	30.03	100.1 ± 0.30
	100%	37.5	37.10	95.9 ± 0.26
	120%	45	43.9	97.5 ± 0.40
PHB	80%	2	1.9	96.0 ± 0.01
	100%	2.5	2.5	100.0 ± 0.01
	120%	3	2.83	98.5 ± 1.15

Table 3: Precision data

S. NO.	INTRA DAY			INTER DAY		
	GRS	MHB	PHB	GRS	MHB	PHB
1	24676438	3399898	139989	24666436.5	3397263	183813.5
2	24837874.5	3436399	190109	24857484.5	3419136.5	184937
3	24707901.5	3485827	189313	24701601.5	3384804	184973.5
4	24454332	3389675	189514	24897930	3328073	181020.5
5	24848298.5	3357928	183757	24445213.5	3362345	181488
6	24165186.5	3389847	184242	24404446	3343961	182129.5
%RSD	0.79	0.79	0.89	0.82	0.99	0.94

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a

series of measurements. A minimum of six replicate sample determinations were carried together with a simple statistical assessment of the results, including the percent relative standard deviation. For intra-day precision, %RSD for GRS, MHB and PHB were found to be 0.79%, 0.79% and 0.89%, respectively. For the inter-day precision, %RSD for GRS, MHB and PHB were found to be 0.82%, 0.99% and 0.94%, respectively (Limit %RSD: < 2.0%). The results are shown in Table 3.

Stability Indicating Study

Force degradation studies

Whole stability indicating RP-HPLC assay method for simultaneous determination of GRS, MHB and PHB were done using above developed method. In order to establish stability-indicating nature of the method, drug product and diluent were subjected to various stress conditions to conduct force degradation studies. Stress studies were carried out under the conditions of acidic, basic, oxidative, thermal and UV exposure [15]. Several trials with different severity of each stressed condition were conducted. Results are shown in Table 4.

Table 4: Force degradation Data

Condition	%Degradation		
	GRS	MHB	PHB
0.1N HCl, 1 hr, 80° C	10.11	14.12	13.17
0.1N NaOH, 1 hr, 80° C	10.15	14.87	12.14
Thermal,80° C 1 hr.	15.88	17.27	16.87
3% H_2O_2, 1 hr, 80° C	7.75	8.76	8.23
UV Light, 2 hr.	13.89	17.57	16.22

Results and Discussion

The present study was aimed at developing a precise, sensitive, rapid and accurate RP-HPLC method for the analysis of GRS, MHB and PHB in oral suspension. The retention time for GRS, MHB and PHB was found to be 12.84 min, 4.192 min and 11.021 min, respectively. UV spectra showed that the drug absorbed maximum at 254 nm, so this wavelength was selected as the detection wavelength. The correlation coefficient (0.999) was found almost equal to one which states that the method was linear in the concentration range selected. On comparison of chromatograms of diluents, standard and sample no interference was observed from the peaks of diluent, standard and sample. It shows that the method is specific. The precision studies were performed and the % RSD of the determinations of GRS, MHB and PHB was found to be 0.79%, 0.79% and 0.89% for intraday precision and 0.82%, 0.99% and 0.94% for inter-day precision respectively which are within the limits. Hence, the proposed method was found to be precise. The accuracy of the method was found to be good with the overall % RSD for recovery at 80%, 100% and 120% levels were all within the limits. This indicates that the proposed method was found to be accurate. Method validation suggests that the developed method had high sensitivity with LOD of GRS, MHB and PHB at 26.653 µg/ml, 1.715 µg/ml and 0.124 µg/ml, respectively and LOQ for GRS, MHB and PHB were found to be 80.768 µg/ml, 5.197 µg/ml and 0.377 µg/ml, respectively. The assay results by applying the RP-HPLC method (Table 5) was found to be within the pharmacopoeial limits and the assay values were found to be 98.7%, 98.1% and 98.6% for of GRS, MHB and PHB respectively.

Table 5: Assay Data

NAME	%ASSAY	SPECIFIC RANGE
GRS	99.9 %	98-102 %
MHB	100.4 %	
PHB	99.7 %	

Conclusion

The developed RP-HPLC method for the quantification of GRS, MHB and PHB has various advantages like less retention time, good peak symmetry and phenomenal linearity, highly sensitive, simple, precise and accurate. The mobile phase can be easily prepared and diluent is economical and readily available and it does not need sample preparation with sophisticated techniques or instruments. The drug employed in the study was stable up to 48 hours. This attributes the high quality of the method. The proposed method can be used for the routine analysis of GRS in oral suspension preparations and for routine application in quality control laboratories without interference of excipients.

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