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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC ASSAY METHOD FOR DETERMINATION OF PHENOBARBITONE IN BULK DRUG AND TABLET FORMULATION

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ABSTRACT: The objective of the current study was to develop simple, precise and accurate stability indicating by isocratic RP-HPLC assay method and validated for determination of Phenobarbitone in solid pharmaceutical dosage forms. Isocratic RP-HPLC separation was achieved on a analytical C18 R column (250 mm × 4.6 mm i.d., 5 μm particle size) using mobile phase of Acetonitrile: Methanol (65:35, v/v) at a UV detector. The drug was subjected to acid degradation, alkaline degradation, oxidation, photolysis and heat to apply stress condition. The method was validated for specificity, linearity, precision, accuracy, robustness and solution stability. The method was linear in the drug concentration range of 10-60 μg/ml with a correlation coefficient 0.999. The method was also found to be robust as indicated by the % RSD values which are less than 2%. The stress degradation studies showed that Phenobarbitone undergoes degradation in acid, base, oxidation, dry heat (4.80%, 1.52%, 2.16%, and 1.81% respectively). Degradation products produced as a result of stress studies did not interfere with detection Phenobarbitone and the assay can thus be considered stability indicating.

INTRODUCTION: Phenobarbital is called a barbiturate that acts by slowing down the activity of brain. Phenobarbital has sedative and hypnotic properties, which will help patients to relax before surgery or help to sleep¹.

Phenobarbitone in tablet is assay by using conductimetric titration was done by indirect determination with NaOH². The structure of Phenobarbitone was shown in **figure 1**.

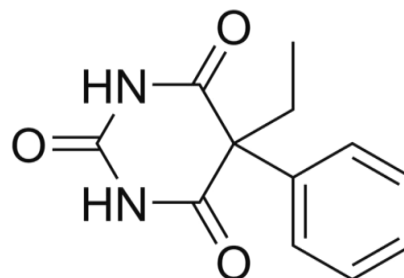


FIGURE 1: STRUCTURE OF PHENOBARBITONE

MATERIALS AND METHODS³⁻⁸:

Phenobarbitone sample was obtained from Abbott Healthcare, Mumbai. Phenobarbitone tablet was purchased from local market. The solvent used Acetonitrile (HPLC grade), Water (HPLC grade), NaOH (AR grade), HCl (AR grade), H₂O₂ (HPLC grade), OPA (HPLC grade).



These chemicals were purchased from Merck Chemicals (Mumbai, India).

Selection of mobile phase and chromatographic conditions: Chromatographic separation studies were carried out on a C-18, column on the working standard solution of Phenobarbitone (50 μ g/ml). Initially, trials were carried out using acetonitrile and methanol in various proportions along with varying pH, to obtain the desired system suitability parameters. After several trials, Acetonitrile: Methanol (pH adjusted to 4.5 with ortho phosphoric acid) (65: 35 v/v), was chosen as the mobile phase, which gave good resolution and acceptable peak parameters.

Preparation of Standard stock solution: 10mg of Phenobarbitone reference standard was weighed accurately and transferred in 100ml volumetric flask. Drug was dissolve in Acetonitrile: Methanol (65: 35

v/v) and volume was made up to 100ml with same solvent. So as to get the concentration 100 μ g/ml. 5ml standard stock solution of Phenobarbitone was then diluted in 10ml Acetonitrile: Methanol (65: 35 v/v) to get working standard solution 50 μ g/ml.

Preparation of mobile phase: Mobile phase was prepared by mixing acetonitrile: methanol (pH adjusted to 4.5 with ortho phosphoric acid) (65: 35 v/v), filtered through 0.45 μ membrane filter paper and then sonicated on ultra sonic water bath for 30min.

Selection of Detection Wavelength: From the standard stock solution further dilutions were done using Acetonitrile: Methanol (65: 35 v/v) and scanned over the range of 200 - 400nm and the spectra was obtained. It was observed that Phenobarbitone showed considerable absorbance at 237 nm (**Figure 2**).

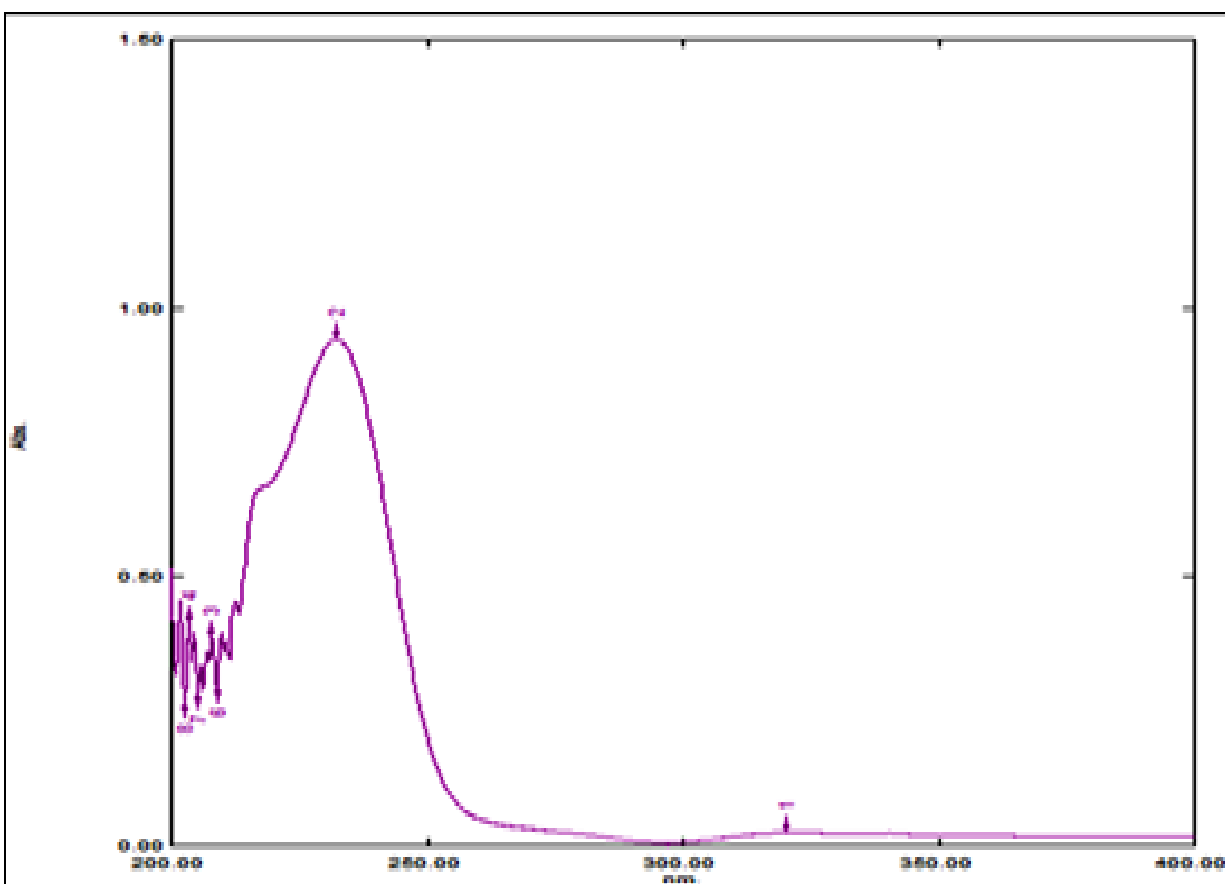


Figure 2: UV spectrum of Phenobarbitone (50 μ g/ml) in acetonitrile: methanol (65: 35 v/v)

Chromatogram of Phenobarbitone: The column was saturated with the mobile phase (indicated by constant back pressure at desired flow rate). Standard solution of Phenobarbitone was injected to get the chromatogram.

The retention time for Phenobarbitone was found to be 4.78 \pm 0.02 min. Chromatogram of Phenobarbitone is shown in (**Figure 3**).

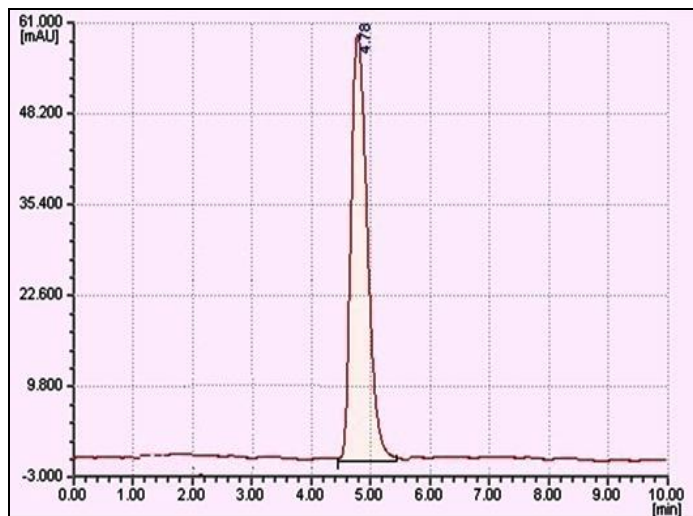


FIGURE 3: REPRESENTATIVE CHROMATOGRAM OF PHENOBARBITONE (50µg/ml, RT = 4.78).

Degradation Studies: The International Conformance on Harmonization (ICH) guidelines entitled stability testing of new drug substance and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies of the Phenobarbitone using the proposed method⁽⁸⁻¹²⁾.

Acidic hydrolysis: 5ml of working standard solution was mixed with 2ml of 2 N HCl and kept for 3 hours. After 3hours solution was neutralized with NaOH then solution was diluted to 10ml with Acetonitrile: Methanol (65:35) and injected in stabilized chromatographic conditions. Under this condition, degradation was observed (Deg-1, RT = 3.24) (Table 1 & Figure 4).

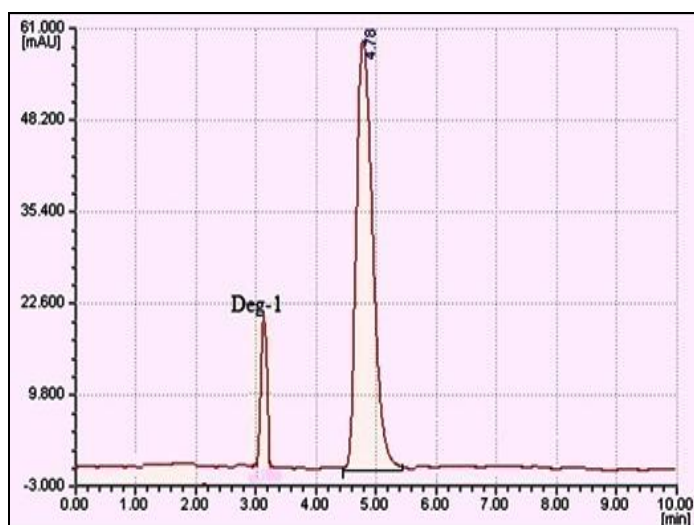


FIG. 4: CHROMATOGRAM OF PHENOBARBITONE (50µg/ml) AFTER ACIDIC HYDROLYSIS INDUCED DEGRADATION WITH DEGRADATION PRODUCT AT RT 3.24

Alkaline hydrolysis: 5ml of working standard solution was mixed with 2ml of 1.2N NaOH and kept for 3 hours. After 3 hours solution was neutralized with HCl then solution was diluted to 10ml with acetonitrile: methanol (65:35) and injected & degradation was observed (Deg-2, RT = 3.94) (Table 1 & Figure 5).

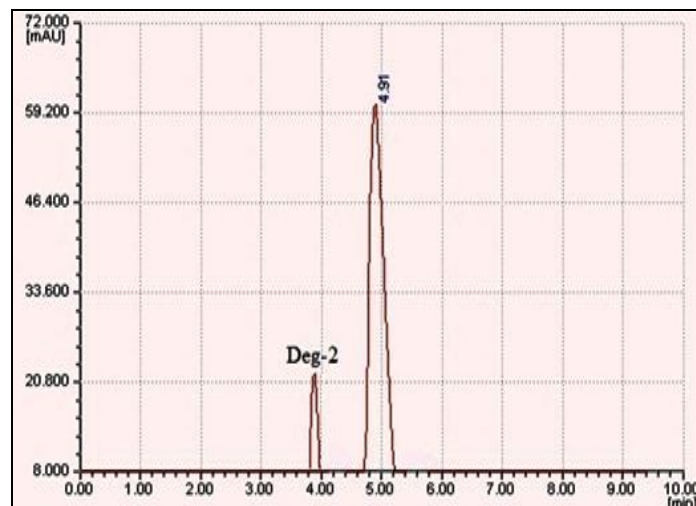


FIG. 5: CHROMATOGRAM OF PHENOBARBITONE (50µg/ml) AFTER ALKALI HYDROLYSIS INDUCED DEGRADATION WITH DEGRADATION PRODUCT AT RT 3.94

Oxidation: 5ml of working standard solution was mixed with 3ml 6% solution of H₂O₂. The solution was diluted to 10ml with Acetonitrile: Methanol (65:35) and refluxed for 3 hours. The solution was injected in stabilized chromatographic conditions. (Deg-3,4,5, RT =2.41,2.50,2.98) (Table 1 & Figure 6).

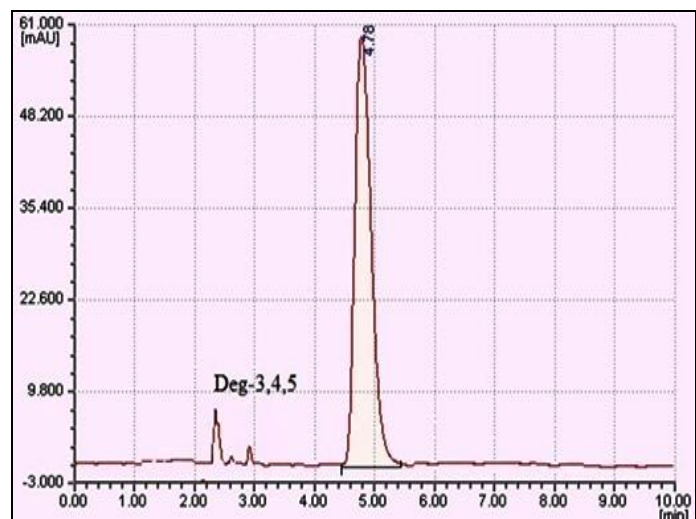


FIG. 6: CHROMATOGRAM OF PHENOBARBITONE (50µg/ml) AFTER OXIDATIVE DEGRADATION WITH DEGRADATION PRODUCT AT RT 2.41, 2.50, 2.98 RESPECTIVELY.

Degradation under Dry Heat: Dry heat studies were performed by keeping drug sample in oven (100°C) for a period of 48 hours. 10mg of exposed drug was weighed accurately and transferred to a 100ml of volumetric flask and dissolved in Acetonitrile: Methanol (65:35), the volume was made up with Acetonitrile: Methanol (65:35) to get conc. of 100µg/ml. 5ml standard stock solution of Phenobarbitone was then diluted in 10ml Acetonitrile: Methanol (65:35) to get working standard solution 50µg/ml. The solution then injected in stabilized chromatographic conditions (**Table 1 & Figure 7**).

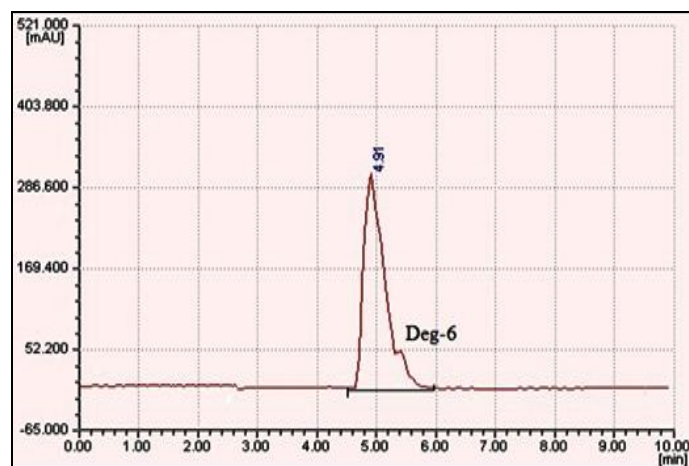


FIG. 7: CHROMATOGRAM OF PHENOBARBITONE (50µg/ml) AFTER DRY HEAT DEGRADATION WITH DEGRADATION PRODUCT AT RT 5.4

Photo-degradation studies:

Long UV-Degradation at 366nm: The photochemical stability of the drug was studied by exposing the drug sample to long UV (366nm) light

for 48 hour 10mg after exposure, accurately weighed 10mg of drug in 100ml of methanol to get concentration 100µg/ml. 5ml standard stock solution of Phenobarbitone was then diluted in 10 ml Acetonitrile: Methanol (65:35) to get working standard solution 50µg/ml and was then injected in stabilized chromatographic conditions (**Table 1 & Figure 8**).

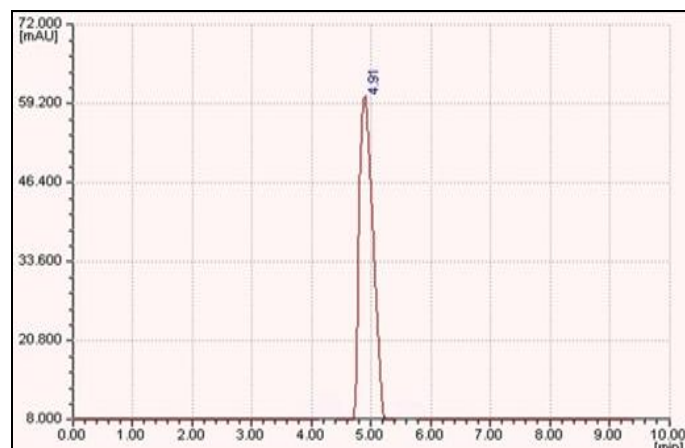


FIG. 8: CHROMATOGRAM OF PHENOBARBITONE (50µg/ml) AFTER EXPOSURE OF 366 NM, NO DEGRADATION PEAK WAS FOUND.

Short UV-Degradation at 256nm: The photochemical stability of the drug was studied by exposing the drug sample to short UV (256nm) light for 48 hour 10mg after exposure, accurately weighed 10mg of drug in 100ml of methanol to get concentration 100µg/ml. 5ml standard stock solution of Phenobarbitone was then diluted in 10ml methanol to get working standard solution 50µg/ml and was then injected in stabilized chromatographic conditions. No degradation peaks were obtained.

TABLE 1: SUMMARY OF STRESS DEGRADATION STUDIES OF PHENOBARBITONE BULK DRUG

Sr. No.	Stress deg. Parameter	Peak Area	Percent Degradation	RT of deg. Product
1.	Initial	455014	-	-
2.	Acid (2N HCl Kept for 3 hrs)	433186	4.80%	3.24
3.	Alkali (1.2N NaOH Kept For 3 hrs)	448135	1.52%	3.94
4.	6% H ₂ O ₂ Kept For 3 hrs	445196	2.16%	2.41, 2.50, 2.98
5.	Dry Heat at 100°C (48 hrs)	446791	1.81%	5.4
6.	Long UV 366 nm	455086	-	-
8.	Short UV 254 nm	455165	-	-

Validation of Analytical methods: The validation for HPLC method development was performed using parameters like Linearity, Precision, Accuracy, Limit of detection (LOD), Limit of quantification (LOQ) and Robustness.

Linearity: The standard stock solution containing 100µg/ml of Phenobarbitone to prepare range of standard solutions containing six different concentrations of analyte. The linearity of the relationship between peak area and concentration was determined by analyzing six standard solutions over the concentration range 10-60µg/ml.

The results obtained are shown in **table 2 & 7**. The peak areas were plotted against the corresponding concentrations to obtain the calibration curve (**Figure 9**).

TABLE 2: LINEARITY STUDIES OF PHENO BARBITONE

Concentration	Peak area
10 µg/ml	51264
20 µg/ml	150140
30 µg/ml	251110
40 µg/ml	357800
50 µg/ml	455014
60 µg/ml	559089

Precision: The precision of the method was demonstrated by intra-day and inter-day variation studies. In the inter day studies, 3 different concentrations 30, 40 and 50µg/ml were injected in stabilized chromatographic conditions and were analyzed in triplicate. The percentage RSD was calculated. The result obtained for intraday variations

are shown in **table 3 & 7**. In the inter day variation studies, 30, 40 and 50µg/ml were injected in stabilized chromatographic conditions and were analyzed. This procedure was repeated once a day for three consecutive days. The percentage RSD was calculated. The result obtained for interday variations are shown in **table 4 & 7**.

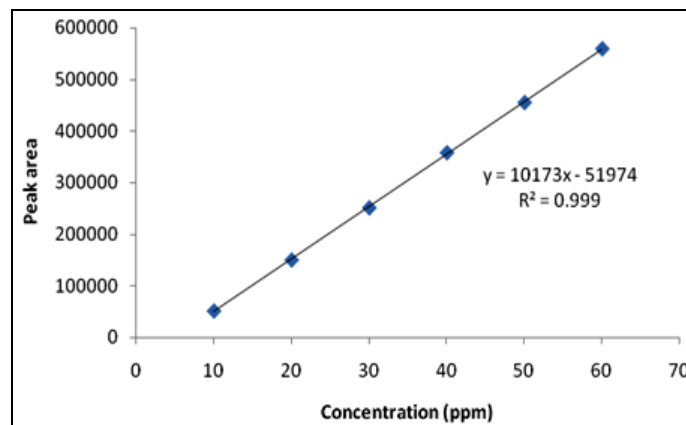


FIG. 9: CALIBRATION CURVE FOR PHENO BARBITONE

TABLE 3: INTRA-DAY PRECISION STUDIES FOR PHENOBARBITONE

Conc. (ng/band)	Peak area			Mean	SD	% RSD
	Trial 1	Trial 2	Trial 3			
30	25110	25132	25392	25211.33	156.84	0.622
40	357800	357640	357319	357586.3	244.94	0.068
50	455014	455039	455054	455035.7	20.207	0.004

TABLE 4: INTER-DAY PRECISION STUDIES FOR PHENOBARBITONE

Conc. (ng/band)	Peak area			Mean	SD	% RSD
	Day 1	Day 2	Day 3			
30	25110	25149	25371	25210	107.33	0.425
40	357800	357335	357210	357448.3	234.44	0.065
50	455014	455011	455089	455038	34	0.747

Accuracy: To check accuracy of the method, recovery studies were carried out by mixing standard drug solution to pre-analyzed sample solution at three different levels 80, 100 and 120%. Basic concentration of sample chosen was 25µg/ml of Phenobarbitone bulk drug solution to which 20, 25 and 30µg/ml of Phenobarbitone tablet solution was

added. These solutions were injected in stabilized chromatographic conditions in triplicate to obtain the chromatograms. The drug concentrations of Phenobarbitone were calculated by using linearity equation. The results obtained are shown in **table 5 & 7**.

TABLE 5: RECOVERY STUDIES OF PHENOBARBITONE

Level	Conc. (µg/ml) Std. Sample	Area	Mean	Recovered Conc.	% Recovery
80	25 + 20	395631	395570	43.99	98.75
		395254			
		395825			
100	25 + 25	461039	461351.3	50.45	100.9
		461182			
		461833			
120	25 + 30	503126	501982.3	54.45	99
		502457			
		500364			

Limit of Detection (LOD): LOD is calculated from the formula:

$$DL = \frac{3.3 \sigma}{S}$$

Where, σ = standard deviation of response for the lowest conc. in the range; S = slope of the calibration curve.

LOD = Phenobarbitone: 0.0035 $\mu\text{g/ml}$

Limit of Quantification (LOQ): The quantitation limit (QL) may be expressed as:

$$QL = \frac{10 \sigma}{S}$$

LOQ = Phenobarbitone: 0.1183 $\mu\text{g/ml}$.

Range: Phenobarbitone: 10-60 $\mu\text{g/ml}$

Robustness: Robustness was performed by injecting the Phenobarbitone standard solution in to the HPLC by altering the flow rate, changing the pH and changing the composition of the organic solvent from the normal chromatographic conditions. The results are tabulated in **table 6 & 7**.

TABLE 6: ROBUSTNESS STUDIES OF PHENOBARBITONE

Flow Rate	Tailing factor	Peak area			Mean	SD	% RSD
		Trial 1	Trial 2	Trial 3			
0.9	1.64	455014	455101	455054	455056.3	43.54691	0.0095
1.0	1.49	455014	455209	455039	455087.3	106.1053	0.0233
1.1	1.50	454089	454256	455014	454453	492.9635	0.1084

Mobile Phase composition	Tailing factor	Peak area			Mean	SD	% RSD
		Trial 1	Trial 2	Trial 3			
(63:37)	1.92	455101	455059	455085	455081.7	21.19748	0.0046
(65:35)	1.49	455014	455209	455039	455087.3	106.1053	0.0233
(67:33)	1.33	455069	455635	455264	455322.7	287.5245	0.0631

pH of Mobile Phase	Tailing factor	Peak area			Mean	SD	% RSD
		Trial 1	Trial 2	Trial 3			
4.4	1.33	457820	456210	456601	456877	839.7363	0.1837
4.5	1.49	455014	455209	455039	455087.3	106.1053	0.0233
4.6	1.89	455128	455520	455632	455426.7	264.6457	0.0581

TABLE 7: SUMMARY OF VALIDATION PARAMETERS OF PHENOBARBITONE

Sr. No.	Validation Parameter	Phenobarbitone
1	Linearity Equation	Y=10173x - 51974
	(r^2)	0.999
	Range	10 – 60 $\mu\text{g/ml}$
2	Precision (% RSD)	
	Intraday	0.2313%
	Inter day	0.4123%
3	Accuracy (% recovery)	98.7%, 100.9%, 99.9%
4	LOD	0.0036 $\mu\text{g/ml}$
5	LOQ	0.1183 $\mu\text{g/ml}$
6	Specificity	Specific
7	Robustness indicated by %RSD	0.0552%

RESULTS AND DISCUSSION: The developed method was found to be precise as the %RSD values for intraday and inter-day were found to be less than 2%. Good recoveries (98.7% to 100.9%) of the drug were obtained at each added concentration, indicating that the method was accurate. The method was also found to be specific indicated by the % recoveries ranging from 98.7% to 100.9%. The LOD and LOQ were found to be 0.0036 μ g/ml and 0.11 μ g/ml indicating the sensitivity of the method.

The method was also found to be robust as indicated by the % RSD values which are less than 2%. The summary of validation parameters of proposed spectrophotometric method is shown in Table 7. The stress degradation studies showed that Phenobarbitone undergoes degradation in acid, base, oxidation, dry heat (4.80%, 1.52% , 2.16%, 1.81% respectively). Summary of the results of stress degradation studies of Phenobarbitone are shown in the Table 1.

CONCLUSION: All the above factors lead to the conclusion that the proposed method as accurate, precise, simple, sensitive, robust and cost effective and can be applied successfully for the estimation of Phenobarbitone bulk and pharmaceutical formulation and percentage degradation. The proposed method is also useful for determination of Phenobarbitone stability in sample of pharmaceutical dosage forms.

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