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Research Article

Development and Validation of a RP-HPLC-PDA Method for Simultaneous Determination of Phenobarbital and Phenytoin in Pure Form and its Pharmaceutical Dosage Form

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Abstract

Aim: A rapid and precise Reverse Phase High Performance Liquid chromatographic method has been developed and validated for Phenobarbital and Phenytoin, in its pure form as well as in combined tablet dosage form.

Study Design: Analytical method development and validation study.

Methodology: Chromatography was carried out on a Symmetry C18 (4.6 x 150mm, 5µm) column using a mixture of Methanol: Phosphate Buffer pH 3.5 (65:35) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 270 nm.

Results: The retention time of the Phenobarbital and Phenytoin was 2.459, 4.322 min respectively. The method produces linear responses in the concentration range of 5-25mg/ml of Phenobarbital and 2.5-12.5mg/ml of Phenytoin. The method's precision for the determination of assay was below 2.0%RSD.

Conclusion: All the system suitability parameters and validation parameters also met the ICH validation acceptance criteria. Hence, the present developed method can be successfully applicable for routine quality control analysis of bulk and pharmaceutical formulations.

Keywords: Phenobarbital, Phenytoin, RP-HPLC, Method development and Validation.

1. INTRODUCTION

Phenytoin sodium is 5,5-diphenylimidazolidine-2,4-dione sodium salt. Phenytoin sodium belongs to the category of drugs referred to as anticonvulsant and anti-epileptic. Phenytoin is one of the most commonly used antiepileptic medications in clinical practice for generalized seizures. It is used to prevent and control seizures. It works by reducing the spread of seizure activity in the brain. Phenytoin acts on sodium channels on the neuronal cell membrane, limiting the spread of seizure activity and reducing seizure propagation. By promoting sodium efflux from neurons, phenytoin tends to stabilize the threshold against hyperexcitability caused by excessive stimulation or environmental changes capable of reducing membrane sodium gradient. This includes the reduction of post-tetanic potentiation at synapses. Loss of post-tetanic potentiation prevents cortical seizure foci from detonating adjacent cortical areas [1-2].

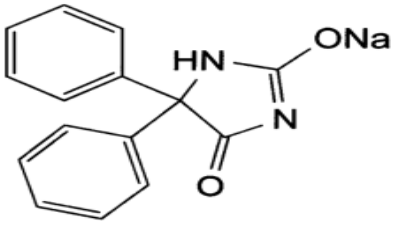

Figure 1: Chemical structure of Phenytoin sodium

Phenobarbital is 5-ethyl-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione. Phenobarbital belongs to a class of drugs known as barbiturate anticonvulsants. It works by controlling the abnormal electrical activity in the brain that occurs during a seizure. Phenobarbital acts on GABA receptors, increasing synaptic inhibition. This has the effect of elevating seizure threshold and reducing the spread of seizure activity from a seizure focus. Phenobarbital may also inhibit calcium channels, resulting in a decrease in excitatory transmitter release. The sedative-hypnotic effects of PHENOBARBITAL are likely the result of its effect on the polysynaptic midbrain reticular formation, which controls CNS arousal [3-5]

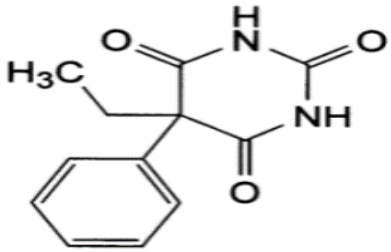

Figure 2: Chemical structure of Phenobarbital

Phenytoin and Phenobarbital both depress the motor cortex, raise the seizure threshold and reduce the spread of seizure. Phenytoin stabilises neuronal membrane, inhibiting movement of sodium and calcium ions during the nerve impulse. Phenobarbital aids GABA mediated inhibition of nerve cells.

A detailed literature survey revealed that there are various methods like UV-Spectrophotometry [6], RP-HPLC [7-10] and LC-MS [11] have been developed for the determination of phenytoin sodium and Phenobarbital in individual and in combination with other drugs. The objective of this study was to develop a simple, Rapid, specific, accurate, precise and cost-effective RP-HPLC assay for the determination of phenytoin sodium and Phenobarbital in combined pharmaceutical tablet dosage form. This method was validated in accordance with ICH guidelines[12-14] and published literature for method development and validation.

2. MATERIALS:

2.1. INSTRUMENTS USED

The present method was quantitatively estimated on a Waters Alliance 2695 separation module HPLC system, and data processing was done using Empower 2 software. The eluates were monitored by 996 Photo-diode array detectors. Sonication's dissolution and degassing of the solvents and the mobile phase were achieved on Labman digital ultra sonicator. The pH of the solution was adjusted by using a Lab India pH meter.

2.2. CHEMICALS USED: The standard drugs of Phenobarbital and Phenytoin were collected as gift samples from Sura labs, Hyderabad. Acetonitrile (HPLC grade), Methanol (HPLC grade), water (HPLC grade), and KH_2PO_4 of analytical grade were used for the preparation of the mobile phase.

3. METHODOLOGY:

3.1. Preparation of standard solution:

Accurately weighed and transferred 10 mg of Phenobarbital and Phenytoin into a 10ml of clean dry volumetric flasks added about 7ml of Methanol and sonicated to dissolve and removal of air completely and made volume up to the mark with the same Methanol.

Further pipetted 0.15ml of Phenobarbital and 0.075ml of Phenytoin from the above stock solutions into a 10ml volumetric flask and diluted up to the mark with diluents.

3.2. Preparation of buffer and mobile phase:

3.2.1. Preparation of Phosphate buffer pH 3.5:

Accurately weighed 6.8 grams of KH_2PO_4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.5.

3.2.2. Preparation of mobile phase:

Accurately measured 650 ml (65%) of Methanol and 350 ml of Phosphate buffer (35%) a were mixed and degassed in digital ultrasonicator for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

4. HPLC METHOD DEVELOPMENT:

Procedure:

Injected the samples by changing the chromatographic conditions and recorded the chromatograms, noted the conditions of proper peak elution for performing validation parameters as per ICH guidelines

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: Phosphate Buffer in proportion 65:35 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, Symmetry and X-Bridge. Symmetry C18 (4.6×150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow. Optimised chromatogram of Phenobarbital and Phenytoin is represented in Figure 1, and the placebo did not interfere with the retention time of Phenobarbital and Phenytoin.

5. METHOD VALIDATION

In order to confirm that the developed analytical method is suitable for routine quality control analysis, Method validation was proceeded by using various analytical parameters like accuracy, precision, linearity, detection limit, quantitation limit and robustness based on guidelines given by ICH.

5.1. SYSTEM SUITABILITY: Before proceeding with validation parameters, a system suitability study was done to ensure that the HPLC system, reagents, chemicals, and column used are capable to give accurate and precise results. The standard solution was injected for

five times, recorded the chromatograms, and calculated the %RSD for the areas of all five replicate injections in HPLC.

5.2. ASSAY OF DRUG:

5.2.1. Preparation of Sample Solution:

Taken average weight of one tablet and crushed in a mortor by using pestle and weighed 10mg equivalent weight of Phenobarbital and Phenytoin sample into a 10mL clean dry volumetric flask and added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent.

Further pipetted 0.15 ml of Phenobarbital and 0.075 ml Phenytoin above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Procedure:

Injected the three replicate injections of standard and sample solutions and calculated the assay by using formula:

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

5.3. LINEARITY: To assure that the developed method obeys the Beers-Lamberts law, a series of aliquots were prepared in the range of 5-25 µg/mL and 2.5-12.5 µg/mL for phenobarbital and phenytoin respectively. Injected the samples into HPLC system, recorded the chromatograms and plotted a calibration curve and calculated correlation coefficient using linear regression analysis.

Preparation of Solutions:

Accurately weighed and transferred 10 mg of Phenobarbital and 10mg of Phenytoin working standard into a 10ml of clean dry volumetric flasks added about 7mL of Diluents and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

5.3.1. Preparation of Level – I (5 ppm of Phenobarbital & 2.5ppm of Phenytoin):

Pipetted out 0.05ml of Phenobarbital and 0.025 ml of Phenytoin stock solutions was take in a 10ml of volumetric flask diluted up to the mark with diluent.

5.3.2. Preparation of Level – II (10 ppm of Phenobarbital &5ppm of Phenytoin):

Pipetted out 0.1ml of Phenobarbital and 0.05 ml of Phenytoin stock solutions was take in a 10ml of volumetric flask diluted up to the mark with diluent.

5.3.3. Preparation of Level – III (15 ppm of Phenobarbital & 7.5 ppm of Phenytoin):

Pipetted out 0.15 ml of Phenobarbital and 0.075ml of Phenytoin stock solutions was take in a 10ml of volumetric flask diluted up to the mark with diluent.

5.3.4. Preparation of Level – IV (20 ppm of Phenobarbital & 10ppm of Phenytoin):

Pipetted out 0.2 ml of Phenobarbital and 0.1 ml of Phenytoin stock solutions was take in a 10ml of volumetric flask diluted up to the mark with diluent.

5.3.5. Preparation of Level – V (25 ppm of Phenobarbital & 12.5ppm of Phenytoin):

Pipetted out 0.25ml of Phenobarbital and 0.125ml of Phenytoin stock solutions was take in a 10ml of volumetric flask diluted up to the mark with diluent.

Procedure: Injected each level into the chromatographic system and measured the peak area.

Plotted a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculated the correlation coefficient.

5.4. PRECISION

The method's precision was determined by carrying out Repeatability and Intermediate precision.

5.4.1. Repeatability

The standard solution was injected for five times and measured the area for all five injections in HPLC under same operating conditions. Recoded the chromatograms and calculated the mean, standard deviation, and %Relative standard deviation.

5.4.2. Intermediate precision:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days under same operating conditions. Recoded the chromatograms and calculated the mean, standard deviation, and %Relative standard deviation.

5.5. ACCURACY: Method's accuracy was confirmed by calculating the recovery of the spiked samples at the concentration level of 50%, 100% and 150%.

5.5.1. Preparation of 50% Standard stock solution: Accurately weighed and transferred 10 mg of Phenobarbital and 10mg of Phenytoin working standard into a 10ml of clean dry volumetric flasks added about 7mL of Diluents and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Pipetted out 0.75ml of Phenobarbital and 0.0375ml of Phenytoin from the stock solutions into a 10ml volumetric flask and diluted up to the mark with diluents.

5.5.2. Preparation of 100% Standard stock solution: Pipetted out 0.15ml of Phenobarbital and 0.075ml of Phenytoin from the stock solutions into a 10ml volumetric flask and diluted up to the mark with diluents.

5.5.3. Preparation of 150% Standard stock solution: Pipetted out 0.225ml of Phenobarbital and 0.1125 ml of Phenytoin from the stock solutions into a 10ml volumetric flask and diluted up to the mark with diluents.

Procedure: Injected the three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculated the Amount found and Amount added for Phenobarbital and Phenytoin and calculated the individual recovery and mean recovery values.

5.6. ROBUSTNESS:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

Pipetted out 0.15ml of Phenobarbital and 0.075ml of Phenytoin from the stock solutions into a 10ml volumetric flask and diluted up to the mark with diluents.

5.6.1. Effect of Variation of flow conditions: The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected twice and chromatograms were recorded

5.6.2. Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. Methanol: Phosphate Buffer was taken in the ratio and 70:30, 60:40 instead (65:35), remaining conditions are same. 10µl of the above sample was injected twice and chromatograms were recorded.

5.7. LIMIT OF DETECTION

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= 3.3 × σ / s

Where, σ = Standard deviation of the response

S = Slope of the calibration curve

5.8. LIMIT OF QUANTITATION

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where, σ = Standard deviation of the response and S = Slope of the calibration curve

6. RESULTS AND DISCUSSION

6.1. METHOD DEVELOPMENT

To optimize the chromatographic conditions for the concurrent assessment of Phenobarbital and Phenytoin, in its pure form as well as in tablet dosage form by RP-HPLC different combinations of mobile phase were tried but better results were obtained using a mixture of Methanol: Phosphate Buffer pH 3.5 (65:35) at a flow rate of 1.0ml/min which exhibited sharp peaks for Phenobarbital and Phenytoin at a retention time of 2.459 and 4.322 min respectively (displayed in Figure 1). By using the Symmetry C18 (4.6 x 150mm, 5µm) column maintained at 40ºC at a flow rate of 1 mL/min, both the drugs showed good absorbance at 270 nm. The method precision for the determination of assay was below 2.0%RSD. As per ICH guidelines the resolution between the 2 peaks was observed to be >2, column efficiency was proved, as the number of theoretical plates was >2500, Peak asymmetry was found to be within the acceptance criteria[<2]. The total analysis time was observed to be less than 5min, which proved that the method can be readily employed for regular analysis due to reduced solvent consumption, more sustainability and cost-effectiveness. As Methanol is employed as mobile phase, the method is also environmentally friendly.

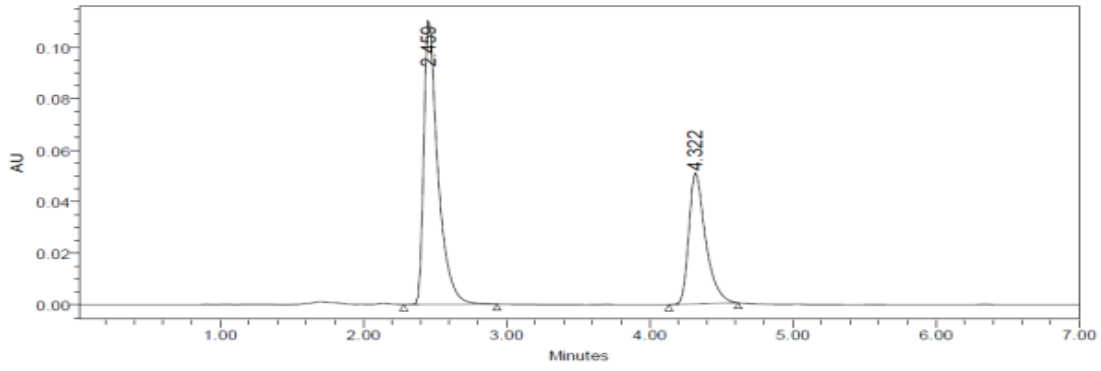

Figure 3: Optimized chromatogram of Phenobarbital and Phenytoin

6.2. METHOD VALIDATION:

6.2.1. SYSTEM SUITABILITY: The standard solution was injected for five times and measured the area for all five injections in HPLC. As per ICH guidelines the key parameters like number of theoretical plates [>2500], Peak Asymmetry [<2.0], resolution [>2.0] and %RSD should be <2.0. The %RSD for the area of five replicate injections, tailing factor and Theoretical plates was found to be within the specified limits which proved the system suitability of the present method. Results are represented in Tables 3 and 4.

Table 3: Results of system suitability for Phenobarbital

S. No.	Rt	Peak Area	USP plate count	USP Tailing
1	2.459	600560	3637	1.7
2	2.459	600331	9643	1.7
3	2.460	600987	8728	1.7
4	2.459	600558	8596	1.8
5	2.460	600966	9627	1.7
Mean		600680.4		
Std. Dev		285.9760479		
% RSD		0.048		

Table 4: Results of system suitability for Phenytoin

S. No.	Rt	Peak Area	USP Resolution	USP plate count	USP Tailing
1	4.322	422674	4.3	8860	1.7
2	4.322	423569	5.4	7833	1.9
3	4.315	424543	6.4	6852	1.7
4	4.322	423834	5.4	7583	1.8
5	4.315	424635	6.4	6985	1.7
Mean		423851			
Std. Dev		799.7909102			
% RSD		0.1887			

6.2.2. SPECIFICITY

Prepared Blank, placebo, standard and sample all had been injected. The blank and placebo did not interfere with the retention time of Phenobarbital and Phenytoin.

6.2.3. LINEARITY

Linearity was assessed by plotting a calibration curve correlating peak response with their corresponding concentrations. A concentration range of 5-25 µg/mL and 2.5-12.5 µg/mL for phenobarbital and phenytoin was used respectively, and linearity curves are represented in Figures 4 and 5. The linear regression equations were y = 30702x + 134361(R^2 = 0.9991) for Phenobarbital and y = 43017x + 97700(R^2 = 0.9991) for Phenytoin. (Table-5 and 6)

Table 5: Linearity study data for Phenobarbital

Concentration (µg/ml)	Average (Peak Area)
5	280199
10	450220
15	600560
20	741230
25	902243

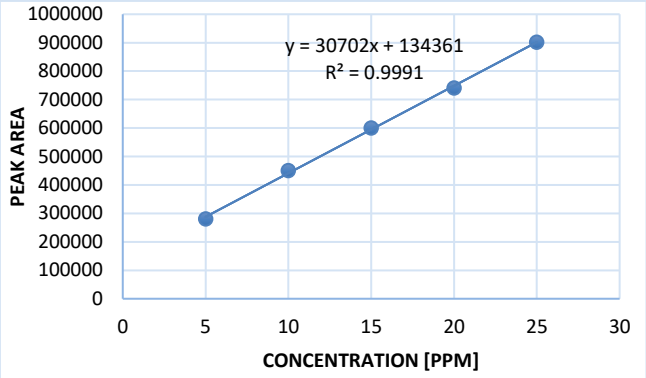

Figure 4: Linearity curve of Phenobarbital

Table 6: Linearity study data for Phenytoin

Concentration (µg/ml)	Average (Peak Area)
2.5	199798
5	319960
7.5	422674
10	523456
12.5	635768

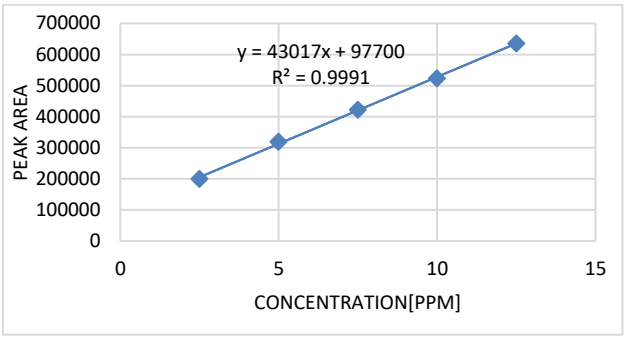

Figure 5: Linearity curve of Phenytoin

6.2.4. ACCURACY

As per ICH guidelines the accuracy range for validating drug substances or products should be within 95-105% of target concentration. The mean recovery values obtained for the three specification levels at 50%, 100%, and 150% for Phenobarbital and Phenytoin were found to be 100.03% and 99.7% meeting the validation criteria proved the method's accuracy. The calculated results were summarized in Table 7 and 8.

Table 7: The accuracy results for Phenobarbital

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	308408	7.5	7.55	100.6	100.3%
100%	600619	15	15	100	
150%	894293	22.5	22.6	100.4	

Table 8: The accuracy results for Phenytoin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	216092	3.75	3.8	101.3	99.7%
100%	423626	7.5	7.45	99.3	
150%	634469.7	11.25	11.1	98.6	

6.2.5. ROBUSTNESS

The robustness results were obtained by varying the flow rate and mobile phase composition. From the data it was observed that the tailing factor and number of theoretical plates, did not vary significantly with deliberate adjustments in flow rate and mobile phase composition. Thus, the obtained results of the developed method were confirmed the robustness. The results were displayed in Table-9 and 10.

Table 9: Results for Robustness of Phenobarbital

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	600122	2.456	5215	1.8
Less Flow rate of 0.9 mL/min	651206	2.741	5199	1.79
More Flow rate of 1.1 mL/min	546820	2.270	5234	1.8
Less organic phase	586420	3.266	5298	1.8
More organic phase	542813	2.147	5287	1.76

Table 10: Results for Robustness of Phenytoin

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	422042	4.312	5648	1.5
Less Flow rate of 0.9 mL/min	453012	4.830	5687	1.6
More Flow rate of 1.1 mL/min	398654	3.979	5602	1.5
Less organic phase	445983	3.266	5643	1.55
More organic phase	402315	2.147	5699	1.51

6.2.6. LIMIT OF DETECTION

The detection limit of Phenobarbital and phenytoin was found to be 0.031 and 0.0613µg/ml which proved the sensitivity of the developed method.

6.2.7. LIMIT OF QUANTIFICATION

The quantification limit of Phenobarbital and phenytoin was found to be 0.09 and 0.185µg/ml respectively.

7. CONCLUSION

In the present investigation, a simple, Rapid, sensitive, precise, accurate and cost-effective RP-HPLC method was developed for the quantitative estimation of Phenobarbital and Phenytoin in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps. The solvent system used in this method was economical. The %RSD values were within 2 and the method was found to be precise. The results expressed in Tables for RP-HPLC method was promising. Moreover, in comparison to the previously reported methods, the retention time is less which proved the rapidity of the present developed method. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. At last, fastest run time, eco-friendliness, low solvent use, better selectivity at 270 nm proved that the newly established method can be successfully employed for testing the retest period of API. It can also be applicable for the routine determination of Phenobarbital and Phenytoin in bulk drug and in pharmaceutical dosage forms.

CONSENT: It is not applicable

ETHICAL APPROVAL: It is not applicable

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COMPETING INTERESTS: Authors have declared that no competing interests exists.

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