

METHOD DEVELOPMENT AND VALIDATION BY RP-HPLC FOR ESTIMATION OF PHENYTOIN IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

In the Current scenario there is a need for a creation of a useful RP-HPLC methods for various types of drug. Thus a easy, reproducible and trustworthy reversed phase high performance liquid chromatographic (RP-HPLC) method is developed and validated for the estimation of Phenytoin. Jasco System was utilized to carry out chromatography with an UV 730 D detector. On C18 (4.6x250 mm) column. The mobile phase used is Methanol: Distill water (50:50 V/v) at a flow rate of 1 ml / min. The pH of mobile phase 3. Detection was done with a UV detector at 254 nm. As per the ICH guidelines, parameters such as precision, linearity, accuracy, ruggedness, LOD and LOQ were studied. The retention of Phenytoin was 5.174. the Linearity range was found to be. The correlation coefficients of phenytoin obtained were 0.979. The developed method was found to

be reliable, accurate and easy for simultaneous estimation of Phenytoin in pharmaceutical dosage forms. The developed method is also useful for quality control of both bulk manufacturing as well as pharmaceutical dosage forms.

KEYWORDS: Phenytoin, method validation, RP-HPLC, ICH guidelines.

1. INTRODUCTION

Name

Phenytoin

Molecular FormulaC₁₅H₁₂N₂O₂**Description**

1. An anticonvulsant that is used in a wide variety of seizures. It is also an anti-arrhythmic and a muscle relaxant. The mechanism of therapeutic action is not clear, although several cellular actions have been described including effects on ion channels, active transport, and general membrane stabilization. The mechanism of its muscle relaxant effect appears to involve a reduction in the sensitivity of muscle spindles to stretch. Phenytoin has been proposed for several other therapeutic uses, but its use has been limited by its many adverse effects and interactions with other drugs.^[1,2]

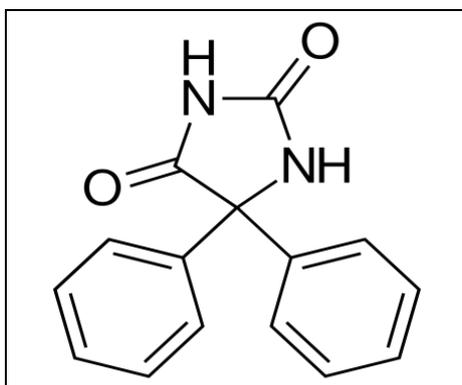
Structure

Fig 1: Structure of PHT.

2. MATERIALS AND METHOD**2.1 Instrumentation**

Jasco HPLC system and C18 column (250 x 4.6 mm) was used. The instrument is equipped with an UV 730 D detector. A 20 microLitre rheodyne injector port was used for injecting the samples.^[2,3]

2.2 Chemicals and solvents

The working standard of Phenytoin was obtained as gift samples from Glenmark Pharmaceutical Ltd, Goa, India. The tablet market formulation was procured from local market. HPLC grade water was purchased from Lupin, Ltd, Mumbai, India. Methanol (HPLC Grade) was obtained.

2.3 Chromatographic conditions

Column: C18 column (250x 4.6 mm)

Mobile phase: Methanol: Distill water in proportion of 50:50 v/v (PH-3) Detector 254

λ_{\max} : 254 nm.

Injection Volume:- 20 μ l.

Flowrate: 1 ml/min.

Temperature: Ambient.

Run Time: 10 min Diluents: CAN.

2.4 Selection of Mobile Phase

Standard solution of Phenytoin (PHT) was injected into the HPLC system and run in different solvent systems. Mixture of different solvents were tried in order to determine optimum chromatographic conditions for effective separation of PHT. After several permutation and combination, it was found that mixture of methanol: water gives satisfactory results as compared to other mobile phases. Finally the optimal composition of the mobile phase Methanol: water in the ratio of 50:50 (ph-3) was selected, as it gave high resolution of PHT with minimal tailing.^[5,6]

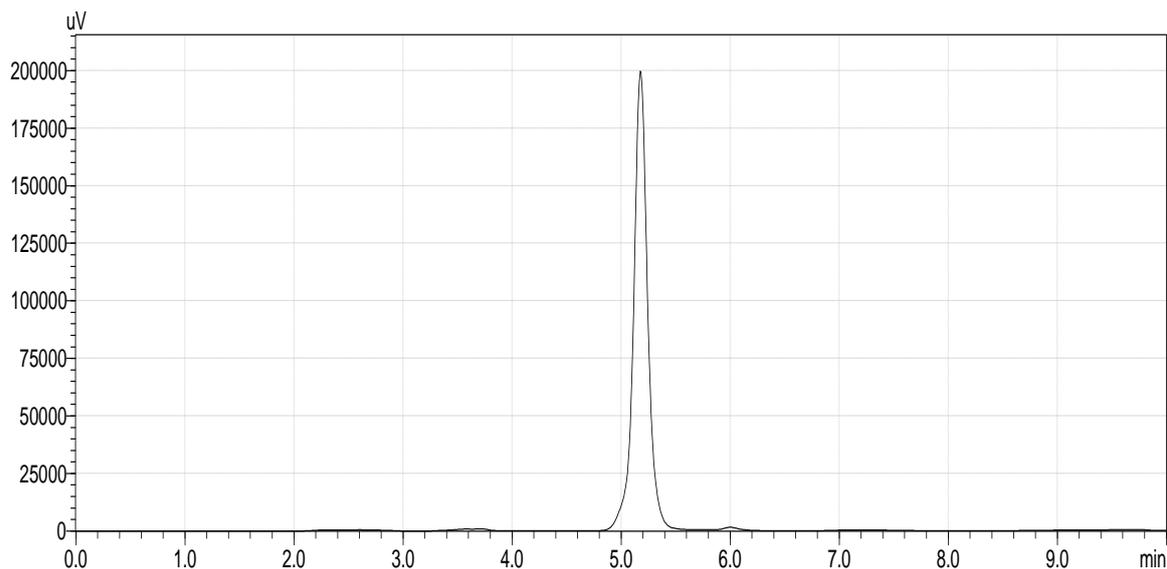
2.5 Preparation of Standard stock solution

Mobile phase was prepared by mixing 50 ml of methanol with 50 ml of Distill water. The pH of mobile phase was adjusted by 0.05% ortho phosphoric acid (PH-3). This mobile phase was filtered through 0.45 μ membrane filter and then it was ultrasonicated for 20 minutes.

2.6 Preparation of standard stock solution

About 10 mg PHN are accurately weighed and transferred to 10 ml volumetric flasks. The drug is dissolved in 5 ml of with shaking. The volume was made up to the mark using the mobile phase, thus getting 1000 μ g/ml of PHT of the standard stock solution. Ultrasonication was done for 20 minutes and filtration was done with the help of membrane filter of pore size 0.154.

Mobile Phase: Methanol: Water 50:50 (v/v) (pH-3)



λ max: 254 nm

Flow rate: 1 ml/min.

Retention Time: 5.174 min

2.7 System suitability parameters

The System suitability parameters were determined as follows.

Table 1: System suitability parameters.

| Sr No. | Parameters | PHT |
|--------|------------------------------|---------|
| 1 | Retention time (min) 5.174 | 4.55 |
| 2 | No. of theoretical plate (N) | 6055.60 |
| 3 | Tailing Factor | 0.70 |

Time(min).

Table 2: Standard Calibration curves.

| Sr No. | Conc. ($\mu\text{g/ml}$) | Mean peak area |
|--------|----------------------------|----------------|
| 1 | 10 | 566.42 |
| 2 | 20 | 1234.50 |
| 3 | 30 | 1463.25 |
| 4 | 40 | 2110.00 |
| 5 | 50 | 2510.00 |

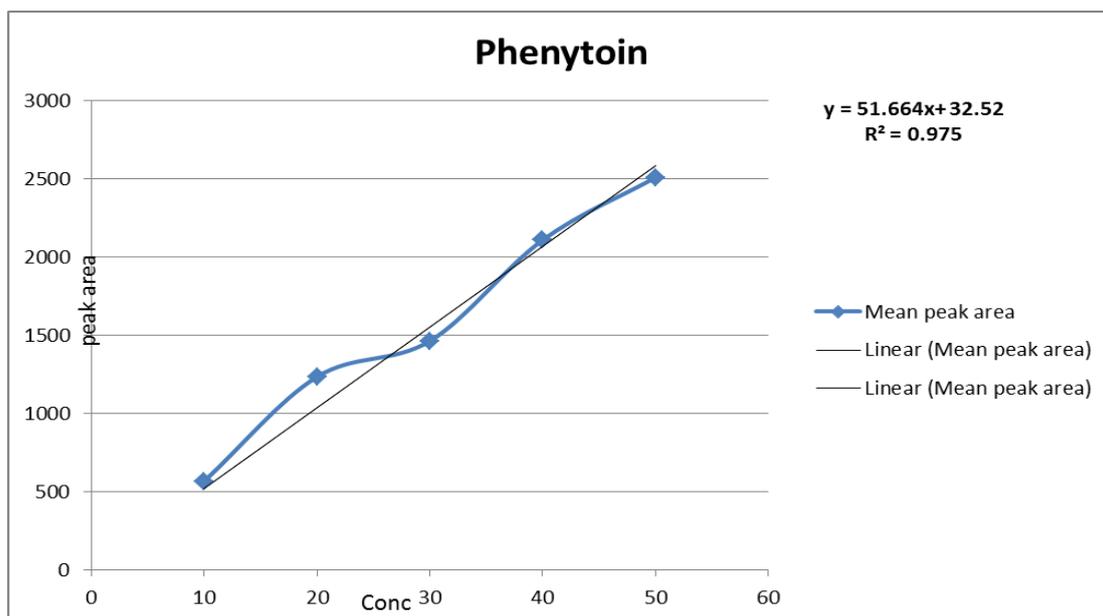


Fig 3: Calibration curve of Phenytoin by HPLC.

2.8 Study of beers-Lambert's law

Preparation of Standard calibration curves and selection of analytical concentration ranges: Appropriate & necessary aliquots were transferred to a series of 10 ml volumetric flasks. Concentration ranges were 10-30 which were made up to mark by the mobile phase. The prepared diluted solutions were filtered through 0.2 μ membrane filter. Optimized chromatographic conditions were used for recording of chromatogram. The Filtrate (20 μ l) was injected into the column. Area for each peaks for PHT were measured at 254 nm. The mean peak area was calculated by each sample solution being chromatographed five times. Standard calibration graph for PHT are given in Fig. no.3.

3. Analysis of marketed formulation

Drug estimation in marketed formulations was done by taking twenty tablets containing 10 mg of PHN are weighed and average weight was calculated. The taken 20 tablets were crushed to powder in a mortar. For the analysis drug quantity of drug equivalent to 10 mg was transferred to 10 ml volumetric flask containing the mobile phase of 5 ml. It is then ultrasonicated for 10 mins. Then mobile phase was utilized to make up the volume up to the mark. The solution was then filtered, This solution was diluted further to obtain mixed sample solution containing 10 μ g/ml of PHT. The solution was filtered through 0.30 μ membrane filter. A 20 μ l of sample solution was injected into sample injector for seven times under chromatographic conditions as described above. Areas of each peak was measured at

254 nm. Peak area of PHT found in the pure mixture was used to determine the amount of each drug present in the sample. The results obtained are provided in table nos. 3 & 4.

Table 3: HPLC Assay of PHN (n=3).

| Amt. taken ($\mu\text{g/ml}$) | Peak Area | Amt. found ($\mu\text{g/ml}$) | % of drug found |
|---------------------------------|-----------|---------------------------------|-----------------|
| 10 | 650.55 | 10:5 | 100:40 |
| 10 | 665.80 | 10:15 | 100:10 |
| 10 | 655.80 | 10:12 | 100:50 |

Table 4: Statistical evaluation of marketed formulation.

| Drug | % Mean* | S.D. | % RSD |
|------|---------|------|-------|
| PHN | 102.52 | 0.32 | 0.31 |

*Average of six determination.

4. Validation

The developed method was validated as per the given ICH guidelines.

4.1 Linearity and Range

The linearity of measurement was evaluated by analysing different concentrations of the standard solutions of PHT. At the conc. range of 10-50 $\mu\text{g/ml}$ for PHT respectively the Beer's law was well obeyed.^[11,12]

Table 5: Linear regression data for calibration curves.

| Parameters | Phenytoin |
|-----------------|------------------------|
| Linearity range | 10-30 $\mu\text{g/ml}$ |
| r^2 | 0.975 |
| Slope | 51.664 |
| Intercept | 32.52 |

r^2 Correlation coefficient; S.E.: Standard error.

4.2 Precision

4.2.1 Repeatability

To check the degree of repeatability of the method, six samples of the marketed formulation were analyzed. The results of the Repeatability are given below in Table no. 6.^[10]

Table 6: Result of repeatability.

| Drug | Conc ($\mu\text{g/ml}$) | Area mean* | Amount found | % Amount found | S.D.* | %RSD* |
|------|---------------------------|------------|--------------|----------------|-------|-------|
| PHN | 10 $\mu\text{g/ml}$ | 657.38 | 10:12 | 100:33 | 1.32 | 1.37 |

*Average of six determination.

Table 7: Results of Intra-day and Inter-day precision studies.

| Parameter | Conc. (µg/ml) | Area mean* | Amount found* | % Amount found* | S.D.* | %RSD* |
|-----------|---------------|------------|---------------|-----------------|-------|-------|
| Intraday | 10 | 657.38 | 10.13 | 100:10 | 1.06 | 0.15 |
| | 20 | 668.75 | 20.15 | 100:10 | 0.65 | 0.12 |
| | 30 | 1080.00 | 30.40 | 100:00 | 1.30 | 0.10 |
| Interday | 10 | 665.40 | 10:14 | 100:20 | 0.8 | 0.10 |
| | 20 | 700.00 | 20:18 | 100:10 | 1.68 | 0.08 |
| | 30 | 1060.01 | 29:48 | 100:30 | 0.60 | 0.06 |

*Average of three determinations

Low %RSD values for intra and inter day confirmed that the method is precise

4.2.2 Intermediate precision

The Intra and Inter-day precision was determined by analysis of the marketed formulation of PHN on the same day at different time intervals and on different days as well respectively. The results are provided in a tabulated form in table no. 7.

4.3 Accuracy

According to ICH guidelines, recovery studies were done to check the accuracy of the proposed method by applying the standard solution method to known amount of TA and EMT corresponding to 80,100 and 120%. Analysis was performed as per the procedure of analysis of PHN tablets. The recovery were done three times at every level. The results of recovery studies are provided below in Table no.8 and 9.

Table 8: Recovery studies results.

| Recovery Level (%) | Conc of Drug (µg/ml) | | Total conc.of drug (µg/ml) | Toatal amt. Recovered (µg/ml) | % Recovery* | %RSD |
|--------------------|----------------------|----|----------------------------|-------------------------------|-------------|------|
| 70 | 5 | 7 | 17 | 17.20 | 100 | 0.50 |
| 90 | 5 | 9 | 19 | 19.05 | 100.20 | 0.36 |
| 110 | 5 | 11 | 20 | 20.10 | 100.30 | 1.20 |

*Average of three determinations.

Table 9: Robustness evaluation of PHN.

| Sr. No. | Factor | Level | Retention time |
|---------|---------------------------|-------|----------------|
| 1 | Flow rate (ml/min) | | |
| | 0.9 | -1 | 5.5 |
| | 1 | 0 | 5.1 |
| | 1.1 | +1 | 5.05 |
| | Mean +/- S.D. (n=6) | | 5.17+/- 0.22 |
| 2 | Mobile phase volume (v/v) | | |
| | 48:50 | -1 | 5.5 |
| | 52:45 | 0 | 5.10 |
| | 50:55 | +1 | 5.05 |
| | Mean +/- S.D. (n=6) | | 5.17+/-0.12 |
| 3 | Wavelength | | |
| | 245 | -1 | 5.17 |
| | 247 | 0 | 5.35 |
| | 254 | +1 | 5.17 |
| | Mean +/- S.D. (n=6) | | 5.11+/- 0.092 |

Table 10: LOD and LOQ of PHN.

| Parameter | TPM |
|--------------------------|------------------------|
| LOD ($\mu\text{g/ml}$) | 0.045 $\mu\text{g/ml}$ |
| LOQ ($\mu\text{g/ml}$) | 0.142 $\mu\text{g/ml}$ |

4.4 Robustness

The robustness of the method is very necessary to check and determine in analytical works. During development robustness was thus studied by small but purposeful and deliberate variations in flow rate, percentage of methanol in the mobile phase and wavelength. Each factor selected to examine and check were changed at three particular levels which are -1,0,1. One factor at a time was changed to estimate the effect and to the effect that it made on the retention time of the drugs. The results thus obtained are provided in table no. 10.^[3,8,9]

Flow rate variation, % of methanol in the mobile phase and wavelength did not affect the results obtained. Rt and tailing factors of both the drugs at different levels of variations were found to be similar. Hence, the method used in the present analytical work was found to be robust.

4.5 Limit of Detection (LOD)

The limit of detection (LOD) is the smallest concentration that can be detected but it is not necessarily quantified as an exact value obtained. LOD can be calculated by the formula;

$$\text{LOD} = 2.4\sigma / S$$

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

4.6 Limit of Quantitation (LOQ)

The limit of quantitation is defined as the lowest amount of analyte in the sample that can be quantitatively determined with proper precision and accuracy. LOQ can be calculated by the formula.

$$\text{LOQ} = 10\sigma / S$$

Where, σ = standard deviation of the response, S = slope of calibration curve LOD,

LOQ are shown in the Table no. 11.

CONCLUSION

From all the observations and its obtained results it was concluded that the developed RP – HPLC method for the estimation of Phenytoin in bulk and pharmaceutical dosage form was accurate, precise, linear, robust, simple, easy, systematic and rapid. Percentage recovery obtained in the study shows that the method was free from interference of excipients used in the formulation.

CONFLICT OF INTERESTS

Declared None.

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