International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 9, Issue 10, 2017

Original Article

# DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR PHENYTOIN SODIUM AND PHENOBARBITONE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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*Received: 12 May 2017 Revised and Accepted: 31 Aug 2017*

**ABSTRACT**

**Objective:** To develop an accurate, simple, rapid, precise, and linear RP-HPLC method for the simultaneous estimation of phenytoin sodium and phenobarbitone in tablet dosage form and validated as per ICH guidelines.

**Methods:** The method used was a reverse phase HPLC (RP-HPLC) method using Hypersil BDS $C_{18,}$ (250×4.6 mm, 5µm) column, mobile phase comprising of methanol: phosphate buffer (pH 5.0) (50:50), flow rate of 1.0 ml/min and a detection wavelength of 215 nm using a UV detector.

**Results:** The retention time for phenytoin sodium and phenobarbitone was found to be 3.97 min and 6.90 min, respectively. The linearity of developed method was achieved in the range of 10-30 µg/ml for phenytoin sodium and 3-9 µg/ml for phenobarbitone. The detection (LOD) and quantitation (LOQ) limits were 1.44 and 4.36 µg/ml for phenytoin sodium and 0.40 and 1.35 µg/ml for phenobarbitone respectively.

**Conclusion:** A simple, accurate, precise, linear and rapid RP-HPLC method was developed for simultaneous quantitative estimation of phenytoin sodium and phenobarbitone in bulk and pharmaceutical formulation. The method was validated as per ICH guidelines. Hence, the method holds good for the routine analysis of phenytoin sodium and phenobarbitone in various pharmaceutical industries as well as in academics.

**Keywords:** Phenytoin Sodium, Phenobarbitone, RP-HPLC, Method Development, Validation


DOI: http://dx.doi.org/10.22159/ijpps.2017v9i10.19917

## INTRODUCTION

Phenytoin sodium (fig. 1) 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 [1-4].

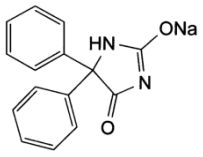


**Fig. 1: Chemical structure of phenytoin sodium [1]**

Phenobarbitone (fig. 2) is 5-ethyl-5-phenyl-2, 4, 6(1H, 3H, 5H)-pyrimidinetrione. Phenobarbitone 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. Phenobarbitone 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 phenobarbitone are likely the result of its effect on the polysynaptic midbrain reticular formation, which controls CNS arousal [5-7].

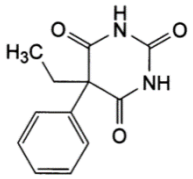


**Fig. 2: Chemical structure of phenobarbitone [5]**

Phenytoin and phenobarbitone 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. Phenobarbitone aids GABA mediated inhibition of nerve cells [8, 9].

A detailed literature survey revealed there are various RP-HPLC methods have been developed for the determination phenytoin sodium and phenobarbitone in individual and in combination with other drugs [10-20]. However, till date there is no RP-HPLC method has been reported for simultaneous estimation of phenytoin sodium and phenobarbitone in combined dosage form. Hence, the objective of this study was to develop a simple, specific, accurate, precise and sensitive RP-HPLC assay for the determination of phenytoin sodium and phenobarbitone in combined pharmaceutical tablet dosage form. This method was validated in accordance with ICH guidelines and published literature for method development and validation [22-24].

## MATERIALS AND METHODS

Pharmaceutical grade phenytoin sodium and phenobarbitone were procured from Intas Pharmaceuticals Ltd., Ahmedabad. The

marketed formulation Epilan C contains phenytoin sodium 100 mg and phenobarbitone 30 mg was purchased from the local market. Methanol, orthophosphoric acid, acetonitrile and HPLC grade water were obtained from Merck. All solvents used in this work are HPLC grade. RP-HPLC Shimadzu (LC 20ATVP) model with Spin chrome (LC SOLUTIONS) software was employed in this method. Analytical column used for the separation of analytes is Hypersil BDS $C_{18}$ (250 X 4.6 mm, 5 µm) was used for separation of analytes.

**Methods**

**Selection of wavelength**

Standard solutions of phenytoin sodium and phenobarbitone were prepared at a concentration of 10 µg/ml and scanned by UV/Visible spectrophotometer at the range of 200-400 nm. Combined UV spectrums of phenytoin sodium and phenobarbitone are depicted in fig. 3. The isosbestic point selected for simultaneous estimation was 215 nm (fig. 3).

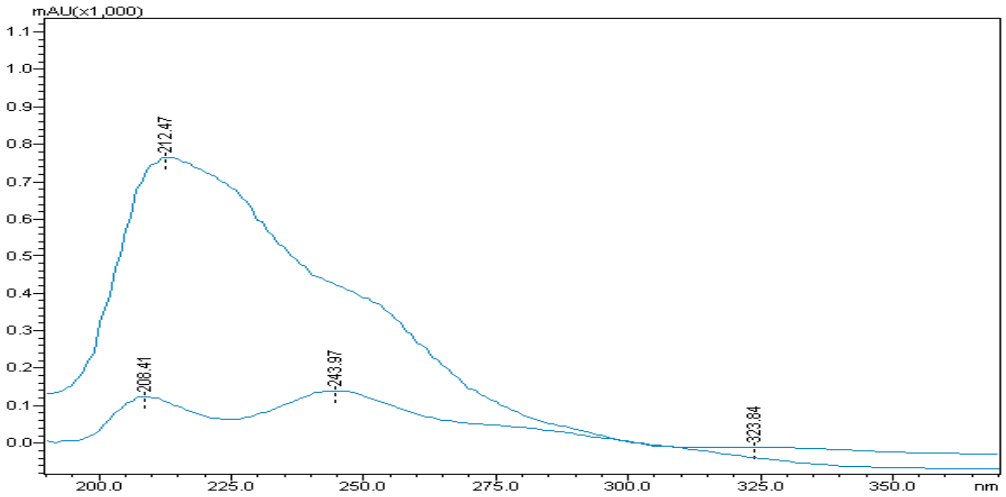


**Fig. 3: UV overlaps spectrum of phenytoin sodium and phenobarbitone**

**Chromatographic conditions**

The developed method used a reverse phase Hypersil BDS $C_{18}$ (250 X 4.6 mm, 5 µm) column, a mobile phase of methanol: phosphate buffer pH 5 adjusted with 0.1 M NaOH (50:50), flow rate of 1.0 ml/min and a detection wavelength of 215 nm using a UV detector.

**Preparation of phosphate buffer (0.05M $KH_2PO_4$)**

Accurately weighed 6.8 g of potassium dihydrogen phosphate ($KH_2PO_4$) taken and dissolved in 800 ml of distilled water. Solution pH was found to be 4.7 which was adjusted to pH 5.0 with 0.1 M NaOH. Final volume was made up to 1000 ml with distilled water.

**Preparation of mobile phase**

A mixture of 50 volumes of HPLC grade methanol and 50 volumes of phosphate buffer was prepared. The mobile phase was sonicated for 10 min to remove gasses.

**Preparation of standard solutions**

A standard stock solution of phenytoin sodium was prepared by dissolving 20 mg of phenytoin sodium insufficient mobile phase. The solution was then filtered and sonicated for 5 min and diluted to 100 ml with mobile phase. From this solution, 1 ml taken and diluted up to 10 ml with a mobile phase containing 20 µg/ml. This is treated as 100% of a standard stock solution of phenytoin sodium. A standard stock solution of phenobarbitone was prepared by dissolving 6 mg of phenobarbitone insufficient mobile phase. The solution was then filtered and sonicated for 5 min and diluted to 100 ml with mobile phase. From this solution, 1 ml taken and diluted up to 10 ml with a mobile phase containing 6 µg/ml. This is treated as 100% of a standard stock solution of phenobarbitone.

**Sample preparation**

Twenty tablets were weighed and finely powdered. The powder equivalent to 200 mg phenytoin sodium and 60 mg phenobarbitone was accurately weighed. This powder was transferred to the volumetric flask of 1000 ml capacity and dissolved in 500 ml of mobile phase. The flask was sonicated for 10 min and volume was made up to the mark with the mobile phase. The above solution was filtered through whatmann filter paper (0.45µ). From this solution, 1 ml taken and diluted up to 10 ml with a mobile phase containing 20 µg/ml of phenytoin sodium and 6 µg/ml of phenobarbitone. This solution was used for the estimation of phenytoin sodium and phenobarbitone.

**RESULTS AND DISCUSSION**

**Method development**

Different chromatographic conditions were tried for better separation and resolution. Hypersil BDS $C_{18}$ (250 X 4.6 mm, 5 µm) column was found satisfactory. Peak purity of phenytoin sodium and phenobarbitone was checked using UV detector and 215 nm was considered satisfactory for detecting both the drugs with adequate sensitivity. A number of solvents in the different ratio over a wide range of pH were tried, but either peak shape was broad or resolution was not good. Repeated trials to obtain good, sharp peak with an efficient resolution between two peaks of phenytoin sodium and phenobarbitone done on a $C_{18}$ column in isocratic HPLC. The runtime was 9 min in isocratic trial with mobile phase consisting of methanol: phosphate buffer (pH5.0) (50:50) and $C_{18}$-Hypersil BDS (250×4.6 mm, 5 µm) column, flow rate 1.0 ml/min and detection wavelength 215 nm gave the satisfactory results in terms of retention time, resolution, symmetry and sensitivity. A typical RP-HPLC chromatogram for simultaneous determination of phenytoin sodium and phenobarbitone from standard preparation was obtained as shown in (fig. 4).

**Method validation**

The developed RP-HPLC method was validated for parameters like system suitability, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness according to ICH guidelines.

**System suitability**

Standard solutions were prepared as per above-mentioned method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated. The system suitability parameters were tabulated in table 1. All the parameters were found to be within the limits.

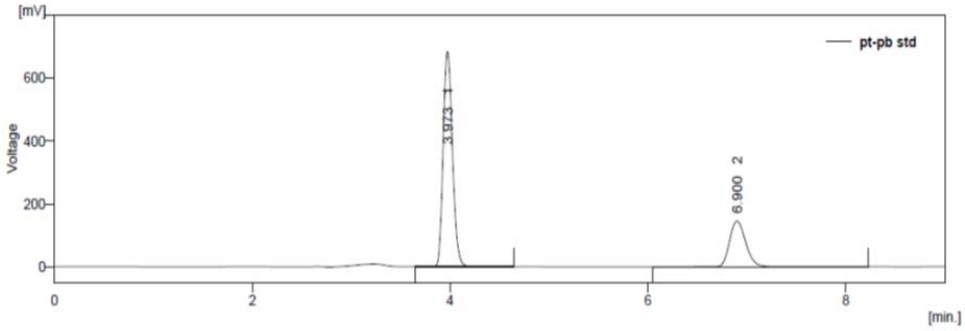


**Fig. 4: Typical chromatogram of standard solution**

**Table 1: Results of system suitability studies**

| Parameters | Acceptance limits | Phenytoin sodium | Phenobarbitone |
|---|---|---|---|
| Retention time | - | 3.973 | 6.900 |
| Theoretical plates | NLT 2000 | 8092 | 8274 |
| Tailing factor (T) | NMT 2 | 1.31 | 1.32 |
| Resolution | NLT 2 | 12.13 | |

#NLT: Not less than. # NMT: Not more than

**Linearity**

The linearity of the test solutions for the assay method was prepared from phenytoin sodium and phenobarbitone standard stock solution at five concentration levels from 50% to 150% of standard concentration. The peak area versus concentration data was treated by least-squares linear regression analysis (fig. 5 and 6). The results have shown an excellent correlation between peak areas and concentration within the concentration range of 10–30 µg/ml for phenytoin sodium, 3–9 µg/ml for phenobarbitone (table 2). The correlation coefficients were found to be 0.997 for phenytoin sodium and 0.998 for phenobarbitone, which meet the method validation acceptance criteria and hence the method was said to be linear for both the drugs.

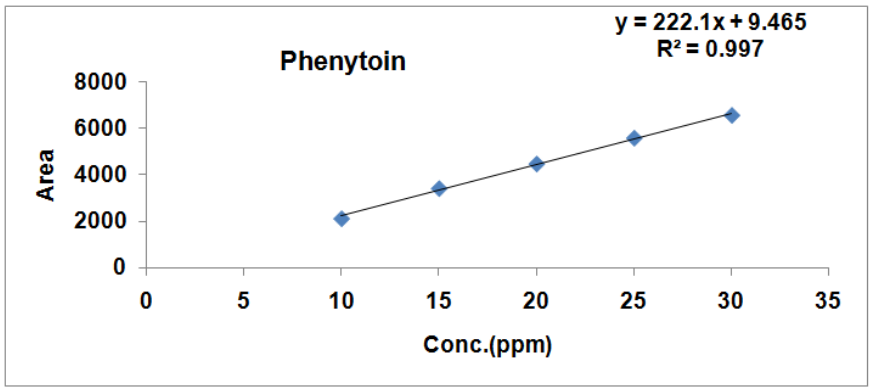


**Fig. 5: Linearity chart for phenytoin sodium**

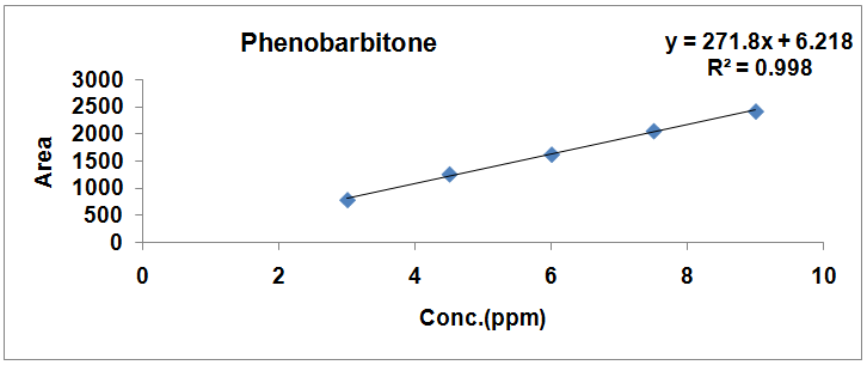


**Fig. 6: Linearity chart for phenobarbitone**

**Table 2: Linearity data for phenytoin sodium and phenobarbitone**

| % level | Phenytoin sodium concentration (µg/ml) | Phenytoin sodium peak area | Phenobarbitone concentration (µg/ml) | Phenobarbitone peak area |
|---|---|---|---|---|
| 50 | 10 | 2130.9 | 3 | 790.956 |
| 75 | 15 | 3432.565 | 4.5 | 1266.038 |
| 100 | 20 | 4492.878 | 6 | 1632.345 |
| 125 | 25 | 5610.012 | 7.5 | 2066.555 |
| 150 | 30 | 6595.911 | 9 | 2429.197 |
| Correlation coefficient | 0.997 | | 0.998 | |
| Slope | 222.1 | | 271.8 | |

**Precision**

The precision of the method was verified by precision method studies. It is done by two methods-repeatability and reproducibility (Intraday and inter-day Precision).

**Repeatability**

The sample solution was prepared at working concentration as per the test method and analysis was performed. The sample solutions of phenytoin sodium and phenobarbitone injected 6 times into the column. The results of repeatability are as tabulated in table 3. The average was taken, and percent relative standard deviation (% RSD) calculated and reported. % RSD values were found within the limits, and the method was found to be precise.

**Reproducibility (Intraday and inter-day precision)**

Intraday and inter-day precision were carried out using three different concentrations for phenytoin sodium (10, 20 and 30 µg/ml) and phenobarbitone (3, 6, 9 µg/ml) injected thrice into the column. The results of intraday and inter-day precision were tabulated in table 4. The average was taken, and % RSD was calculated and reported. % RSD values were within the limits, and the method was found to be precise.

**Table 3: Repeatability data for phenytoin sodium and phenobarbitone**

| S. No. | Phenytoin sodium 20 µg/ml n = 6 peak area | Phenobarbitone 6 µg/ml n = 6 peak area |
|---|---|---|
| 1 | 4426.225 | 1631.509 |
| 2 | 4403.994 | 1623.362 |
| 3 | 4381.889 | 1615.218 |
| 4 | 4408.186 | 1624.932 |
| 5 | 4421.056 | 1629.75 |
| 6 | 4407.577 | 1616.7 |
| Mean | 4408.1545 | 1623.579 |
| SD | 15.4952407 | 6.634317 |
| %RSD | 0.351513104 | 0.408623 |

# n: number of injections =6, # SD= standard deviation, # % RSD: % relative standard deviation

**Table 4: Intraday and inter-day precision data for phenytoin sodium and phenobarbitone**

| Drug | Conc.(µg/ml) | Intra-day precision | | Intra-day precision | |
|---|---|---|---|---|---|
| | | mean±SD (n=3) | % RSD | mean±SD (n=3) | % RSD |
| | 10 | 2247.89±9.39 | 0.418 | 2248.67±21.57 | 1.35 |
| **Phenytoin Sodium** | 20 | 4433.55±16.63 | 0.375 | 4420.20±35.88 | 1.81 |
| | 30 | 6699.39±25.42 | 0.379 | 6678.78±71.05 | 1.06 |
| | 3 | 822.29±6.13 | 0.746 | 825.80±8.16 | 1.58 |
| **Phenobarbitone** | 6 | 1628±4.33 | 0.266 | 1624.60±20.92 | 1.28 |
| | 9 | 2462.86±18.39 | 0.747 | 2462.64±24.76 | 1.19 |

# n: number of injections =3, # SD= standard deviation, # % RSD: % relative standard deviation

**Accuracy**

The accuracy of the method was determined by recovery studies by the determination of % mean recovery of both the drugs at three different levels (80 %, 100 % and 120%). At each level, three determinations were performed. The percentage recovery and mean percentage recovery were calculated for the drug was shown in table 5. The observed data were within the required range, which indicates good recovery values and hence the accuracy of the method developed.

**Table 5: Results of accuracy**

| Level (%) | Phenytoin sodium % recovery | % mean | Phenobarbitone % recovery | % mean |
|---|---|---|---|---|
| 80 | 99.12912961 | 99.27810819 | 98.69885722 | 98.88293264 |
| 80 | 100.0278808 | | 99.58154956 | |
| 80 | 98.67731416 | | 98.36839113 | |
| 100 | 100.5330846 | 100.8659537 | 100.8960369 | 100.9071524 |
| 100 | 101.7390348 | | 101.1372592 | |
| 100 | 100.3257419 | | 100.6881612 | |
| 120 | 100.7030532 | 100.3468355 | 100.9922779 | 100.1039046 |
| 120 | 99.59680407 | | 99.88472671 | |
| 120 | 100.7406491 | | 99.43470906 | |

**Robustness**

To determine the robustness of the developed method, experimental conditions were deliberately altered, and the system suitability parameters were evaluated. The solutions prepared as per the test method and injected at different variable conditions like flow rate (0.8, 1.2 ml/min.), mobile phase ratio of methanol: phosphate buffer (52:48, 48:52) and pH (4.8, 5.2). At the flow rate of 1.0 ml/min shows, a sharp peak with good resolution and rest of the flow rates were found to be not satisfactory. The method passed all system suitability parameters indicating that the method was robust.

**Table 6: Robustness study for phenytoin sodium and phenobarbitone**

| Parameter | Method condition | Phenytoin sodium | | Phenobarbitone | |
|---|---|---|---|---|---|
| | | Plate count | Tailing | Plate count | Tailing |
| Mobile phase (Methanol: phosphate buffer) | 52:48 | 8267 | 1.38 | 8012 | 1.34 |
| | 48:52 | 8395 | 1.35 | 8145 | 1.29 |
| pH | 4.8 | 8254 | 1.34 | 8426 | 1.28 |
| | 5.2 | 8412 | 1.31 | 8247 | 1.34 |
| Flow Rate | 0.8 | 8342 | 1.35 | 8371 | 1.31 |
| | 1.2 | 8246 | 1.30 | 8367 | 1.32 |

**Detection limit and quantification limit**

Limit of detection (LOD) which represents the concentration of the analyte at S/N ratio of 3.3 and limit of quantification (LOQ) at which S/N was 10 were determined experimentally for the proposed methods and results were given in table 7. Hence, the detection limits and quantitation limits of the drugs were given S/N ratios of 3.3 and 10 respectively.

**Table 7: LOD and LOQ**

| Drug | LOD(µg/ml) | LOQ(µg/ml) |
|---|---|---|
| Phenytoin sodium | 1.44 | 4.36 |
| Phenobarbitone | 0.40 | 1.22 |

## DISCUSSION

The developed method can be used for routine analysis because the linearity found is nearing 1 that is 0.997 and 0.998 for phenytoin sodium and phenobarbitone respectively which shows the good regression for linearity. Maximum recovery is obtained by this developed method and the mean percentage recovery for each component is nearing 98% to 100%. Therefore this method can be used for the routine analysis and one most important reason is that the developed method does not involve the use of expensive reagents. The method we developed involves chemicals like methanol and buffer, which are easily available. There are various RP-HPLC methods have been reported for the determination phenytoin sodium and phenobarbitone in individual and in combination with other drugs [10-20]. However, till date there was no RP-HPLC method has been reported for simultaneous estimation of phenytoin sodium and phenobarbitone in combined dosage form. So this method is first of its kind.

## CONCLUSION

The proposed RP-HPLC method was found to be simple, specific, accurate, precise, robust, rapid and economical. This method gives good resolution between all the two compounds with a short analysis time. The proposed RP-HPLC method can be useful for routine analysis of phenytoin sodium and phenobarbitone in the tablet dosage form.

## ACKNOWLEDGEMENT

The authors would like to thank Arihant School of Pharmacy and Bioresearch Institute, Adalaj, Gandhinagar, Gujarat for providing necessary facilities. The authors are also grateful to Intas Pharmaceuticals, Ahmedabad for providing gift samples of phenytoin sodium and phenobarbitione.

## AUTHORS' CONTRIBUTION

Principal author: Planned the experimental setup, performed lab work, interpreted data, and wrote the manuscript.

Co-author contribution: Supervised the development of work and helped in the evaluation of the manuscript.

Both authors read and approved the final manuscript.

## CONFLICT OF INTERESTS

Authors have no conflict of interest

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**How to cite this article**

- Radhika Shah, Ragin Shah. Development and validation of RP-HPLC method for phenytoin sodium and phenobarbitone in bulk and pharmaceutical dosage form. Int J Pharm Pharm Sci 2017;9(10):224-229.