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SIMULTANEOUS DETERMINATION OF PHENOBARBITAL AND PHENYTOIN BY USING RP-HPLC METHOD IN PURE AND ITS PHARMACEUTICAL DOSAGE FORM

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Abstract:

A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Phenobarbital and Phenytoin, in its pure form as well as in tablet dosage form. 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. The retention time of the Phenobarbital and Phenytoin was 2.456, 4.312 ±0.02min respectively. The method produce linear responses in the concentration range of 5-25mg/ml of Phenobarbital and 2.5-12.5mg/ml of Phenytoin. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: *Phenobarbital, Phenytoin, RP-HPLC, validation.*

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INTRODUCTION:

Analytical chemistry is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and the quantitative measurement of the substances present in bulk and pharmaceutical preparation.

Measurements of physical properties of analytes such as conductivity, electrode potential, light absorption or emission, mass to charge ratio, and fluorescence, began to be used for quantitative analysis of variety of inorganic and biochemical analytes. Highly efficient chromatographic and electrophoretic techniques began to replace distillation, extraction and precipitation for the separation of components of complex mixtures prior to their qualitative or quantitative determination. These newer methods for separating and determining chemical species are known collectively as instrumental methods of analysis. Most of the instrumental methods fit into one of the three following categories viz spectroscopy, electrochemistry and chromatography

Advantages of instrumental methods:

- Small samples can be used
- High sensitivity is obtained
- Measurements obtained are reliable
- Determination is very fast
- Even complex samples can be handled easily

Limitations of instrumental methods:

- An initial or continuous calibration is required
- Sensitivity and accuracy depends on the instrument
- Cost of equipment is large
- Concentration range is limited
- Specialized training is needed
- Sizable space is required

HPLC Basic Instrumentation [6,7]:

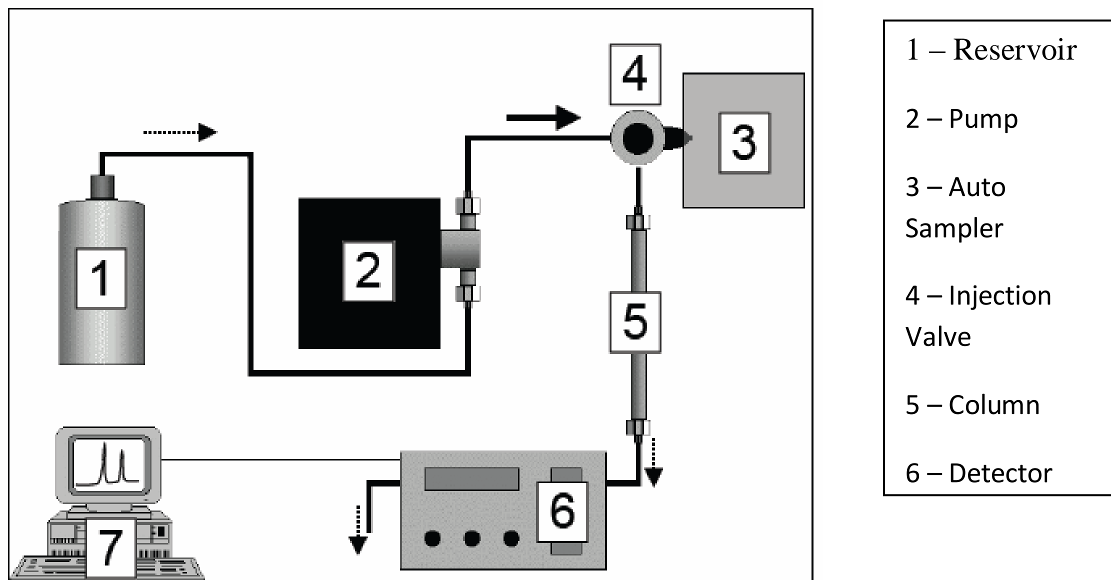
High Performance Liquid Chromatography [4,5]:

HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch.

The rate of distribution of drugs between Stationary and mobile phase is controlled by diffusion process. If diffusion is minimized faster and effective separation can be achieved .The techniques of high performance liquid chromatography are so called because of its improved performance when compared to classical column chromatography advances in column chromatography into high speed,efficient ,accurate and highly resolved method of separation.

For the recent study metformin and Sitagliptin was selected for estimation of amount of analyte present in formulation and bulk drug. The HPLC method is selected in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages

- Speed many analysis can be accomplished in 20min (or) less.
- Greater sensitivity(various detectors can be employed).
- Improved resolution(wide variety of stationary phases).
- Re usable columns(expensive columns but can be used for many analysis).
- Ideal for the substances of low viscosity.
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labour).
- Precise and reproducible.
- Integrator itself does calculations.
- Suitable for preparative liquid chromatography on a much larger scale.



HPLC components:
The essential components[4] of a complete HPLC system are solvent delivery system (Pump), detector, fixed volume injector loop or autosampler, solvent reservoirs, packed column, data system and recorder. A schematic of a simplified HPLC system is shown in above Figure.

Column:
The column is probably the heart of HPLC system. The development of this column technology leads to the evolution of the HPLC instrumentation systems used today.The conventionally used HPLC columns are particle packed columns. The key of column selection when previous separation is not available resides in knowing the chemistry of the sample. Columns should never be dry. A dry column will eventually have voids because the packing will shrink away from the wall, which would result in band broadening. Before running a sample in HPLC the column should be equilibrated. Usually column equilibrium is achieved after passage of 10 – 20 column volumes of the new mobile phase through the column. Insufficient column equilibrium usually leads to retention difference.

Pump:
The solvent delivery system or as it is commonly called the pump includes two major types, constant volume or flow and constant pressure. Constant volume pumps are mechanically driven systems, most commonly using screw driven syringes or reciprocating pistons. On the other hand, constant pressure pumps are driven or controlled by gas pressure.

Injector or Auto sampler
Samples are usually introduced by syringe injection via a manual injector into the mobile phase stream or by the use of an auto sampler. The important aspects in sample introduction are precise and reproducible injections. This is especially important with quantitative analysis where the reproducibility of the peak response is dependant on the precision of the sample introduction. Direct syringe injection through a manual injector was the first popular method of sample introduction. As HPLC instrumentation evolved, many auto sampler techniques were applied so that sample introduction has become more precise and rapid.

Detector:
HPLC detectors include ultraviolet-visible, fluorescence, electrochemical, refractometer, mass spectrometer and others. The UV visible absorption detector is the most widely used detector in liquid chromatography, since most organic compounds show some useful absorption in the UV region. This detector is fairly universal in application, although sensitivity depends on how strongly the sample absorbs light at a particular wavelength.

Solvent reservoir:
Different containers are used as a solvent delivery system reservoir. The best material from which the containers are made is glass. Plastic containers are not recommended as it leads to plasticizer leaching. The container should be covered to prevent solvent evaporation. The tubing from the reservoir can be

made of stainless steel or Teflon, and both are satisfactory.

Data handling and analysis [16]
Data handling in HPLC is as important to the success of any experiment or analysis as any other components in the system. It is part of good HPLC techniques to properly label and document the analytical results. The advanced computer softwares used now in data handling and analysis allow easy recording and storage of all chromatographic data.

MATERIALS AND METHODS:

Phenobarbital from Sura labs, Phenytoin from Sura labs, Water and Methanol for HPLC from LICHROSOLV (MERCK). Acetonitrile for HPLC from Merck,

Hplc method development:
Trails
Preparation of standard solution:
Accurately weigh and transfer 10 mg of Phenobarbital and Phenytoin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

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

Procedure:
Inject the samples by changing the chromatographic conditions and record the chromatograms, note 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.

Optimized chromatographic conditions:

Instrument used : Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature : 40ºC
Column : Symmetry C18 (4.6×150mm, 5µ)
Buffer : Accurately weighed 6.8 grams of KH2PO4 was taken in a 1000 ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.5
pH : 3.5
Mobile phase : Methanol: Phosphate buffer pH 3.5 (65:35v/v)
Flow rate : 1ml/min
Wavelength : 270nm
Injection volume : 10 µl
Run time : 7 min

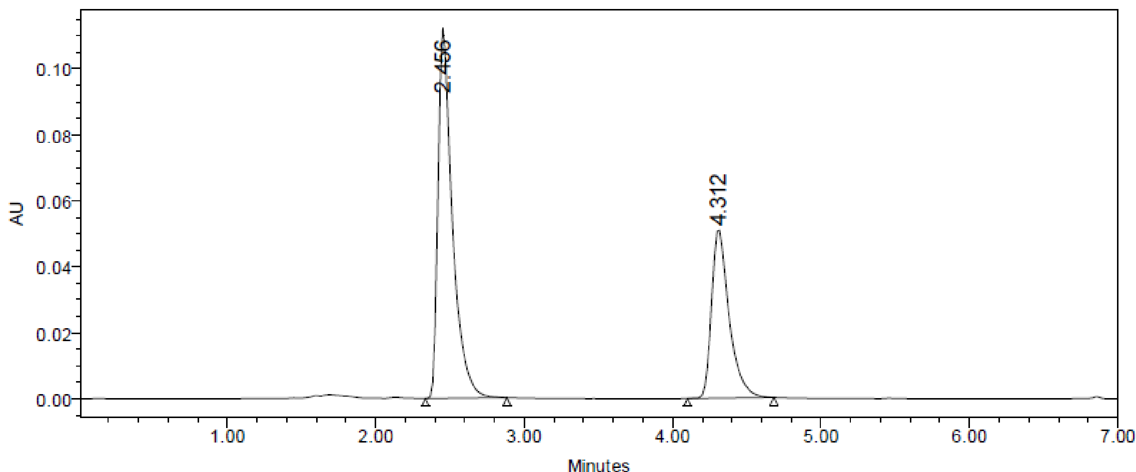
Validation
Preparation of buffer and mobile phase:
Preparation of Phosphate buffer pH 3.5:
Accurately weighed 6.8 grams of KH2PO4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.5.

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 ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent.

RESULTS AND DISCUSSION:

Optimized Chromatogram (Standard)
Mobile phase : Methanol: Phosphate Buffer pH 3.5 (65:35)
Column : Symmetry C18 (4.6×150mm, 5.0 µm)
Flow rate : 1 ml/min
Wavelength : 270 nm
Column temp : 40ºC
Injection Volume : 10 µl
Run time : 7 minutes



Optimized Chromatogram

Table: - peak results for optimized

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Phenobarbital	2.456	600122	112157		1.6	5215
2	Phenytoin	4.312	422042	51068	3.2	1.5	5648

Observation: From the above chromatogram it was observed that the Phenobarbital and Phenytoin peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Optimized Chromatogram (Sample)

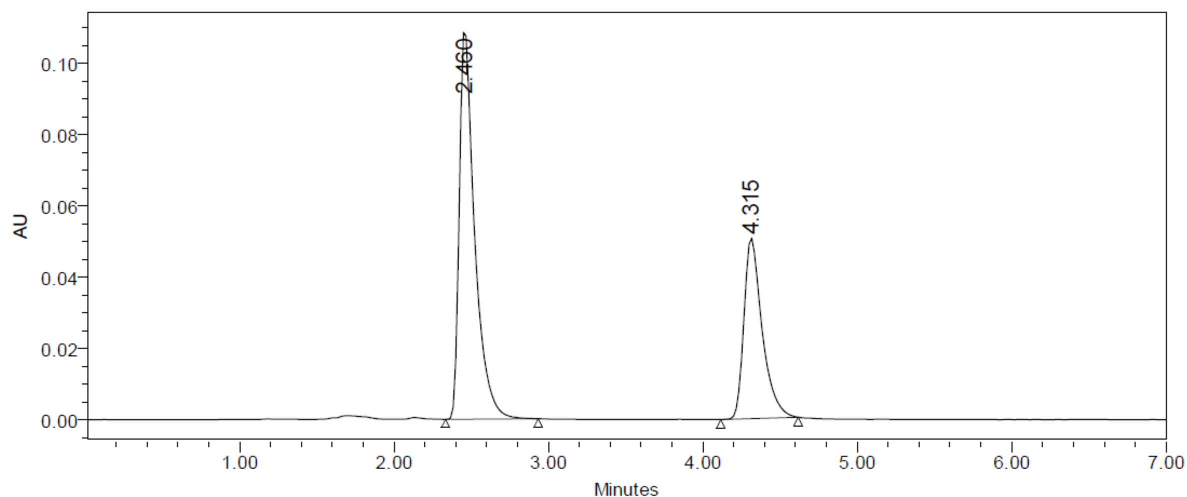


Figure: Optimized Chromatogram (Sample)

Table: Optimized Chromatogram (Sample)

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Phenobarbital	2.460	600123	112157		1.6	5011
2	Phenytoin	4.315	422041	51068	3.3	1.5	5947

Acceptance criteria:

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

Assay (Standard):

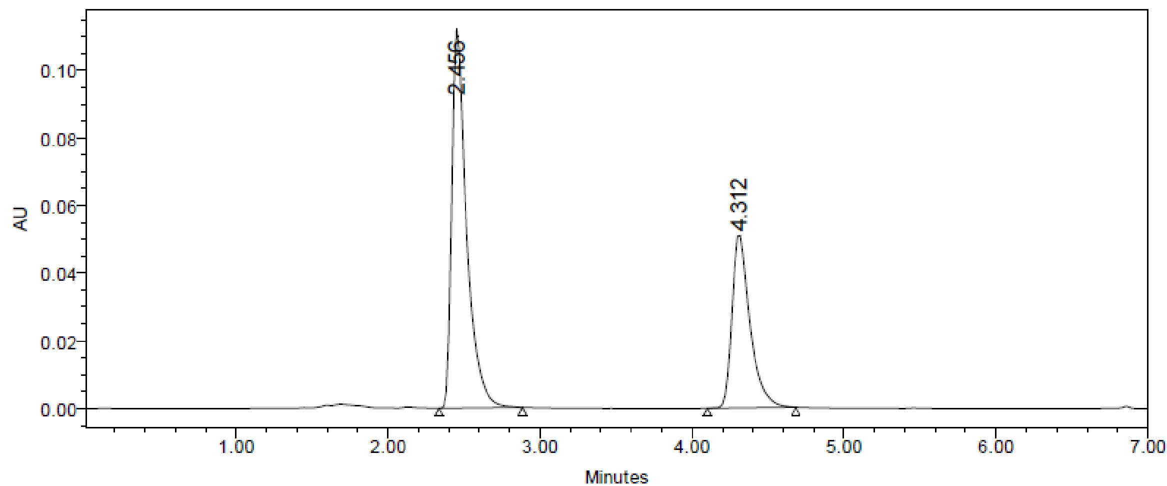


Fig: Chromatogram showing assay of standard injection -1

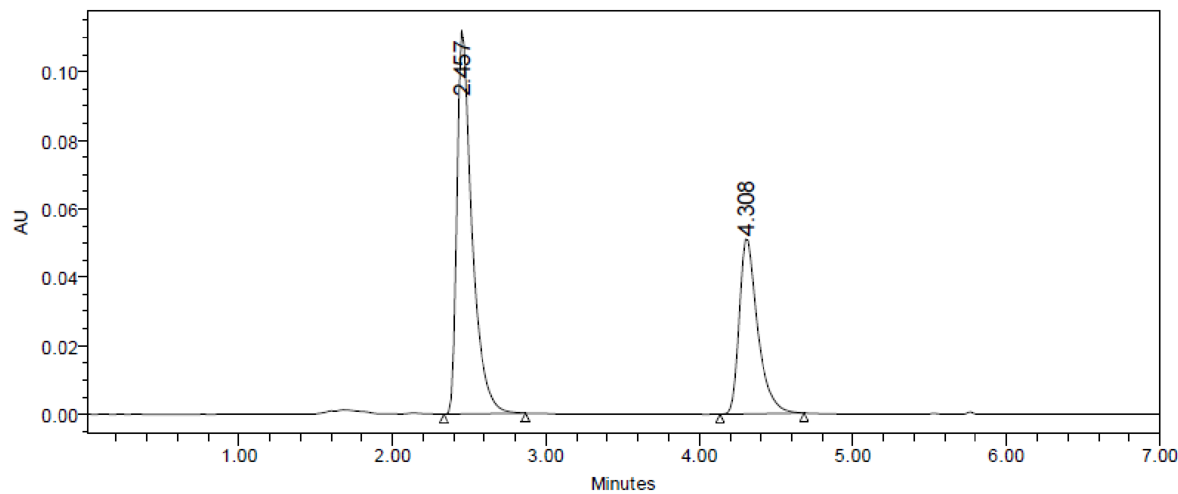


Fig. Chromatogram showing assay of standard injection -2

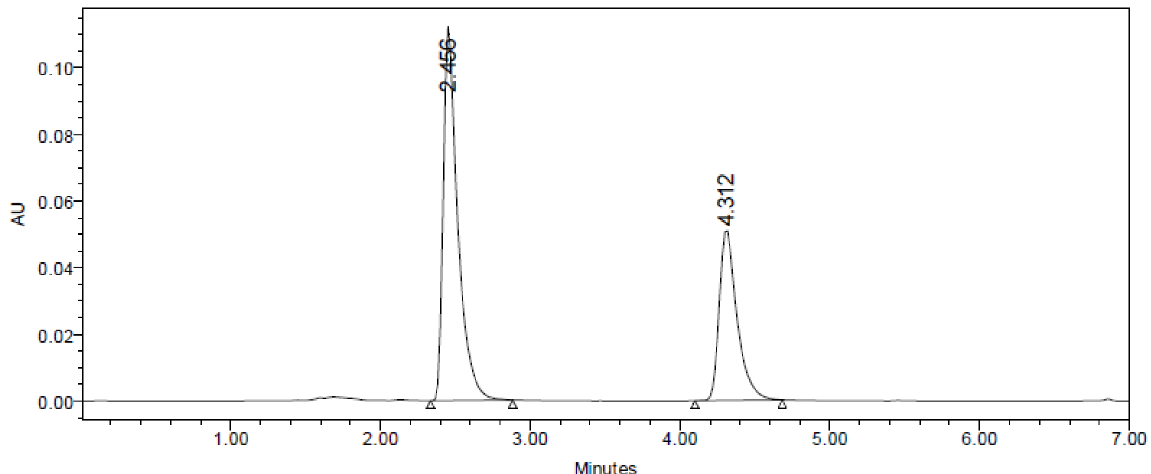


Fig. Chromatogram showing assay of standard injection -3

Table: Peak results for assay standard

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Phenobarbital	2.456	600122	112157		1.5	5023	1
2	Phenytoin	4.312	420842	51068	3.3	1.4	5946	1
3	Phenobarbital	2.457	600205	112399		1.2	5149	2
4	Phenytoin	4.308	422034	51511	3.3	1.4	5848	2
5	Phenobarbital	2.456	600213	11201		1.5	5046	3
6	Phenytoin	4.312	420191	52014	3.2	1.5	5941	3

Assay (Sample):

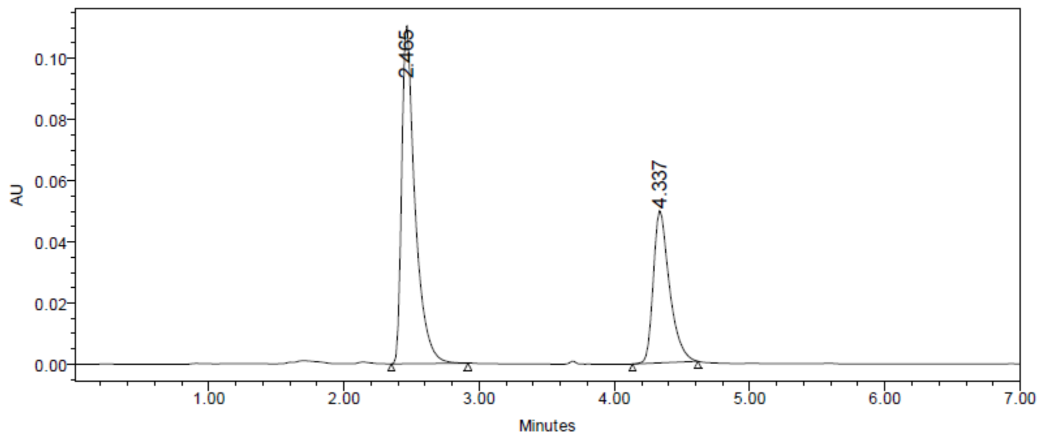


Fig: Chromatogram showing assay of sample injection-1

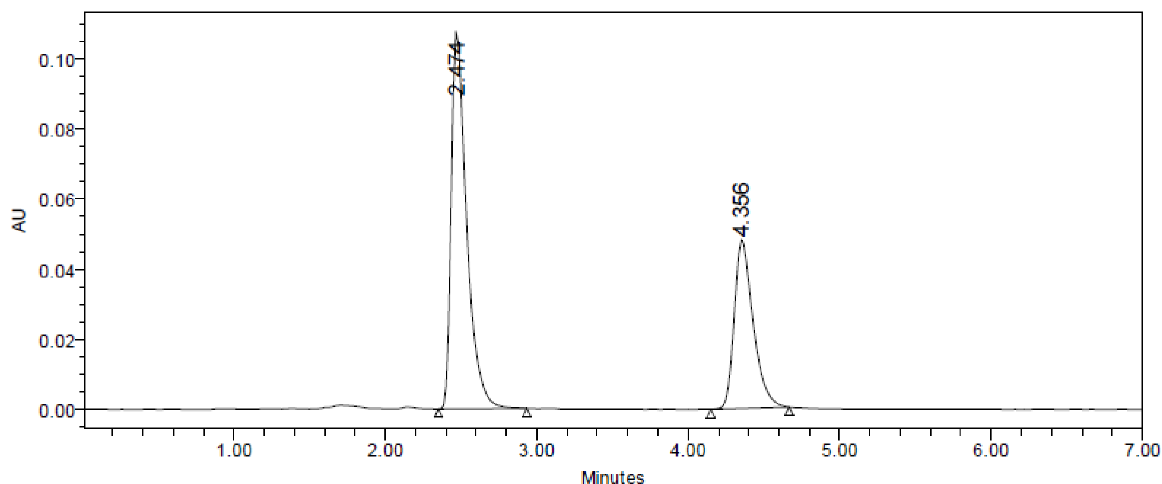


Fig. Chromatogram showing assay of sample injection-2

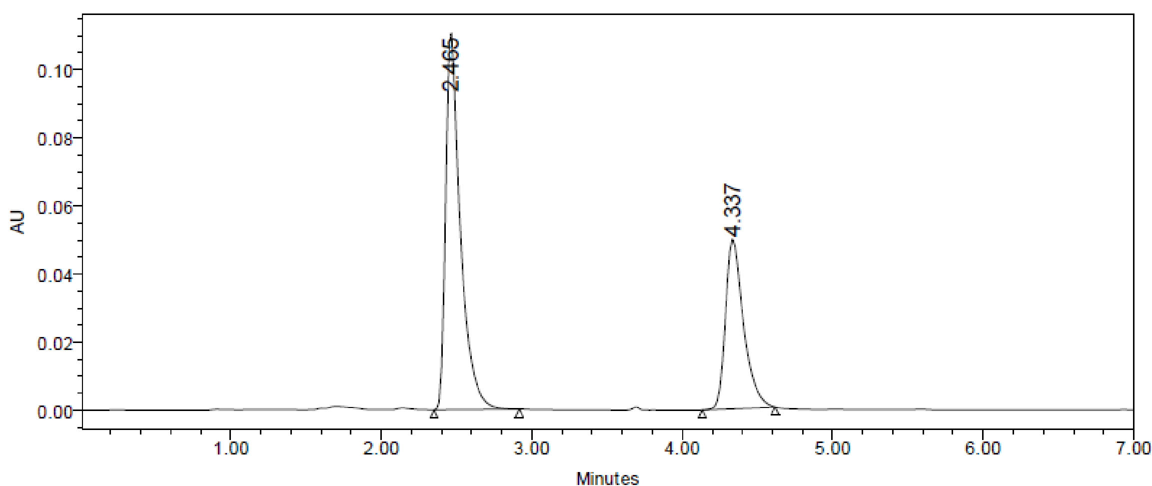


Fig: Chromatogram showing assay of sample injection-3

Table: Peak results for Assay sample

S.No	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Phenobarbital	2.465	601812	110102		1.6	5028	1
2	Phenytoin	4.337	414764	49842	3.2	1.5	5949	1
3	Phenobarbital	2.474	600435	108333		1.6	5189	2
4	Phenytoin	4.356	418130	48360	3.3	1.5	5818	2
5	Phenobarbital	2.465	600212	112453		1.6	5061	3
6	Phenytoin	4.337	413645	48641	3.2	1.5	5812	3

%ASSAY =

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

The % purity of Phenobarbital and Phenytoin in pharmaceutical dosage form was found to be 99.8 %.

Linearity
Chromatographic data for linearity study:
Phenobarbital:

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33.3	5	215760
66.6	10	417001
100	15	600435
133.3	20	791969
166.6	25	974736

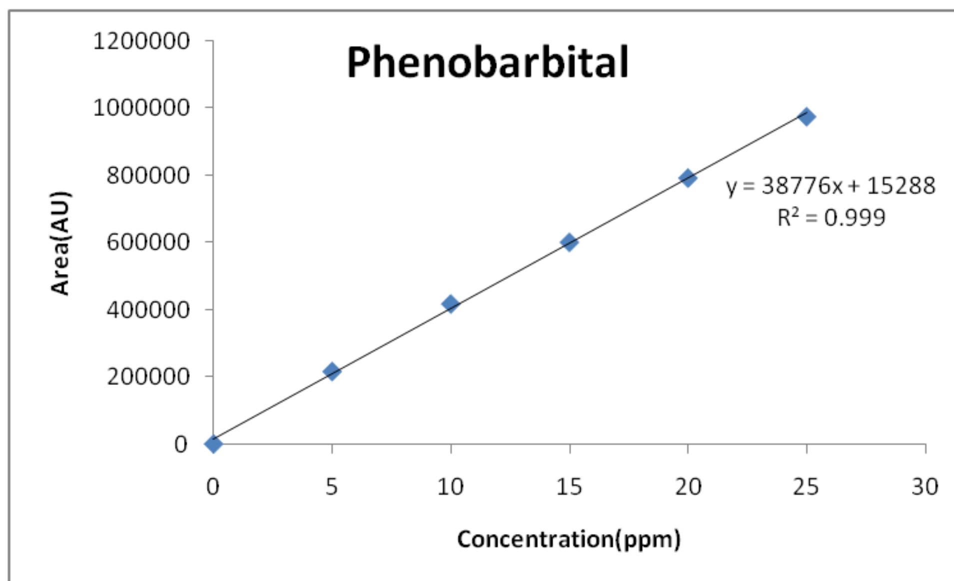


Figure 6.3.4 calibration graph for Phenobarbital

Linearity plot:
The plot of Concentration (x) versus the Average Peak Area (y) data of Phenobarbital is a straight line.

Y = mx + c
Slope (m) = 38776
Intercept (c) = 15288
Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.
Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 15288. These values meet the validation criteria.
Phenytoin

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33	2.5	145474
66	5	279372
100	7.5	421045
133	10	562151
166	12.5	721671

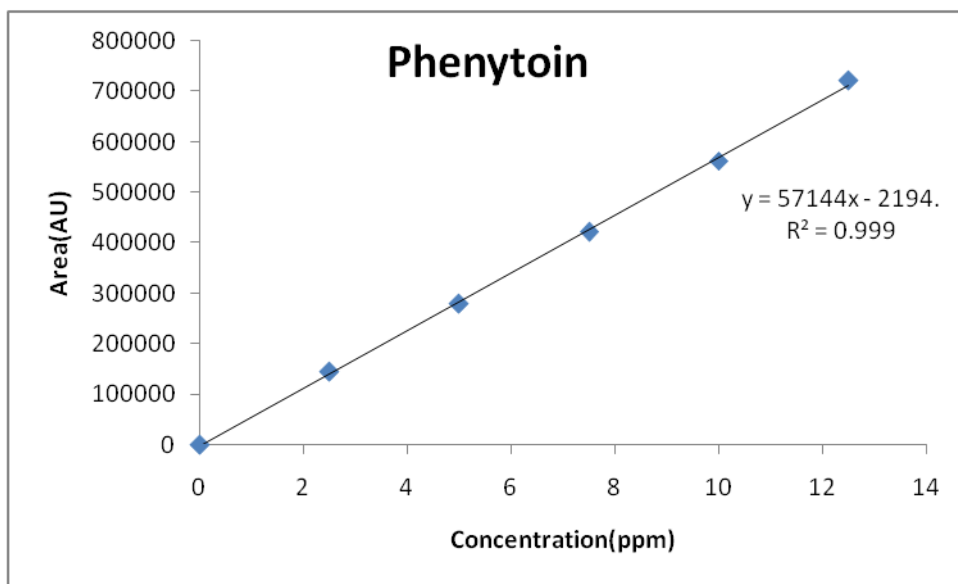


Figure 6.3.4 calibration graph for Phenytoin

Linearity plot:
The plot of Concentration (x) versus the Average Peak Area (y) data of Phenytoin is a straight line.

Y = mx + c
Slope (m) = 57144
Intercept (c) = 2194
Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.
Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 2194. These values meet the validation criteria.

Accuracy:
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	

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	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

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Robustness

Table: Results for Robustness

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

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

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

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CONCLUSION:

In the present investigation, a simple, sensitive, precise and accurate 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.

Phenobarbital and Phenytoin was freely soluble in ethanol, methanol and sparingly soluble in water.

Methanol: Phosphate Buffer pH 3.5 (65:35) was chosen as the mobile phase. 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. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.

This method can be used for the routine determination of Phenobarbital and Phenytoin in bulk drug and in Pharmaceutical dosage forms.

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