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Research Article

A RELIABLE AND COST EFFECTIVE LIQUID CHROMATOGRAPHIC METHOD FOR THE ESTIMATION OF PHENOBARBITAL AND PHENYTOIN IN PHARMACEUTICAL FORMULATIONS

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Abstract

A simple, reliable and cost effective RP-HPLC method for the simultaneous determination of Phenobarbital and Phenytoin in pharmaceutical formulations was developed and validated. Chromatographic separation of two drugs was achieved on a Kromasil C18, (250x4.6nm, 5µm) column by using the mobile phase consisted of methanol: water in the ratio 75:25 (v/v) at a flow rate of 0.9mL/min and the wavelength of detection was at 238 nm. The retention time for Phenobarbital and Phenytoin were found to be 6.10 and 8.10 min respectively. The linearity of the developed method was tested over a concentration range of 6-42µg/mL for Phenobarbital and 10-70µg/mL and the correlation coefficient (r^2) of regression was 0.998 for phenobarbital and 0.9979 for phenytoin which are almost equal to 1. The limit of quantification was 1.5µg/mL for Phenobarbital and 0.7µg/mL for Phenytoin and the limit of detection was 0.45µg/mL for Phenobarbital and 0.2µg/mL for Phenytoin. The mean percentage recoveries were 98.93% for Phenobarbital and 100.16% for Phenytoin respectively.

Keywords: Phenobarbital, Phenytoin, RP-HPLC, Simultaneous estimation.

Introduction

Phenobarbital (Fig. 1) is 5-ethyl-5-phenyl barbiturate. It is a white powder; odourless. Soluble in 3 parts of water and in 25 parts of ethanol (96%) [1]. It is commonly used for convulsive disorders and is the drug of choice for seizures in infants' upto 2 months old [2]. **Phenytoin (Fig. 2)** is 5, 5-diphenylimidazolidine-2,4-dione. A white crystalline powder and is odourless; it is very slightly soluble in water; soluble in 70 parts of ethanol (96%); slightly soluble in chloroform and in ether [1]. It is the prototype and most commonly prescribed member of the hydantoin family of drugs. Bioequivalency is a problem with the hydantoins because of their very poor water solubility and a low therapeutic ratio [2].

Epilepsy is a chronic neurological disorder that affects approximately 50 million people worldwide [3]. This neurological disorder can be controlled with a single anti epileptic drug in the majority of cases but when monotherapy fails, two antiepileptic drugs should be combined [4]. Phenobarbital and phenytoin belong to the most used antiepileptic drugs [5].

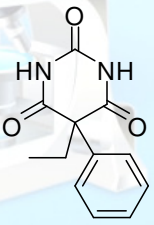


Fig1: Phenobarbital

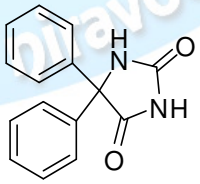


Fig2: Phenytoin

According to the literature survey there are several analytical methods for the determination of Phenobarbital *viz*. liquid chromatographic methods [6-9], capillary electrophoresis method [10], ion-selective electrode potentiometric method [11], spectrophotometric method [12], and conductometrical analysis [13]. Similarly for phenytoin also there were methods using HPLC [9,14-19], LC-MS[20,21], GC[22,23], spetrophtometry[24,25]. According to the knowledge of the author there is only one method for the determination of Phenobarbital and phenytoin in combined dosage form by using HPLC [26].

Objective: To develop a simple, reliable, precise and accurate RP-HPLC method for the simultaneous estimation of Phenobarbital and phenytoin in comined dosage forms.

MATERIALS AND METHODS

Chemicals and reagents

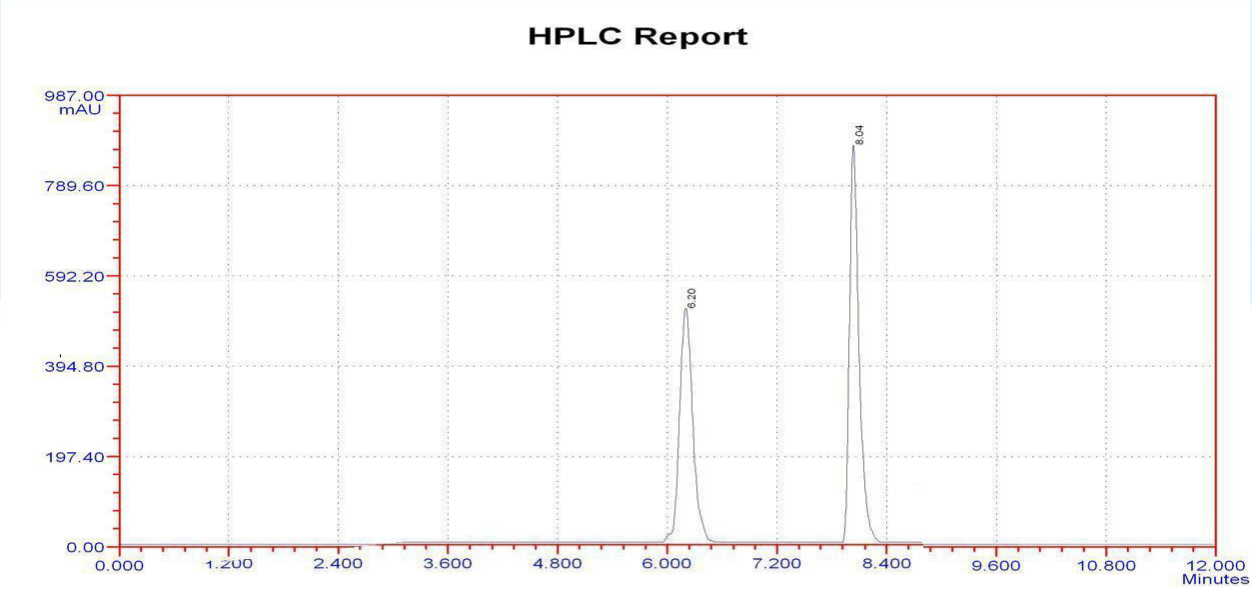
Methanol and water of HPLC grade were produced from Merck specialties Pvt.ltd. Mumbai. A working standard of Phenobarbital and Phenytoin was provided by V.V.Med laboratories, Hyderabad.

Equipment

Chromatographic separation was performed on a PEAK chromatographic system equipped with LC-P7000 isocratic pump; Rheodyne injector with 20µl fixed volume loop, variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Teccomp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Sonicator (1.5L) Ultrasonicator was used to sonicating the mobile phase and samples. Standard and sample drugs were weighed by using Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted by using Systronics digital pH meter.

Year of Experiment: 2013.

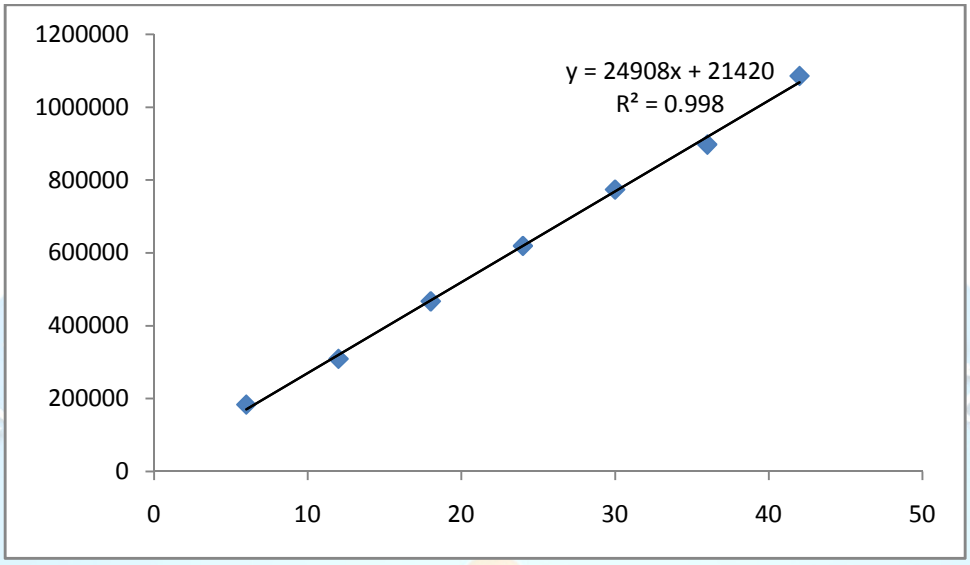
Site: Department of chemistry, Acharya Nagarjuna University, Nagarjunanagar, Guntur 522510, Andhra Pradesh (INDIA).



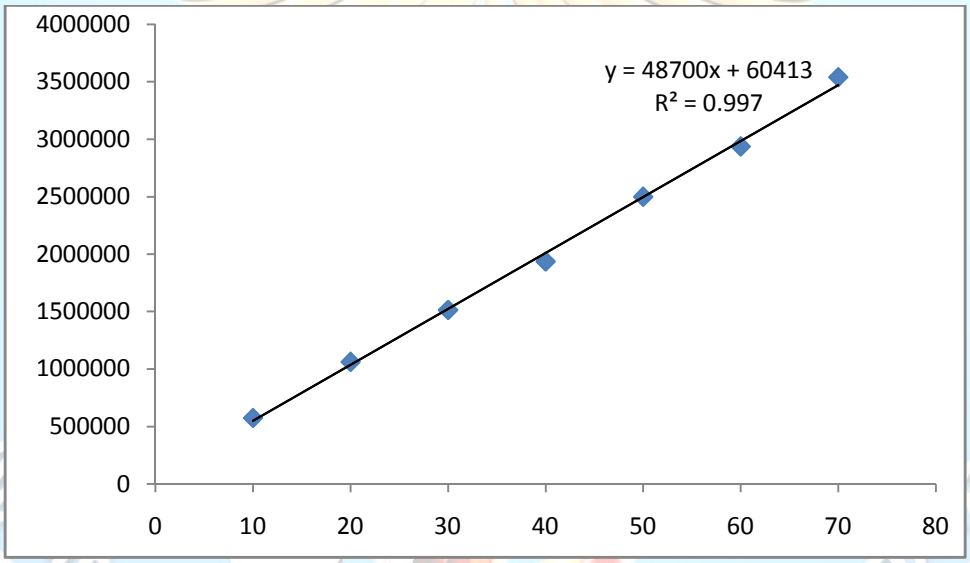
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		6.202	52781	616702.1	24.078	1.63	5615
2		8.037	96644	1944589.0	75.922	1.77	3180
	Sum:		149425	2561291.2	100.0000		

Fig.3: Standard Chromatogram of Phenobarbital (t_R 6.10min) and Phenytoin (t_R 8.10min).

Fig. 4. Calibration curves of Phenobarbital and Phenytoin:



Phenobarbital



Phenytoin

Chromatographic conditions

Kromasil C18 (250X4.6mm) column was used for the separation; a mobile phase of methanol, and water in the ratio 75:25 at pH 4.9. The mobile phase was filtered through 0.45µm membrane filter paper and delivered at a flow rate of 0.9mL/min and the wavelength of detection was at 238 nm. The injection volume was 20µL, and the total run time 12min. The resulting chromatogram was shown in Fig.3.

Preparation of standard stock solution:

10mg of the standard drugs were weighed accurately and was dissolved in 10ml methanol separately to get a concentration of 1000µg/ml of both Phenobarbital and Phenytoin separately. It was sonicated to dissolve completely. Then it was filtered through membrane filter paper. This standard stock solution used to prepare necessary concentrations to construct calibration curve by proper dilution.

Preparation of sample solution

Twenty tablets of Phenobarb (Phenobarbital -60mg, Phenytoin-100mg) taken and powdered. A quantity of powder equivalent to 10 mg of Phenobarbital, Phenytoin taken and transferred in to 100 mL light resistant flask and made up the required volume by using mobile phase. Pipetted out 1mL of resulting solution in to the 10 mL volumetric flask and made up the required volume by using mobile phase and sonicated for 15 min, then filtered through 0.45µm filter. From this Phenobarbital-24 µg/mL, Phenytoin-40 µg/mL sample solution was prepared.

Method development

Different mobile phases containing of methanol and water in different proportions were tried, and finally, methanol: H_2O in the ratio 75:25 was found to be an appropriate mobile phase, which gave acceptable peak parameters for both Phenobarbital and Phenytoin.

Method validation

The validation of the method was carried out in terms of parameters like linearity, precission, accuracy, limit of detection, limit of quantification, specificity and robustness.

Linearity

The linearity of the method was tested over a concentration range of 6 to 42µg/mL of PHB and 10-70 µg/mL PHT. 20µL of each concentration was injected in duplicate into the HPLC system. The response was read at 238nm and the corresponding chromatograms were recorded. The regression value of the plots was computed by least square regression method.

Precision

The precision of the method was determined by inter day and intraday studies, solutions of standard and sample were repeated thrice in a day and percentage relative standard deviation (%RSD) was calculated.

Accuracy

The accuracy of the method was determined by recovery experiments. The recovery studies were performed by the regular addition method. At 50%, 100%, 150% level, the percentage recovery was calculated. For both the drugs, recovery was performed in the same way. The recovery studies were performed in triplicate.

Robustness

Robustness of the method was studied by making slight changes in chromatographic conditions, such as mobile phase ratio, pH and wavelength of detection.

System suitability and Specificity

The system suitability and specificity was established by analyzing the number of theoretical plates, retention time and tailing factor.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) is the smallest concentration of the analyte that gives the measurable response. LOD was calculated using the following formula:

$$LOD = \frac{3.3 \times \text{standard deviation}}{\text{Slope of calibration curve}}$$

The limit of quantification (LOQ) is the smallest concentration of the analyte, which gives a response that can be accurately quantified. LOQ was calculated using the formula:

$$LOQ = \frac{10 \times \text{standard deviation}}{\text{Slope of calibration curve}}$$

RESULTS AND DISCUSSION

All the validation parameters for the proposed method were determined according to Conference on Harmonization (ICH) guidelines. In the present study the combination of the two drugs for the selected mobile phase methanol and water in the ratio 75:25 was found to be much prominent. The order of elution of the two drugs is in good agree-

ment with the earlier reported method [26], which was developed, with the mobile phase CH_3CN and water in the ratio 25:75. The retention times(t_R) observed are 1.63 for Phenobarbital and 1.77 for phenytoin, moreover, the tailing factors obtained were also good(<2). The present method was developed by using simple methanol and water as mobile phase and is cheaper than the earlier reported method[26] and the validated techniques discussed below are also more reliable. So, the method is simple, reliable and cost effective.

Linearity

The linearity of the method was tested over a concentration range of 6 to 42µg/mL of PHB and 10-70 µg/mL PHT. Peak area (A) and concentration (c) of each drug substance was subjected to regression analysis to calculate the correlation coefficients. A correlation coefficient r^2=0.998 for PHB and 0.9979 for PHT of the regression was found to be almost equal to 1. Linearity results were presented in Table 1 and graph in Fig.4.

Precision

The intraday %RSD of Phenobarbital and Phenytoin were found to be 0.35% and1.54%, respectively. The inter day %RSD of Phenobarbital and Phenytoin were found to be 0.61% and 1.25% respectively, shown in table 2 and 3. The values of %RSD within a day, day to day variation <2% proves that the method is precise.

Accuracy

The accuracy of the method was determined by recovery experiments. The recovery studies were performed by the regular addition method. At 50%, 100%, 150% level, the percentage recovery was calculated and presented in table 4. Recovery was found to be within the range of 98.93% to 100.16% which indicates the accuracy of the method. For both the drugs, recovery was performed in the same way. The recovery studies were performed in triplicate. The results of recover studies were found to be satisfactory.

Robustness

Robustness of the method was studied by making slight changes in chromatographic conditions, such as mobile phase ratio, pH and wavelength of detection (table 5). It was observed that there were no marked changes in the chromatograms obtained. So the developed RP-HPLC method was robust.

System suitability and Specificity

The system suitability and specificity was established by analyzing the number of theoretical plates, retention time and tailing factor.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ values of PHB and PHT have shown in table 6 supports the sensitivity of the developed method.

Table 1: Results of Linearity

S.NO	Concentration in µg/ml	Phenobarbital Peak Area	Concentration in µg/ml	Phenytoin Peak Area
1	6	183996	10	573296
2	12	309178	20	1061416
3	18	467026	30	1513233
4	24	619035	40	1935290
5	30	773472	50	2499031
6	36	896918	60	2936830
7	42	1084873	70	3539733
		Slope: 25205 Intercept: 12495 Cc:0.998		Slope: 49203 Intercept: 35241 Cc:0.998

Table 2: Results of precision study (Intra-day)

S.NO	Concentration in µg/ml	Phenobarbital Peak Area	Concentration in µg/ml	Phenytoin Peak Area
1	24	612975	40	1910664
2	24	616676	40	1971899
3	24	614821	40	1934319
4	24	613790	40	1997288
5	24	619380	40	1989964
6	24	614048	40	1965391
		RSD:0.35		RSD:1.54

Table 3: Results of precision study (Inter-day)

S.NO	Concentration in µg/ml	Phenobarbital Peak Area	Concentration in µg/ml	Phenytoin Peak Area
1	24	616702	40	1944589
2	24	610135	40	1933964
3	24	617737	40	1940370
4	24	619412	40	1943386
5	24	609676	40	1990386
6	24	612267	40	1993886
		RSD:0.61		**RSD:1.25**

Table 4: Accuracy data of the developed method

	Phenobarbital				Phenytoin			
% of Recovery	Final Concentration (µg/ml)	Concentration Obtained	% Recovery	R.S.D	Final Concentration (µg/ml)	Concentration Obtained	% Recovery	R.S.D
50%	459770	17.72	98.45		1543269	30.59	101.98	
	461242	17.8	98.76		1519626	30.13	100.42	
	460199	17.7	98.54	0.16	1532365	30.38	101.26	0.77
100%	612179	23.73	98.89		1953056	40.37	100.92	
	614390	23.82	99.25	0.30	1954082	40.39	100.97	1.69

Table 5: Results of Robustness

Condition	Phenobarbital	% difference	Phenytoin	% difference
Standard	**619035**	**0.0**	**1935290**	0.0
Mp Changes Methanol:Water 60:30	610723	-1.3	1945437	0.52
Methanol:Water 80:20	612400	-1.07	1932779	-0.12
WL Changes-240nm	617802	-0.19	1935614	0.016
WL Changes- 236nm	617384	-0.26	1950874	0.80
PH changes-4.7	617432	-0.25	1946717	0.59

Table 6: LOD and LOQ

Parameter	Phenobarbital	Phenytoin
LOD	0.45µg/ml	0.2µg/ml
LOQ	1.5µg/ml	0.7µg/ml

CONCLUSION:

The developed RP-HPLC method for the simultaneous estimation of Phenobarbital and phenytoin in combined dosage form is more precise, robust, and accurate in respect of validation and the quality of separation achieved of the two drugs is also good in terms of retention times and tailing factor. Hence the proposed method is applicable to the determination of Phenobarbital and phenytoin in pharmaceutical formulations.

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