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VALIDATION OF STABILITY INDICATING HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF ASSAY OF CARBAMAZEPINE DRUG IN THE PHARMACEUTICALS TABLET FORMULATIONS USING PHENYTOIN AS AN INTERNAL STANDARD

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ABSTRACT

Carbamazepine is an anticonvulsant is used to treat partial seizures, tonic-clonic seizures, pain of neurologic origin such as trigeminal neuralgia, and psychiatric disorders including manic-depressive illness and aggression due to dementia. Validation of stability indicating Simple, Specific, Precise, Accurate, Linear, Rugged, Robust High Performance Liquid Chromatographic method of analysis for determination of assay of Carbamazepine drug in the pharmaceuticals tablet formulations using Phenytoin as an internal standard was performed. The assay was accomplished using a mixture of Methanol, Acetonitrile and Mill-Q water in the volume ratio of 45:20:35v/v/v as mobile phase on Zorbax SB C18, 150 mm x 4.6mm, 5µ as chromatographic column at a flow rate of 1.000 ml per min and a wavelength of 230 nm with a UV detector. The temperature of auto injector and column oven was 10^0C and 30^0C receptively. The Injection volume kept as 10 µL. Linearity of the analytical method was evaluated at concentration range of 5.4142 µg/ml to 451.1792 µg/ml with Correlation coefficient (r) value more than 0.999. The LOD and LOQ were 0.3466 µg/mL and 1.0503 µg/mL respectively. The retention time found to be 5.65 min for Carbamazepine and 3.82

min for internal standard respectively. Specificity, Method Precision, System Precision, Ruggedness, Robustness, Recovery, Stability of analytical solution, Filter paper selection study, Stress testing(Force Degradation) at various conditions were performed as per the ICH (Q2) recommendations. All the results were found with in acceptance criteria.

KEYWORDS: Carbamazepine, Phenytoin, High Performance Liquid Chromatographic, Force degradation studies, Assay.

INTRODUCTION

Carbamazepine is an anticonvulsant is used to treat partial seizures, tonic-clonic seizures, pain of neurologic origin such as trigeminal neuralgia, and psychiatric disorders including manic-depressive illness and aggression due to dementia. The response to Carbamazepine is variable and may be due to its variable transport, especially across the blood-brain-barrier. Carbamazepine is available for oral administration as 100 mg, 200 mg, and 300 mg and 400mg strength. The chemical name is 5H-dibenz[b,f]azepine-5-carboxamide. The molecular formula for Carbamazepine is $C_{15}H_{12}N_2O$. The molecular weight of Carbamazepine is 236.27. Carbamazepine is a white to off-white powder, practically insoluble in water and soluble in alcohol and in acetone. The pKa of Carbamazepine is 5.9.[1-6]

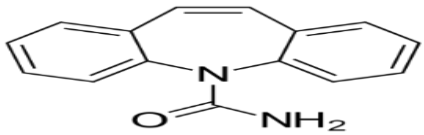

Figure. 1: Chemical structure of Carbamazepine.

Phenytoin is 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. The chemical name is 5,5-Diphenyl-imidazolidine-2,4-dione. The molecular formula is $C_{15}H_{12}N_2O_2$. The molecular weight of Phenytoin is 252.27. Phenytoin is white to powder and has a pKa of 9.47. Phenytoin is very slightly soluble in water and soluble in organic solvents such as methanol.[1-6]

Figure. 2: Chemical structure of Phenytoin.

While Reviewing Literature for analytical method of analysis it was observed that many methods have been reported for determination of Carbamazepine in combination and individually[7-24] but none of the reported HPLC methods have not been validated using internal standard to compensate any processing related and method related variability. Most of the published method[7-24] not performed stability-indicating studies (Acid, Alkali, Peroxide, Thermal, Photolytic, Humidity degradation,) which are mandatory as per the ICH (Q2) recommendations.

The main objective of the work is to develop and validate stability indicating HPLC method[25-28] of analysis which is Simple, Specific, Precise, Accurate, Linear, Rugged, Robust etc. for determination of assay of Carbamazepine drug in the pharmaceutical tablet formulations using Phenytoin as an internal standard.

MATERIAL AND METHODS

Instrumentation

Shimadzu Prominence HPLC system equipped with dual pump, SIL-HTc auto-sampler with cooler, column oven, variable wavelength UV detector and a data acquisition system (Lab Solution Software) were used for the determination of assay of Carbamazepine drug in the pharmaceutical tablet formulations using Phenytoin as an internal standard.

Reagents and Materials: The reagents used during analysis include Methanol [HPLC Grade], Acetonitrile [HPLC Grade], Water [Milli-Q /HPLC Grade], Carbamazepine and Phenytoin was used obtained from Wockhardt Pharmaceutical limited. Fixed dose tablets containing 400 mg of Carbamazepine of Novartis Ltd. was purchased from Local medical, Aurangabad (Maharashtra).

Analytical solutions: Stock solutions having concentrations approximately, 197.4568 µg/mL of Carbamazepine in methanol and 600.0390 µg/mL of Phenytoin in methanol were prepared and solutions were filtered through 0.45µm nylon membrane filter with discarding first 2 mL

of the filtrate before use. The solution of Phenytoin was used as internal standard dilution solution during various experiments performed in an analytical method validation and assay calculations of pharmaceutical formulation. Standard solution having concentrations approximately, 100.0000 µg/ml of Carbamazepine and 75.0000 µg/mL of Phenytoin in mixture were prepared in mobile phase and use as a reference solution for related activities and system suitability. Filter the solution through 0.45µm nylon membrane filter with discarding first 2 mL of the filtrate before use.

Sample solution having concentrations 100.0000 µg/ml of Carbamazepine and 75.0000 µg/mL of Phenytoin was prepared in mobile phase by dissolving a quantity of powder equivalent to Strength of 400 mg of Carbamazepine and use as a sample solution for related activities. Filter the solution through 0.45µm nylon membrane filter with discarding first 2 mL of the filtrate before use.

RESULT AND DISCUSSION

Method development: Primarily, numerous trials for optimization of method was performed using different mobile phases composition, different ratios of organic to buffer, different organic solvents, different buffer with different pH, different stationary phases, different internal standards and variable chromatographic settings in an effort to achieve the finest peak resolution and separation between Carbamazepine and internal standard as depicted in Figure No.3.

A summarized chromatographic condition was as follows

Mobile phase:	Methanol, Acetonitrile and Mill-Q water (45:20:35v/v/v)
Rinsing Solution:	Methanol : Mill-Q water (50:50v/v)
Chromatographic Column:	Zorbax SB C18, 150 mm x 4.6mm, 5µ
Wavelength:	230 nm
Column Oven Temperature:	30 ^{0}C
Sample cooler Temperature:	10 ^{0}C
Flow rate:	1.000 ml per minute
Injection Volume:	10 µl
Run Time:	7.5 minute
Retention Time (minute):	Carbamazepine -5.65 Phenytoin-3.82

Analytical method validation

The Analytical method was optimized and validated in accordance with the current ICH guidelines and recommendations by means of a vision to accomplish Simple, Specific, Precise, Accurate, Linear, Rugged, Robust method.[25-28]

Specificity

For the evaluation of specificity; Blank solution, placebo solutions, sample solution, standard solution in triplicate were injected into HPLC system. No interference was observed from blank solution and placebo at the retention time of chromatographic peak of Carbamazepine and internal standard. Peak purity was passes (purity angle was less than purity threshold) for Carbamazepine and % assay difference with respect to method precision found to be 0.10%. The typical chromatograms of various samples under optimized HPLC conditions was depicted in Figure No.3.

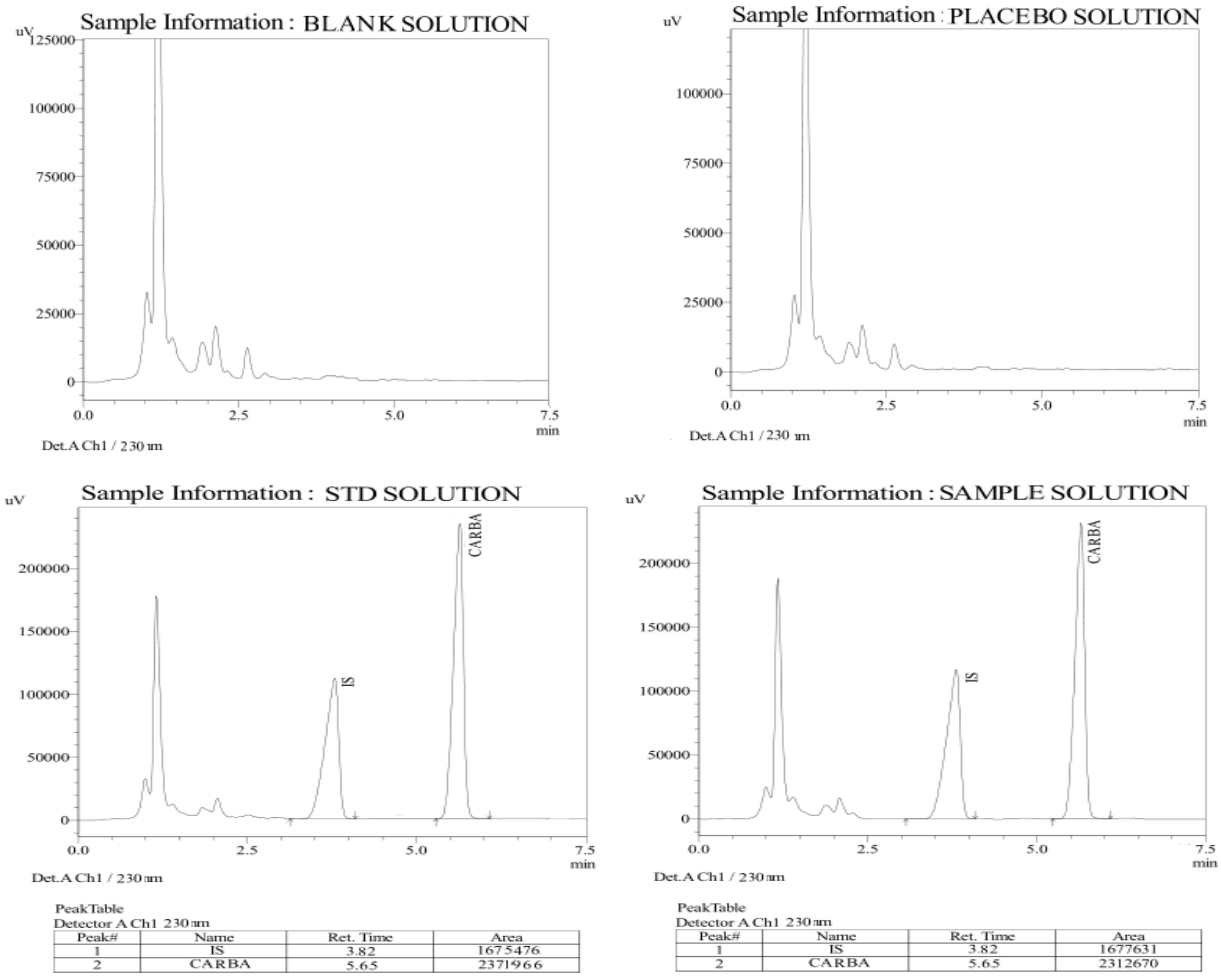

Figure. 3: Typical chromatograms of Blank solution, Placebo solution, Sample solution & standard Solution.

System Precision: Six replicates injections of standard solution were injected in to the HPLC system and the chromatograms and area ratio of Carbamazepine to the Phenytoin recorded. For Carbamazepine Theoretical plates and Tailing factor found to be 13245 and 0.95 respectively. % RSD for area ratio of Carbamazepine to the Phenytoin of six replicate injections of standard solution found to be 0.19% implies that system is précises as tabulated in Table No.1.

Table No. 1. Result of System precision for Carbamazepine.

Injection No.	Area ratio (Carbamazepine to Phenytoin)
1	1.4157
2	1.4144
3	1.4155
4	1.4172
5	1.4217
6	1.4150
Mean	**1.4166**
Standard Deviation	**0.00268**
% R.S.D.	**0.19**

Method Precision: For the evaluation of Method precision of the analytical method, six samples from homogenous mixture of single batch were prepared as per the test procedure of methodology and analyzed on HPLC system .%RSD for % assay of Carbamazepine of six samples found to be 0.33% as tabulated in Table No.2.

Table No. 2. Result of Method precision for Carbamazepine.

Sample No.	% Assay of Carbamazepine
1	99.3
2	99.6
3	99.4
4	99.9
5	100.2
6	99.6
Mean	**99.7**
Standard Deviation	**0.33**
% R.S.D.	**0.33**

Method Ruggedness: The ruggedness was evaluated through analysis of six samples from a homogenous mixture of single batch by different analyst by using different column, different system and on different day. %RSD for % assay of ruggedness samples found to be 0.35 % and Overall %RSD of ruggedness and method precision samples found to be 0.33 % for Carbamazepine as tabulated in Table No.3.

Table No. 3. Result of Ruggedness for Carbamazepine.

Sr. No.	Carbamazepine	
	% Assay of Carbamazepine Method precision	% Assay of Carbamazepine Ruggedness
1	99.3	99.5
2	99.6	99.4
3	99.4	99.2
4	99.9	100.1
5	100.2	99.6
6	99.6	100.0
Mean	**99.7**	**99.6**
Standard Deviation	**0.33**	**0.35**
% R.S.D.	**0.33**	**0.35**
Overall Mean	**99.7**	
Overall S.D.	**0.33**	
Overall R.S.D.	**0.33**	

Accuracy (Recovery): Accuracy of the analytical method was evaluated at a known concentration of Carbamazepine at about 50%, 100% and 150% of test concentration of sample solution and 50% (1X Blend) and 150% (3x Blend) was calculated. % accuracy at individual level and overall average of % Recovery at all level for Carbamazepine found to be in the range 99% to 101 % and %RSD for %assay of all levels found to be 0.21 % as tabulated in Table No.4.

Table No. 4. Result of Recovery for Carbamazepine.

Spike level in %	Carbamazepine			
	% Recovery	Mean	SD	% RSD
50% (Assay)	100.2	100.1	0.06	0.06
	100.1			
	100.1			
100% (Assay)	100.0	99.9	0.15	0.15
	99.7			
	99.9			
150% (Assay)	99.9	100.1	0.20	0.20
	100.3			
	100.1			
50% (1X Blend)	100.1	100.0	0.17	0.17
	100.1			
	99.8			
150% (3X Blend)	100.4	100.1	0.36	0.36
	100.2			
	99.7			
Overall Mean	100.0			
Overall S.D.	0.21			
Overall % R.S.D.	0.21			

Linearity

For the evolution of the linearity of the analytical method, standard dilutions of Carbamazepine in a concentration range of 5.4142 µg/ml to 451.1792 µg/ml for Carbamazepine prepared as per the test procedure of methodology and analyzed on the HPLC system.

Correlation coefficient (r) value for Carbamazepine using a regression equation with a 1/ (concentration^2) of weighting factor was calculated over above mentioned concentration range.

Lower limit of Detection (LOD) and Lower limit of Quantification (LOQ) calculated using following formulas.

Limit of detection (LOD) =3.3 X S.D. of Y intercept / Slope of the calibration curve.

Limit of Quantification (LOQ) =10 X S.D. of Y intercept / Slope of the calibration curve.

The LOD and LOQ were found to be 0.3466 µg/ml and 1.0503 µg/ml respectively.

Correlation coefficient (r) value was found to be more than 0.9999 for Carbamazepine.

Results were tabulated in Table No.5 and the linearity plot was depicted in Figure No.4.

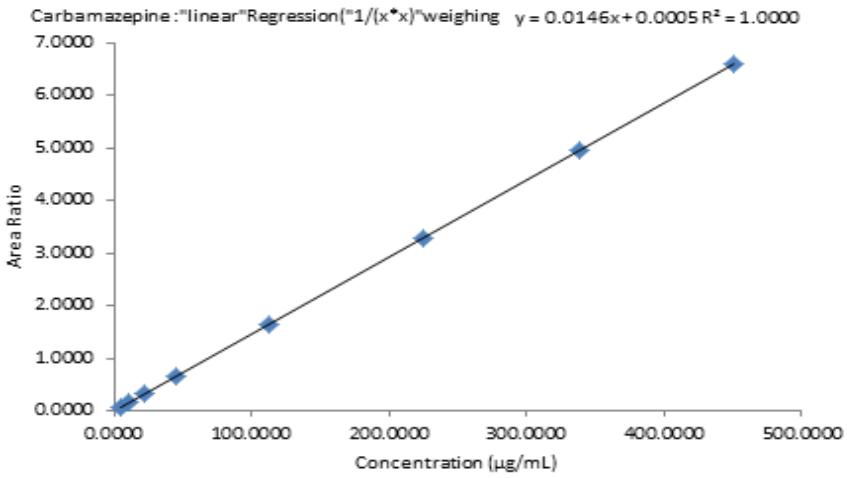

Figure. 4: Linearity plot for Carbamazepine.

Table No. 5. Result of Linearity for Carbamazepine.

Sample no.	Carbamazepine	
	Concentration in µg/mL	Area Ratio
1	5.0150	0.0790
2	10.3508	0.1648
3	20.7015	0.3294
4	41.4030	0.6596
5	103.5076	1.6478
6	207.0151	3.2945
7	311.3009	4.9363
8	380.2380	6.5866
Slope	**0.0146**	
Intercept	**0.0005**	
CC (r)	**1.0000**	

The results of the linearity confirmed that an excellent correlation was exists between area ratio and concentration of drug within the specified concentration range.

Stability in analytical solution

For the evolution of stability in analytical solution; freshly prepared standard solution and sample solution injected on the HPLC system at initially and different time intervals up to 52 hours and 48 hours respectively and the results of standard solution and sample solution were recorded. Absolute % difference and similarity factor were calculated.

For sample solution; absolute % difference between the assay of initial result and assay obtained at different time intervals found to be in the range 0.10% to 0.40%.

For standard solution; similarity factor between the initial result and results obtained at different time intervals found to be in the range of 99.8% to 100.0%.

The sample solution and standard solution are stable up to 48 hours and 52 hours respectively on bench top at room temperature.

Filter paper study: Filter paper study was performed to measure the analysis impact of filter paper used during various experiments of analytical method validation. For the evolution of the filter paper study of the analytical method, standard solution was prepared as per test procedure of methodology and distributed the standard solution in two different portions. One portion centrifuged at 4000 rpm for 5 minutes and second portion was filter through 0.45-µm nylon membrane filter with discarding first 2mL of the filtrate and all the samples were analyzed on HPLC system.

Similarity factor between as such standard solution and filtered standard solution was found to be 100.4. Absolute difference between average % assay of centrifuged sample solution and filtered sample solution was found to be 0.10%.

Form the results it was concluded that the 0.45-µm nylon membrane filter with discarding first 2mL of the filtrate is suitable for the determination of Assay of Carbamazepine in tablet formulation.

Forced degradation study

Forced degradation study was performed by treating sample solution of tablet containing 400 mg Carbamazepine under acidic, basic, peroxide, thermal, photolytic and humidity conditions but somewhat degradation of the carbamazepine observed under acid treated and alkali treated stress condition as tabulated in Table No.6**.**

Table No. 6. Results of Force degradation for carbamazepine.

Degradation Condition	% Degradation
Acid Treated	8.4
Alkali Treated	9.2
Peroxide Treated	2.2
Thermal Treated	0.2
Photolytic Treated	0.1
Humidity Treated	0.1

Method robustness

Robustness of the analytical method was evaluated by accomplishment of analysis under marginally changed in the chromatographic method of analysis such as change in detection wavelength, change in flow rate, change in composition of the mobile phase and change in column oven temperature, and the assay results were compared with the assay result of method precision i.e. with finalized chromatographic conditions. The analytical method used is robust for change in flow rate, change in column oven temperature, and change in wavelength and change organic component of mobile phase. Overall %RSD between % assay at original parameters and changed parameters were calculated as tabulated in Table No.7.

Table No.7 Result of robustness for Carbamazepine.

Sr. No.	Method precision	Minus Flow	Plus Flow	Minus Temp	Plus Temp	Minus Least comp. (Methanol)	Plus Least comp. (Methanol)	Minus Least comp. (Acetonitrile)	Plus Least comp. (Acetonitrile)	Minus nm	Plus nm
1	99.3	99.3	99.4	99.5	98.9	99.3	99.8	99.4	99.6	99.4	99.2
2	99.6	99.3	99.7	99.6	99.7	99.7	100.1	99.8	99.7	99.6	99.6
3	99.4	99.5	99.3	99.7	99.4	99.6	99.7	99.3	99.4	99.3	99.4
4	99.9	Not Applicable									
5	100.2										
6	99.6										
Overall mean		**99.6**	**99.6**	**99.6**	**99.6**	**99.6**	**99.7**	**99.6**	**99.6**	**99.6**	**99.6**
Overall SD		**0.31**	**0.30**	**0.27**	**0.37**	**0.29**	**0.30**	**0.31**	**0.28**	**0.30**	**0.31**
Overall		**0.31**	**0.30**	**0.27**	**0.37**	**0.29**	**0.30**	**0.31**	**0.28**	**0.30**	**0.31**

Range: From the analytical procedure data of precision, accuracy and linearity, the range of the analytical method used for determination of assay of Carbamazepine drug in the pharmaceutical tablet formulations using Phenytoin as an internal standard was tabulated in Table No.8.

Table No.8. Range for Carbamazepine.

Name of Analyte	Concentration (µg/mL)
Carbamazepine	5.4152 µg/ml to 451.1792 µg/ml

Analysis of Marketed Products: The potency test of marketed tablet products were performed after the complete validation of the method for determination of assay of Carbamazepine drug in the pharmaceutical tablet formulations using Phenytoin as an internal standard was performed by the proposed validated method.

The potency of tested brands was found to be within the limit of 98.00-102.00%.The results are tabulated in Table No.9.

Table No.9 Potency of Marketed Products for Carbamazepine.

Sr. No.	Carbamazepine			
	Brand name code	Label Claimed (mg)	Amount found (mg)	Potency (%)
1	CARBA(A)	400	402	100.25
2	CARBA(B)	400	398	99.50

CONCLUSIONS

This is the first reported High Performance Liquid Chromatographic method developed used for determination of assay of Carbamazepine drug in the pharmaceuticals tablet formulations using Phenytoin as an internal standard was stability indicating as recommended by ICH guidelines and validated for Specificity, System precision, Method precision, Ruggedness,

Robustness, Accuracy etc. The present analytical method has a widespread linear concentration range augmenting its applicability to different strength of Carbamazepine tablet formulations. The chromatographic method may also be applied for estimation of drugs in plasma, serum, urine after using appropriate sample extraction technique. Thus the method is Simpler, Accurate and Economical as compare to the previous methods.

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