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Research Article

DEVELOPMENT ANDVALIDATION OF A STABILITY INDICATING ANALYTICAL METHOD FORDETERMINATION OF RELATED SUBSTANCES BY RPHPLC OFPHENYTOIN SODIUM IN PHENYTOIN SODIUM CAPSULES

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ABSTRACT

Phenytoin, approved by the Food and Drug Administration in 1999 as a sedative for use in the intensive care unit, is a potent and highly selective α2-adrenoceptor agonist with significant sedative, analgesic and anxiolytic effects mostly used in the intensive care units. This article describes validation for the determination of related substances of Phenytoin Sodium in Phenytoin Sodium Capsules by usinga high performance liquid chromatography. The high performance liquid chromatography resolution was achieved on an Inertsil ODS 3, 150 x 4.6mm, 5µm, column with an gradient elution at a flow rate of 1.0 mL/min using a mobile phase A as buffer and mobile phase B as acetonitrile. The detection was performed by a photo diode array Detector. The method was validated in the concentration range of Limit of quantitation to 150% of working concentration. The intra and inter-day precision and accuracy were within Limit (10 % Relative Standard Deviation). The overall mean recoveries of Phenytoin were 97.5% for Limit of Quantitation and 95.6 % for 50% to 150%.

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INTRODUCTION

Phenytoin is an antiepileptic drug approved in the United State Food and Drug Administration, Europe and several other countries. Phenytoin is currently used to manage partial onset seizures in humans suffering from epilepsy. Phenytoin has the molecular formula $C_{15}H_{12}N_2O_2$ and the chemical name 5,5-diphenylimidazolidine- 2,4-dione with molecular weight of 252.268 gram per mol[1-2].The primary site of action appears to be the motor cortex where spread of seizure activity is inhibited. Possibly by promoting sodium efflux from neurons, phenytoin tends to stabilize the threshold against hyper excitability caused by excessive stimulation or environmental changes capable of reducing membrane sodium gradient. This includes the reduction of posttetanic potentiation at synapses. Loss of posttetanic potentiation prevents cortical seizure foci from detonating adjacent cortical areas. Phenytoin reduces the maximal activity of brain stem centers Unfortunately, Phenytoin has narrow therapeutic window, and careful monitoring of the drug-plasma level is necessary during therapy to avoid undesirable effects[3-6]. The analysis by HPLC is more significant than using other methods like UV, liquid chromatography and immunoassays for the estimation of Phenytoin sodium[7-8]. Analytical method is validated that allows the determination of Related Substances of phenytoin sodium in Phenytoin sodium capsules. The validation parameters, Specificity, Forced degradation, linearity, repeatability, precision, Accuracy, Solution Stability and robustness were validated [9-10].

MATERIAL AND METHODS

Working standard and Impurity standard used in Experiments reported in table No.1. Apparatus and instruments used in experiment are listed in table No 2. . Reagents and solvents used: Water (HPLC grade, Milli Q), Acetonitrile (HPLC grade, JT Baker) Methanol (HPLC grade, JT Baker), Ortho-Phosphoric acid (AR grade).

Table No.1 working Standard and Impurity Standard

S No.	Name
1	Phenytoin Sodium
2	Impurity C standard
3	Impurity D
4	Impurity E standard

Development Trials: Standard, impurities and spiked sample were injected in to HPLC using following trials

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Table No 2 List of Instrument Used

Sr No	Instrument	Make	Software	Detector/Model No
1	HPLC	Waters	Empower Software	2489 dual wavelength
2	HPLC	Waters	Empower Software	2998 PDA Detector
3	Sonicator	Lab India	NA	NA
4	Weight balance	Mettler Toledo	NA	ML204
5	Oven	Thermo lab	NA	GMP
6	Photolytic Chamber	Thermo lab	NA	GMP

Table 3 Development Trials 01 and 02

Chromatography Parameters	Trial 01			Trial 02		
Column	Inertsil, ODS 3V C18, 250 x 4.6mm, 5µ,					
Buffer	Mix 1.0 mL of Orthophosphoric Acid in 1000 mL of water					
Mobile phase	Mobile phase A: Buffer (100%) Mobile phase B: Acetonitrile (100%)					
Diluent	Methanol			Water: Methanol (70:30)		
Flow Rate	1.0 mL/min.			1.0 mL/min.		
Injection Volume	10 µL			10 µL		
Wavelength	220 nm			220 nm		
Column Temp.	30°C			40°C		
Elution	Gradient Elution			Gradient Elution		
Standard Concentration	5.0 ppm			5.0 ppm		
Sample Concentration	1000 ppm			1000 ppm		
Gradient	**Time (min)**	**MP A (Buffer)**	**MP B (ACN)**	**Time (min)**	**MP A (Buffer)**	**MP B (ACN)**
	0	85	15	0	82	18
	25	50	50	3	82	18
	40	20	80	28	60	40
	45	5	95	40	60	40
	60	5	95	45	45	55
	65	85	15	50	45	55
	75	85	15	52	82	18
				60	82	18
Conclusion	All Known impurities were eluted and well separated from before Phenytoin peak.			Impurity D and unknown impurity are closely eluting rest of the impurities and Phenytoin peak were found satisfactory.		

Table 4 Development Trials 03 and 04

Chromatography Parameters	Trial 03			Trial 04		
Column	Inertsil, ODS 3V C18, 250 x 4.6mm, 5µ,					
Buffer	Mix 1.0 mL of Orthophosphoric Acid in 1000 mL of water					
Mobile phase	Mobile phase A: Buffer (100%) Mobile phase B: Acetonitrile (100%)					
Diluent	Water: Methanol (70:30)					
Flow Rate	1.0 mL/min.					
Injection Volume	10 µL					
Wavelength	220 nm					
Column Temp.	40°C					
Elution	Gradient Elution					
Standard Concentration	5.0 ppm					
Sample Concentration	1000 ppm					
Gradient	**Time (min)**	**MP A (Buffer)**	**MP B (ACN)**	**Time (min)**	**MP A (Buffer)**	**MP B (ACN)**
	0	80	20	0	83	17
	4	80	20	4	83	17
	15	60	40	20	60	40
	20	60	40	30	60	40
	30	45	55	35	45	55
	40	45	55	45	45	55
	43	80	20	48	83	17
	55	80	20	55	83	17
Conclusion	Impurity D and unknown impurities are separated, but Impurity C is eluting close to negative peak at void volume. As a part of development initial gradient composition slowed till 20 minutes and evaluated as Trial-04.			Impurity C is eluting very close to negative peaks at void volume. As a part of development initial gradient composition slowed and gradient runtime increased to 60minutes and evaluated as Trial-05		

Hence Trial 05 was considered as final optimised method and validation was performed on following final methodology (Trail-05).

Final Developed Methodology is as follows

Preparation of Mobile phase: Mobile Phase A: Transfer 1.0 mL of Orthophosphoric acid in 1000 mL of water and filter through 0.45 µ nylon filters. Mobile Phase B: Used Acetonitrile 100%.

Diluent: Prepare a mixture of water and methanol in the ratio of 70:30 v/v and mix well.

Preparation of Diluted standard solution: Weigh and transfer accurately about 25 mg of Phenytoin Sodium working standard to a 100 mL volumetric flask, add about 30 mL of methanol sonicate to dissolve. Cool to room temperature and make up to the mark with methanol. Dilute 2 mL of this solution to 100 mL with diluent.

Table 5 Development Trials 05

Chromatography Parameters	Trial 05 Final optimised		
	Time (min)	MP A (Buffer)	MP B (ACN)
Gradient	0	85	15
	3	85	15
	30	65	35
	36	65	35
	41	40	60
	50	40	60
	53	85	15
	60	85	15
Conclusion	Impurity C separation was found satisfactory and Trial-05 methodology is finalised as optimised method		

Preparation of System suitability solution: Weigh accurately about 2 mg of Impurity E into a 20 mL volumetric flask, add 10 mL of Methanol. Sonicate to dissolve. Cool to room temperature and dilute up to the mark with methanol. (Solution A). Weigh and transfer accurately about 100 mg of Phenytoin Sodium working standard into 100 mL volumetric flask, add 30 mL of methanol. Sonicate to dissolve. Cool to room temperature. Accurately Transfer 3 mL of Solution A into it and dilute upto the mark with water.

Preparation of Placebo solution: Weigh and transfer accurately placebo equivalent to about 100 mg of Phenytoin Sodium in to 100 mL volumetric flask, add about 30 mL of methanol, sonicate for 15 minutes with occasional swirling. Cool to room temperature, make up to volume with water and mix. Filter through 0.45µ Teflon Filter

Preparation of Sample solution: Pool the contents of 10 capsules. Weigh and transfer accurately sample equivalent to about 100 mg of Phenytoin Sodium in to 100 mL volumetric flask, add about 30 mL of methanol, sonicate for 15 minutes with occasional swirling. Cool to room temperature, make up to volume with water and mix. Filter through 0.45µ Teflon filter. Inject separately Resolution solution and Standard solution into the chromatograph, record the chromatograms, and measure the peak responses. The resolution between Phenytoin and impurity 1 should be more than 6.0. The Relative standard deviation for six replicate injections should not be more than 10%, for Standard solution.

Chromatographic conditions:

Column	Inertsil ODS 3, 150 x 4.6mm, 5µm
Wavelength	220 nm
Flow rate	1.0 mL/min
Injection volume	10 µL
Column Temperature	40°C
Runtime	60mins

Gradient programme

Time (min)	Mobile phase A	Mobile phase B
0.0	85	15
3	85	15
30	65	35
36	65	35
41	40	60
50	40	60
53	85	15
60	85	15

RESULT AND DISCUSSION

Specificity: Specificity is the ability of the method to measure the analyte in the presence of process related and the degradation impurities. All known impurity solutions individually, sample solution and spiked sample solution with all known impurities at specification level were prepared and injected into the HPLC equipped with a photodiode array detector and analysed. Peak purity passed for Phenytoin, Impurity C, Impurity D and Impurity E in control sample and spiked sample. Data is reported in Table no 3 & 4 and Figure No 1, 2&3.

Table No 6 Peak purity of standard and Control sample

Sample	Phenytoin	
	Purity angle	Purity Threshold
Standard solution	Standard preparation	1.008
Control sample	Control sample – 25 mg	2.368

Table No 7 Peak purity of spiked sample

Sample	Purity angle	Purity Threshold
Phenytoin	2.469	3.930
Impurity C	0.714	16.063
Impurity D	2.958	61.178
Impurity E	0.907	18.632

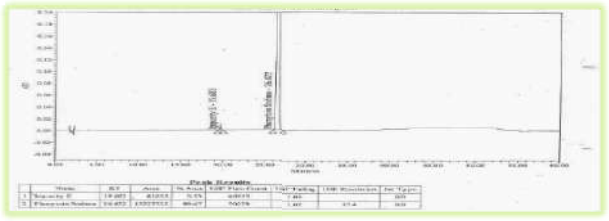


Figure No 1 System Suitability solution

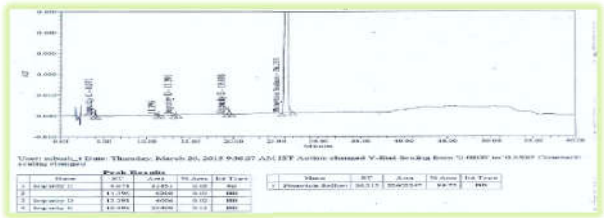


Figure No 2 Control Sample

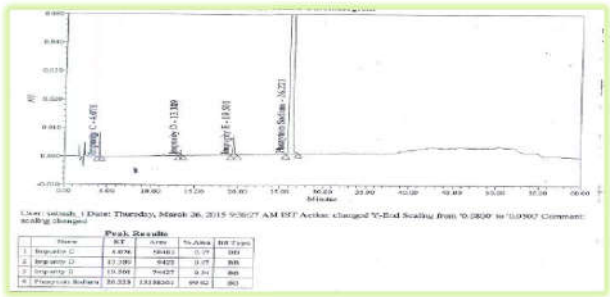


Figure No 3 Spiked Sample

Forced Degradation Studies: Summary of Forced degradation data is reported in Table no 5.

Table No 8 Table for impurities in Forced Degradation Studies

Experiment	Degradation Condition	% Imp C	% Imp D	% Imp E	% Single max	% Total
Control	--	0.052	0.028	0.221	0.030	0.331
Acid Degradation	5N HCl – 70°C/3hrs	0.060	0.024	0.079	0.027	0.190
Base Degradation	2N NaOH –70°C/3 hrs	0.057	0.023	0.264	0.016	0.360
Peroxide Degradation	50% H_2O_2 70°C/3 hrs	ND	0.026	0.218	6.552	12.816
Thermal Degradation	105°C – 72 hours	0.049	0.023	0.205	0.027	0.313
Humidity Degradation	25°C/92%RH – 72 hours	0.051	0.027	0.212	0.029	0.357
Photolytic Degradation	1.2 million lux hours	0.050	0.023	0.214	0.029	0.316

Limit of Detection and Limit of Quantification: Based on determination of Prediction linearity, six replicate injections were made for LOD & LOQ. Data is summarized in the given Table no 6.

Table No 9 Limit of Detection and Limit of Quantitation

	Phenytoin	Impurity C	Impurity D	Impurity E
Limit of Detection				
(%)	0.004	0.004	0.003	0.007
(µg/mL)	0.035	0.043	0.031	0.071
% RSD	2.980	1.870	7.140	4.110
Limit of Quantitation				
(%)	0.012	0.014	0.009	0.024
(µg/mL)	0.116	0.144	0.094	0.237
% RSD	1.310	0.640	2.000	1.820

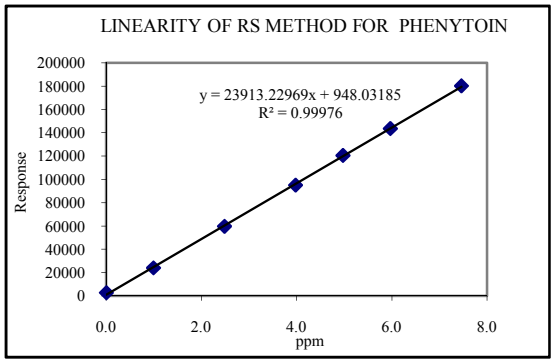
Linearity: Excellent correlation was achieved for the regression line of Phenytoin and its related impurities over a range from LOQ to 150 % of the limit level. The correlation coefficient obtained for all the plots was greater than 0.999. The linearity results are tabulated in Table No. 7 & 8.

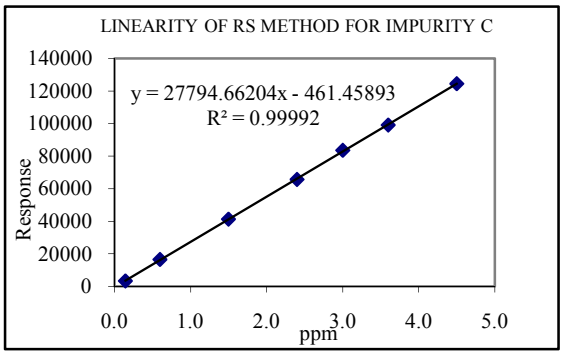
Table No 10 Table for Linearity of Phenytoin and Impurity C

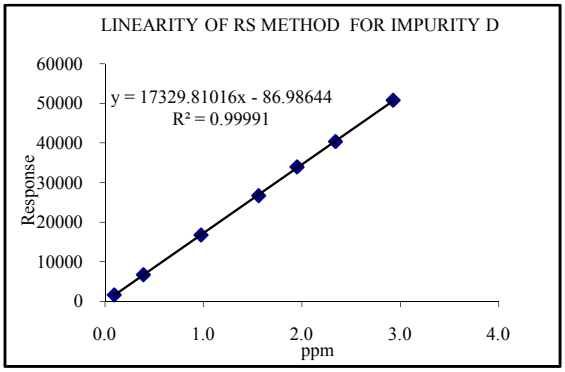
Level	Concentration (µg/ml)	Phenytoin	Concentration (µg/ml)	Impurity C
LOQ	0.116	2660	0.144	3306
Lin-1	0.953	24019	0.600	16506
Lin-2	2.382	59712	1.499	41278
Lin-3	3.811	94942	2.398	65694
Lin-4	4.763	120485	2.998	83538
Lin-5	5.716	143572	3.598	99210
Lin-6	7.145	180288	4.497	124559
	Slope	25233	Slope	27795
	Intercept	-327	Intercept	-461
	Correlation Coefficient	1.0000	Correlation Coefficient	1.0000

Table No 11 Table for Linearity of Impurity D and Impurity E

Level	Concentration (µg/ml)	Impurity D	Concentration (µg/ml)	Impurity E
LOQ	0.094	1623	0.237	3386
Lin-1	0.390	6729	0.988	17681
Lin-2	0.976	16767	2.469	44266
Lin-3	1.562	26720	3.951	70374
Lin-4	1.952	33942	4.939	89602
Lin-5	2.343	40352	5.927	106449
Lin-6	2.928	50802	7.408	133812
	Slope	17330	Slope	18132
	Intercept	-87	Intercept	-628
	Correlation Coefficient	1.0000	Correlation Coefficient	1.0000







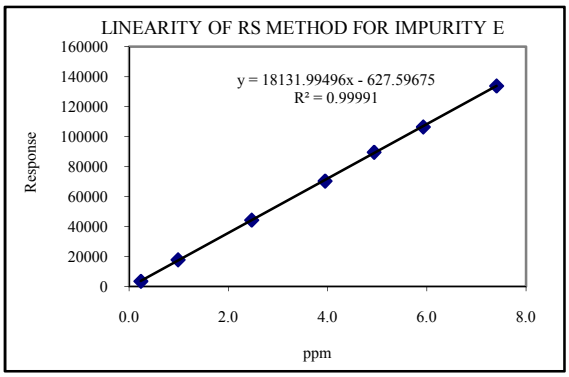


Figure No.4 Linearity graph of Phenytoin, Impurity C, D & E

Accuracy: The studies were carried out at four different levels: LOQ, 50%, 100%, and 150% of limits. The percentage of recoveries of Imp-C, Imp-D and Imp-E were calculated with respect to amount spiked and amount recovered. The percentage recovery at each level was calculated against the Phenytoin sodium standard. Mean recovery should be in the range of 90.0% to 110.0% for 50%, 100% and 150% levels and 85% to 115% for LOQ level. Mean recovery in percentage is reported in Table no. 9.

Table No 12 Accuracy of Impurity of Phenytoin Sodium Capsules

	Mean Recovery (%)			
Name of Impurity	LOQ	50	100	150
Impurity C	87.52	93.7	93.2	94.6
Impurity D	96.5	101.4	101.9	101.8
Impurity E	108.5	96.0	92.6	95.0

Precision: Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of same sample under the prescribed conditions. Quantification of individual impurities and Phenytoin Sodium Capsule was performed for each of the preparations and the percent relative standard deviation (RSD) was determined for the content of the impurities.

To evaluate the intermediate precision, the same experiment was repeated with a different lot of column and a different instrument in the same laboratory. Precision data reported in table no.10.

Table No. 13 Over all %RSD Comparison for Impurities in Precision and Ruggedness study

Sr. No.	% Impurity C	% Impurity D	% Impurity E	% Unk Max	% Total Imp
Precision-1	0.053	0.028	0.210	0.029	0.291
Precision-2	0.053	0.028	0.209	0.029	0.290
Precision-3	0.053	0.028	0.212	0.030	0.293
Precision-4	0.053	0.028	0.214	0.030	0.295
Precision-5	0.053	0.028	0.216	0.030	0.297
Precision-6	0.053	0.028	0.214	0.027	0.295
Ruggedness-1	0.052	0.027	0.215	0.029	0.294
Ruggedness-2	0.053	0.028	0.216	0.029	0.297
Ruggedness-3	0.053	0.028	0.215	0.029	0.296
Ruggedness-4	0.053	0.028	0.216	0.029	0.297
Ruggedness-5	0.053	0.027	0.215	0.029	0.295
Ruggedness-6	0.053	0.027	0.215	0.029	0.295
Mean	0.053	0.028	0.214	0.029	0.295
SD	0.000	0.000	0.002	0.001	0.002
% RSD	0.000	0.000	0.93	3.45	0.68

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. Deliberate changes were made from original experimental conditions to record the tailing factor and theoretical plates of the Phenytoin Sodium Capsule to determine the robustness of the developed method. Data reported in Table no.11.

Table No. 14 Robustness, RRT

Sr. no.	Parameters	Variations	RRT of Related Compounds		
			Impurity-C	Impurity-D	Impurity-E
1	Control-1	-	0.16	0.51	0.75
	Control-2		0.16	0.51	0.74
2	Column Temperature	-5°C	0.16	0.51	0.75
		+5°C	0.16	0.51	0.75
3	Flow rate	-0.1ml/min	0.17	0.52	0.76
		+0.1ml/min	0.15	0.50	0.75
4	Wavelength	-5 nm	0.16	0.51	0.74
		+5 nm	0.16	0.51	0.74

Stability of Analytical solution: The solution stability of sample and standard solution provide an indication of the method's reliability in normal usage during the storage of the solutions used in the method. No significant changes were experienced in the content of any of the impurities during solution stability. The % Cumulative RSD of Standard solution and sample Solution Reported in Table No.12 & 13.

Table No. 15 Table for solution stability for diluted standard at room temperature

Sr. No.	Time (hrs)	Response (Area) Phenytoin
1	Initial	109489
2	31.00	109901
3	43.00	109900
4	56.00	109934
5	64.00	109708
6	75.00	109758
7	86.00	110127
8	96.00	109238
9	107.00	109863
Cumulative % RSD		0.24

Table No. 16 Table for solution stability for sample solution preparation at Room Temperature

Sr. No.	Time (hrs)	Area Phenytoin	Area Imp C	Area Imp D	Area Imp E
1	Initial	23063582	13060	4225	34388
2	40.00	22881482	12974	4191	35267
3	51.00	19929588	12948	4212	35299
4	61.00	19005600	12932	4263	35304
Cumulative % RSD		9.71	0.44	0.72	1.29

Table No 17 Table for System Suitability

S.No	Experiment	% RSD of standard	Theoretical plates	Tailing Factor	Resolution between Phenytoin & Impurity E
1	Forced degradation	0.95	70079	1.02	17.4
2	Linearity, LOD and LOQ	0.66	77296	1.0	18.1
3	Accuracy, Method Precision, Solution Stability	0.07	72076	1.01	17.7
4	Ruggedness	0.11	94474	1.00	20.0

SUMMARY AND CONCLUSION

The Validated HPLC method for related substance of Phenytoin Sodium is linear, precise, accurate and specific. The results of the validation carried out for the method satisfied the ICH requirements. This method can be used for the detection and quantification of known, unknown and degradation impurities in the Phenytoin Sodium Capsules during routine analysis and also for stability studies in view of its capability to separate degradation products.

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List of Abbreviations

No.	Number
LOQ	Limit of Quantitation
LOD	Limit of Detection
Imp	Impurity

Unk	Unknown
Max	Maximum
Hrs	Hours
HPLC	High performance Liquid Chromatography
RSD	Relative Standard Deviation
RRT	Relative retention time

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