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Importance of a rapid and accurate UPLC method for *in-vitro* dissolution testing of a narrow therapeutical index molecule

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ABSTRACT

A rapid, accurate and precise Ultra Performance Liquid Chromatographic (UPLC) method was developed for generating an exhaustive In-Vitro Dissolution profiles of phenytoin sodium capsules in an Immediate Release formulations. The method has been validated. The method employs Waters UPLC system on Acquity BEH C18, 100 x 2.1mm, 1.7µm column with a flow rate of 0.3 mL/min using a mobile phase of 50-50% of Buffer and Acetonitrile. The UPLC was equipped with a UV-Visible Detector and the measurements were taken at 229nm. The immediate release formulations label claim were 300mg, 100mg, 50mg and 25mg for which the injection volume was appropriately selected. The total runtime for each injection was 2mins only with the retention time of the phenytoin peak at about 1.4mins.The method was validated for Linearity, Specificity, precision, Solution Stability and Accuracy. The method validation shows the linearity correlation 0.999. The intra and inter-day precision are within acceptance criteria. Phenytoin sodium dissolution shows the stability of sample and standard solution. The accuracy was within Limit and data was generated for In-vitro Dissolution studies of phenytoin sodium capsules and Tablets.

Keywords: Phenytoin Sodium, Analytical Method, Validation, In-vitro Dissolution, Ultra performance Liquid Chromatography.

INTRODUCTION

Phenytoin is an approved antiepileptic drug which is a white to off white crystalline powder with the molecular formula C15H12N2O2 and the chemical name 5,5-diphenylimidazolidine- 2,4-dione and molecular weight of 252.268 gram per mol[1-3]. The primary site of action appears to be the motor cortex where spread of seizure activity is inhibited.

Phenytoin sodium being an anti-epileptic/ anti-convulsant drug, it falls under the category of Narrow Therapeutic Index (NTI)and has a non- linear kinetics. The bioavailability range lies between 90-111.11% as against other drugs with the range of 80-120%. Loss of post tetanic potentiation prevents cortical seizure foci from detonating adjacent cortical areas. Phenytoin reduces the maximal activity of brain stem centers [4-7].This NTI molecule needs exhaustive In-vitro dissolution profiles for matching with the reference formulation. In such cases, the analysis by UPLC becomes more significant than using other methods like HPLC[8], UV, liquid chromatography and immunoassays for the estimation of Phenytoin sodium [9]. The UPLC method is developed, equivalency between HPLC and UPLC methodology was established and UPLC method has been validated. The ICH validation parameters linearity, repeatability, precision, Accuracy, Solution Stability and robustness were validated[10-11].

The aim describes evaluation of a validated UPLC method for performing the Dissolution analysis phenytoin sodium capsules.

MATERIALS AND METHODS

Sr No	Instrument	Make	Software	Detector/Model No
1	UPLC Acquity H class	Waters	Empower Software	TUV Detector
2	UPLC Acquity H class	Waters	Empower Software	PDA Detector
3	Dissolution	Electrolab	NA	TDT-14L
4	Sonicator	Lab India	NA	NA
5	Weight balance	Mettler Toledo	NA	ML204

Methodology:

Preparation of Buffer: Accurately transfer 1 mL of O-Phosphoric acid in 1000 mL of water and filter through 0.2µm filter.

Preparation of Mobile Phase: Prepare a mixture of Buffer and Acetonitrile in the ratio 50:50 v/v and degas.

Preparation of Dissolution medium: (Water) Use deaerated and degassed water as dissolution medium.

Dissolution Parameters:

Apparatus	:	Basket
Dissolution Medium	:	Water
Temperature	:	37 ± 0.5°C
RPM	:	50 rpm
Volume	:	900 mL
Time Point	:	45 minutes

Note: For dissolution profile withdraw 10 mL of samples at 10, 15, 30, 45 and 60 minutes time interval. Replenish with 10 mL of dissolution medium previously maintained at 37°C ± 0.5°C at every time interval.

Preparation of Standard stock solution: Weigh and transfer accurately about 33 mg of Phenytoin Sodium working standard into a 50 mL volumetric flask, add about 15 mL methanol and sonicate it to dissolve. Cool to room temperature and make up to the mark with methanol and mix.

Standard solution for 25 mg capsules: Transfer 2 mL of Standard stock solution in to 50 mL volumetric flask and dilute up to mark with dissolution medium.

Standard solution for 50 mg capsules: Transfer 2 mL of Standard stock solution in to 25 mL volumetric flask and dilute up to mark with dissolution medium.

Standard solution for 100 mg capsules: Transfer 4 mL of Standard stock solution in to 25 mL volumetric flask and dilute up to mark with dissolution medium.

Standard solution for 300 mg capsules: Transfer 5 mL of Standard stock solution in to 10 mL volumetric flask and dilute up to mark with dissolution medium.

Preparation of Sample solution: Place one capsule in each of the six dissolution vessel containing 900 mL of dissolution media. Carry out the dissolution test. Withdraw 10 mL of aliquots, after each time interval replenish it with fresh dissolution media previously maintained at 37°C. Filter the solution through 0.45µ Nylon filter.

Chromatographic Condition:

Column	: Acquity BEH C18, 100 x 2.1mm, 1.7µm
Flow Rate	: 0.3 mL / min.
Detection	: 229 nm.
Column Temp	: 40°C.
Injection Volume	: 0.5 µL for 300 mg, 1.0 µL for 100 mg and 2.0 µL for 50 and 25mg

Run Time : 2 min.
Retention time : Between 1.0 to 1.6 minutes

Evaluation of System Suitability: Inject the five replicate injections of standard solution into the chromatograph and record the chromatograms. Measure the area counts for Phenytoin peak. The RSD of five replicate injections of standard solution should not be more than 2.0%. Number of theoretical plates should not be less than 5000.

Procedure: Separately inject equal volume of Blank (Dissolution medium) and Sample solution into the chromatograph and record the chromatograms. Measure the area counts for Phenytoin peak.

RESULTS AND DISCUSSION

Specificity: Prepared a representative Placebo solution, Sample solution of Phenytoin Sodium Capsules and Standard solutions as per the Methodology. Injected each of the Dissolution media, Placebo solution, Sample solution and Standard solution into the UPLC using the Chromatographic system as per the Methodology utilizing a photodiode array detector. No interference was observed from Blank and Placebo at the retention time of Phenytoin peak. Also, the peak purity data of Phenytoin peak shows that Phenytoin peak is homogeneous and there are no coeluting peaks. Therefore, the UPLC method for the dissolution of Phenytoin in Phenytoin Sodium Capsules is specific. Specificity reported in table no.1.

Table 1: Table for Specificity

Sr. No.	Name	Purity Angle	Purity Threshold
1	Standard solution	0.541	1.457
2	Sample solution	0.548	1.210

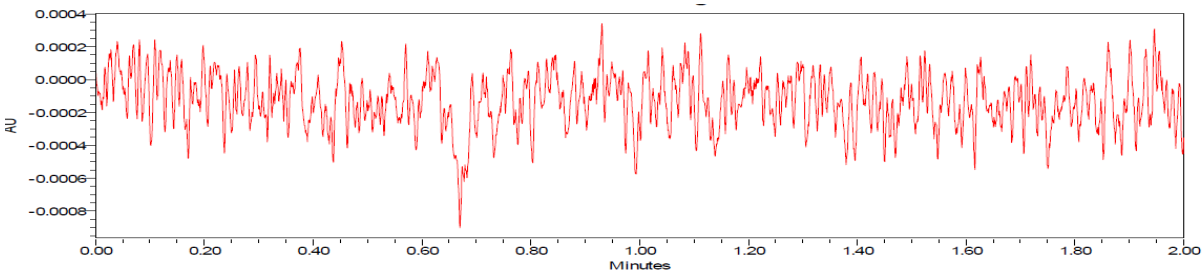

Figure No: 1 Blank Chromatogram

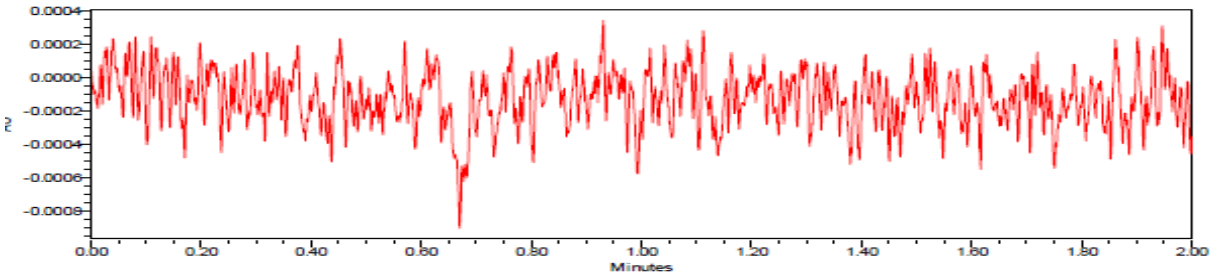

Figure No: 2 Placebo Chromatogram

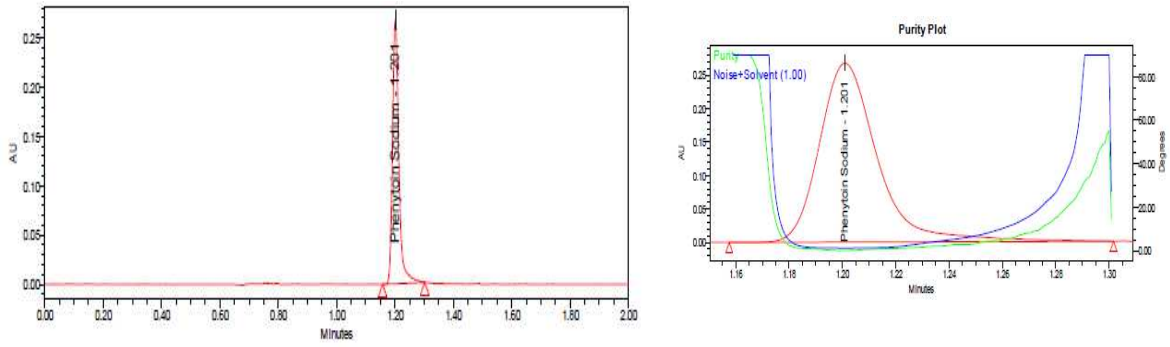

Figure No: 3 Standard Chromatogram and peak purity plot

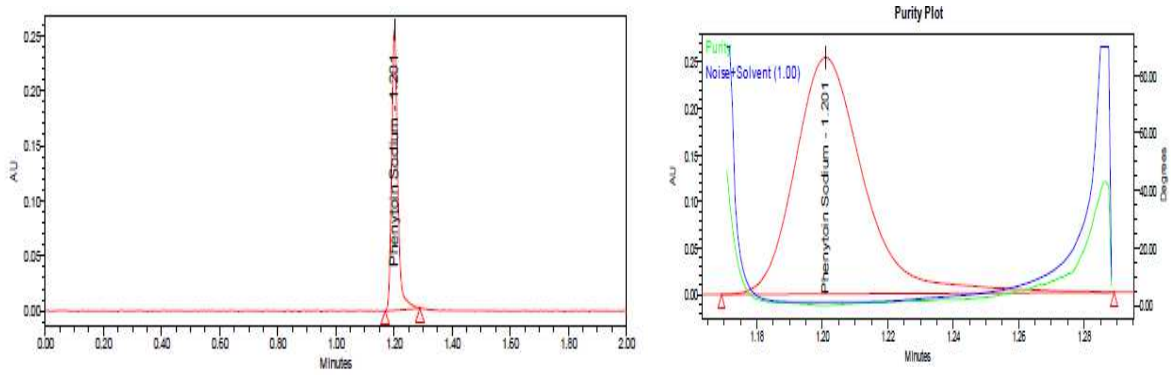

Figure No: 4 Sample Chromatogram and peak purity plot

Linearity and Range: A series of Standard preparations of Phenytoin were prepared over a range of 20% to 150% of the working concentration of Phenytoin in Phenytoin Sodium Capsule. The Correlation coefficient is 0.999. Therefore, the UPLC method for the dissolution of Phenytoin in Phenytoin Sodium Tablets is linear. Linearity reported in table no.2.

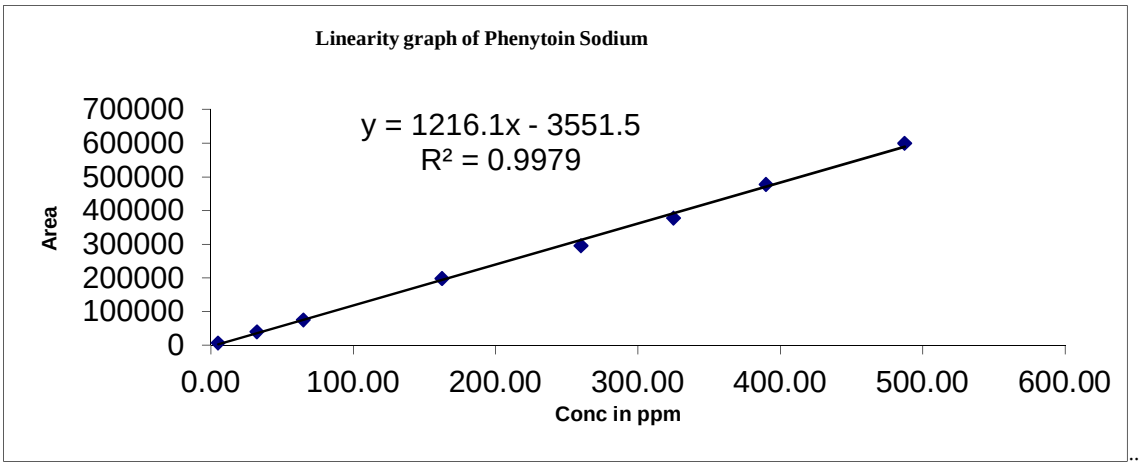

Figure No: 5Linearity plot

Table 2: Linearity Table

% Linearity Range	Concentration (ppm)	Response (Area)	Statistical analysis	
20	5.20	6626	Slope	1252
50	32.50	42243		
80	65.01	77997		
90	162.52	188975	Intercept	-5050
100	260.04	319463		
110	325.04	383917		
120	390.05	483945	Correlation Coefficient	0.999
150	487.57	619956		

Accuracy (Recovery): Weighed placebo of Phenytoin Sodium Tablets equivalent to 1 tablet of 20 mg in separate 1000 ml volumetric flasks & spiked Phenytoin API at 60% of 25 mg, 60%, 80%, 100% and 120% in triplicate of 300 mg, added dissolution medium and sonicated for 30 mins. The mean recovery is 93.4%Thereforethe UPLC method for the dissolution of Phenytoin in Phenytoin Sodium Capsule is accurate. Accuracy reported in table no.3.

Table 3: Accuracy Table

Sample No.	Amount added (mg)	Amount recovered (mg)	% Recovery
60%-25mg Sample-1	15.09	15.47	102.5
60%-25mg Sample-2	15.40	15.46	100.4
60%-25mg Sample-3	15.01	15.21	101.3
60 %-300mg Sample-1	179.11	162.81	90.9
60 %-300mgSample-2	178.98	165.21	92.3
60 %-300mg Sample-3	179.11	164.61	91.9
80 %-300mg Sample-1	238.81	226.32	94.8
80 %-300mg Sample-2	238.89	223.19	93.4
80 %-300mg Sample-3	238.92	219.49	91.9
100%-300mg Sample-1	298.57	273.50	91.6
100%-300mg Sample-2	298.33	278.80	93.5
100%-300mg Sample-3	298.58	276.46	92.6
120%-300mg Sample-1	358.02	343.96	96.1
120%-300mg Sample-2	357.92	342.12	95.6
120%-300mg Sample-3	358.16	344.12	96.1
Mean SD % RSD			96.1 3.216 3.35

Precision

System Precision: Five replicate injections of the Standard Preparation for Phenytoin Sodium Capsule were chromatographed into the UPLC using the method as described under Methodology. The RSD of system precision is reported in Table no. 4.

Table 4: System precision

Sr. No	Response
1	403949
2	400143
3	397293
4	401708
5	391381
Mean	398895
SD	4848.711
%RSD	1.216

Method Precision: Experiment: Six Sample Preparations of Phenytoin Sodium Capsule 300 mg was analyzed using the method as described under Methodology. The RSD of method precision is 2.409% refer Table 7. Therefore; the UPLC method for the dissolution of Phenytoin in Phenytoin Sodium Capsule is precise

Ruggedness: Six Sample preparations of the same lot as used in method precision of Phenytoin Sodium Capsule were analyzed by a different analyst, using different column, on a different day, on a different UPLC. The Over all

%RSD of intermediate precision is 2.186%. Therefore, the UPLC method for the dissolution of Phenytoin in Phenytoin Sodium Capsule is reproducible. Comparison of Precision and Ruggedness reported in table no.5.

Table 5: Table for Precision and Ruggedness

Sample	Analyst -1(Precision) % Drug release	Analyst -2 (Ruggedness) % Drug release
1	92	100
2	97	97
3	98	97
4	97	100
5	95	98
6	98	97
Mean	96.2	98.2
SD	2.317	1.472
% RSD	2.409	1.499
Overall Mean	97.2	
Overall SD	2.125	
Overall % RSD	2.186	

Stability of Analytical solution: The sample and standard preparations for Phenytoin Sodium Capsule were analyzed initially and at different time intervals stored at room temperature. The cumulative area RSD of Standard solution and sample Solution Reported in Table No.6.

Table 6a: Stability of Analytical Solution–(Standard)

No.	Time (hr.)	Area
1	INITIAL	415989
2	3 HRS	417151
5	10 HRS	429773
9	20 HRS	420355
13	30 HRS	421795
17	40 HRS	416753
% RSD		1.03

Table 6b: Stability of Analytical Solution– (Sample)

No.	Time (hr.)	Area
1	INITIAL	421380
2	3 HRS	418407
3	4 HRS	426435
4	7 HRS	426509
5	9 HRS	414699
6	12 HRS	418329
7	14 HRS	420916
8	17 HRS	418690
9	20 HRS	403463
%RSD		1.65

The validated method has been used to perform dissolutions of phenytoin sodium capsules across the biological pH of the body in different dissolution media like pH 1.2 (0.1N HCL), pH 4.5 Acetate Buffer, pH 6.8 Phosphate buffer and water.

The Test product in-vitro values were put together against the reference product values to access the theoretical bio-availability. These values were statistically compared using by using F2 factors.

At pH values where sink conditions may not be achievable for all strengths in vitro dissolution may differ between different strengths and similar profiles at the same dose could be compared. Thus multiple units of lower strengths equivalent to 300 mg were used for dissolution and data was compared to bio strength 300 mg.

Table 7: Comparative dissolution profiles of Reference Product and Test Product of 300 mg

Time (min)	% Drug Release in Water		% Drug Release in 0.1N HCl		% Drug Release in pH 4.5 Acetate Buffer		% Drug Release in pH 6.8 Phosphate buffer	
	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product
10	100	93	0	2	8	10	6	8
15	102	100	1	3	10	10	9	9
30	103	102	1	5	11	12	12	12
45	103	102	3	7	11	13	13	12
60	104	102	4	9	11	14	13	13
90			5	9	12	14	14	13
120			6	10	12	15	15	11

In water, since dissolution was more than 85% at 15 minute time point F2 factor were not calculated.

Since phenytoin sodium has low solubility (conversion to base) in acidic media pH 1.2, incomplete release was observed at the end of 60 minutes; dissolution was further carried till 120 minutes. Even after that no improvement observed in cumulative dissolution values. However dissolution profiles were found to be comparable for both Reference and Test Product, since dissolution was incomplete, F2 values could not be calculated.

For pH 4.5 acetate Buffer and pH 6.8 Phosphate buffer, incomplete drug release was observed for both Reference Product and Test Product and dissolution profiles were found to be comparable.

Lower strengths of the formulation were evaluated for the comparability in Water and it was found that Test Products are comparable with respective Reference Products. The % Drug release in water is tabulated below.

Table 8: Dissolution comparison table for Test and reference product

Time (min)	300 mg		100 mg		50 mg		25mg	
	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product
10	100	93	78	98	85	90	67	91
15	102	100	92	96	94	93	85	95
30	103	102	96	101	97	94	89	97
45	103	102	97	99	100	94	90	99
60	104	102	98	99	101	94	91	98

Similar Dissolutions were carried out for the other physiological pH [pH 1.2, 4.5 and 6.8] for lower strengths namely 100mg, 50mg, 25mg.

Table 9: Dissolution Profiles of 300mg, 100mg, 50mg and 25mg in 0.1N HCl

Time (min)	300 mg		100 mg		50 mg		25mg	
	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product
10	0	2	2	1	1	6	3	3
15	1	3	4	3	1	7	4	5
30	1	5	7	8	3	11	8	9
45	3	7	9	11	4	14	10	13
60	4	9	10	13	5	22	12	17
90	5	9	10	16	8	29	15	24
120	6	10	10	19	11	35	20	32

Due to less solubility in acidic media (pH 1.2) incomplete release was observed. This could be due to sink condition of the strengths.

Table 10: Dissolution Profiles of 300mg, 100mg, 50mg and 25mg in pH 4.5 Acetate Buffer

Time (min)	300 mg		100 mg		50 mg		25mg	
	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product
10	8	10	21	34	24	48	19	61
15	10	10	25	33	31	52	31	65
30	11	12	29	34	43	58	42	72
45	11	13	31	35	46	61	47	76
60	11	14	31	36	48	62	50	80
90	12	14	32	37	49	64	54	82
120	12	15	32	37	51	65	57	85

Table 11: Dissolution Profiles of 300mg, 100mg, 50mg and 25mg in pH 6.8 Phosphate Buffer

Time (min)	300 mg		100 mg		50 mg		25mg	
	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product
10	6	8	10	21	9	17	9	43
15	9	9	16	24	15	23	15	54
30	12	12	25	31	28	29	26	60
45	13	12	30	34	34	33	33	63
60	13	13	31	34	37	35	36	63
90	14	13	33	36	39	36	40	69
120	15	11	34	37	41	39	42	74

Incomplete drug release was observed for all the strengths due to less solubility of drug in this media. As strengths decreases, drug release was found to increases. This could be due to lack of sink condition across the strengths.

Further to ascertain the low release of the drug in the physiological media, dissolution profiles were performed on multiple units, since sink condition was not achievable.

Multiple units of lower strengths of Test Product were taken equivalent to 300 mg to generate the data. Three capsules of 100mg, six capsules of 50mg and twelve capsules of 25mg were taken to perform the dissolution profile.

Table 12: Dissolution Profiles of Multi-units of 100mg, 50mg and 25mg equivalent to 300mg in 0.1N HCl

Time (min)	300 mg (x1capsule)		100 mg (x3 capsule)		50 mg(x 6 capsule)		25mg(x 12 capsule)	
	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product
10	0	2	2	3	0	4	0	4
15	0	3	2	4	0	5	0	6
30	1	5	4	7	1	9	1	12
45	1	7	7	10	3	10	2	14
60	3	9	9	10	6	11	6	14
90	4	9	10	11	10	12	9	16
120	5	10	10	12	12	13	12	17

Based on the observed values, a dissolution profile of all lower strengths was found to be comparable to the bio strength 300 mg at pH 1.2

Table 13: Dissolution Profiles of Multi-units of 100mg, 50mg and 25mg equivalent to 300mg in pH 4.5 Acetate Buffer

Time (min)	300 mg (x1capsule)		100 mg (x3 capsule)		50 mg(x 6 capsule)		25mg(x 12 capsule)	
	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product
10	8	10	15	11	6	14	14	16
15	10	10	15	12	15	16	16	15
30	11	12	17	15	18	15	16	15
45	11	13	17	16	16	16	16	15
60	11	14	16	16	18	15	15	15
90	12	14	17	17	18	15	15	15
120	12	15	16	16	19	15	15	15

Based on the observed values, a dissolution profile of all lower strengths was found to be comparable to the bio strength 300 mg at pH 4.5 Buffer

Table 14: Dissolution Profiles of Multi-units of 100mg, 50mg and 25mg equivalent to 300mg in pH 6.8 Phosphate Buffer

Time (min)	300 mg (x1capsule)		100 mg (x3 capsule)		50 mg(x 6 capsule)		25mg(x 12 capsule)	
	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product	Reference Product	Test Product
10	6	8	9	16	3	10	2	12
15	9	9	14	16	6	11	4	12
30	12	12	17	17	14	11	9	13
45	13	12	20	18	17	11	11	13
60	13	13	21	18	18	12	14	14
90	14	13	21	18	20	12	16	15
120	15	11	23	21	21	12	16	14

Based on the observed values, a dissolution profile of all lower strengths was found to be comparable to the bio strength 300 mg at pH 6.8

Hence the Overall Inference for Multiunit data generation ascertains that for all lower strengths [25, 50 and 100 mg] multiunit data were found to be comparable to bio strength [300 mg] in physiological pH [pH1.2, 4.5 and 6.8] Reference Products and Test Products.

CONCLUSION

An exhaustive data has been generated with the help of a rapid and accurate UPLC method of analysis for the dissolution profiles of 300mg, 100mg, 50mg and 25mg in physiological pH's for single unit profile and Multi-unit profile. The single unit data in Water is comparable for all strengths. In other pH's (0.1N HCl, pH 4.5 and pH 6.8) the data are not comparable within the strengths. The Multi-unit dissolution profiles for all strengths are comparable.

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