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CHROMATOGRAPHIC METHOD DEVELOPMENT AND VALIDATION STABILITY-INDICATING THREE IMPURITIES AND ITS DEGRADATION PRODUCTS IN TOLVAPTAN 15/30MG TABLETS

M. Naresh Chandra Reddy†, K. B. Chandra Sekhar† and A. Kavitha††

†Department of Chemistry, Jawaharlal Nehru Technological University, Anantapur, India.

††Richer Pharmaceuticals, IDA, Prasanthi Nagar, Kukatpally, Hyderabad, (A.P) India – 500072.

†††Department of Chemistry, College of Engineering, JNT University, Kukatpally, Hyderabad 500085, India.

††††Department of Metallurgical and Materials Engineering, Mahatma Gandhi Institute of Technology, Gandipet, Hyderabad – 500 075. A.P. India.

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***Corresponding Author**
M. Naresh Chandra Reddy
Department of Chemistry, Jawaharlal Nehru Technological University, Anantapur, India.

ABSTRACT

A Novel RPHPLC Quantification method was developed for estimation of Tolvaptan known impurities like its Amino Hydroxy, Amino and Keto which, were separated on Phenomenex Kinetex C18 column (75 mm x 4.6 mm; 2.6µ). Using a mixture of phosphate buffer, Acetonitrile and Methanol as a gradient mobile phase with a flow rate of 1.0 ml/min; λ max at 240 nm. The developed method was validated all the parameters like linearity, specificity, LOD, LOQ, accuracy, robustness, ruggedness, precision, filter variation, solution stability and forced degradation studies.

KEYWORDS: Method development and validation, Tolvaptan, Related substances, Stability-indicating, Tablets.

INTRODUCTION

Tolvaptan is chemically N-(4-{[(5R)-7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl]carbonyl}-3-methylphenyl)-2-methylbenzamide (fig. 1). Tolvaptan is non peptide vasopressin V2 receptor antagonist inhibits water re-absorption in the kidney blocking VP binding resulting in water dieresis without significantly changing electrolyte excretition.[1-2] Tolvaptan is available as a tablet for administration. Many techniques have

been reported quantitave estimation including Spectrophotometric[3-4], liquid chromatographic[5-6], UPLC[7], LC/MS method for human plasma.[8]

Since no method has been developed for the separation and estimation of impurities in Tolvaptan tablets and the drug is being marketed in domestic and international market the present study by the author describes a rapid, accurate and precise RP – HPLC method for the estimation of known related impurities, i.e., Amino Hydroxy impurity((4-Amino-2-methylphenyl) (7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1H-benzo[b]azepin-1-yl)methanone), Amino Impurity(1-(4-amino-2-methylbenzoyl)-7-chloro-3,4- dihydro-iH-benzo[b]azepin-5(2H)-one) and Keto Impurity (N-(4-(7-chloro-5-oxo- 2,3,4,5-tetrahydro-1H-benzo[b]azepine-1-carbonyl)-3-methylphenyl)-2-methylbenzamide) and degrading products under stress conditions present in Tolvaptan tablets. The method was validated as per ICH guidelines.[9]

EXPERIMENTAL

Material and Methods

Chromatographic Conditions

Agilent 1200 series HPLC consisting pump, Auto sampler, VWD & photo diode array detector, thermostatic column compartment connected with Open lab and EZ Chrom software connected with a Phenomenex Kinetex C_{18} 75 x 4.6mm, 2.6µ, 100A.

Chemicals and reagents

Tolvaptan pure drug and impurities, Acetonitrile (HPLC Grade), water (HPLC Grade), Methanol(HPLC Grade), orthophosphoric acid 85% pure were AR grade from SD Fine Chem., was used in the present study. The tablet formulations purchased from local market Hyderabad, India.

Mobile phase

Accurately transfer 1ml of orthophosphoric acid in 1000ml of water, Filter the solution through 0.22µ nylon filter and sonicate to degas it. The buffer was used as mobile phase preparation A, Acetonitrile and methanol in the ratio (80:20 v/v) used as mobile phase mobile preparation B, Tolvaptan and its impurities were separated and eluted in a gradient program represented in Table-1. The flow rate of the mobile phase was maintained at 1.0ml/min. The column temperature was maintained at 35ºC and the detection was carried out at 240nm with an injection volume of 10µl.

Diluent

Prepare a filtered and degassed mixture of acetonitrile and water (60:40 v/v).

Standard solution preparation

Weigh accurately 25mg of Tolvaptan working standard into 50 ml volumetric flask, add 30 ml of acetonitrile and dissolve, further make up the volume with water. Further dilute 1 ml to 100ml with diluent.

Placebo preparation

Weigh and transfer the amount of placebo present in equal to 50 mg of Tolvaptan and transfer into 100 ml volumetric flask add 60 ml of acetonitrile, sonicate to dissolve for 10 minutes and dilute to volume with water. Further filtrate the solution through 0.22µ filter.

Sample preparation

Weigh tablet powder equivalent to 50 mg of Tolvaptan into 100 ml volumetric flask add 60 ml of acetonitrile, sonicate to dissolve for 10 minutes and dilute to volume with water. Further filtrate the solution through 0.22µ filter.

Impurities Calculation

$$\% \text{ of Impurity} = \frac{\text{Impurity Area x Standard weight x 1 x 100 x 1 x standard Potency}}{\text{Average Standard Area x 50 x 100 x sample weight x Label amount}} x\ RF$$

% of Total Impurities = Sum of % Individual impurities,

RF – Response Factor

RESULTS AND DISCUSSION

System Suitability

System suitability was evaluated from the standard solution preparation by injecting six times into the HPLC. The parameters measured were Theoretical plates, asymmetry, %RSD, the observed results asymmetry is about 1.18, theoretical plates about 55000, % RSD is 0.18 and the resolution between two peaks greater than 2.0 indicates the method suitable for related substances estimation.

Placebo and impurities interference

Interference from placebo and impurities was carried out by preparing the following specificity samples. Performed related substances on Placebo equivalent to the amount present in test preparation and injected into the chromatography. By preparing and inject

impurities at 1.0% of test concentration, by preparing active sample as per test concentration, by spiking the active sample with individual known impurities at 1.0% of test concentration. The above samples were injected and observed for any interference from blank and placebo at the retention time of analyte and known impurity peaks. This was further demonstrated by determining the peak purity of analyte and known impurity peaks. Since no interference of blank, placebo and known impurities was observed at the retention time of analyte. Individual impurity peaks are separated from the analyte peak. Peak purity of analyte peak and known impurity peaks are greater than 0.99, so the method is specific for Tolvaptan tablets. The chromatogram of spiked impurities with Tolvaptan tablet preparation shown in (fig. 2).

Limit of Quantitation and Detection

The limit of quantitation (LOQ) and detection (LOD) were conducted on the basis of signal to noise ratio method. Different concentrations of impurities with sample solution were injected, LOQ established the values which give the signal to noise ratio about 10.0, for LOD of impurities were established which give the signal noise ratio about 3.0; the results of both LOQ & LOD values were tabulated in Table-2.

Linearity and Detector Response

The linearity of detector response for impurities was demonstrated by prepared solutions of Lacosamide and its impurities over the range of LOQ to 200% level and the detector response was found to be linear and the correlation coefficient was more than 0.998, proves Tolvaptan and its impurities are linear, the results were tabulated in Table-3 and the chromatogram shown in (fig. 3).

Establishment of RRT's and RF Values for Impurities

The RRT's and RF values were calculated from the linearity levels of 50%, 100% and 200% i.e., 0.5%, 1.0% and 2.0% of test concentration. The RRT's and RF values were calculated and the results were tabulated in Table-4.

Precision

Six sample preparations representing a single batch were injected, the each impurity area were determined and the precision was evaluated, the %RSD of each impurity results was less than 10.0 indicates the method is precise, the results are tabulated in Table-4.

Intermediate Precision

The ruggedness of the method was injected six preparations of a single batch sample by different analyst (analyst-2), different column (column-2) and different instrument (instrument-2). The %RSD of each impurity was calculated; the results were less than 10.0. consider the precision results for analyst-1, column-1 and system-1, the mean %RSD values of both precision and intermediate calculated, the results were less than 15.0 shows the method is rugged and the results were tabulated in Table-4.

Accuracy

The accuracy of the test method was prepared recovery samples (i.e. test sample with known quantities of Amino hydroxide Impurity, Amino Impurity and Keto Impurity) at the level of LOQ, 50%, 100%, 150% and 200% of target concentration, as the recovery results were found between 90 to 110% the method is accurate for the estimation of Tolvaptan tablets and its impurities over the range of LOQ to 200% level of target concentration and the results were tabulated in Table-5.

Robustness

The solution stability & mobile phase stability

The standard and sample solution kept for bench top, under refrigerator were injected initially, after 24 hours and 48 hours. The difference between initial, 24hrs and 48hrs of individual impurity less than 0.03% and total impurities less than 0.1% and the similarity factor after 24 hours and after 48 hours is between 0.95 to 1.05 indicates the solution is stable up to 48hrs and the results were tabulated in Table 8.for mobile phase stability the standard and sample solutions injected initially, after 24 hours and after 48 hours, a slight variation of parameters like theoretical plates, asymmetry and % RSD indicates the mobile phase is stable up to 48 hours.

Extraction time of analyte

The difference between as such condition and different extraction samples for % of individual impurity less than 0.03% and % of total impurities 0.1% found within the limits.

Filter variation

The filter variation was injected the test solution of centrifuged and filtered through 0.22µ nylon filter 0.45µnylon filter and 0.22µ PVDF, 0.45µ PVDF filter and the difference between

filtered portions of individual impurity less than 0.03% and total impurities were less than 0.1% with respect to centrifuged sample shows no effect of filter variation.

Effect of Column Temperature and Flow Variation

The standard preparation was injected under normal condition (i.e. as such condition) and of the altered conditions column temperature 35±5°C and flow rate 1±0.1ml the difference between as such for all changed conditions parameters like theoretical plates, asymmetry and % RSD within the limits proves the method is robust.

FORCED DEGRADATION STUDIES

Acid Hydrolysis Stress study

Weighed equivalent to 50 mg of Tolvaptan Tablet powder and transferred into 250 ml round bottom flask, added 50 ml of acetonitrile sonicate to dissolve for 15 minutes and added 50 ml of 5N HCl. Forcibly degrades the sample at 60°C and collected the sample after 5 hours. Filtrate the solution through 0.45 micron nylon filter and injected into the chromatography. The homogeneity of the peak is pure, the sample is highly sensitive to acid the results are tabulated in Table-6 and the chromatogram shown in (fig. 4a).

Base Hydrolysis Stress Study

Weighed equivalent to 50 mg of Tolvaptan Tablet powder and transferred into 250 ml round bottom flask, add 50 ml acetonitrile sonicate to dissolve for 15 minutes and added 50ml of 0.1N NaoH and mixed. Forcibly degrade the sample at 60°C and collect the sample after 48 hours. Filtrate the solution through 0.45 micron nylon filter and injected into the chromatography. The homogeneity of the peak is pure, the sample is not sensitive to base the results are tabulated in Table-6 and the chromatogram shown in (fig. 4b)

Peroxide Oxidation Stress Study

Weighed equivalent to 50 mg of Tolvaptan Tablet powder and transferred into 250 ml round bottom flask, add 50 ml acetonitrile sonicate for 15 minutes and added 50ml of 10% H_2O_2. Forcibly degrade the sample at 60°C and collect the sample after 5 hours. Filtrate the solution through 0.45 micron nylon filter and injected into the chromatography. The homogeneity of the peak is pure, the sample is highly sensitive to peroxide the results are tabulated in Table-6 and the chromatogram shown in (fig. 4c)

Water degradation Stress study

Weighed equivalent to 50 mg of Tolvaptan Tablet powder and transferred into 250 ml round bottom flask, add 50 ml acetonitrile sonicate for 15 minutes and added 50ml of purified water. Forcibly degrade the sample at 60°C and collect the sample after 48 hours. Filtrate the solution through 0.45 micron nylon filter and injected into the chromatography. The homogeneity of the peak is pure, the sample is not sensitive to water the results are tabulated in Table-6 and the chromatogram shown in (fig. 4d).

Heat Stress Study

Forcibly degrade the sample exposed to heat under oven at 60°C temperature, collect the sample after 10^{th} day and equivalent to 50 mg of Tolvaptan Tablet powder and transferred into 100 ml volumetric flask, add 50 ml acetonitrile sonicate for 15 minutes and dilute to volume with purified water. Filtrate the solution through 0.45 micron nylon filter and injected into the chromatography. The homogeneity of the peak is pure, the sample is not sensitive to heat the results are tabulated in Table-6 and the chromatogram shown in (fig. 4e)

Photolytic Stress study

UV Light

Forcibly degrade the sample exposed to UV light for 200watt hours/m^2, collect the sample and equivalent to 50 mg of Tolvaptan Tablet powder and transferred into 100 ml volumetric flask, add 50 ml acetonitrile sonicate for 15 minutes and dilute to volume with purified water. Filtrate the solution through 0.45 micron nylon filter and injected into the chromatography. The homogeneity of the peak is pure, the sample is not sensitive to UV the results are tabulated in Table-6 and the chromatogram shown in (fig. 4f)

Sun light

Forcibly degrade the sample exposed to Sun light for 1.2 million lux hours, collect the sample and equivalent to 50 mg of Tolvaptan Tablet powder and transferred into 100 ml volumetric flask, add 50 ml acetonitrile sonicate for 15 minutes and dilute to volume with purified water. Filtrate the solution through 0.45 micron nylon filter and injected into the chromatography. The homogeneity of the peak is pure, the sample is not sensitive to sun light the results are tabulated in Table-6 and the chromatogram shown in (fig. 4g)

Degradation at 75% Relative Humidity

Forcibly degraded the sample exposed under 75% relative humidity and collect the sample after 10th day Weighed equivalent to 50 mg of Tolvaptan Tablet powder and transferred into 100 ml volumetric flask, added 50 ml of acetonitrile sonicate to dissolve for 15 minutes and diluted to the volume with purified water. Filtrate the solution through 0.45 micron nylon filter and injected into the chromatography. The homogeneity of the peak is pure, the sample is not sensitive to 75% relative humidity the results are tabulated in Table-6 and the chromatogram shown in (fig. 4h).

Table 1: HPLC Gradient Program.

Time (min)	Mobile phase A	Mobile phase B
0	65	35
1.5	65	35
4.5	50	50
15	50	50
17	65	35
20	65	35

Table 2: LOD & LOQ results.

S. No	Name of the Component	LOD RESULTS		LOQ RESULTS	
		S/N Ratio	% level of component w.r.t to sample concentration	S/N Ratio	% level of component w.r.t to sample concentration
1	Tolvaptan	2.84	0.0034	10.30	0.0112
2	Amino Hydroxy impurity	2.72	0.0031	9.92	0.0103
3	Amino impurity	2.70	0.0030	9.93	0.0099
4	Keto impurity	2.46	0.0032	9.96	0.0106

Table 3: Linearity Results.

Compound Name	Correlation coefficient	Slope	Y-Intercept	Residual sum square	Residual standard deviation
Tolvaptan	0.9999	376690.17	2882.93	1.2910×10^{9}	17965
Amino hydroxy Impurity	1.0000	378692.33	-257.60	1.0861×10^{9}	16478
Amino impurity	1.0000	375985.48	527.11	9.7337×10^{8}	15599
Keto Impurity	0.9999	362889.56	1286.70	1.2922×10^{9}	17974

Table 4: Precision, Intermediate Precision, RF and RRT Results.

Parameter	Amino Hydroxy Impurity	Amino Impurity	Keto Impurity
Precision(n=6)			
Tolvaptan 15mg tablets	**0.32**	**0.15**	**0.36**

Tolvaptan 30mg tablets	**0.26**	**0.11**	**0.17**
Intermediate Precision(n=6)			
Tolvaptan 15mg Tablets	**0.42**	**0.54**	**0.15**
Tolvaptan 30mg Tablets	**0.19**	**0.66**	**0.16**
Mean method precision and Intermediate precision			
Tolvaptan 15mg Tablets	**0.95**	**1.02**	**0.95**
Tolvaptan 30mg Tablets	**0.94**	**0.88**	**0.95**
RRT&RF Values			
RRT values	**~ 0.21**	**~ 0.33**	**~ 1.20**
RF values	**0.99**	**0.99**	**0.98**

the number of repetitions

Table 5: Accuracy results.

Spike Level	***Amount added(ppm)***	***Mean Amount recovered(ppm)***	***% Mean Recovery***	***%RSD***
Recovery of Amino Hydroxy impurity				
LOQ level	0.051	0.05109	100.19	0.59
50%	2.53	2.5163	99.46	0.21
100%	5.05	5.0545	100.09	0.27
150%	7.58	7.511	99.09	0.40
200%	10.11	10.1939	100.83	0.43
Recovery of Amino impurity				
LOQ level	0.048	0.0466	97.12	0.92
50%	2.51	2.5481	101.52	0.23
100%	5.03	5.0239	99.88	0.30
150%	7.54	7.5128	99.64	0.71
200%	10.05	10.1253	100.75	0.20
Recovery of Keto impurity				
LOQ level	0.051	0.0502	98.58	5.52
50%	2.53	2.5494	100.77	0.32
100%	5.05	4.9974	98.96	0.15
150%	7.58	7.4815	98.7	1.0
200%	10.11	10.1332	100.23	0.22

Table 6: Degradation Results.

TOLVAPTAN TABLETS							
30 mg							
S.No:	**Stress conditions**	**Duration**	**% of Total imp's**	**% of Amino hydroxy imp**	**% of Amino impurity**	**% of Keto imp**	**% of major unknown imp**
1	Normal	NA	0.11	0.01	ND	ND	0.02 (1.17)
2	Thermal at 60°C	10^{th} day	0.11	0.01	ND	ND	0.02(1.17)
3	75% RH	10^{th} day	0.12	0.01	ND	ND	0.02(1.17)
4	UV	200 watt hours/m^2	0.11	0.01	ND	ND	0.02(1.17)

5	Sunlight	1.2 million Lux hours	0.11	0.01	ND	ND	0.02(1.17)
6	5N HCl at 60°C	5 hours	9.40	0.60	ND	0.01	2.21(0.77)
7	0.1N NaOH at 60°C	48 hours	0.17	0.06	ND	0.03	0.02(1.17)
8	10% H2O2 at 60°C	5 hours	6.90	0.19	ND	1.17	1.01(0.73)
9	Water at 60°C	48 hours	0.12	0.01	ND	ND	0.02(1.16)

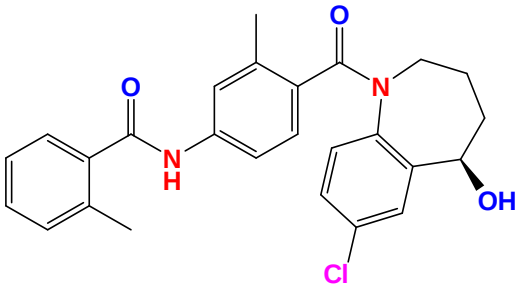


Fig 1. Tolvaptan chemical structure.

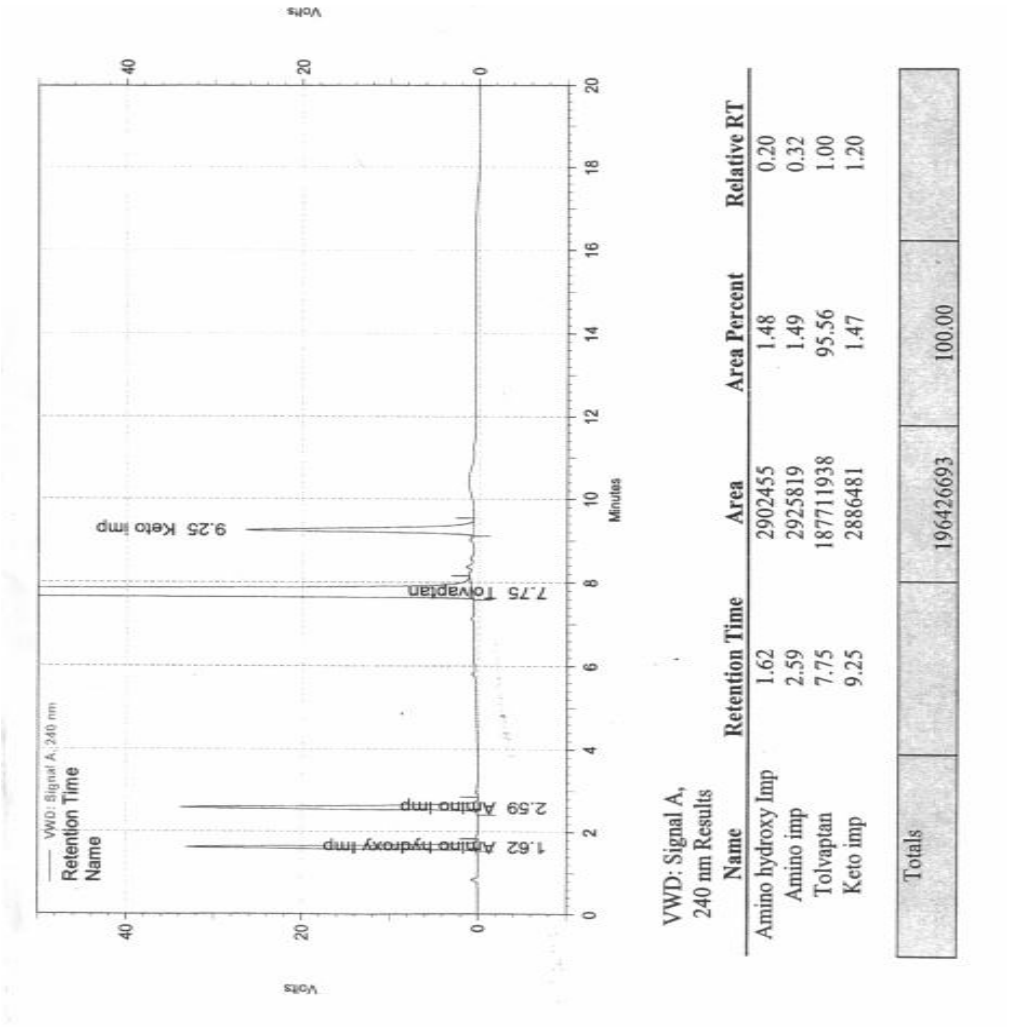
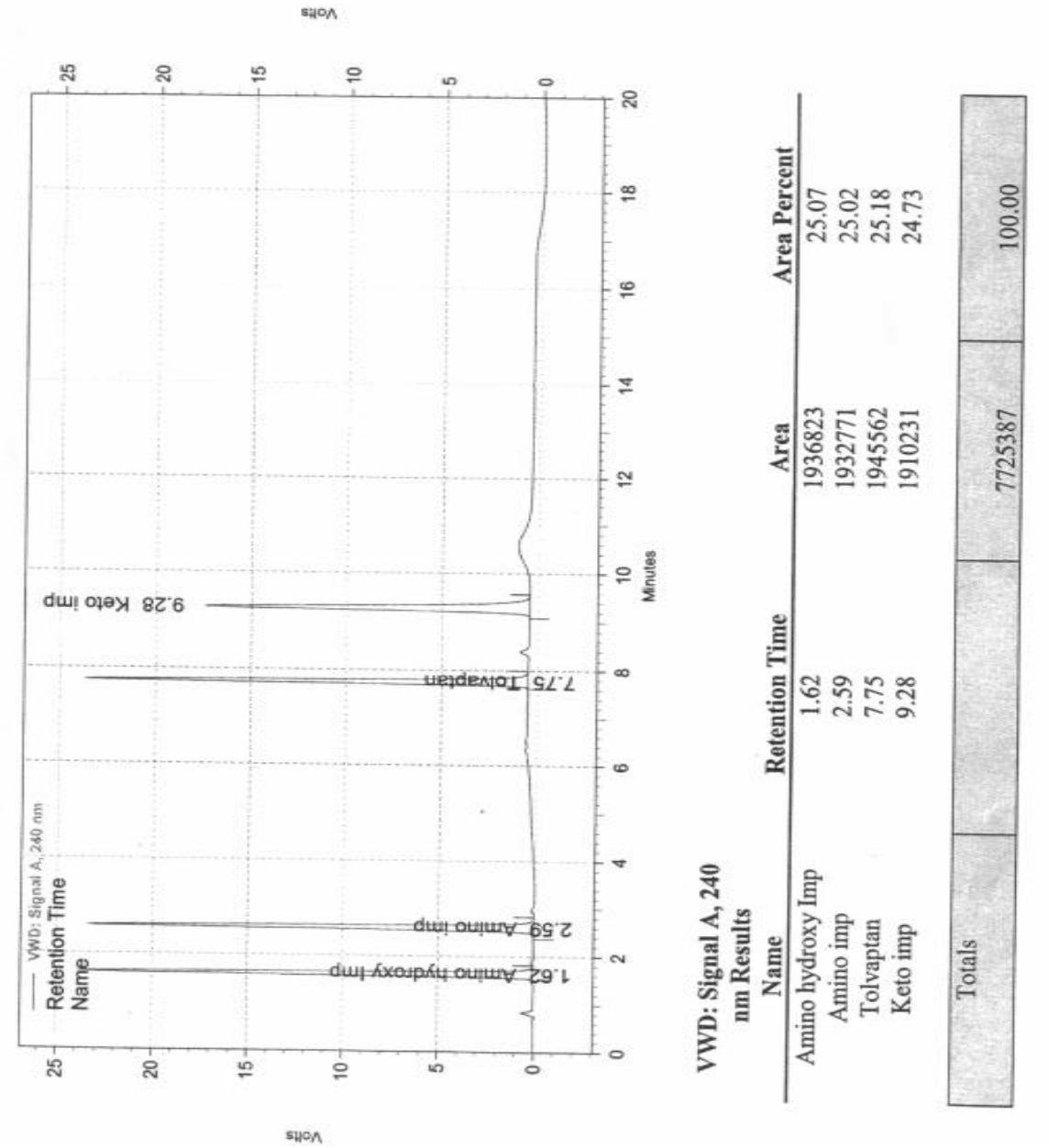


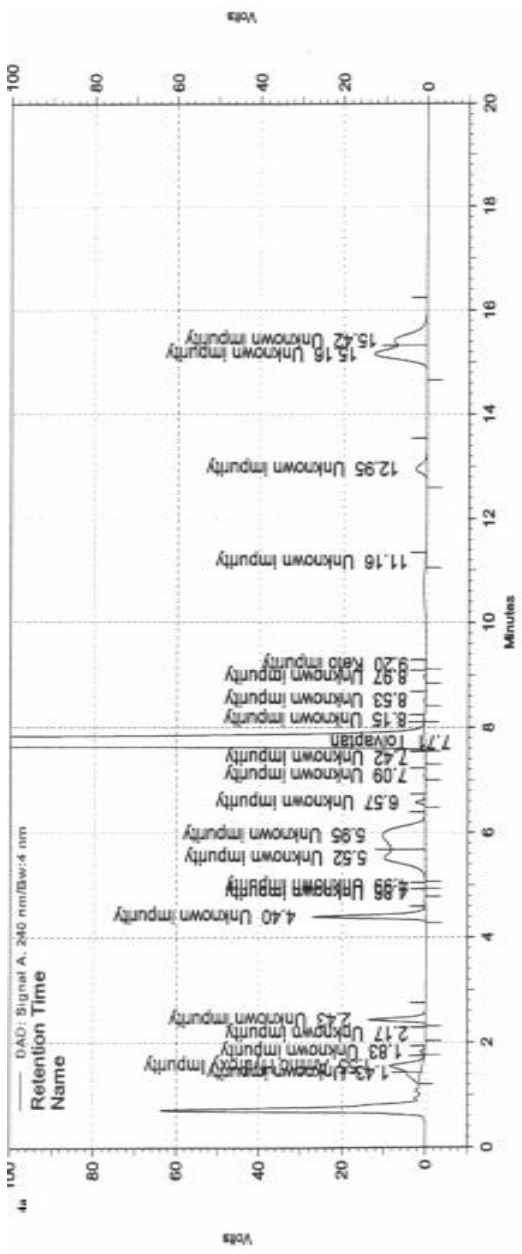
Fig 2. Spiked mpurities with Tolvaptan tablets.



VWD: Signal A, 240 nm Results

Name	Retention Time	Area	Area Percent
Amino hydroxy Imp	1.62	1936823	25.07
Amino imp	2.59	1932771	25.02
Tolvaptan	7.75	1945562	25.18
Keto imp	9.28	1910231	24.73
Totals		7725387	100.00

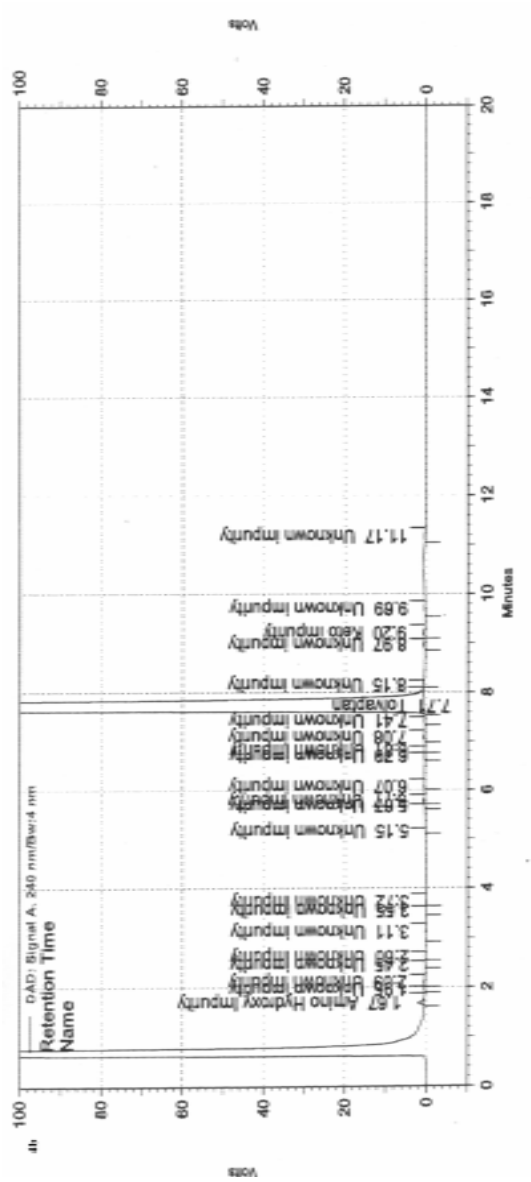
Fig 3. Linearity chromatogram of Tolvaptan and its impurities.



DAD: Signal A, 240 nm/Bw:4 nm
Results

Name	Retention Time	Area	Area Percent	Relative RT
Unknown impurity	1.43	50956	0.21	0.17
Amino Hydroxy Impurity	1.55	143072	0.60	0.19
Unknown impurity	1.83	9621	0.04	0.23
Unknown impurity	2.17	1185	0.00	0.27
Unknown impurity	2.43	136650	0.57	0.31
Unknown impurity	4.40	269981	1.12	0.57
Unknown impurity	4.86	607	0.00	0.63
Unknown impurity	4.99	295	0.00	0.64
Unknown impurity	5.52	350095	1.46	0.71
Unknown impurity	5.95	529807	2.21	0.77
Unknown impurity	6.57	20126	0.08	0.85
Unknown impurity	7.09	2569	0.01	0.92
Unknown impurity	7.42	1369	0.01	0.96
Tolvaptan	7.71	21765366	90.68	1.00
Unknown impurity	8.15	1046	0.00	1.06
Unknown impurity	8.53	3277	0.01	1.11
Unknown impurity	8.97	4642	0.02	1.17
Keto impurity	9.20	1817	0.01	1.20
Unknown impurity	11.16	1649	0.01	1.45
Unknown impurity	12.95	68949	0.29	1.69
Unknown impurity	15.16	392706	1.64	1.98

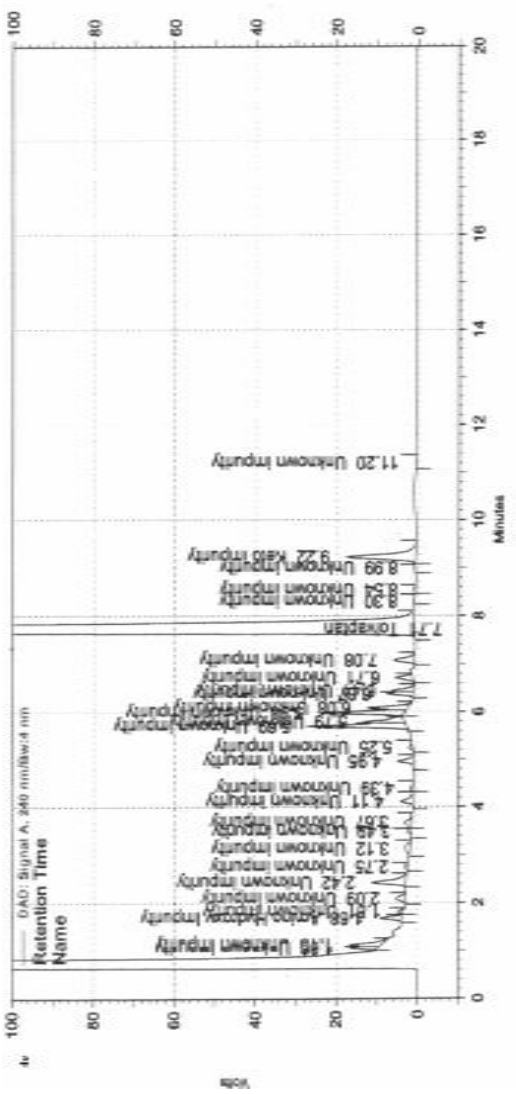
Fig 4a) Chromatogram of Tolvaptan acid degradation (5N HCl at 60°C).



DAD: Signal A, 240 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Relative RT
Amino Hydroxy Impurity	1.67	12950	0.06	0.21
Unknown impurity	1.95	1031	0.00	0.24
Unknown impurity	2.09	3042	0.01	0.26
Unknown impurity	2.45	345	0.00	0.31
Unknown impurity	2.60	355	0.00	0.33
Unknown impurity	3.11	1351	0.01	0.40
Unknown impurity	3.55	292	0.00	0.45
Unknown impurity	3.72	1529	0.01	0.48
Unknown impurity	5.15	143	0.00	0.66
Unknown impurity	5.67	219	0.00	0.73
Unknown impurity	5.77	3146	0.01	0.75
Unknown impurity	6.07	1197	0.01	0.78
Unknown impurity	6.70	297	0.00	0.87
Unknown impurity	6.81	235	0.00	0.88
Unknown impurity	7.08	3386	0.01	0.92
Unknown impurity	7.41	1142	0.00	0.96
Tolvaptan	7.71	23482331	99.80	1.00
Unknown impurity	8.15	1280	0.01	1.06
Unknown impurity	8.97	4786	0.02	1.17
Keto impurity	9.20	6857	0.03	1.20
Unknown impurity	9.69	1756	0.01	1.26

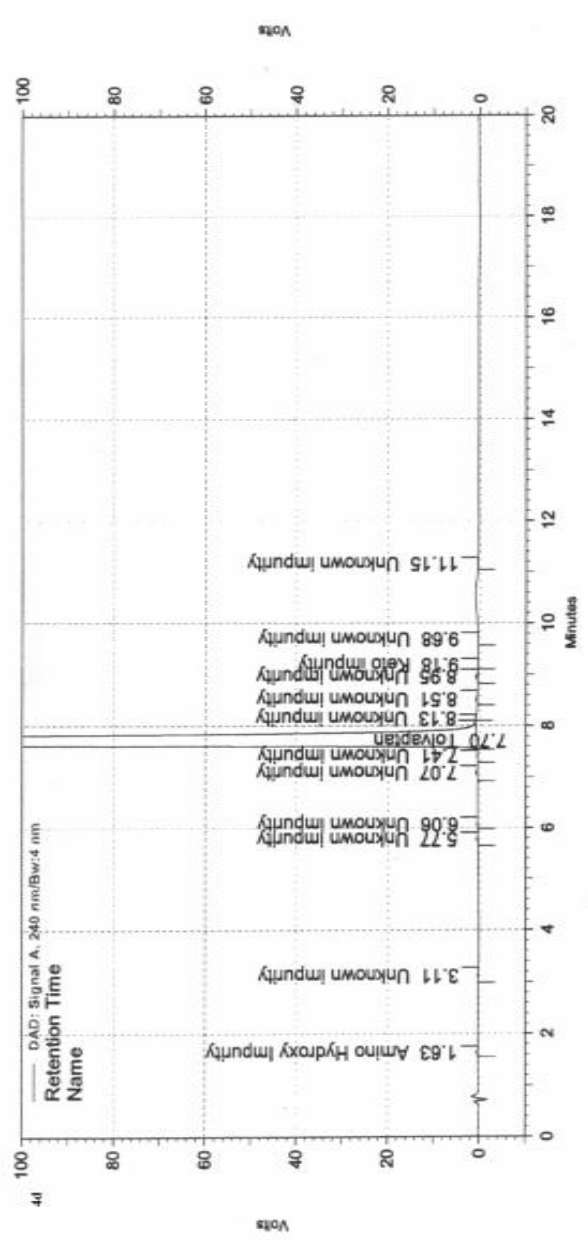
Fig 4b) Chromatogram of Tolvaptan base degradation (0.1N NaOH at 60°C).



DAD: Signal A, 240 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Relative RT
Unknown impurity	1.06	42721	0.19	0.13
Unknown impurity	1.13	72432	0.33	0.13
Amino Hydroxy Impurity	1.68	42360	0.19	0.21
Unknown impurity	1.81	10109	0.05	0.23
Unknown impurity	2.09	31380	0.14	0.26
Unknown impurity	2.42	105985	0.48	0.30
Unknown impurity	2.75	7365	0.03	0.35
Unknown impurity	3.12	24532	0.11	0.40
Unknown impurity	3.49	11561	0.05	0.44
Unknown impurity	3.67	31376	0.14	0.47
Unknown impurity	4.11	36584	0.17	0.53
Unknown impurity	4.39	6582	0.03	0.56
Unknown impurity	4.95	38857	0.18	0.64
Unknown impurity	5.25	11344	0.05	0.68
Unknown impurity	5.69	223271	1.01	0.73
Unknown impurity	5.79	89958	0.41	0.75
Unknown impurity	5.98	182713	0.83	0.77
Unknown impurity	6.08	86390	0.39	0.79
Unknown impurity	6.37	50487	0.23	0.82
Unknown impurity	6.44	45773	0.21	0.83
Unknown impurity	6.71	42173	0.19	0.87

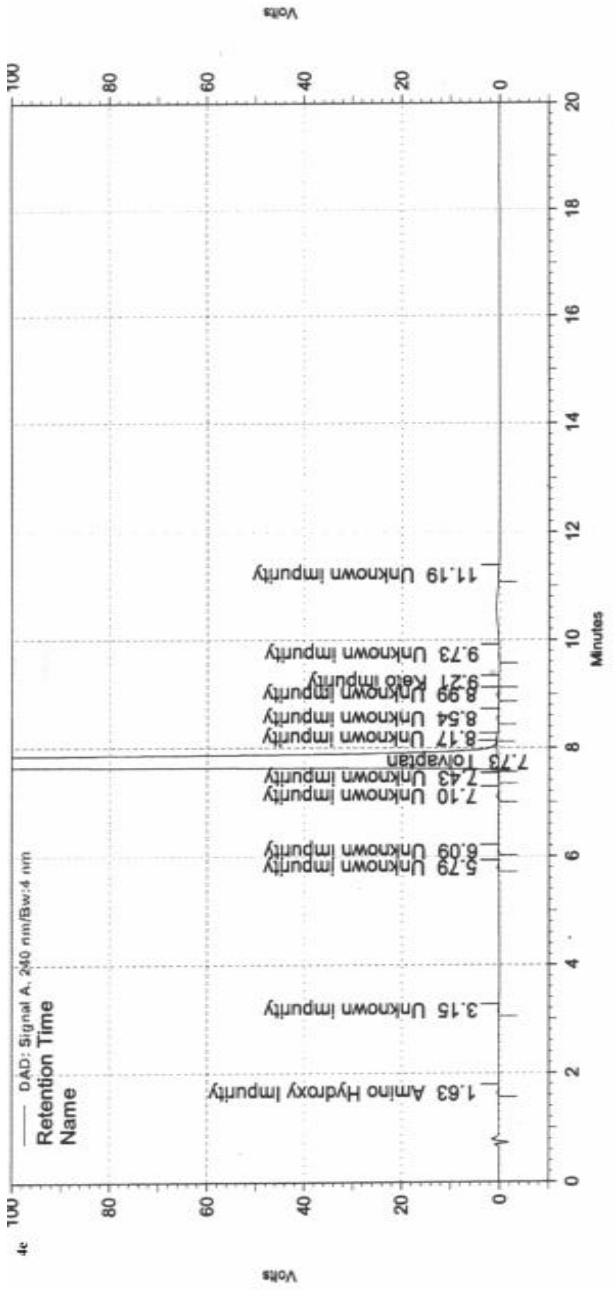
Fig 4c) Chromatogram of Tolvaptan peroxide degradation (10% H2O2 at 60°C)



DAD: Signal A, 240 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Relative RT
Amino Hydroxy Impurity	1.63	3048	0.01	0.20
Unknown impurity	3.11	2083	0.01	0.40
Unknown impurity	5.77	2945	0.01	0.75
Unknown impurity	6.06	1167	0.00	0.78
Unknown impurity	7.07	3408	0.01	0.92
Unknown impurity	7.41	1258	0.01	0.96
Tolvaptan	7.70	23506605	99.88	1.00
Unknown impurity	8.13	1048	0.00	1.06
Unknown impurity	8.51	3613	0.02	1.11
Unknown impurity	8.95	5256	0.02	1.16
Keto impurity	9.18	787	0.00	1.19
Unknown impurity	9.68	997	0.00	1.26
Unknown impurity	11.15	1827	0.01	1.45

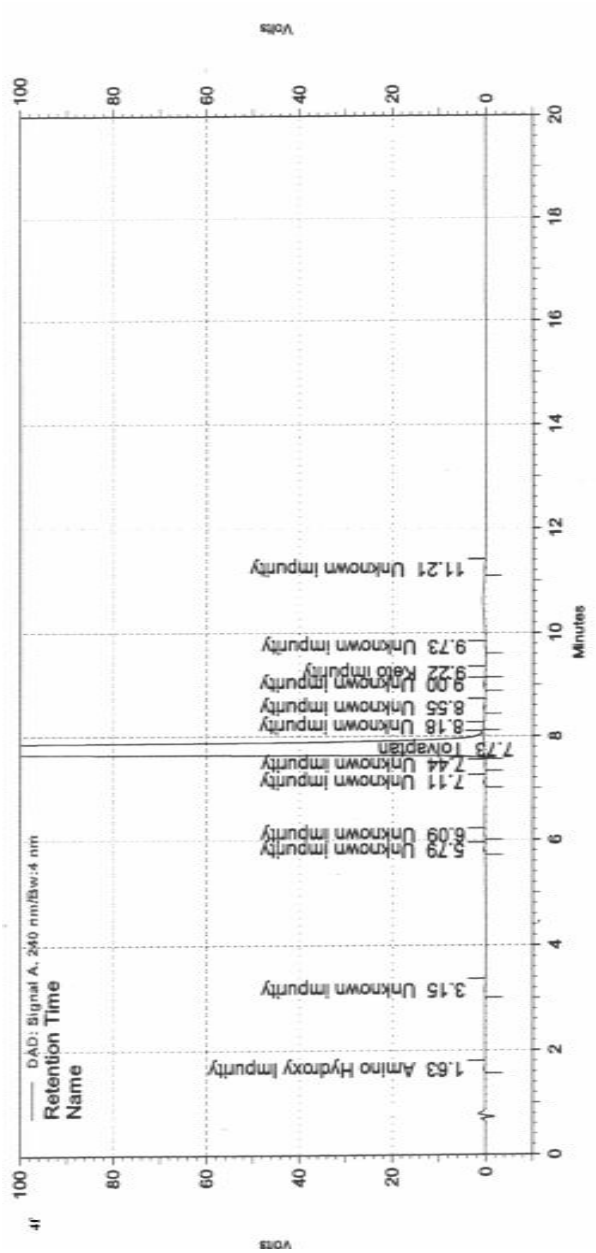
Fig 4d) Chromatogram of Tolvaptan water degradation (Water at 60°C).



DAD: Signal A, 240 nm/Bw:4 nm
Results

Name	Retention Time	Area	Area Percent	Relative RT
Amino Hydroxy Impurity	1.63	2691	0.01	0.20
Unknown impurity	3.15	1741	0.01	0.40
Unknown impurity	5.79	2840	0.01	0.75
Unknown impurity	6.09	1143	0.00	0.78
Unknown impurity	7.10	3270	0.01	0.92
Unknown impurity	7.43	1170	0.00	0.96
Tolvaptan	7.73	23540305	99.89	1.00
Unknown impurity	8.17	1255	0.01	1.06
Unknown impurity	8.54	3603	0.02	1.11
Unknown impurity	8.99	5058	0.02	1.17
Keto impurity	9.21	718	0.00	1.19
Unknown impurity	9.73	1023	0.00	1.26
Unknown impurity	11.19	2162	0.01	1.45

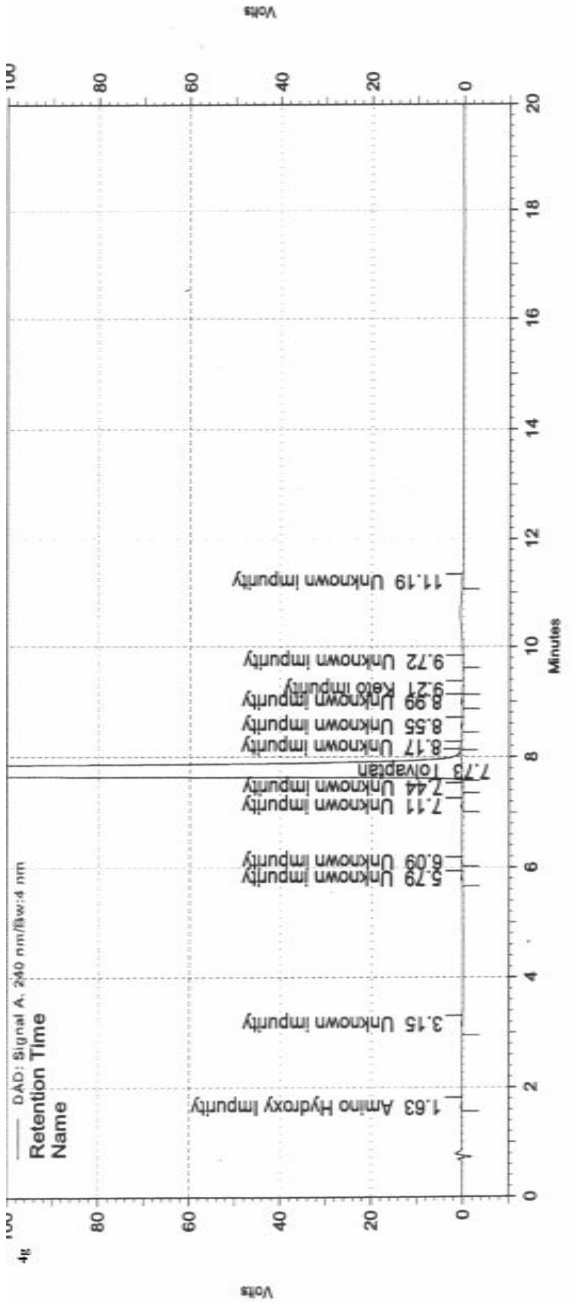
Fig 4e) Chromatogram of Tolvaptan thermal degradation (under oven at 60°C).



DAD: Signal A, 240 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Relative RT
Amino Hydroxy Impurity	1.63	2632	0.01	0.20
Unknown impurity	3.15	2188	0.01	0.40
Unknown impurity	5.79	2912	0.01	0.75
Unknown impurity	6.09	1182	0.01	0.78
Unknown impurity	7.11	3286	0.01	0.92
Unknown impurity	7.44	1124	0.00	0.96
Tolvaptan	7.73	23527326	99.89	1.00
Unknown impurity	8.18	1291	0.01	1.06
Unknown impurity	8.55	3614	0.02	1.11
Unknown impurity	9.00	5042	0.02	1.17
Keto impurity	9.22	729	0.00	1.19
Unknown impurity	9.73	990	0.00	1.26
Unknown impurity	11.21	2085	0.01	1.46

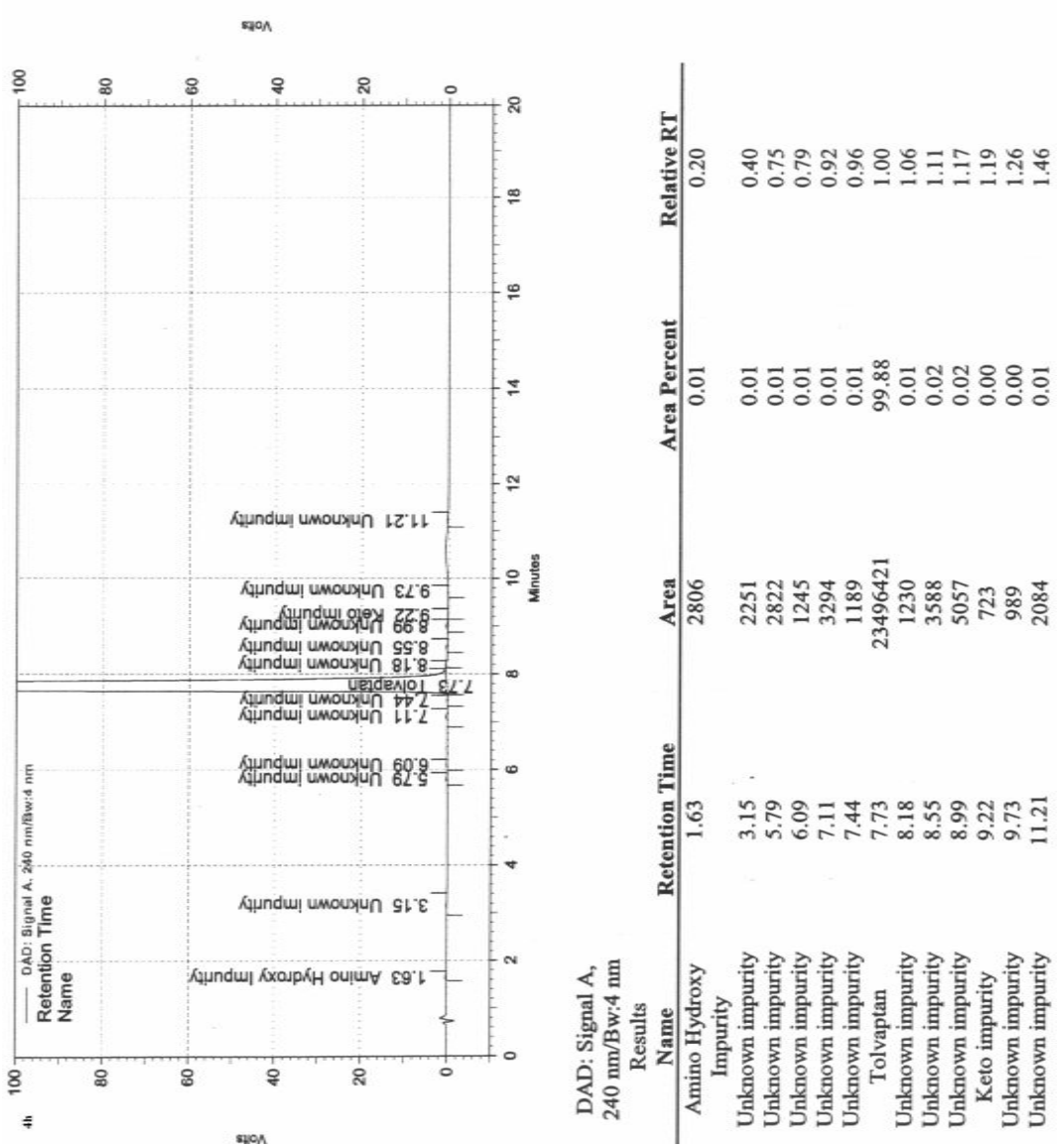
Fig 4f) Chromatogram of Tolvaptan Photolysis degradation (UV light for 200watt hours/m^2)



DAD: Signal A, 240 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Relative RT
Amino Hydroxy Impurity	1.63	2817	0.01	0.20
Unknown impurity	3.15	2123	0.01	0.40
Unknown impurity	5.79	2897	0.01	0.75
Unknown impurity	6.09	1077	0.00	0.79
Unknown impurity	7.11	3344	0.01	0.92
Unknown impurity	7.44	1144	0.00	0.96
Tolvaptan	7.73	23516758	99.89	1.00
Unknown impurity	8.17	1189	0.01	1.06
Unknown impurity	8.55	3472	0.01	1.11
Unknown impurity	8.99	5030	0.02	1.17
Keto impurity	9.21	773	0.00	1.19
Unknown impurity	9.72	1005	0.00	1.26
Unknown impurity	11.19	2001	0.01	1.45

Fig 4g) Chromatogram of Tolvaptan Photolysis degradation (Sun light1.2 million Lux hours).



DAD: Signal A, 240 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Relative RT
Amino Hydroxy Impurity	1.63	2806	0.01	0.20
Unknown impurity	3.15	2251	0.01	0.40
Unknown impurity	5.79	2822	0.01	0.75
Unknown impurity	6.09	1245	0.01	0.79
Unknown impurity	7.11	3294	0.01	0.92
Unknown impurity	7.44	1189	0.01	0.96
Tolvaptan	7.73	23496421	99.88	1.00
Unknown impurity	8.18	1230	0.01	1.06
Unknown impurity	8.55	3588	0.02	1.11
Unknown impurity	8.99	5057	0.02	1.17
Keto impurity	9.22	723	0.00	1.19
Unknown impurity	9.73	989	0.00	1.26
Unknown impurity	11.21	2084	0.01	1.46

Fig 4h) Chromatogram of Tolvaptan humidity degradation (75% humidity).

CONCLUSIONS

The proposed RP-HPLC method satisfies the parameters like system suitability, specificity, precision, accuracy, linearity, and robustness, ruggedness. The obtained results from the validation as per the ICH guidelines and drug stability were indicates this method is accurate, sensitive and best suitable Method for determination of known and unknown impurities in Tolvaptan regular laboratory analysis.

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