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CHROMATOGRAPHIC METHOD DEVELOPMENT AND VALIDATION STABILITY-INDICATING TWO IMPURITIES AND ITS DEGRADATION PRODUCTS IN PYRIDOSTIGMINE BROMIDE ORAL SOLUTION, 60 MG/5 ML

***Naresh Chandra Reddy Male and Kavitha Male**

India.

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***Corresponding Author**
Naresh Chandra Reddy Male
India.

ABSTRACT

A Novel RPHPLC Quantification method was developed for estimation of Pyridostigmine known impurities like its Hydroxy N-Methyl Impurity, Pyridine Dimethyl Carbamate Impurity which, were separated on Phenomenex Kinetex C18 column (150 mm x 4.6 mm; 5µ). Using a mixture of Phosphate Monobasic, 1.74 g of Potassium Phosphate Dibasic and 2.16 g of 1-Octane Sulfonic Acid Sodium Salt Anhydrous, Acetonitrile as a gradient mobile phase with a flow rate of 1.0 ml/min; λ max at 220 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, Pyridostigmine, Related substances, Stability-indicating.

INTRODUCTION

Pyridostigmine 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). Pyridostigmine 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] Pyridostigmine is available as a tablet for administration. Many techniques have been reported quantitave estimation including Spectrophotometric.[3-4] liquid chromatographic,[5-6] UPLC.[7]

Since no method has been developed for the separation and estimation of impurities in Pyridostigmine oral solution 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., Hydroxy N-MethylImpurity (3-Hydroxy-1-methylpyridin-1-ium bromide) and Pyridine Dimethyl CarbamateImpurity (Pyridin-3-yl dimethyl carbamate) and degrading products under stress conditions present in Pyridostigmine oral solution. The method was validated as per ICH guidelines.[9]

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

EXPERIMENTAL

MATERIAL AND METHODS

Chromatographic Conditions

Waters HPLC consisting pump, Auto sampler, VWD & photo diode array detector, Empower software connected with a Phenomenex Kinetex C18, 150 x 4.6 mm, 5 µm, 100A.

Chemicals and reagents

Pyridostigmine 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 oral solution formulations purchased from local market Hyderabad, India.

Mobile phase

Dissolve2.72 g of Potassium Phosphate Monobasic, 1.74 g of Potassium Phosphate Dibasic and 2.16 g of 1-Octane Sulfonic Acid Sodium Salt Anhydrous in 1000 mL of water. Adjust pH of the solution to pH2.5 ± 0.05 with Diluted Phosphoric Acid Solution. Mix well, filter the solution through a Nylon membrane filter, further degas for at least 10 minutes. Label as Mobile Phase A, 100% Acetonitrile used as mobile phase-B, Pyridostigmine 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 25ºC and the detection was carried out at 220nm with an injection volume of 20µl.

Diluent

Dissolve 2.72 g of Potassium Phosphate Monobasic in 1000 mL of water. Mix well and label

as Buffer Solution.

Mix 950 mL of Buffer Solution with 50 mL of Acetonitrile, mix well and label as Diluent.

Standard solution preparation

Accurately weigh and transfer 25 mg of Pyridostigmine Bromide standard into a 250-mL volumetric flask. Add diluent about 60% volume of the flask, sonicate to dissolve, dilute to volume with diluent and mix well. The concentration is about 100 µg/mL of Pyridostigmine Bromide.

Pipet 2.0 mL of Standard Stock Solution into a 100-mL volumetric flask, dilute to the volume with diluent and mix well. The concentration is about 2.0 µg/mL of Pyridostigmine Bromide.

Placebo preparation

Prepare Placebo Sample Solution, if required, by weighing and transferring about 5.13 g of Placebo of Pyridostigmine Bromide Oral Solution into a 50-mL volumetric flask. Add diluent to about 60% volume of the flask, sonicate for 10 minutes with intermittent shaking. Dilute to volume with diluent and mix well. Label as Placebo Sample Solution.

Sample preparation

Accurately weigh and transfer about 5.13 g of Pyridostigmine BromideOral Solution, USP, 60mg/5 mL into a 50-mL volumetric flask. Add diluentto about 60% volume of the flask, sonicate for 10 minutes with intermittent shaking. Dilute to volume with diluent and mix well. Label as Sample Solution. The concentration is about 1000 µg/mL of Pyridostigmine Bromide.

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 1.0, theoretical plates about 55000, % RSD is 1.3 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 purity angle less than purity threshold, so the method is specific for Pyridostigmine oral solution. The chromatogram of spiked impurities with Pyridostigmine 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 Pyridostigmine 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 i.e., 0.04%, 0.10%, 0.20%, 0.32%, 0.40% and 1.2% 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 Hydroxy N-Methyl Impurity, and Pyridine Dimethyl Carbamate Impurity) at the level of LOQ, 100% and 200% of target concentration, as the recovery results were found between 90 to 110% the method is accurate for the estimation of Pyridostigmine oral solution 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 25±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 and transferred 5.13 g of Pyridostigmine Bromide Oral Solution or 5.13 g of Placebo Oral Solution into a separate 50-mL volumetric flask.

Pipetted 5.0 mL of 0.1 N Hydrochloric Acid Solution into the flask, mixed well and tightly closed the flask. Kept the solution under 60°C for 24 hours.

After 24 hours of acid hydrolysis, pipetted 5.0 mL of 0.1 N Sodium Hydroxide solution into the flask to neutralize the solution. 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. 8).

Base Hydrolysis Stress Study

Weighed and transferred 5.13 g of Pyridostigmine Bromide Oral Solution or 5.13 g of Placebo Oral Solution into a separate 50-mL volumetric flask.

Pipetted 5.0 mL of 0.1 N Sodium Hydroxide Solution into the flask, mixed well and tightly closed the flask. Kept the solution under room temperature for 30 minutes.

After 30 minutes of base hydrolysis, pipetted 5.0 mL of 0.1 N Hydrochloric Acid solution into the flask to neutralize the solution. 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. 9)

Peroxide Oxidation Stress Study

Weighed and transferred 5.13 g of Pyridostigmine Bromide Oral Solution or 5.13 g of Placebo Oral Solution into a separate 50-mL volumetric flask.

Pipetted 5.0 mL of 3% Hydrogen Peroxide into the flask, mixed well and tightly closed the flask. Kept the solution under room temperature condition for 24 hours.

After 24 hours of oxidation, followed the impurity sample preparation as described in attached test method. 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. 11).

Water degradation Stress study

Weighed and transferred 5.13 g of Pyridostigmine Bromide Oral Solution or 5.13 g of Placebo Oral Solution into a separate 50-mL volumetric flask.

Pipetted 5.0 mL of Water into the flask, mixed well and tightly closed the flask. Kept the solution under 60°C for 24 hours.

After 24 hours of water hydrolysis, followed the impurity sample preparation as described in attached test method. 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. 10).

Heat Stress Study

Weighed and transferred 5.13 g of Pyridostigmine Bromide Oral Solution or 5.13 g of Placebo Oral Solution into a separate 50-mL volumetric flask. Tightly closed the flask and kept the flask under 70°C for 24 hours.

After exposure under 70°C for 24 hours, prepared the sample solutions following the impurity sample preparation as described in attached test method. 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. 12)

Table 1: HPLC Gradient Program.

Time (Minutes)	Mobile phase-A (%)	Mobile phase-B (%)
0	94	6
5	94	6
10	90	10
15	90	10
20	85	15
24	85	15
30	80	20
35	80	20
40	94	6
45	94	6

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	Pyridostigmine	4.0	0.011	14.0	0.045
2	HydroxyN-Methyl Impurity	4.0	0.011	11.0	0.044
3	PyridineDimethyl Carbamate Impurity	3.0	0.010	12.0	0.039

Table 3: Linearity Results.

Compound Name	Correlation coefficient	Slope	Y-Intercept	Residual sum square	Residual standard deviation
Pyridostigmine	0.9998	24043	-0.63%	1562970.377	19285
Hydroxy N-Methyl Impurity	0.9997	31138	0.07%	3102838.623	12548
Pyridine Dimethyl Carbamate Impurity	1.0000	30266	1.42%	3099847.163	14289

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

Parameter	Hydroxy N-Methyl Impurity	Pyridine Dimethyl Carbamate Impurity
Precision(n=6)	**0.42**	**0.52**
Intermediate Precision(n=6)	**0.24**	**0.15**
Mean	**0.52**	**0.64**

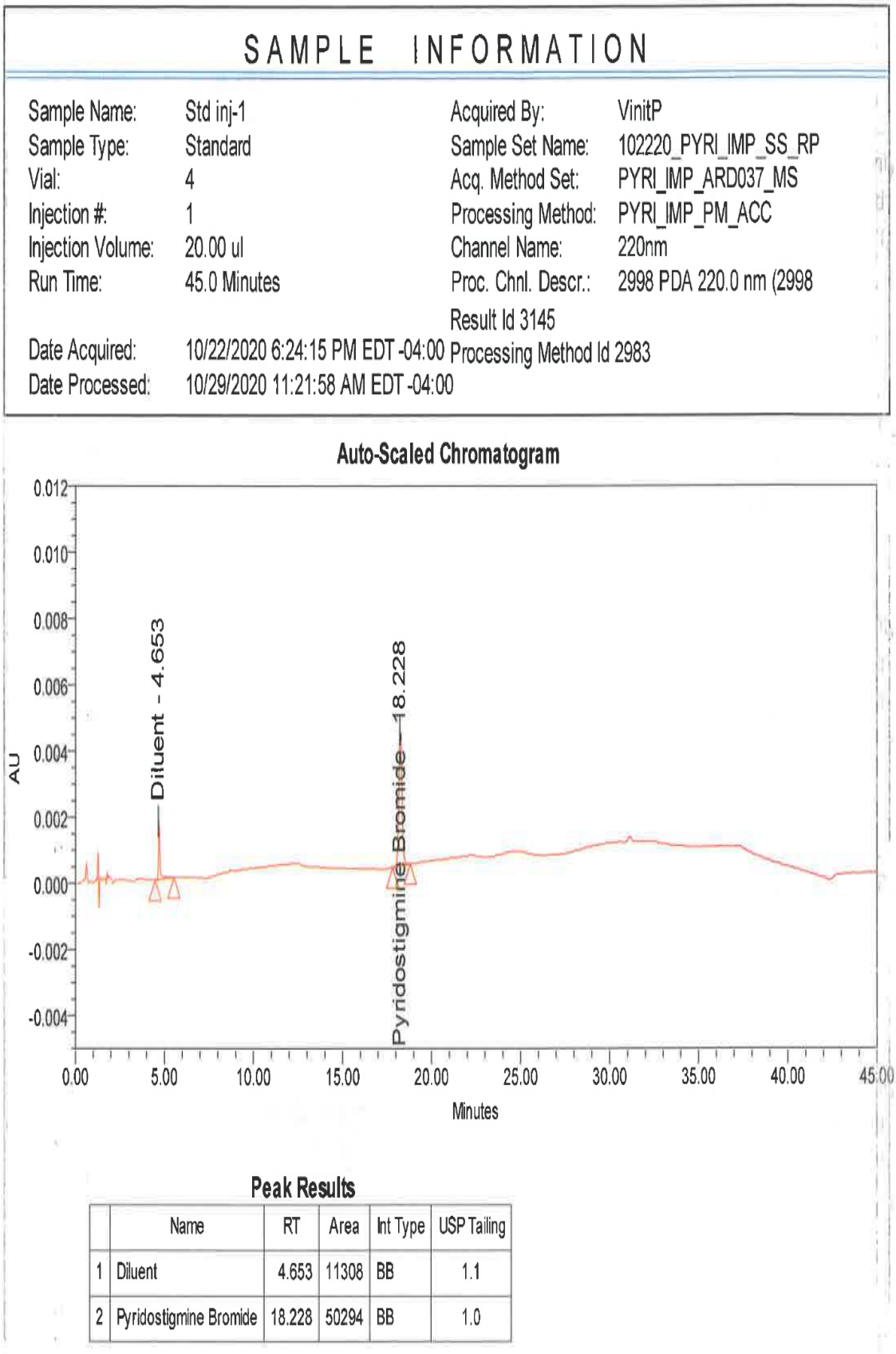
RRT&RF Values		
RRT values	**~ 0.624**	**~ 1.158**
RF values	**1.31**	**1.17**

Table 5: Accuracy results.

Spike Level	***Amount added(µg/mL)***	***Amount found(µg/mL)***	***% Mean Recovery***	***%RSD***
Recovery of Hydroxy N-Methyl Impurity				
LOQ level	0.4353	0.4352	100.25	0.65
100%	2.1766	2.1526	100.10	0.72
200%	4.3533	4.2433	100.52	0.54
Recovery of Pyridine Dimethyl Carbamate Impurity				
LOQ level	0.4235	0.4224	100.01	0.21
100%	2.1241	2.1321	99.92	0.15
200%	4.2451	4.2151	99.52	0.12

Table-6: Degradation Results.

Sample Name	**Condition**	**% Rec Based on Control**	**Total Impurity (%)**	**Mass Balance**	**Purity Angle**	**Purity Threshold**
Control	Not Stressed	100.0	0.000	100.0	0.515	0.669
Acid Hydrolysis	5.0 mL of 0.1 N HCl, 60°C for 24 hours	98.6	0.438	99.0	0.511	0.657
Base Hydrolysis	5.0 mL of 0.1 N NaOH, Room Temperature for 30 minutes	87.2	6.120	93.3	0.387	0.540
Water Hydrolysis	5.0 mL of Water, 60°C for 24 hours	100.0	0.025	100.0	0.501	0.671
Oxidation	5.0 mL of 3% H_2O_2, Room Temperature for 24 hours	100.3	0.116	100.4	0.556	0.685
UV/White Light	SUNTEST CPS+, Room Temperature for 8 hours*	100.0	0.000	100.0	0.512	0.670
Elevated Temperature	70°C for 24 hours	100.2	0.068	100.3	0.493	0.673



	Name	RT	Area	Int Type	USP Tailing
1	Diluent	4.653	11308	BB	1.1
2	Pyridostigmine Bromide	18.228	50294	BB	1.0

Fig. 1: Representative Chromatogram of Standard Solution.

SAMPLE INFORMATION

Sample Name:	System suitability solution	Acquired By:	VinitP
Sample Type:	Control	Sample Set Name:	102220_PYRI_IMP_SS_RP
Vial:	2	Acq. Method Set:	PYRI_IMP_ARD037_MS
Injection #:	1	Processing Method:	PYRI_IMP_PM_ACC
Injection Volume:	20.00 ul	Channel Name:	220nm
Run Time:	45.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
		Result Id 3114	
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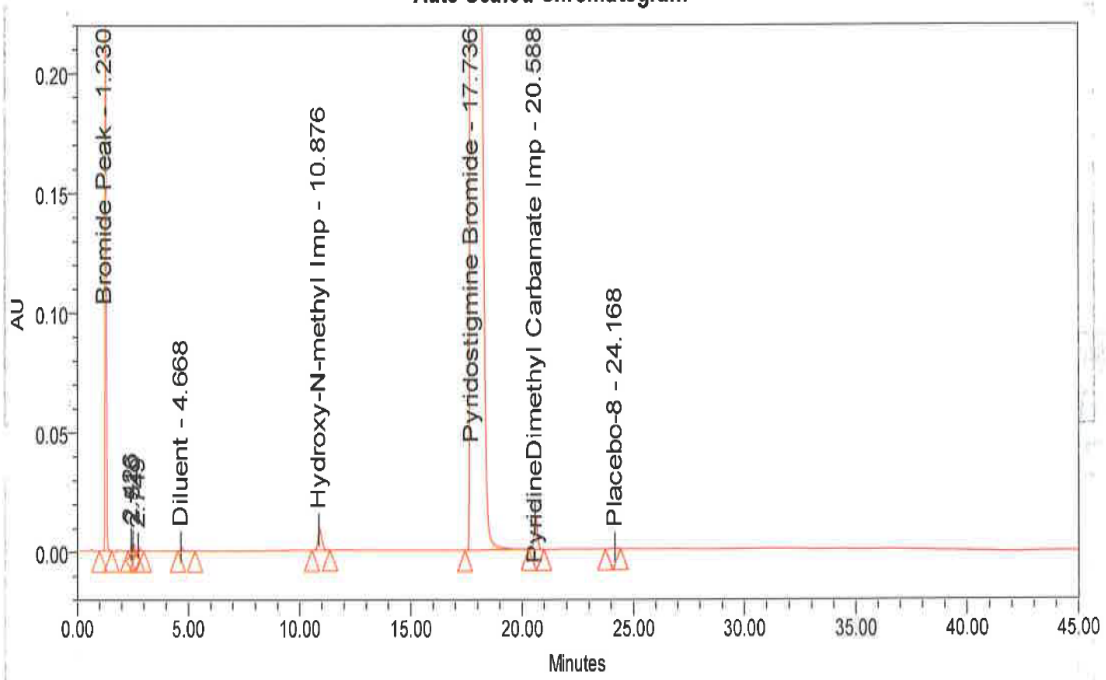
Date Processed:	10/29/2020 11:21:55 AM EDT -04:00		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Int Type	RT Ratio
1	Bromide Peak	1.230	877937	BB	
2		2.426	16379	BV	
3		2.510	12937	VB	
4		2.749	8402	BB	
5	Diluent	4.668	10773	BB	
6	Hydroxy-N-methyl Imp	10.876	106482	BB	0.613
7	Pyridostigmine Bromide	17.736	24952024	BB	1.000
8	PyridineDimethyl Carbamate Imp	20.588	142390	BB	1.161
9	Placebo-8	24.168	4207	BB	

Fig. 2: Representative Chromatogram of System Suitability Solution.

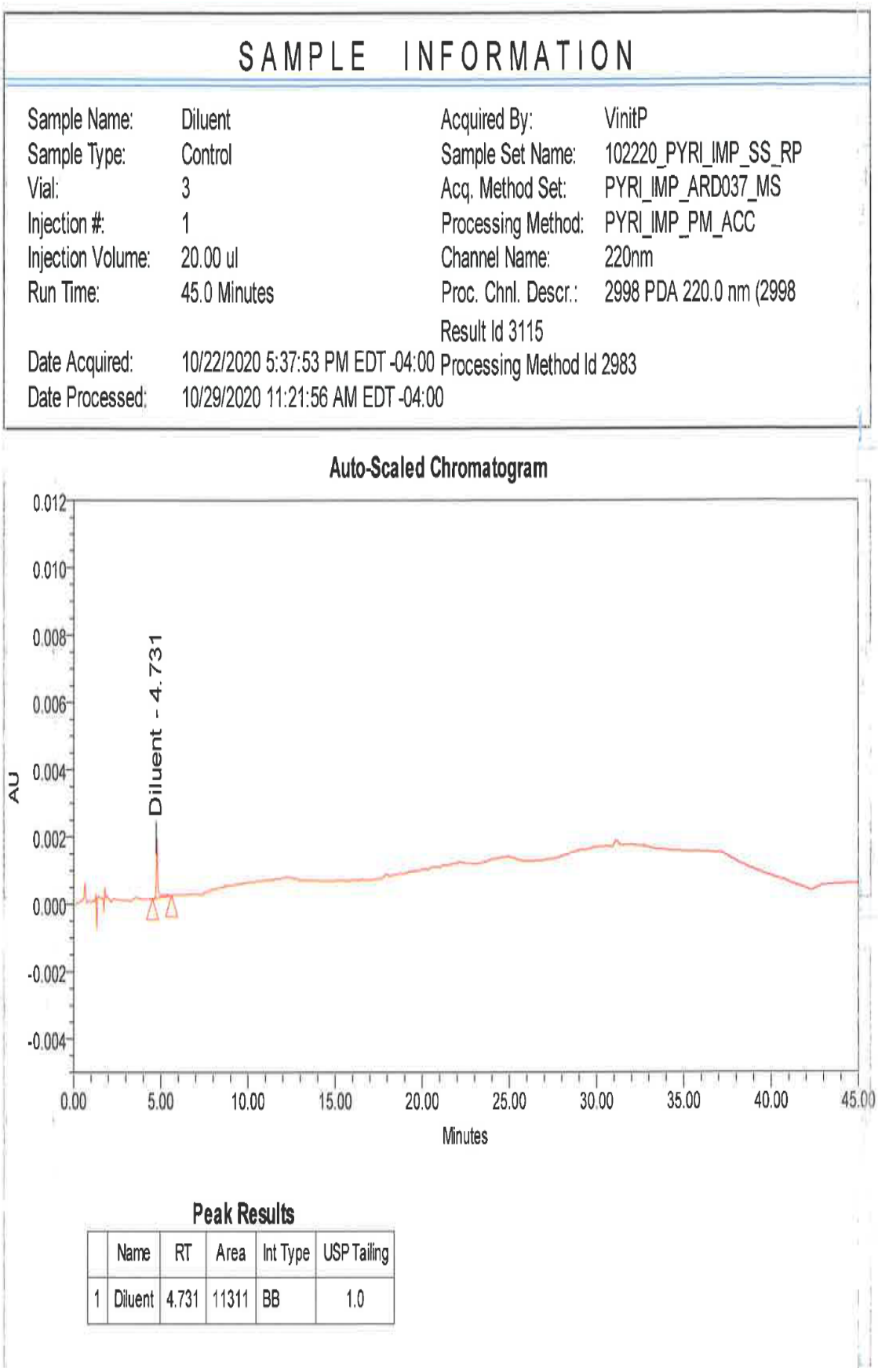
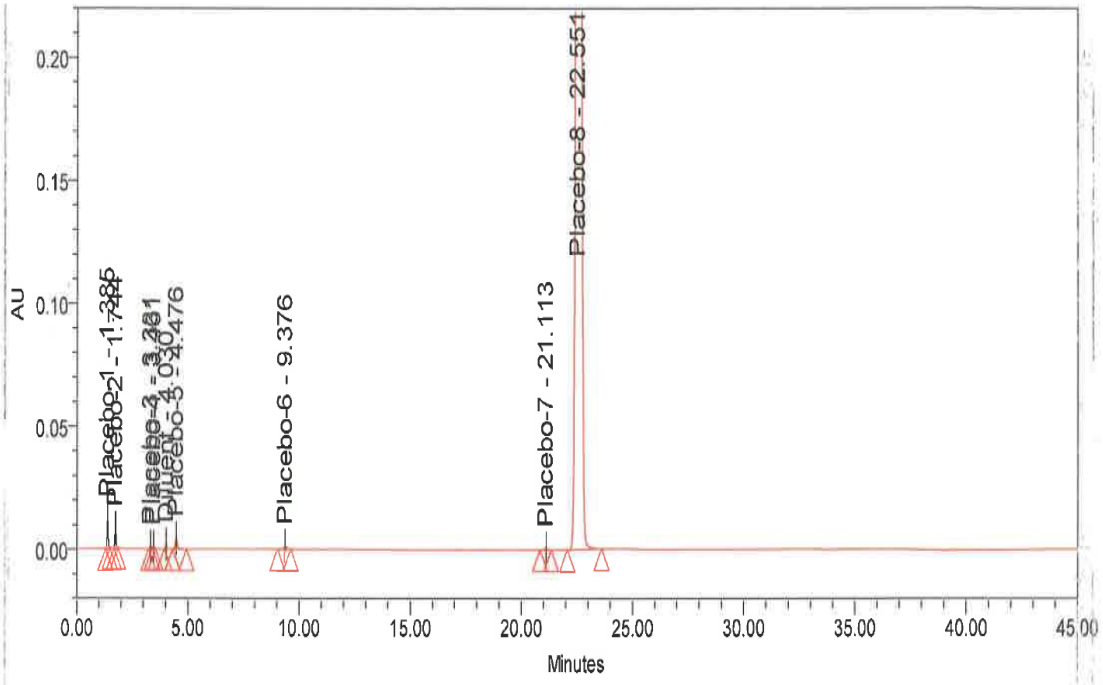


Fig. 3: Representative Chromatogram of Diluent.

SAMPLE INFORMATION

Sample Name:	Placebo interference solution	Acquired By:	VinitP
Sample Type:	Control	Sample Set Name:	102220_PYRI_IMP_SS
Vial:	12	Acq. Method Set:	PYRI_IMP_ARD001_MS
Injection #:	1	Processing Method:	PYRI_IMP_PM
Injection Volume:	20.00 ul	Channel Name:	220nm
Run Time:	45.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
		Result Id 2142	
Date Acquired:	10/23/2020 12:16:16 AM EDT -04:00	Processing Method Id 1937	
Date Processed:	10/27/2020 11:06:04 AM EDT -04:00		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Int Type	RT Ratio
1	Placebo-1	1.385	39179	BB	
2	Placebo-2	1.744	30806	BB	
3	Placebo-3	3.331	3499	BB	
4	Placebo-4	3.461	2770	BB	
5	Diluent	4.030	7913	BB	
6	Placebo-5	4.476	30426	BB	
7	Placebo-6	9.376	11446	BB	
8	Placebo-7	21.113	3828	BB	
9	Placebo-8	22.551	12302963	BB	

Fig. 4: Representative Chromatogram of Placebo Solution.

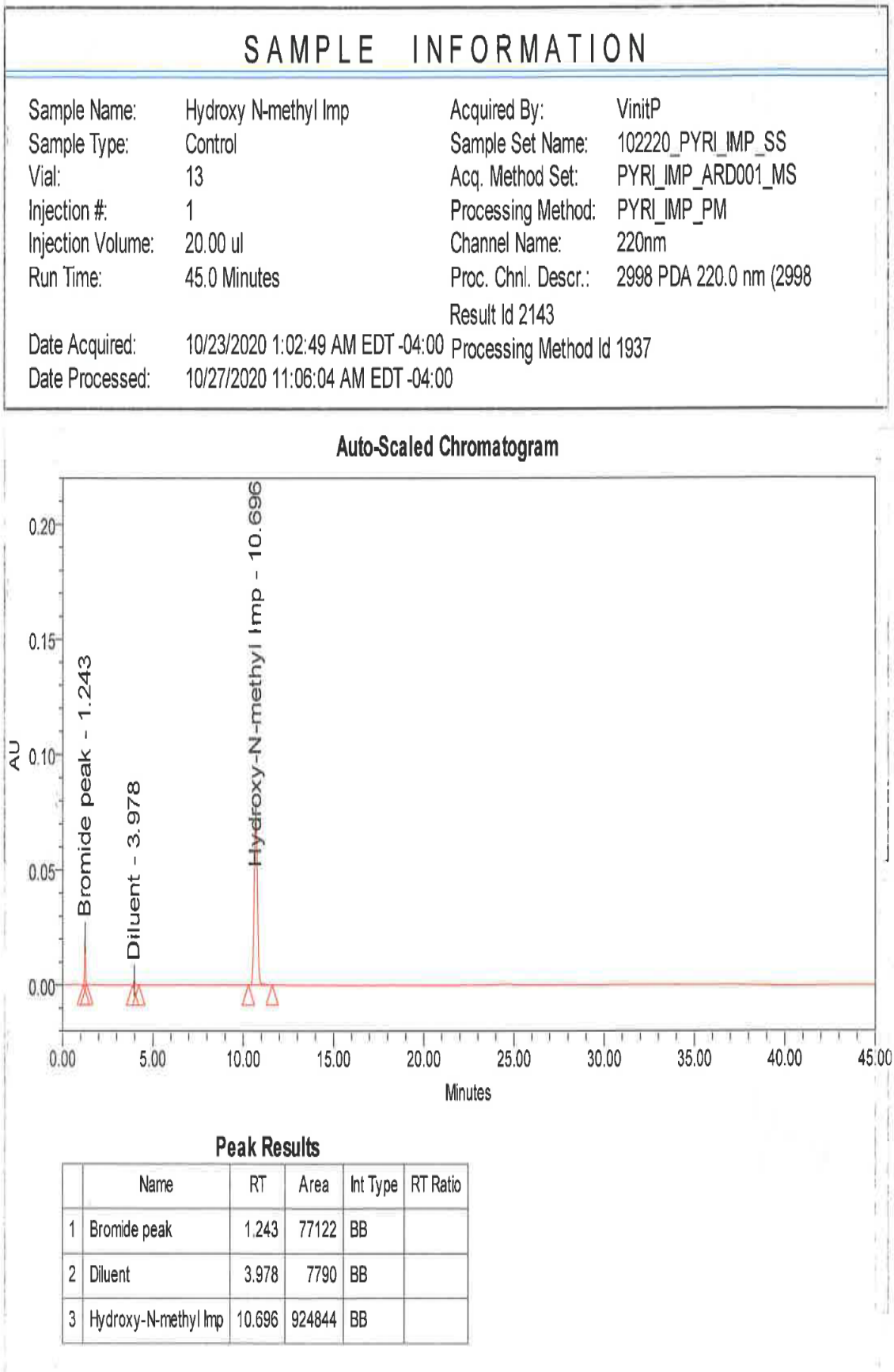
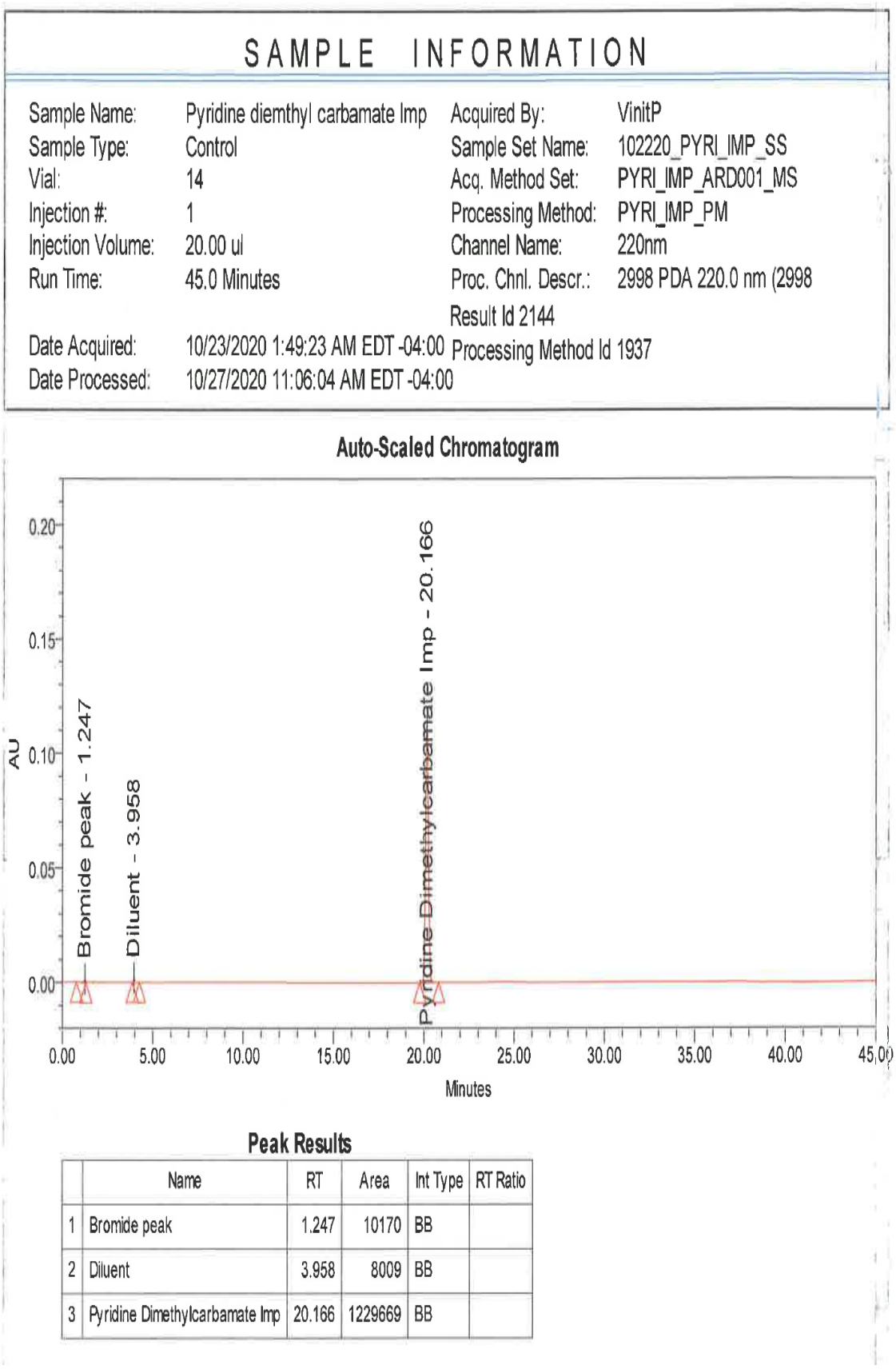


Fig. 5: Representative Chromatogram of Hydroxy N-Methyl Impurity Solution.



	Name	RT	Area	Int Type	RT Ratio
1	Bromide peak	1.247	10170	BB	
2	Diluent	3.958	8009	BB	
3	Pyridine Dimethylcarbamate Imp	20.166	1229669	BB	

Fig. 6: Representative Chromatogram of Pyridine Dimethyl Carbamate Impurity Solution.

SAMPLE INFORMATION

Sample Name:	Selectivity solution	Acquired By:	VinitP
Sample Type:	Control	Sample Set Name:	102220_PYRI_IMP_SS
Vial:	19	Acq. Method Set:	PYRI_IMP_ARD001_MS
Injection #:	1	Processing Method:	PYRI_IMP_PM
Injection Volume:	20.00 ul	Channel Name:	220nm
Run Time:	45.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
		Result Id 2149	
Date Acquired:	10/23/2020 5:41:49 AM EDT -04:00	Processing Method Id 1937	
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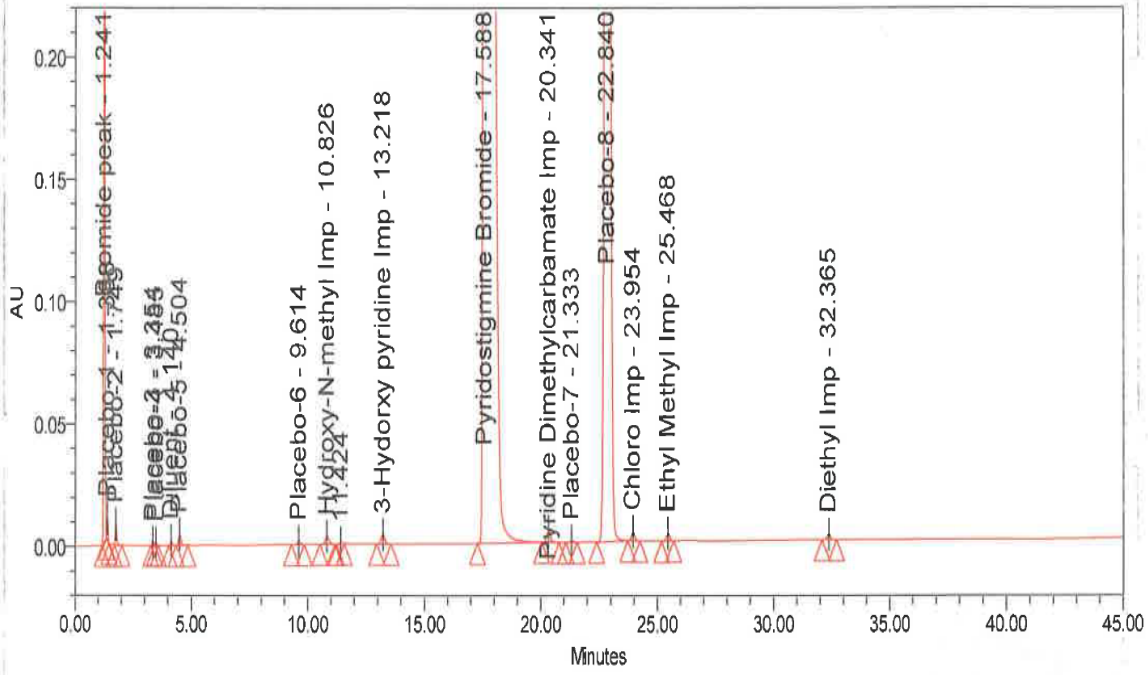
Auto-Scaled Chromatogram



Peak Results

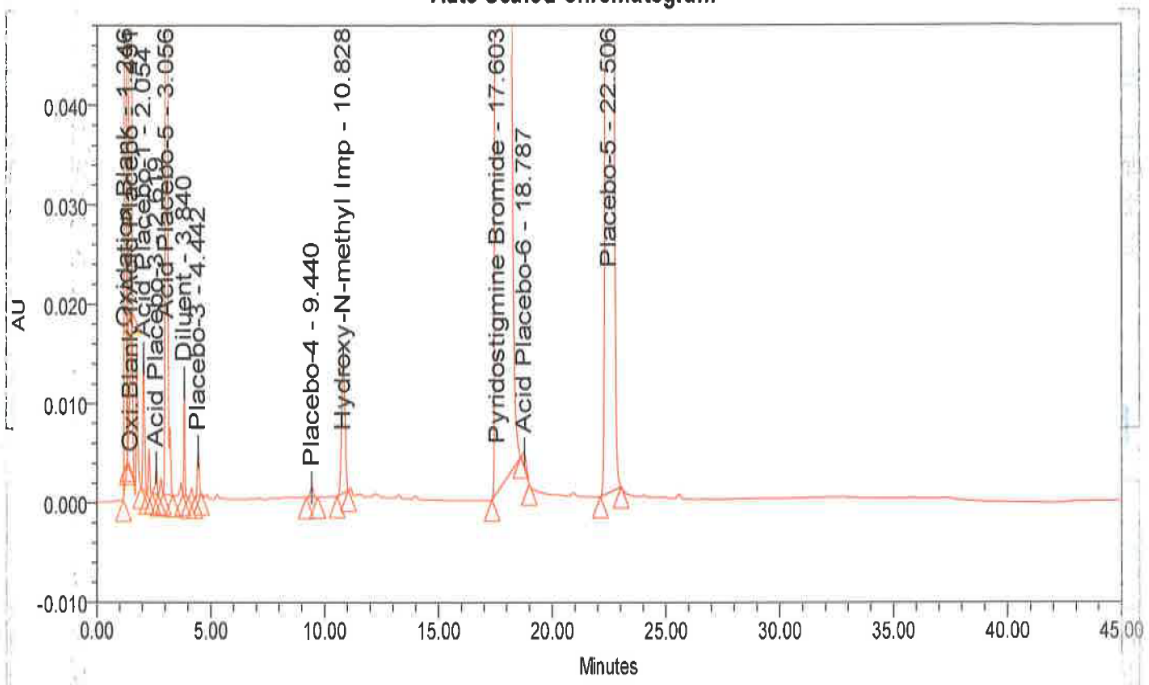
	Name	RT	Area	Int Type	RT Ratio
1	Bromide peak	1.241	1022532	BB	0.071
2	Placebo-1	1.388	25245	BB	0.079
3	Placebo-2	1.749	33983	BB	0.099
4	Placebo-3	3.354	3546	BB	0.191
5	Placebo-4	3.485	2759	BB	0.198
6	Diluent	4.140	7584	BB	0.235
7	Placebo-5	4.504	29749	BB	0.256
8	Placebo-6	9.614	11331	BB	0.547
9	Hydroxy-N-methyl Imp	10.826	38100	BB	0.616
10		11.424	2651	BB	
11	3-Hydorxy pyridine Imp	13.218	41700	BB	0.752

Fig. 7: Representative Chromatogram of Selectivity Solution.

SAMPLE INFORMATION

Sample Name:	Sample_0.1 N HCl @60°c-24H	Acquired By:	VinitP
Sample Type:	Unknown	Sample Set Name:	102820_PYRI_IMP_SS_SIS
Vial:	12	Acq. Method Set:	PYRI IMP ARD001 SIS MS
Injection #:	1	Processing Method:	PYRI_IMP_SIS_PM
Injection Volume:	20.00 ul	Channel Name:	220nm
Run Time:	45.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
		Result Id 5811	
Date Acquired:	10/28/2020 11:49:42 PM EDT -04:00	Processing Method Id 5508	
Date Processed:	11/5/2020 11:08:31 AM EST -05:00		

Auto-Scaled Chromatogram

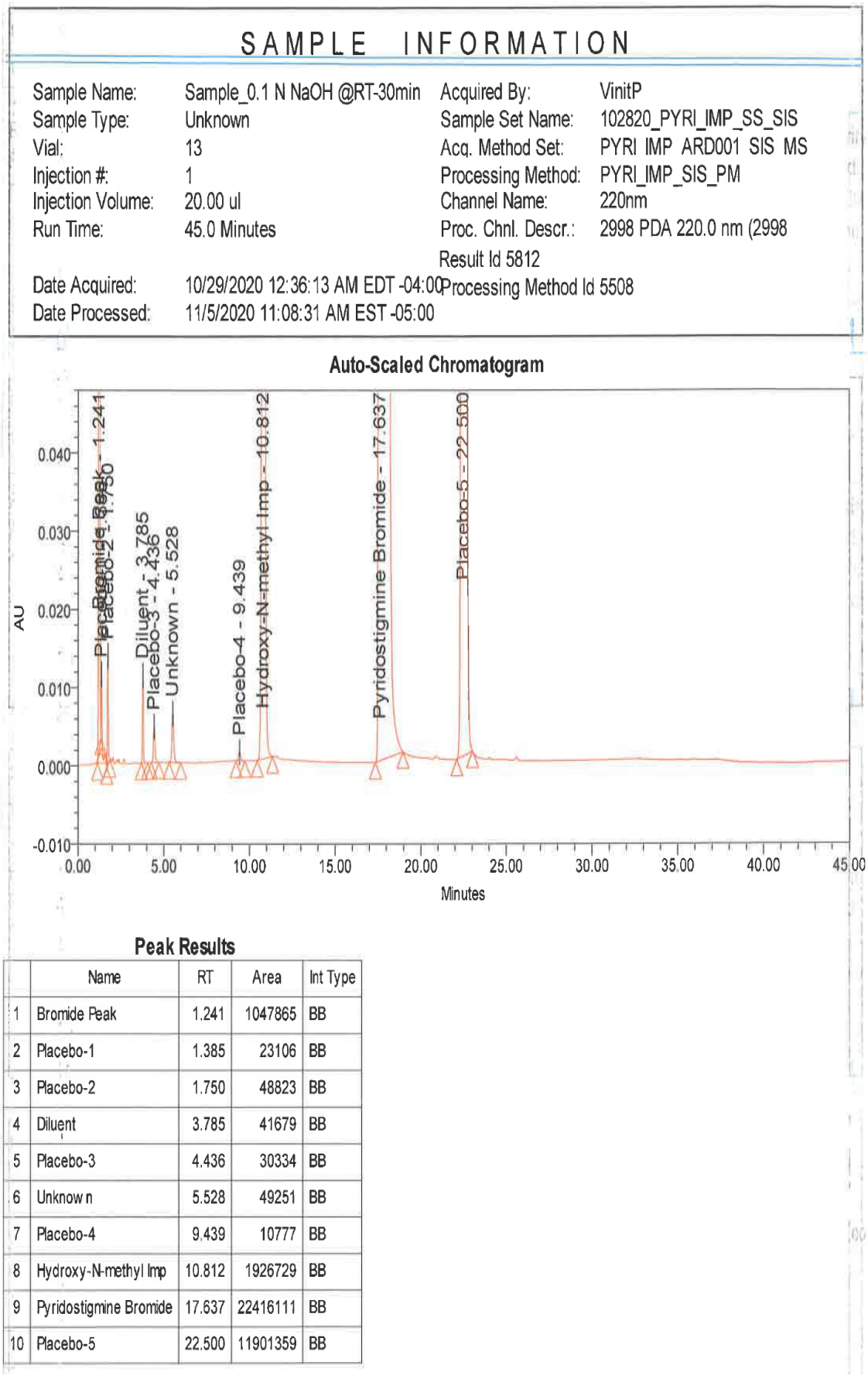


Peak Results

	Name	RT	Area	Int Type
1	Oxidation Blank	1.246	1045138	BB
2	Oxi.Blank-1/Acid Placebo	1.491	509114	BB
3	Acid Placebo-1	2.054	71035	BB
4	Acid Placebo-3	2.619	15316	BB
5	Acid Placebo-5	3.056	1169775	BB
6	Diluent	3.840	39793	BB
7	Placebo-3	4.442	26843	BB
8	Placebo-4	9.440	10183	BB
9	Hydroxy-N-methyl Imp	10.828	142257	BB
10	Pyridostigmine Bromide	17.603	25274044	BB
11	Acid Placebo-6	18.787	17260	BB

	Name	RT	Area	Int Type
12	Placebo-5	22.506	11758279	BB

Fig. 8: Representative Chromatogram of Acid Hydrolysis Sample Solution.



Peak Results

	Name	RT	Area	Int Type
1	Bromide Peak	1.241	1047865	BB
2	Placebo-1	1.385	23106	BB
3	Placebo-2	1.750	48823	BB
4	Diluent	3.785	41679	BB
5	Placebo-3	4.436	30334	BB
6	Unknown	5.528	49251	BB
7	Placebo-4	9.439	10777	BB
8	Hydroxy-N-methyl Imp	10.812	1926729	BB
9	Pyridostigmine Bromide	17.637	22416111	BB
10	Placebo-5	22.500	11901359	BB

Fig. 9: Representative Chromatogram of Base Hydrolysis Sample Solution.

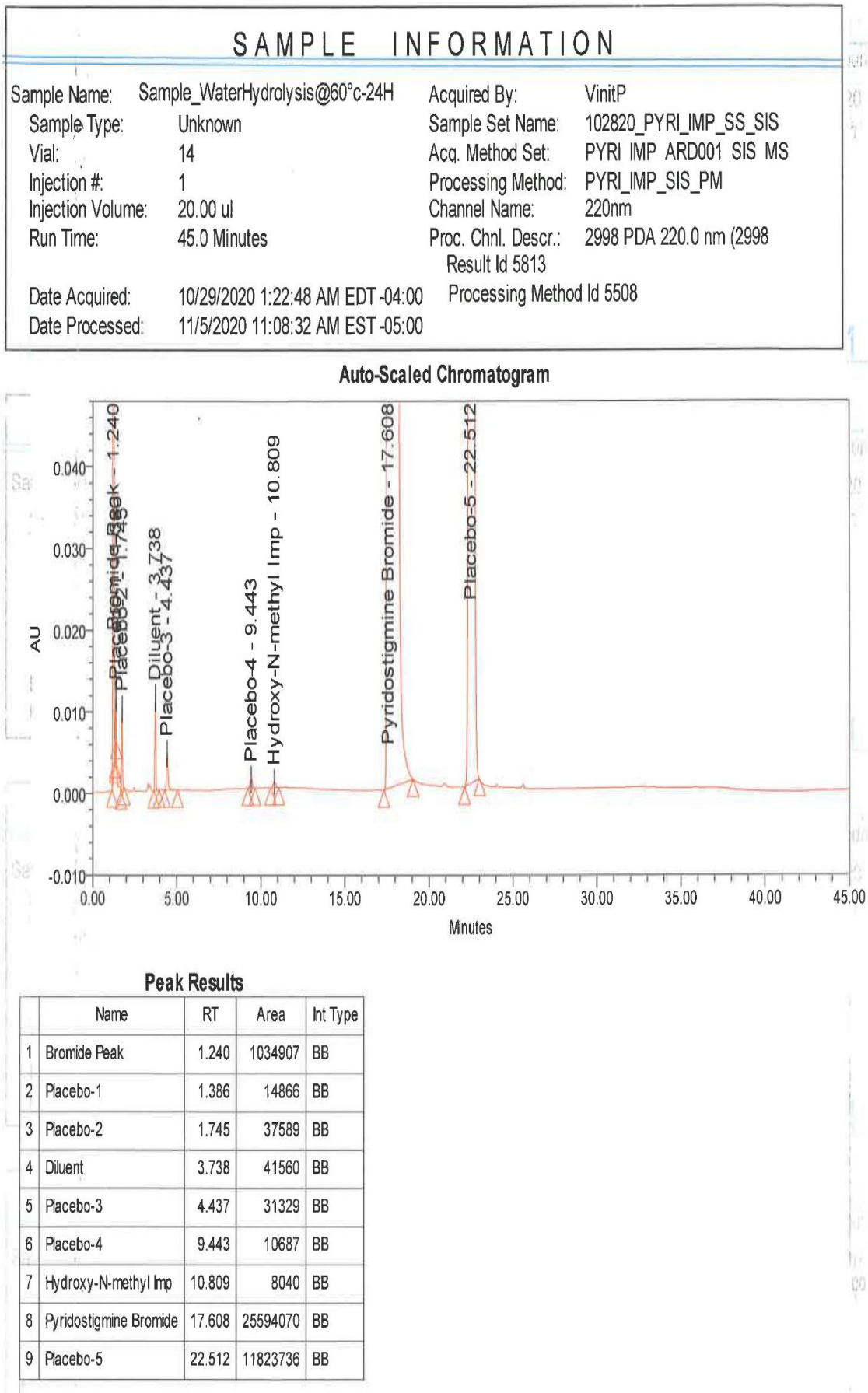
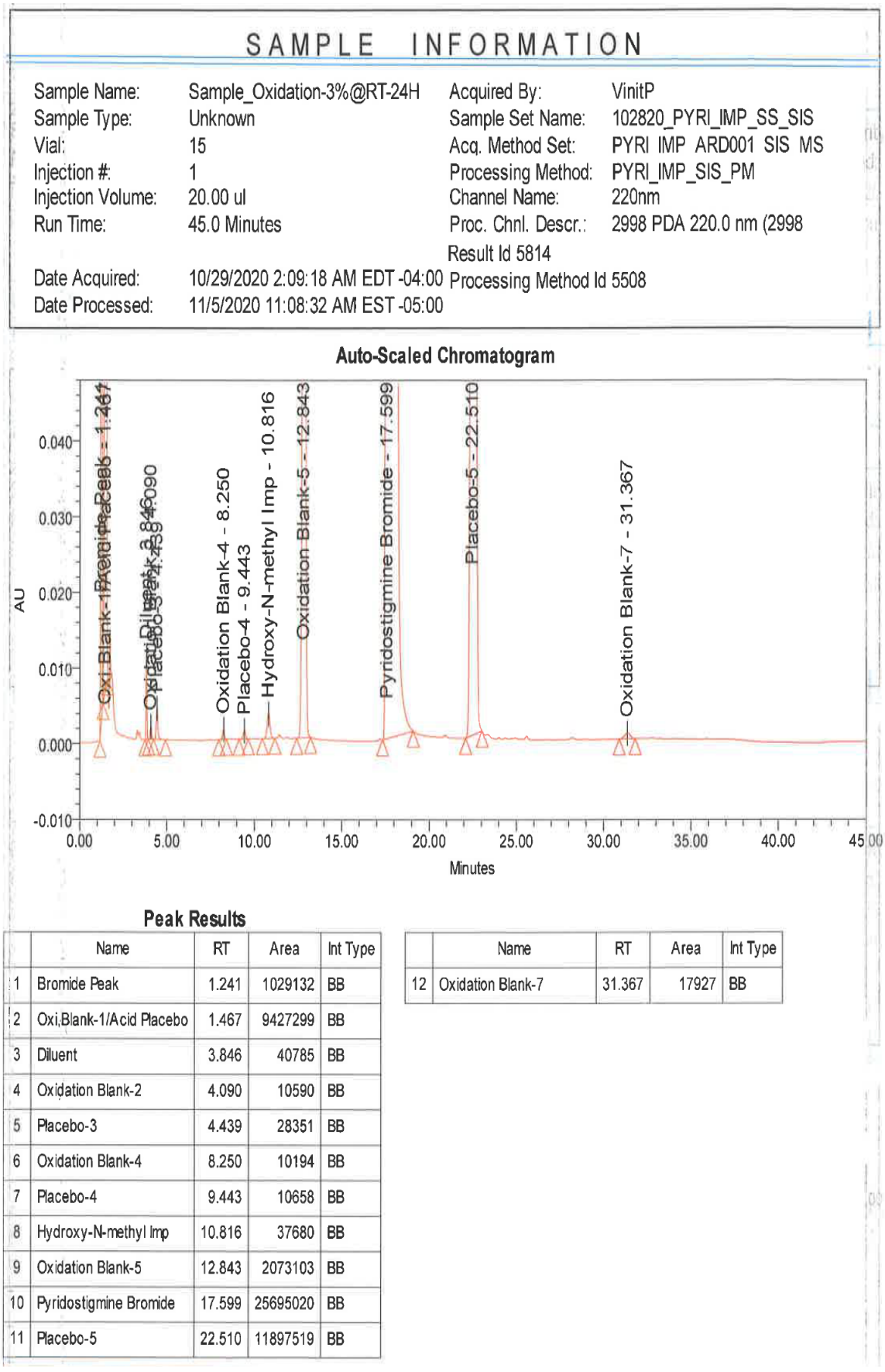


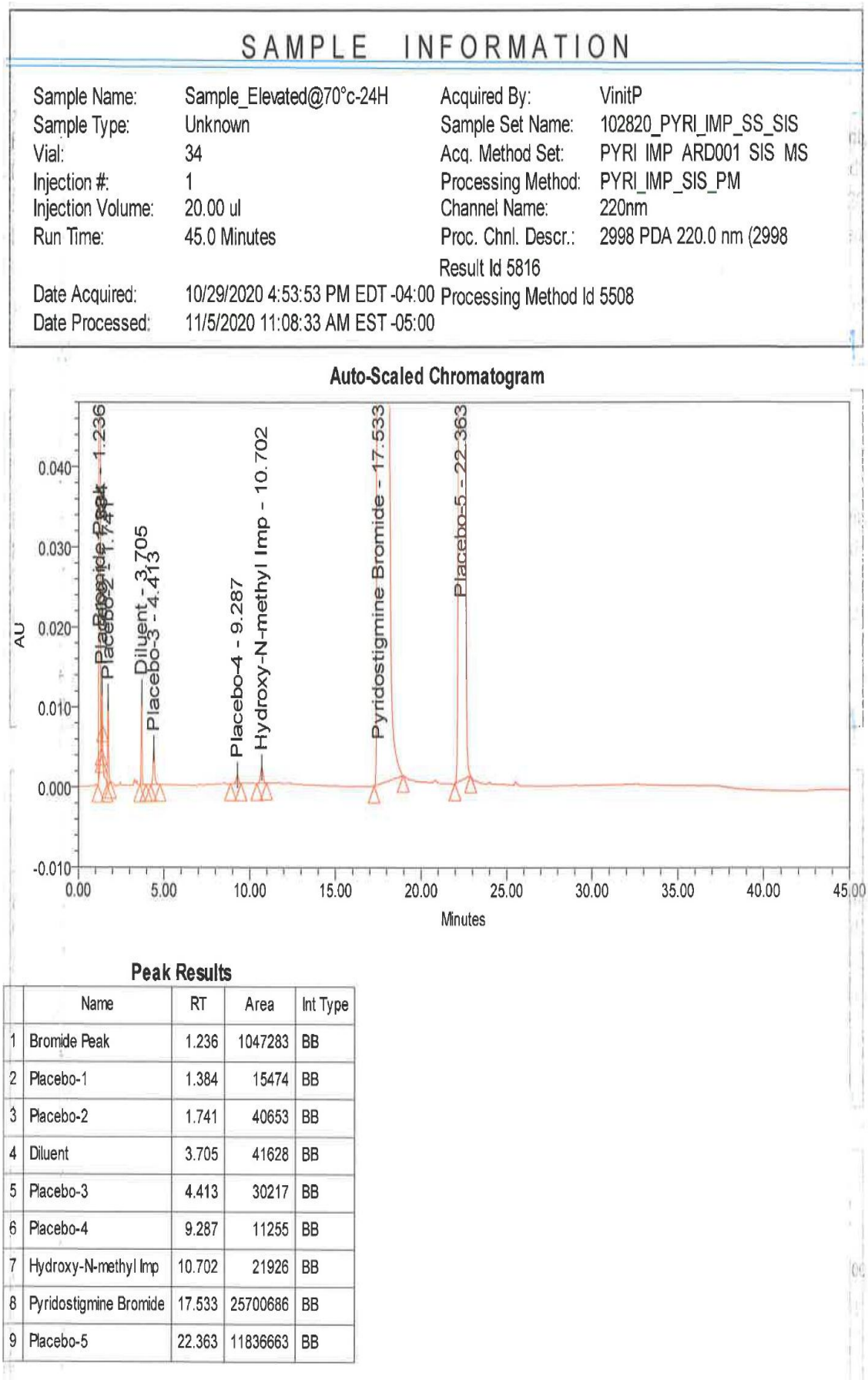
Fig. 10: Representative Chromatogram of Water Hydrolysis Sample Solution.



	Name	RT	Area	Int Type
1	Bromide Peak	1.241	1029132	BB
2	Oxi,Blank-1/Acid Placebo	1.467	9427299	BB
3	Diluent	3.846	40785	BB
4	Oxidation Blank-2	4.090	10590	BB
5	Placebo-3	4.439	28351	BB
6	Oxidation Blank-4	8.250	10194	BB
7	Placebo-4	9.443	10658	BB
8	Hydroxy-N-methyl Imp	10.816	37680	BB
9	Oxidation Blank-5	12.843	2073103	BB
10	Pyridostigmine Bromide	17.599	25695020	BB
11	Placebo-5	22.510	11897519	BB

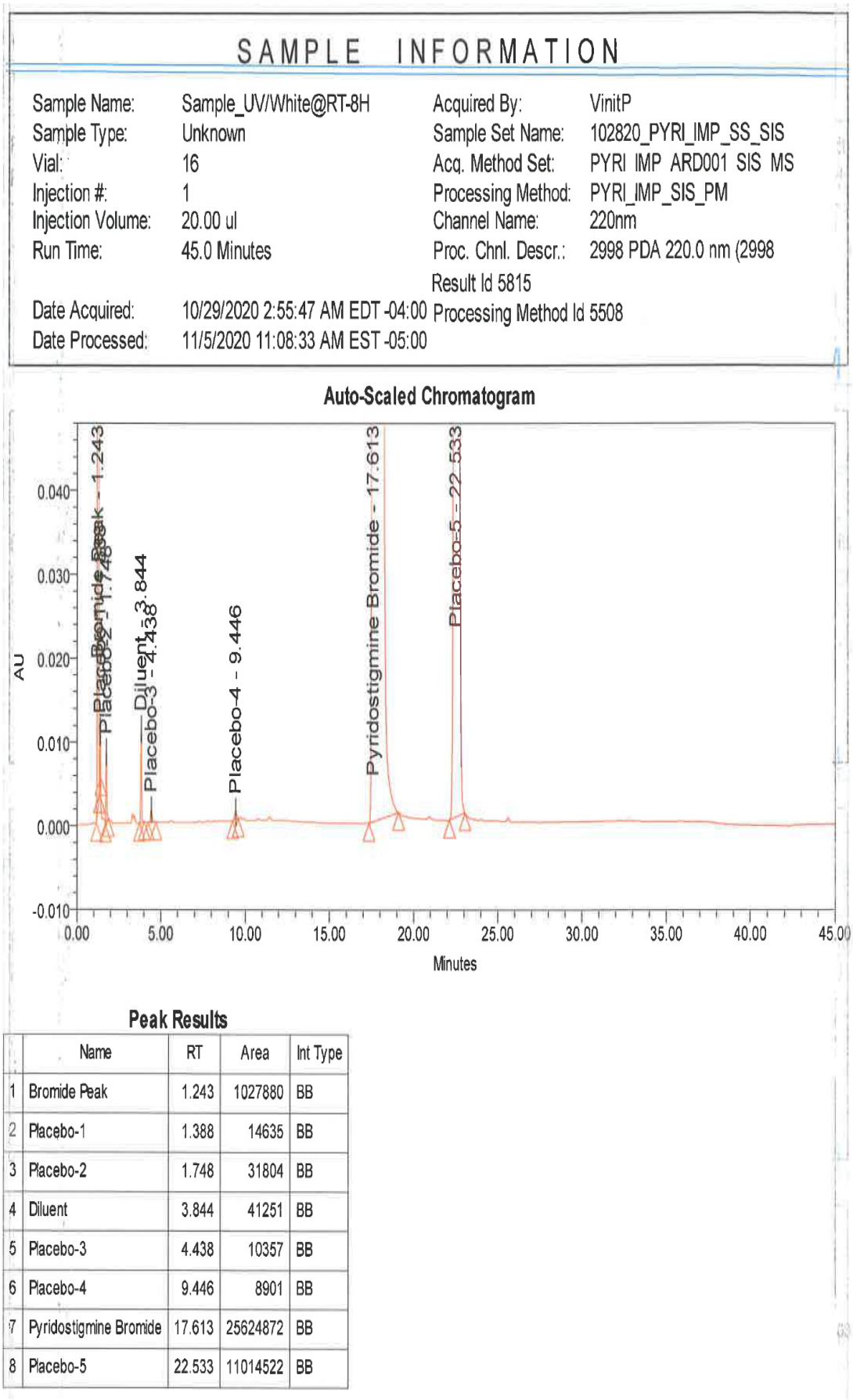
	Name	RT	Area	Int Type
12	Oxidation Blank-7	31.367	17927	BB

Fig. 11: Representative Chromatogram of Oxidation Sample Solution



	Name	RT	Area	Int Type
1	Bromide Peak	1.236	1047283	BB
2	Placebo-1	1.384	15474	BB
3	Placebo-2	1.741	40653	BB
4	Diluent	3.705	41628	BB
5	Placebo-3	4.413	30217	BB
6	Placebo-4	9.287	11255	BB
7	Hydroxy-N-methyl Imp	10.702	21926	BB
8	Pyridostigmine Bromide	17.533	25700686	BB
9	Placebo-5	22.363	11836663	BB

Fig. 12: Representative Chromatogram of 70°C Sample Solution.



Peak Results

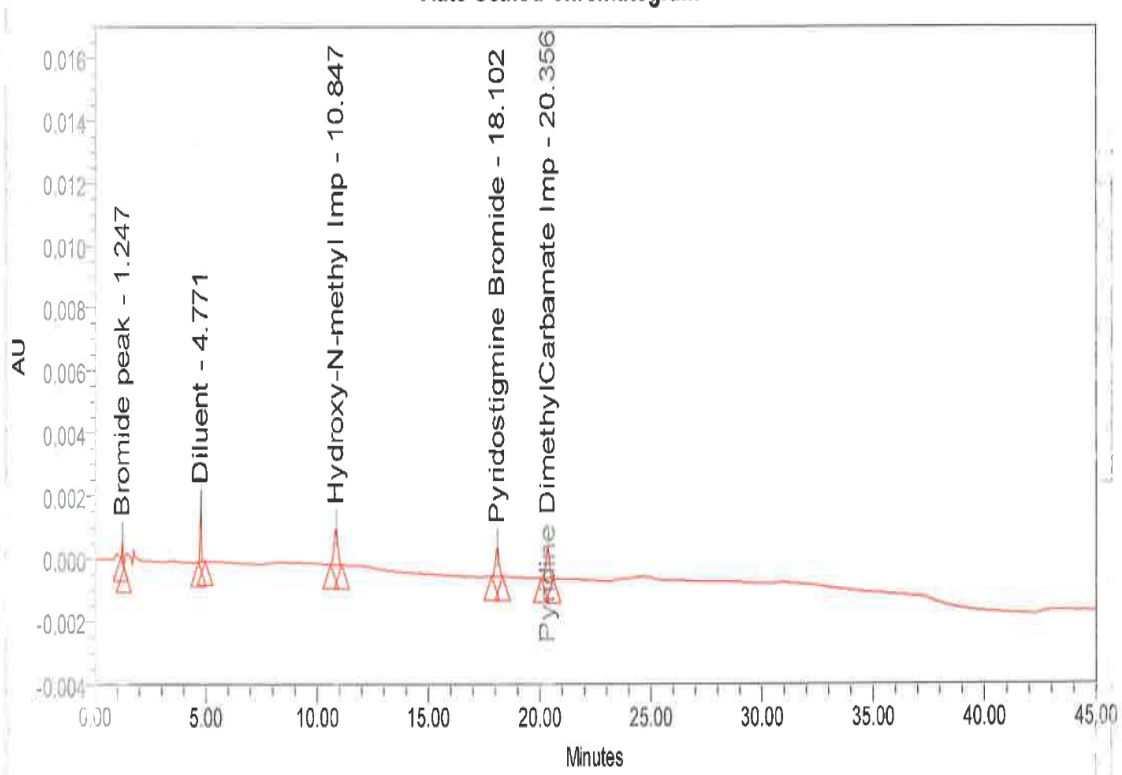
	Name	RT	Area	Int Type
1	Bromide Peak	1.243	1027880	BB
2	Placebo-1	1.388	14635	BB
3	Placebo-2	1.748	31804	BB
4	Diluent	3.844	41251	BB
5	Placebo-3	4.438	10357	BB
6	Placebo-4	9.446	8901	BB
7	Pyridostigmine Bromide	17.613	25624872	BB
8	Placebo-5	22.533	11014522	BB

Fig. 13: Representative Chromatogram of UV/White Light Sample Solution.

SAMPLE INFORMATION

Sample Name:	QL-1	Acquired By:	VinitP
Sample Type:	Control	Sample Set Name:	102220_PYRI_IMP_SS_LIN
Vial:	42	Acq. Method Set:	PYRI_IMP_ARD001_MS_LIN
Injection #:	1	Processing Method:	PYRI_IMP_PM_LIN
Injection Volume:	20.00 ul	Channel Name:	220nm
Run Time:	45.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
		Result Id 2541	
Date Acquired:	10/23/2020 7:27:27 PM EDT -04:00	Processing Method Id 2497	
Date Processed:	10/28/2020 9:28:40 AM EDT -04:00		

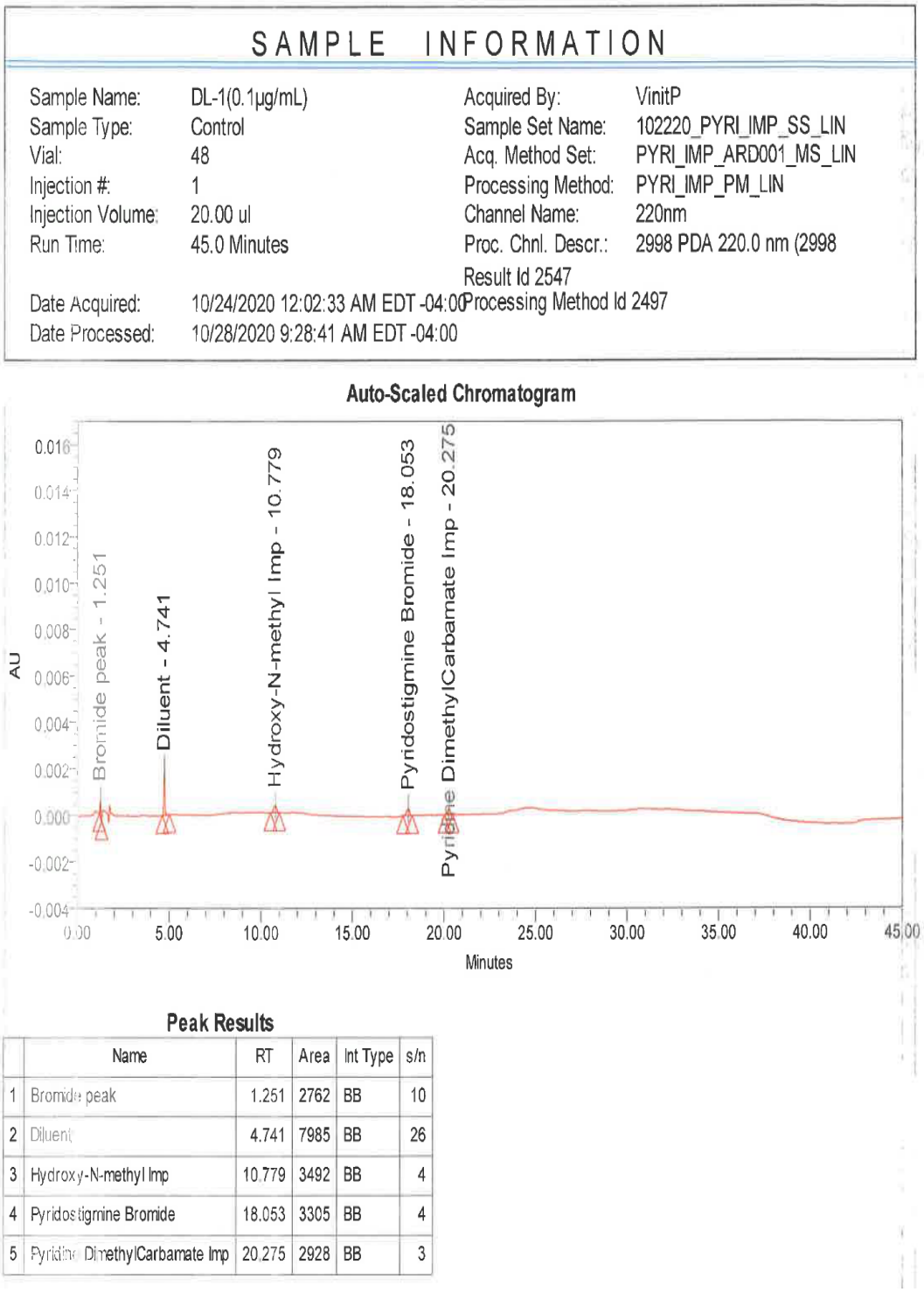
Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Int Type	s/n
1	Bromide peak	1.247	2813	BB	9
2	Diluent	4.771	6929	BB	21
3	Hydroxy-N-methyl Imp	10.847	13270	BB	14
4	Pyridostigmine Bromide	18.102	10506	BB	11
5	Pyridine DimethylCarbamate Imp	20.356	10935	BB	12

Fig. 14: Representative Chromatogram of Quantitation Limit Solution.



	Name	RT	Area	Int Type	s/n
1	Bromide peak	1.251	2762	BB	10
2	Diluent	4.741	7985	BB	26
3	Hydroxy-N-methyl Imp	10.779	3492	BB	4
4	Pyridostigmine Bromide	18.053	3305	BB	4
5	Pyridine DimethylCarbamate Imp	20.275	2928	BB	3

Fig. 15: Representative Chromatogram of Detection Limit Solution.

Photolytic Stress study

UV Light

Weighed and transferred 5.13 g of Pyridostigmine Bromide Oral Solution or 5.13 g of Placebo Oral Solution into a separate quartz culture dish with cover. Kept the dish under UV/White light (SUNTEST CPS+) for 8 hours.

After exposure under UV/White light, with aid of the diluent, transferredPyridostigmine Bromide Oral Solution or Placebo Oral Solution into a separate 50-mL volumetric flask and followed the impurity sample preparation as described in attached test method. 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. 13).

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 Pyridostigmine bromide regular laboratory analysis.

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