ANALYTICAL METHODS

5.1 Introduction

Development and validation of analytical methods are critical for any product development. It helps in establishing measurement systems at various stages of formulation development. Validation of these developed method provide confidence that the developed method is suitable and reliable to measure the target moiety in the final dosage form [1]. Any method is validated for linearity, accuracy, precision, specificity, range, limit of detection (LOD) and limit of quantitation (LOQ) [1].

In the current study, robust analytical methods were developed to measure percent drug loading, entrapment efficiency and *in-vitro* drug release. The details of the developed analytical methods are described here.

5.2 Materials:

The list of all materials and chemicals is presented in following table 5-1.

Table 5-1 Details of materials and chemicals

No.	Material details	Manufacturer/Supplier, Place
1	Hydrochloric acid	Merck, Germany
2	1-octane sulphonic acid sodium salt	Spectro chem, India
3	Triethylamine	Merck, Germany
4	Orthophosphoric acid	Merck, Germany
5	Methanol	Merck, Germany
6	Milli-Q Water	Merck, Germany
7	Amisulpride	Sun Pharmaceutical Industries Ltd, India
8	Granisetron HCl	Sun Pharmaceutical Industries Ltd, India
9	Polylactic-co-glycolic acid (PLGA)	Evonik, Germany
10	Polycaprolactone (PCL)	Sigma-Aldrich, USA
11	Polyvinyl alcohol (PVA)	CDH Fine chemicals, India
12	Dichloromethane	Merck, Germany
13	Mannitol	Roquette, France

5.3 Instrument's:

The list of all instruments used is presented in table 5-2.

Table 5-2 Instrument List

Sr. No.	Name of Instrument	Company, place
1	Analytical balance	Mettler Toledo, USA
2	pH meter	Mettler Toledo, USA
3	Vacuum pump	Millipore, Germany
4	Micropipette	Eppendorf, Germany
5	Ultrasonic bath	PCI Analytics, India
6	Analytical centrifuge	Remi, India
7	UV Spectrophotometer	Shimadzu, Japan
8	High Performance Liquid Chromatographic (HPLC) system	Waters, USA

5.4 Reagents, Solutions preparations:

5.4.1 Preparation of 0.1N HCl

2.1 mL of concentrated HCl was diluted to 250 mL with distilled water in a 250mL-volumetric flask to obtain 0.1 N HCl.

5.4.2 Buffer solution for HPLC

1.0 gm sodium salt of 1-octane sulphonic acid was dissolved in 500 mL water and 2.0 mL of triethylamine was added. This was further diluted to 1000 mL with water. pH was adjusted to 2.5 ± 0.05 with dilute orthophosphoric acid.

5.4.3 Mobile phase for HPLC

Buffer solution and methanol in the ratio of 600: 400 was prepared and mixed well. The mobile phase was filtered and degassed.

5.4.4 Diluent for HPLC

Buffer solution and methanol in the ratio of 600: 400 was prepared and mixed well. The diluent was filtered and degassed.

5.5 Validation of analytical methods:

5.5.1 Linearity and range

Linearity is the ability of analytical method to detect proportionality of concentration of analyte. The workable range of method lies between the higher and lower concentration of analyte for which linearity has been established [2]. Linearity was checked for studied concentrations using statistical tool such as regression coefficient (R2). The R2 value close to 1 will ensure the linearity.

5.5.2 Robustness

Effect of any small changes in developed method which affect the system suitability is known as robustness of the method [1]. The robustness was validated at respective analytical wavelength at initial time point and after 24 h of storage at room temperature for any change in the absorbance of standard solutions.

5.5.3 Method sensitivity

It is determined using following equations Eq. 5-1 and Eq. 5-2 [3],

$$\text{Limit of detection} = 3 \text{ x } \left(\frac{\text{Stadard deviation}}{\text{Slope of curve}}\right) \text{…….. Eq. 5-1}$$

$$\text{Limit of quantification} = 10 \text{ x } \left(\frac{\text{Stadard deviation}}{\text{Slope of curve}}\right) \text{…….. Eq. 5-2}$$

5.5.4 Precision

Consistency and reproducibility of developed analytical method which reflect closeness of measurement after multiple sampling from the same homogeneous sample is known as precision [3]. Intraday and interday precision was determined using 4 µg/mL standard solutions. Percent relative standard deviation (% RSD) was sued to report the precision of method.

5.5.5 Accuracy

The accuracy is nothing but closeness of measurement and the true value [4]. Accuracy was determined using addition of standard drug amounts to the samples analyzed previously. Accuracy was estimated using Eq. 5-3 [5]

$$\% \text{ Recovery} = \left[\frac{(\text{Total conc.after addition} \quad \text{Total conc.before addition})}{\text{Theorotical increase in conc.}}\right] \text{X } 100 \text{…….. Eq. 5-3}$$

5.5.6 Specificity

The ability of the developed analytical method to accurately measure drug in formulation was determined by evaluation of interference by excipients (PLGA, PCL and PVA) using formulation [3]. The formulation was prepared by spiking standard 4 µg/mL solutions with other formulation components. The absorption spectrum of the prototypes was compared with that of standard drug solutions.

5.6 Estimation of Amisulpride by UV Visible spectrophotometer:

UV spectrophotometric method in 0.1N HCl was adopted for estimation of Amisulpride in formulations [6].

5.6.1 Amisulpride stock solutions preparation

100 µg/mL of Amisulpride stock solution was prepared in 0.1N HCl by dissolving 10 mg Amisulpride in 100 ml of 0.1N HCl. [5].

5.6.2 Standard Amisulpride Solutions preparation

1 to 5 µg/mL concentrations of amisulpride solution were prepared from amisulpride stock solution (100 µg/mL) by transferring 0.1 ml to 0.5 ml to 10 ml of volumetric flasks and volume was made up to 10 ml using 0.1N HCl [7].

5.6.3 Analytical Wavelength determination

2 µg/mL standard amisulpride solutions were scanned from 200-800 nm wavelength (λ) using UV Visible spectrophotometer against blank 0.1NHCl to determine (λmax).

5.6.4 Calibration Curve preparation

Standard amisulpride solutions prepared at prespecified concentrations were used to construct calibration curve.

5.7 Estimation of Granisetron by UV Visible spectrophotometer

UV spectrophotometric method in 0.1N HCl was developed for estimation of Granisetron in formulations [8].

5.7.1 Granisetron stock solutions preparation

100 µg/mL of Granisetron stock solution was prepared in 0.1N HCl by dissolving 10 mg Granisetron in 100 ml of 0.1N HCl [5].

5.7.2 Standard Granisetron Solutions preparation

2 to 10 µg/mL concentrations of granisetron solutions were prepared from granisetron stock solution (100 µg/mL) by transferring 0.2 ml to 1 ml to 10 ml of volumetric flasks and volume was made up to 10 ml using 0.1N HCl [7].

5.7.3 Analytical Wavelength determination

4 µg/mL standard granisetron solutions were scanned from 200-800 nm wavelength (λ) using UV Visible spectrophotometer against blank 0.1NHCl to determine (λmax) [5].

5.7.4 Calibration Curve preparation

Standard granisetron solutions prepared at prespecified concentrations were used to construct calibration curve [5].

5.8 Test sample preparation for UV

Sample was weighed accurately and transferred to 10 mL glass tube. 1 mL of dichloromethane was added to completely dissolve polymer. Further, 2 ml of 0.1N HCl was added and sonicated until the dichloromethane was completely volatilized. Further, volume was made up to 100 mL using 0.1N HCl. The sample was then centrifuged for 10 min at 21,000 rpm. The supernatant solution was filtered using 0.22 mm Millipore filters. 1 mL of supernatant was diluted to 10 mL using 0.1N HCl for further analysis.

5.9 Simultaneous estimation by UV Visible spectrophotometer:

No previous simultaneous method has been reported to estimate amisulpride and granisetron in combination dosage form. Hence, Simultaneous estimation method was developed for both the drugs in pure and microsphere dosage form.

In brief, standard stock solutions of amisulpride and granisetron were prepared as per individaul methods described earlier. The λmax for amisulpride and granisetron is 227 and 302 nm respectively. From overlain spectra shown in figure 5-3, it is evident that there are two isosbestic points (at 220 nm and 290.4 nm). However, the granisetron has shoulder peak at 220 nm isosbestic point which can result in erroraneous results. Hence, estimation was caried out using simultaneous equation method [9].

This method of estimation of both drugs is based on the absorption of amisulpride and granisetron at their wavelength maximas. Two λmax selected are 227 nm and 302 nm. The absorptivity values were determined for both the drugs at respective wavelengths. The absorbances and absorptivity at these wavelengths were substituted in equations 5-4 and 5-5 to obtain the concentration of drugs [9].

$$Cx = (A2\ x\ Ay1 - A1\ x\ Ay2)/(ax2\ x\ ay1 - ax1\ x\ ay2) \text{..... Eq. 5-4}$$

$$Cy = (A1\ x\ Ax2 - A2\ x\ Ax1)/(ax2\ x\ ay1 - ax1\ x\ ay2 \text{..... Eq. 5-5}$$

A stand for absorbance of sample solutions of amisulpride at 227 nm and granisetron at 302 nm, respectively. C stands for amisulpride and granisetron concentrations in sample solution [9].

5.10 Simultaneous estimation of Amisulpride and granisetron by HPLC:

The HPLC method developed was adopted from literature after slight modifications [10]

Mobile phase and diluent preparation are mentioned in section 5.4. Standard preparation process is described as follows. The gradient program is shown in table 5-1.

5.10.1 Standard preparation

20 µg/mL standard solutions of amisulpride and granisetron were prepared separately.

5.10.2 Test sample preparation for HPLC

Formulation was weighed accurately and added to 100 mL volumetric flask. 1 mL of Dichloromethane (DCM) was added to completely dissolve the polymers. 2 mL of diluent was added and sonicated to evaporate DCM. Further, volume was made up to 100 mL using diluent. The sample was then centrifuged for 10 min at 21,000 rpm. The supernatant solution was filtered using 0.22 mm Millipore filters. 1 mL of supernatant was diluted to 10 mL using diluent for analysis.

5.10.3 Instrumental conditions

High-Performance Liquid Chromatograph (HPLC) with the following conditions.

Column	:	Symmetry Shield RP-18 150 X 4.6, 3.5 micron
Flow rate	:	1.0 mL/min.
Detection by UV	:	Detection wavelength 227 nm, 302 nm
Run time	:	About 30 min.
Column temp	:	30°C
Injection volume	:	20 µl
Retention time	:	Amisulpride: 10-12 min Granisetron: 14-16 min
Mode	:	Gradient

Table 5-3 Gradient HPLC programme details

Time (min)	% Mobile phase A	% Mobile phase B
0	80	20
20	40	60
22	80	20
30	80	20

The chromatographic analysis shows that the developed method is able to quantify both the drugs individually and simultaneously.

5.10.4 Calculations

$$\%\ Amisulpride = \frac{AT\ X\ WS\ X\ 1\ X\ 100\ X\ 10\ X\ A\ X\ P}{AS\ X\ 100\ X\ 10\ X\ WT\ X\ 1\ X\ LC}\ where:$$

$AT = Average\ area\ count\ of\ amisulpride\ peak\ in\ test\ preparation$

$AS = Average\ area\ count\ of\ amisulpride\ peak\ in\ standard\ preparation$

$WS = Weight\ of\ amisulpride\ WRS\ in\ mg$

$WT = Weight\ of\ sample\ in\ mg$

$A = Average\ weight\ of\ sample\ in\ mg$

$P = Percentage\ purity\ of\ amisulpride$

$LC = Label\ claim\ in\ mg$

$$\%\ Granisetron = \frac{AT\ X\ WS\ X\ 1\ X\ 100\ X\ 10\ X\ A\ X\ P}{AS\ X\ 100\ X\ 10\ X\ WS\ X\ 1\ X\ LC}\ where$$

$AT = Average\ area\ count\ of\ granisetron\ peak\ in\ test\ preparation$

$AS = Average\ area\ count\ of\ granisetron\ peak\ in\ standard\ preparation$

$WS = Weight\ of\ granisetron\ WRS\ in\ mg$

$WT = Weight\ of\ sample\ in\ mg$

$A = Average\ weight\ of\ sample\ in\ mg$

$P = Percentage\ purity\ of\ granisetron$

$LC = Label\ claim\ in\ mg$

5.10.5 Calibration Curve preparation

Standard solutions prepared at prespecified concentrations were used to construct calibration curve [5].

5.11 Results & Discussion

5.11.1 UV Spectrophotometric methods

The observations and results obtained for UV method have been discussed below. The absorption spectra are shown in figure 5-1, 5-2 and 5-3.

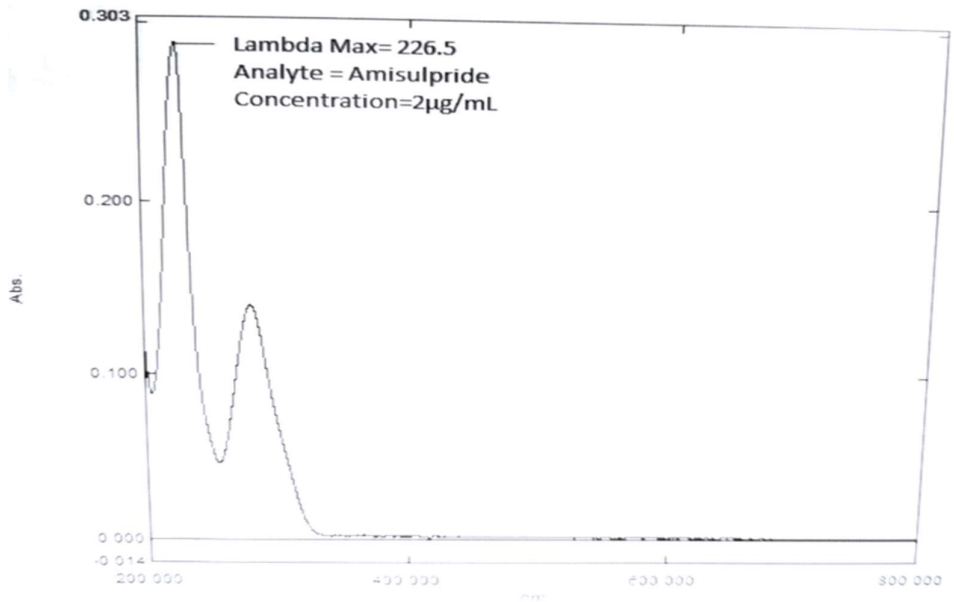


Figure 5-1 Absorption spectrum of Amisulpride in UV

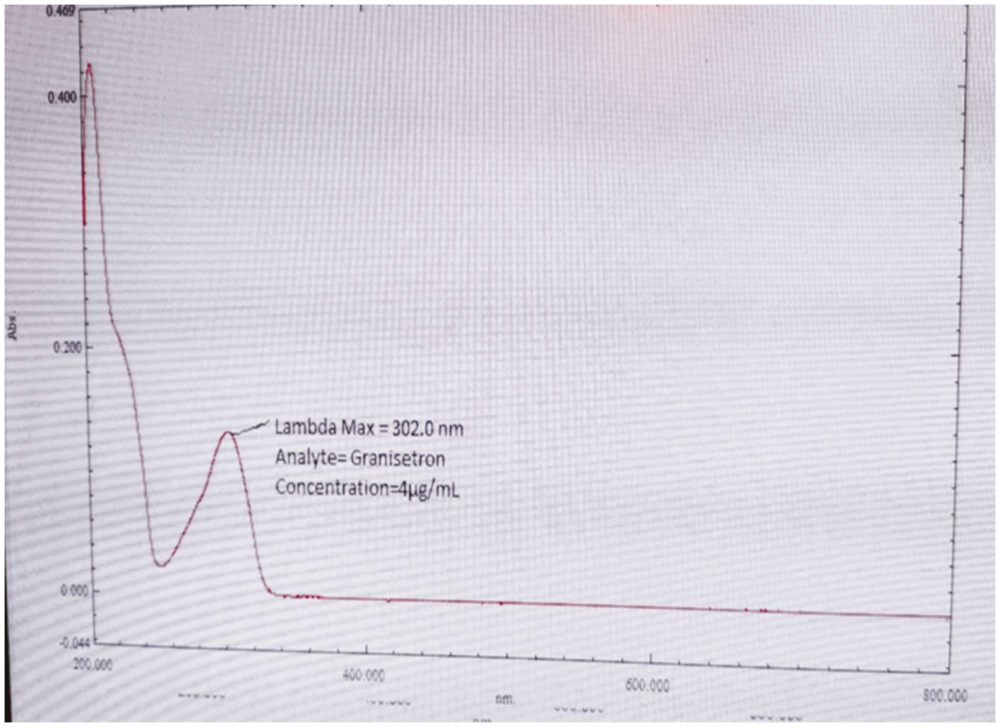


Figure 5-2 Absorption spectrum of Granisetron in UV

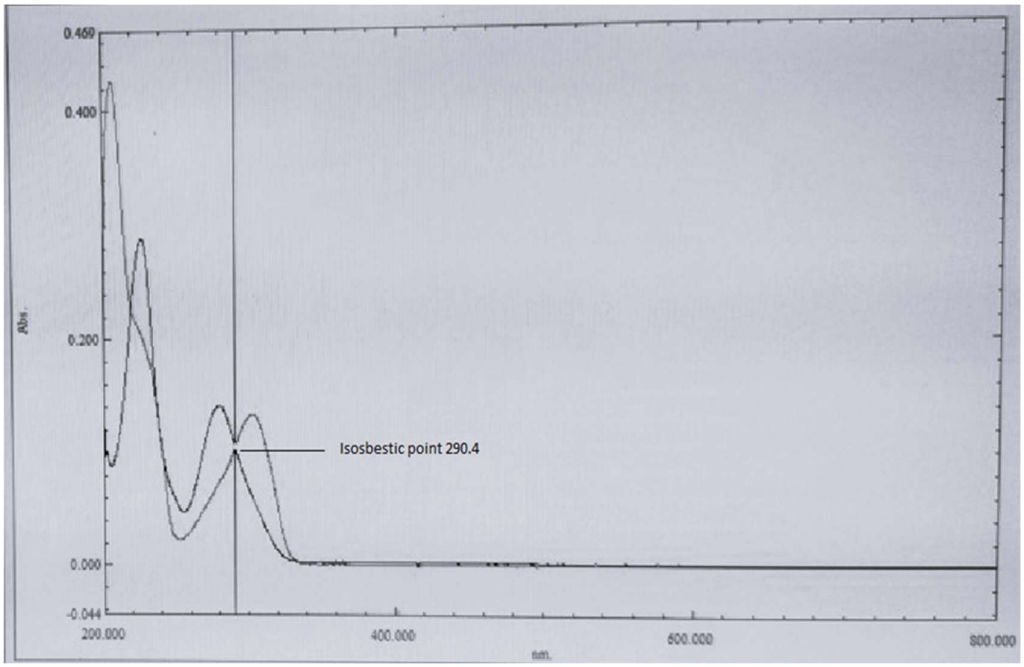


Figure 5-3 Absorption spectrum of Amisulpride and Granisetron in UV

Table 5-4 and 5-5 present the LOD and the LOQ values of UV spectrophotometric method for amisulpride. The LOD and LOQ values suggest the sensitivity of method, as the values were below the concentration range selected for calibration. Calibration curve at initial and after 24 hours for amisulpride are presented in figure 5-4 and 5-5 respectively.

Precision data are presented in Table 5-6. The results were within the acceptable range (< 2%) and thus suggest that the developed method is precise over the selected time interval [3].

The mean % recovery and % RSD values for low, medium and high concentration are presented in Table 5-7. The mean % recovery values near to 100% with % RSD ≤ 1% suggested high accuracy of the developed methods [3].

Table 5-4 Calibration data of Amisulpride in 0.1N HCl at initial

Conc. (µg/mL) Initial	**Absorbance ± SD (n=3)**	**%RSD**
1	0.1547 ± 0.0006	0.3733
2	0.2813 ± 0.0006	0.2052
3	0.4210 ± 0.0010	0.2375
4	0.5597 ± 0.0006	0.1032
5	0.7023 ± 0.0006	0.0822

Mean SD	**Slope**	**LOD**	**LOQ**
0.0007	0.1374	0.0153	0.0509

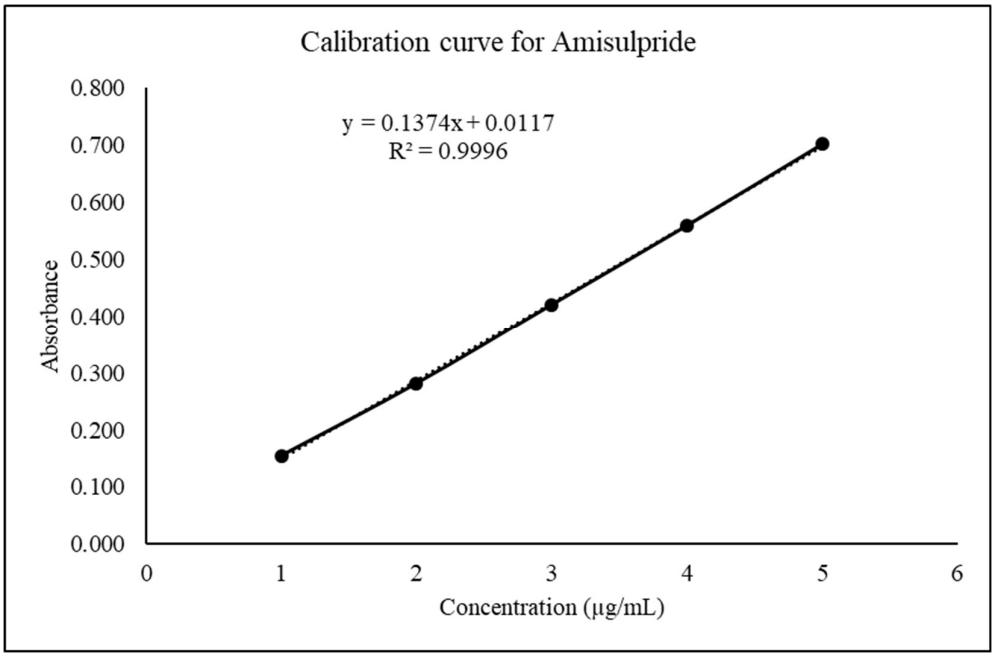


Figure 5-4 Calibration curve of Amisulpride in 0.1N HCl at initial in UV

Table 5-5 Calibration data of Amisulpride in 0.1N HCl after 24 hours

Conc. (µg/mL) After 24 Hours	**Absorbance ± SD (n=3)**	**%RSD**
1	0.1560 ± 0.0008	0.5234
2	0.2807 ± 0.0012	0.4444
3	0.4203 ± 0.0017	0.4044
4	0.5603 ± 0.0012	0.2226
5	0.7017 ± 0.0012	0.1778

Mean SD	**Slope**	**LOD**	**LOQ**
0.0013	0.1371	0.0274	0.0913

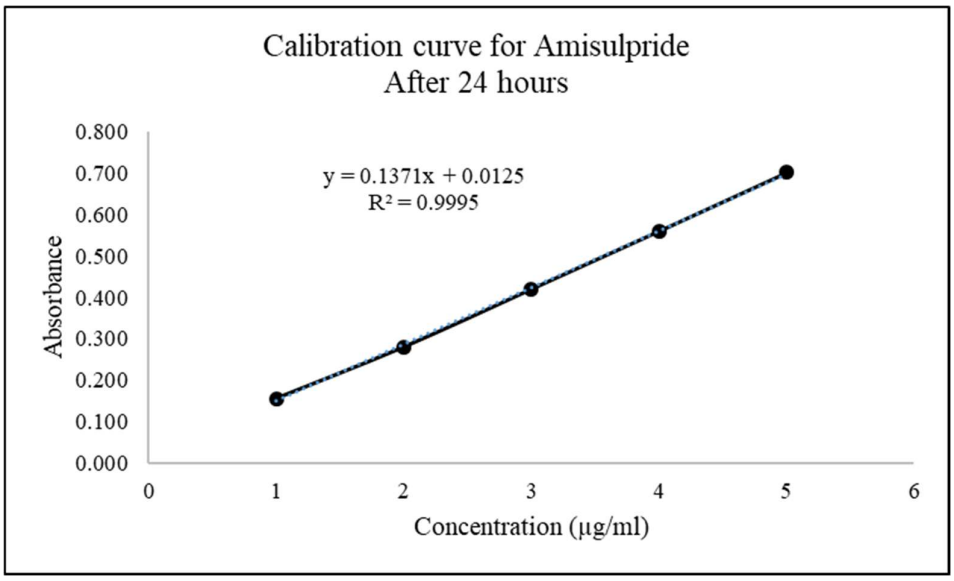


Figure 5-5 Calibration curve of Amisulpride in 0.1N HCl after 24 hours in UV

Table 5-6 Intraday and Interday precision for amisulpride

Conc. Prepared (µg/mL)	**Intraday Precision**			**Interday Precision**		
	µg/mL	Mean	% RSD	µg/mL	Mean	% RSD
4	4.05	4.013	0.714	3.97	3.993	0.515
	3.98			3.99		
	4.01			4.02		

Table 5-7 Accuracy of UV method for Amisulpride

Drug spiked (%)	Recovery			
	µg/mL	%	Mean	% RSD
80	7.15	99.31	99.72	0.341
	7.21	100.14		
	7.18	99.72		
100	8.1	101.25	100.84	1.051
	7.9	99.38		
	8.15	101.88		
120	8.75	99.43	100.08	0.476
	8.85	100.57		
	8.82	100.23		

Table 5-8 and 5-9 present the LOD and the LOQ values of UV spectrophotometric method for granisetron. The LOD and LOQ values suggest the sensitivity of method, as the values were below the concentration range selected for calibration. Calibration curve at initial and after 24 hours for amisulpride are presented in figure 5-6 and 5-7 respectively.

Precision data are presented in Table 5-10. The results were within the acceptable range (< 2%) and thus suggest that the developed method is precise over the selected time interval [3].

The mean % recovery and % RSD values for low, medium and high concentration are presented in Table 5-11. The mean % recovery values near to 100% with % RSD < 1% suggested high accuracy of the developed methods [3].

The absorption spectra of the standard amisulpride and granisetron solution were compared with that obtained for formulation and placebo in same buffers.

Table 5-8 Calibration data of Granisetron in 0.1N HCl at Initial

Conc. (µg/mL) Initial	Absorbance ± SD (n=3)	%RSD
2	0.0730 ± 0.0010	1.3699
4	0.1310 ± 0.0010	0.7634
6	0.1970 ± 0.0010	0.5076
8	0.2690 ± 0.0010	0.3717
10	0.3263 ± 0.0006	0.1769

Mean SD	Slope	LOD	LOQ
0.0009	0.0322	0.0838	0.2795

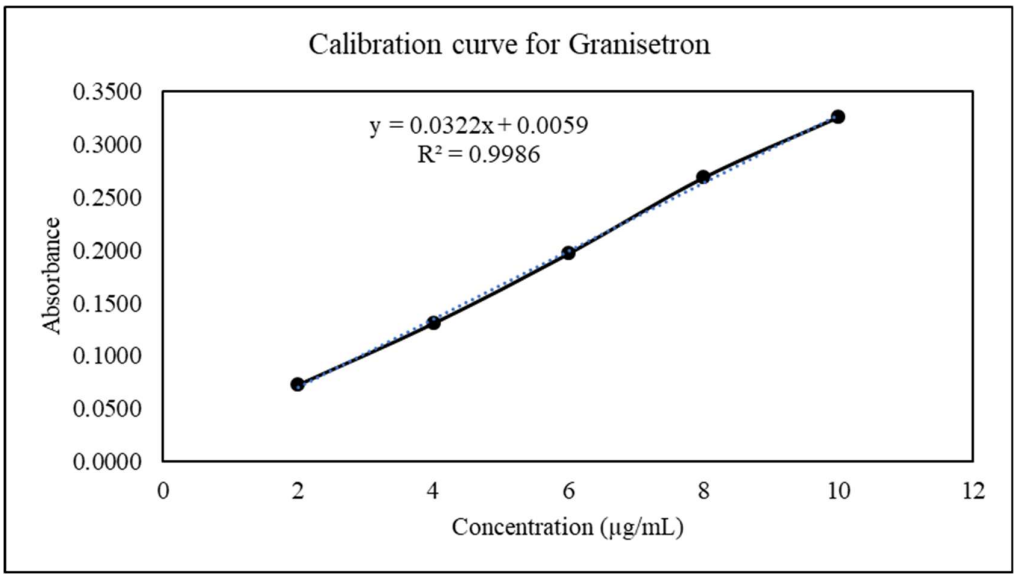


Figure 5-6 Calibration curve of Granisetron in 0.1N HCl at initial in UV

Table 5-9 Calibration data of Granisetron in 0.1N HCl after 24 hours

Conc. (µg/mL) After 24 Hours	Absorbance ± SD (n=3)	%RSD
2	0.0720 ± 0.0008	1.1340
4	0.1327 ± 0.0009	0.7107
6	0.1947 ± 0.0005	0.2422
8	0.2630 ± 0.0008	0.3105
10	0.3252 ± 0.0006	0.1709

Mean SD	Slope	LOD	LOQ
0.0007	0.0318	0.0679	0.2263

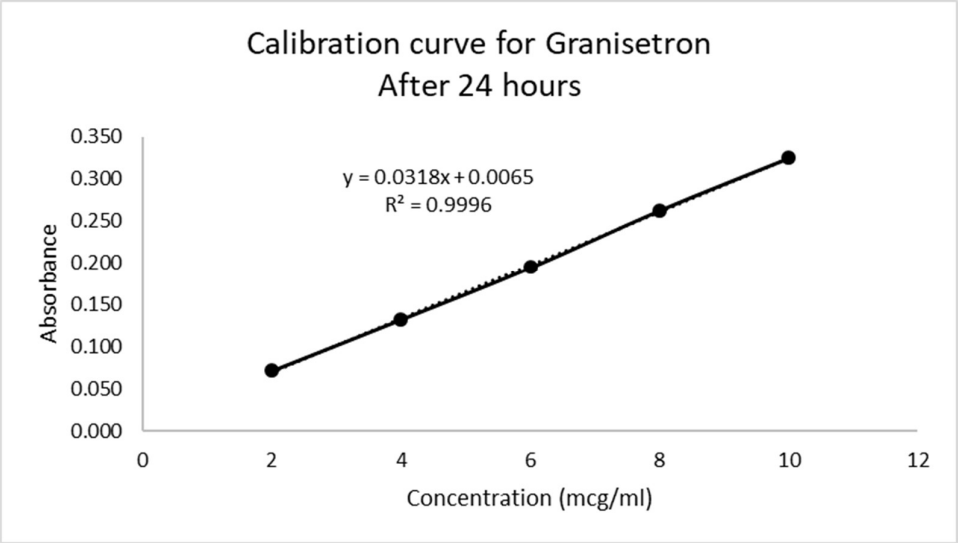


Figure 5-7 Calibration curve of Granisetron in 0.1N HCl after 24 hours in UV

Table 5-10 Intraday and Interday precision for granisetron

Conc. Prepared (µg/mL)	**Intraday Precision**			**Interday Precision**		
	µg/mL	mean	% RSD	µg/mL	mean	% RSD
4	4.02	4.013	0.822	3.98	4.010	0.539
	3.97			4.03		
	4.05			4.02		

Table 5-11 Accuracy of UV method for Granisetron

Drug spiked (%)	Recovery			
	µg/mL	%	Mean	% RSD
80	7.12	98.89	99.31	0.343
	7.18	99.72		
	7.15	99.31		
100	8.05	100.63	100.42	0.776
	7.95	99.38		
	8.1	101.25		
120	8.88	100.91	100.57	0.277
	8.85	100.57		
	8.82	100.23		

As shown in Fig. 5-8 and 5-9, drug's peak position and intensity was not changed in formulation prototypes when compared to standard amisulpride and granisetron solutions which indicates absence of any interference by formulation components at analytical wavelengths (227 nm for amisulpride and 302 nm for granisetron). There were no overlapping or extra peaks observed in excipient mixtures at selected analytical wavelengths which confirmed the specificity of the methods [5].

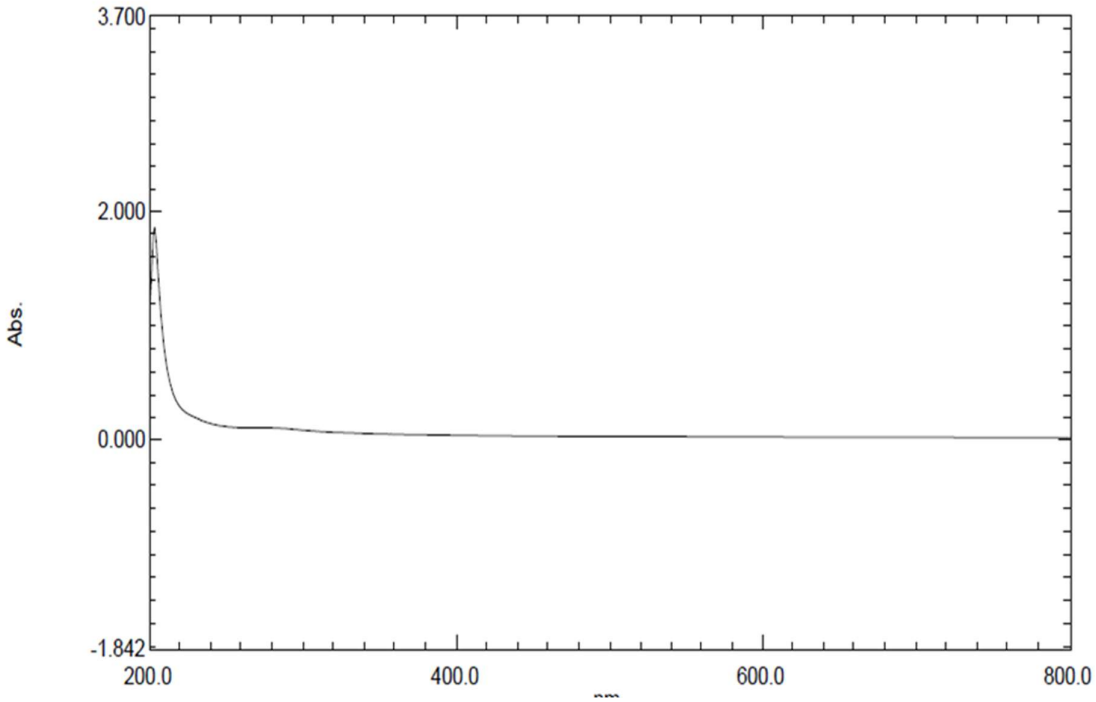


Figure 5-8 Placebo Spectrum in UV

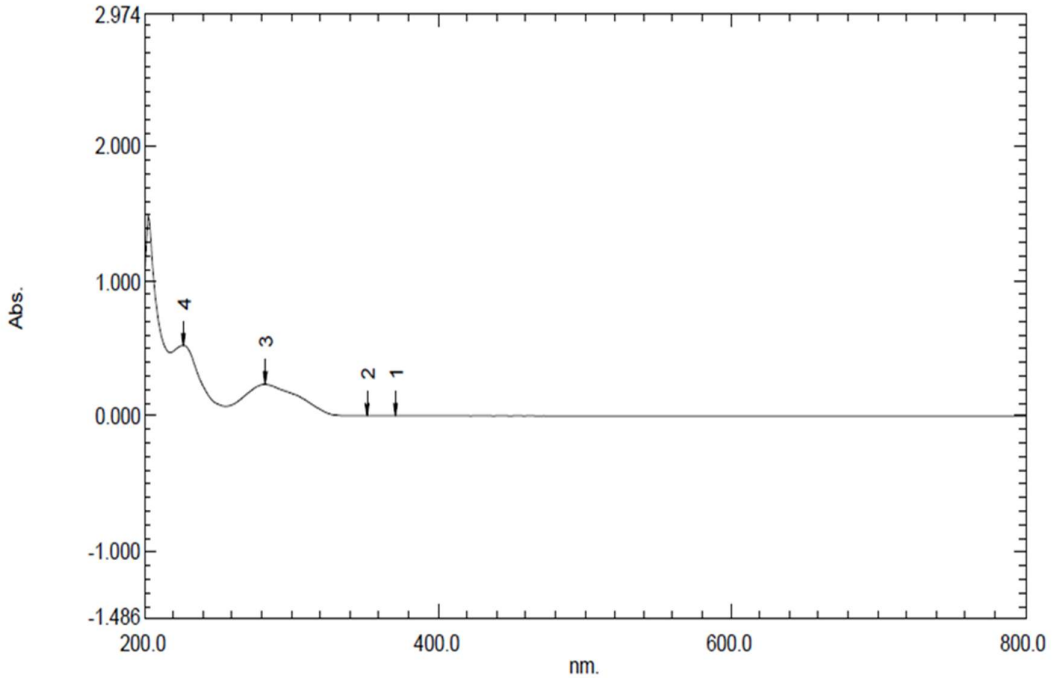
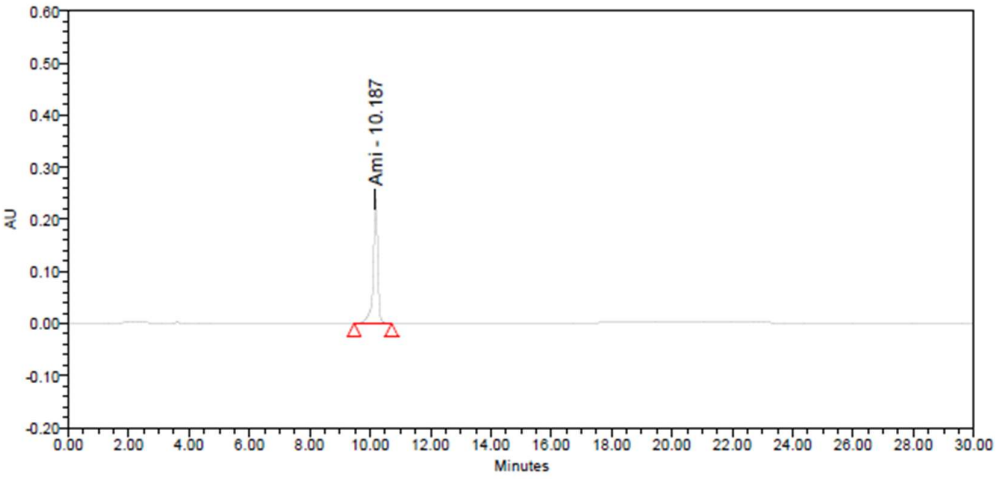


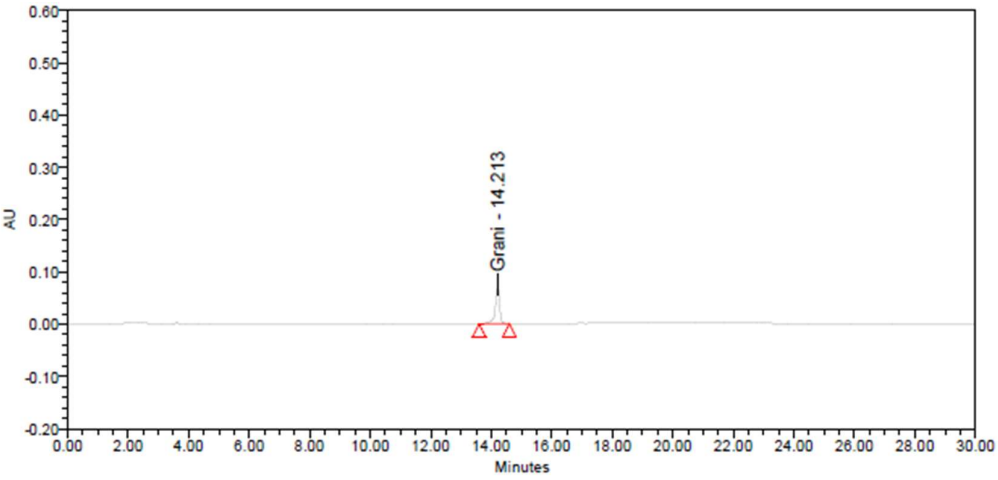
Figure 5-9 Formulation spectrum in UV

5.11.2 HPLC Method

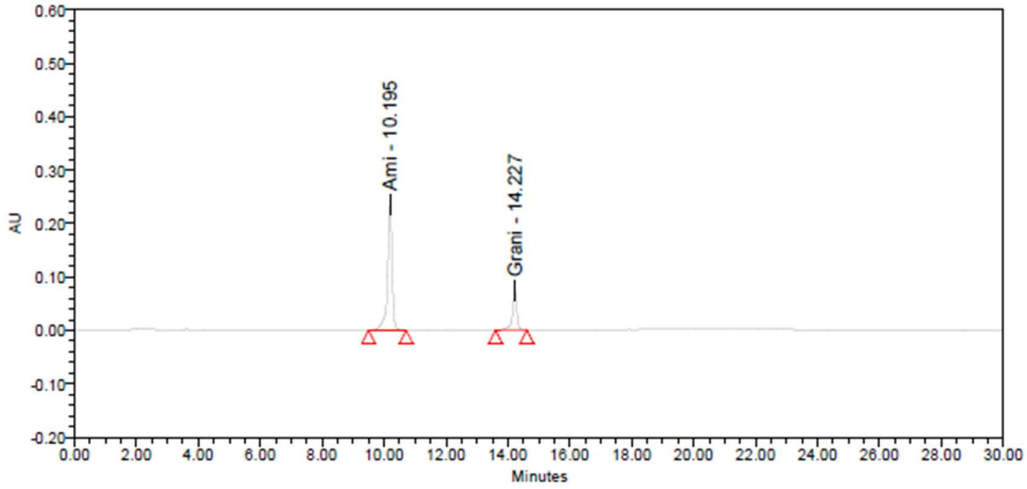
The chromatogram data is presented in figure 5-10 (A to C)



A)



B)



C)

Figure 5-10 HPLC Chromatogram data A) Amisulpride B) Granisetron C) Mixture

The chromatographic analysis shows that the developed method is able to quantify both the drugs individually and simultaneously.

Table 5-12 and 5-13 present the LOD and the LOQ values HPLC method for amisulpride and granisetron. The LOD and LOQ values suggest the sensitivity of method, as the values were below the concentration range selected for calibration. Calibration curve at initial and after 24 hours are presented in figure 5-11A,5-11B, 5-12A and 5-12B.

Precision data are presented in Table 5-14. The results were within the acceptable range (< 2%) and thus suggest that the developed method is precise over the selected time interval [3].

The mean % recovery and % RSD values for low, medium and high concentration are presented in Table 5-15. The mean % recovery values near to 100% with % RSD < 1% suggested high accuracy of the developed methods [3].

Table 5-12 Calibration data of Amisulpride and Granisetron at Initial

Conc. (µg/mL) Initial	Amisulpride Peak Area	Granisetron Peak Area
10	1210033 ± 48	581373 ± 42
12.5	1500441 ± 57	720903 ± 66
15	1827150 ± 61	842991 ± 74
17.5	2105457 ± 84	970893 ± 102
20	2421338 ± 124	1100636 ± 125
LOD	0.002	0.005
LOQ	0.0062	0.0158

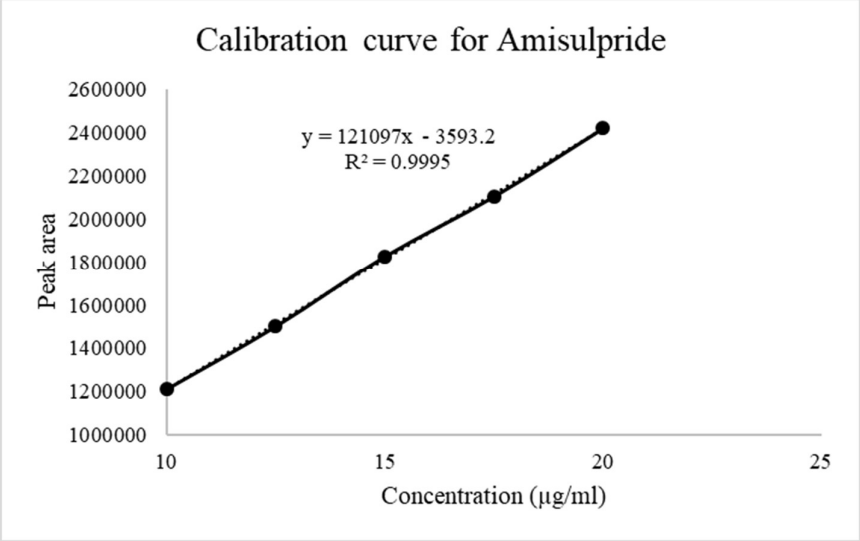


Figure 5-11A Calibration curve at Initial for Amisulpride in HPLC

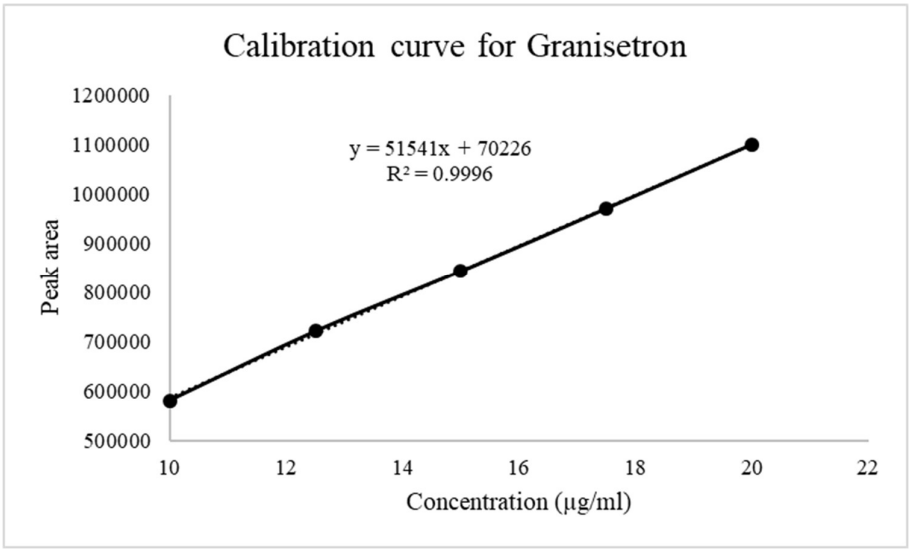


Figure 5-11B Calibration curve at Initial for Granisetron in HPLC

Table 5-13 Calibration data of Amisulpride and Granisetron after 24 hours

Conc. (µg/mL) Initial	Amisulpride Peak Area	Granisetron Peak Area
10	1210078	581330
12.5	1500338	720848
15	1827125	843150
17.5	2105438	970990
20	2421378	1100625
LOD	0.002	0.007
LOQ	0.0080	0.0226

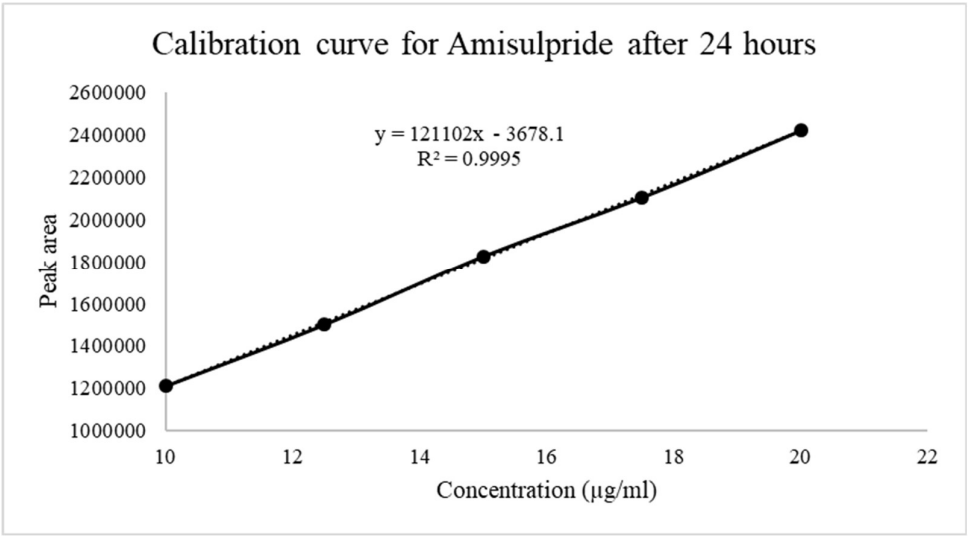


Figure 5-12A Calibration curve at 24h for Amisulpride in HPLC

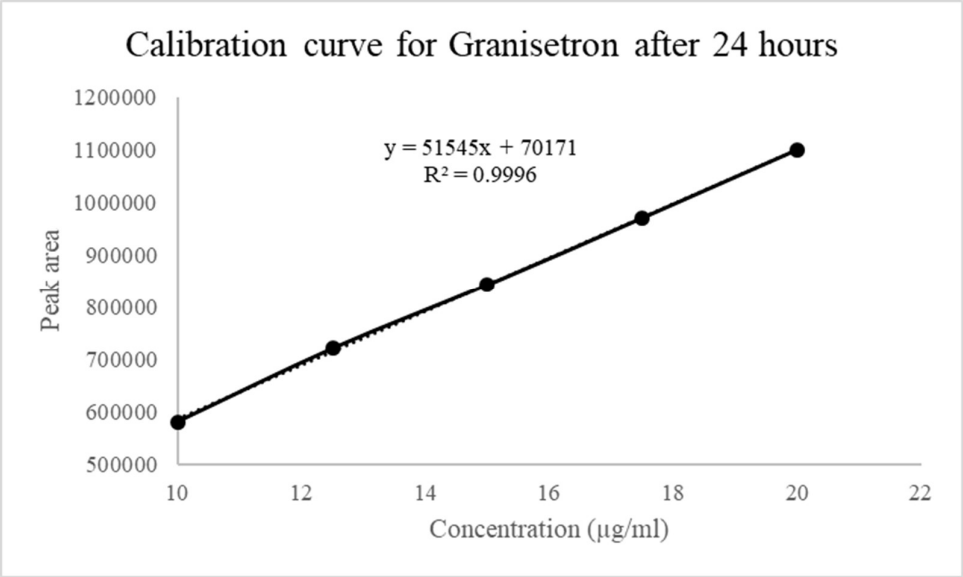


Figure 5-12B Calibration curve at 24h for Granisetron in HPLC

Table 5-14 Intraday and Interday precision

Conc. Prepared (µg/mL)	**Intraday Precision**			**Interday Precision**		
	µg/mL	mean	% RSD	µg/mL	mean	% RSD
Amisulpride 10	9.92	9.98	0.532	9.96	9.99	0.245
	10.05			10.02		
	9.98			9.99		
Granisetron 10	9.95	9.99	0.419	9.92	9.98	0.536
	9.98			9.97		
	10.05			10.05		

Table 5-15 Accuracy evaluation of HPLC method for Amisulpride and Granisetron

Drug	Drug spiked (%)	Recovery			
		µg/mL	%	Mean	% RSD
Amisulpride	80	18.05	100.28	100.19	0.346
		17.95	99.72		
		18.1	100.56		
	100	20.1	100.50	100.22	0.332
		20.08	100.40		
		19.95	99.75		
	120	22.1	100.45	99.94	0.381
		21.9	99.55		
		21.96	99.82		
Granisetron	80	18.1	100.56	100.09	0.472
		17.9	99.44		

Drug	Drug spiked (%)	Recovery			
		µg/mL	%	Mean	% RSD
		18.05	100.28		
	100	20.14	100.70	100.23	0.524
		20.1	100.50		
		19.9	99.50		
	120	22.15	100.68	100.18	0.427
		22.05	100.23		
		21.92	99.64		

Fig. 5-13, shows the drug peaks in formulation and shows no interference from excipients in the chromatogram.

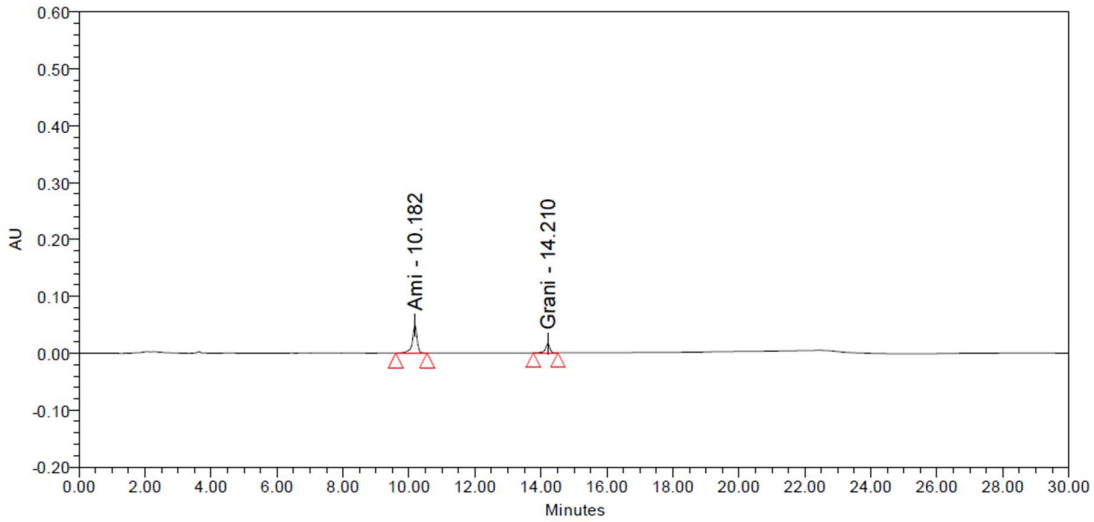


Figure 5-13 Formulation chromatograph in HPLC

5.12 Conclusion:

UV-vis method as well as HPLC method for quantification of amisulpride and granisetron in formulation was successfully developed. Validation results showed that all the methods were linear, precise, accurate, robust, sensitive, and specific to the studied parameters.

5.13 References:

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