Chapter 3

ANALYTICAL METHODS

3.1 Materials

Anastrozole and Exemestane were received as gift samples from Sun Pharma Advance Research Centre, Vadodara, India. Monobasic potassium phosphate, tri ethyl amine (TEA), sodium hydroxide, hydrochloric acid and acetonitrile were of HPLC grade and procured from S.D. Fine Chemicals, Vadodara, India. Double distilled water (DDW) was purified by passing through 0.45µ Millipore filters (Millipore, Bangalore, India).

3.2 Estimation method for anastrozole

3.2.1 High Performance Liquid Chromatography (HPLC)

Quantitative estimation of anastrozole (ATZ) was done by HPLC as described by Mendes et al. with slight modifications [Mendes et al. 2007]. The HPLC system (Shimadzu, Japan) composed of a UV-visible spectrophotometric detector. The separation was performed on a reversed phase C-18 HPLC column (Lichro Cart – RP8, 250 mm × 4.6 mm, 5 µ). Column temperature was maintained at 25 °C throughout the experiment using column oven. A filtered and degassed mixture of buffer (phosphate buffer, 2 g monobasic potassium phosphate in 1000 ml of double distilled water and pH adjusted to 6.0 with TEA) and acetonitrile (60:40) was used as mobile phase. The run time was 10 min and the retention time was 5.2 min. The mobile phase was delivered at a flow rate of 1.5 ml/min, the injection volume was 20 µl and the effluent was monitored at ultraviolet detection at 215 nm. Data processing was done using Spinchrom CFR (Spinchotech, Japan).

3.2.2 Preparation of standard stock solutions

Stock solution of ATZ was prepared in acetonitrile by accurately weighing 10 mg of ATZ in 10 ml (1000 µg/ml) acetonitrile. Further dilution was performed using mobile phase.

3.2.3 Preparation of calibration curve

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml volumetric flask to prepare a working stock solution of ATZ (100 µg/ml). Suitable aliquots of working stock solution were accurately measured and transferred to 10 ml volumetric flasks. The final volume was made up to 10 ml with the mobile phase to give final concentrations of 8, 16, 24, 32 and 40 µg/ml. Standards were analyzed by RP HPLC at UV detection wavelength 215 nm and mobile phase flow rate 1.5 ml/min. After 10 min elution, results were processed using data processing software Spinchrom CFR. The above procedure was repeated three times and results recorded in

the table 3.1 and shown in figure 3.1 and 3.2. All the estimations were carried out at 25 °C, and care was taken to prevent solvent evaporation at every stage of estimation. Calibration plot was constructed for the measured area against drug concentration. Accuracy and precision of the method was determined by performing recovery studies after addition of known concentration of ATZ.

Table 3.1 Calibration curve of ATZ by HPLC at 215 nm, Data presented as Mean ± SD, n=3.

Concentration (µg/ml)	Retention time (min)	Area (mV.s)
8	5.17	117.58 ± 4.97
16	5.17	244.27 ± 8.86
24	5.16	396.70 ± 7.93
32	5.17	519.24 ± 7.80
40	5.18	640.81 ± 10.52

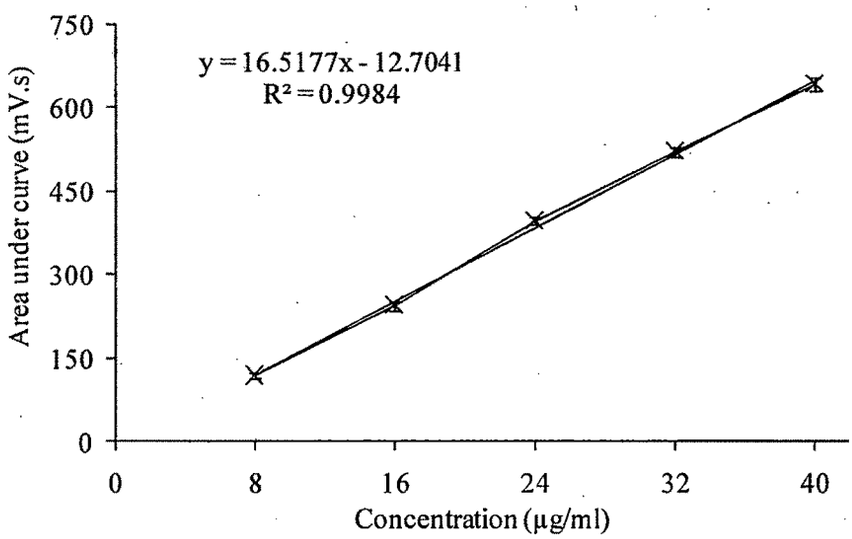

Figure 3.1 Regressed calibration curve of ATZ by HPLC at 215 nm. Data presented as Mean ± SD, n=3 (some error bars are too small to be shown).

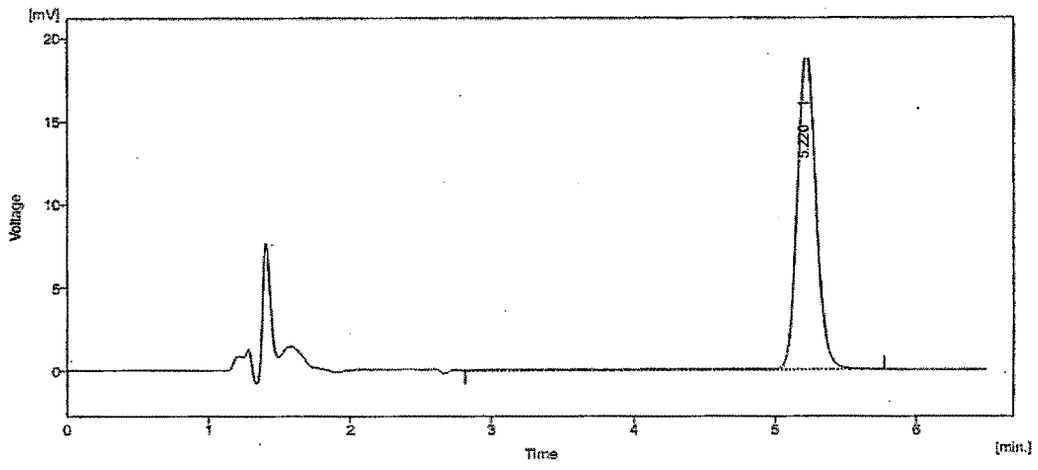

Figure 3.2 Chromatogram of ATZ solution by HPLC at 215 nm.

3.2.4 Analytical method validation

The method was validated for accuracy, precision and linearity.

3.2.4.1 Linearity

The linearity of an analytical method is its ability to elicit, test results that are directly or by a well defined mathematical transformation proportional to the concentration of analyte in samples with a given range (Rifino 2003). A calibration curve is prepared by plotting a dependent variable (AUC, Y) as a function of an independent variable (concentration, X). For evaluation of the linearity of the HPLC method of ATZ, the standard solutions were prepared at 8, 16, 24, 32 and 40 µg/ml concentrations (n = 3) and AUC was calculated. The method was said to be linear for estimation of ATZ if it R^2 was near to 1. Least square regression method was used to determine the regression coefficient, r and the equation for the best fitting line.

This relation if found with a series of measurements, which in practice is often linear one.

$$Y = mX + C$$

Where,

m is a slope of line and C is the intercept on the Y axis.

Linearity of an analytical method for ATZ in mobile phase was established by the regression coefficient.

3.2.4.2 Accuracy

Accuracy of an analytical method is the closeness of test results obtained by that method to true value (The United States Pharmacopoeia 27 NF 22, 2004). Accuracy is calculated from the test results as the % analyte recovered by assay. Accuracy was

calculated by analysis of three replicate samples for the above described methods. The observed concentrations of the drug were then back calculated using the equation of standard calibration curve and compared with actual concentrations.

3.2.4.3 Precision

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of homogenous sample (The United States Pharmacopoeia 27 NF 22, 2004). Precision may be measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions. The precision of an analytical method is usually expressed as the standard deviation or confidence limit. The standard deviation is calculated from following formula (Rifino 2003).

$SD = [\Sigma(X-x)/ n-1]^{½}$

Where, X – an individual measurement in a set.

x – Arithmetic mean of the set.

n – Total number of replicated measurement taken in set.

Precision between different samples can be compared with RSD as follows:

% RSD = [SD/ Mean]* 100

The intra- and inter day precision of the assay were calculated by replicate analysis of the solutions of known concentrations of ATZ at three quality control concentration (Low quality control concentration [LQC], Medium quality control concentration [MQC] and High quality control concentration [HQC] levels). The observed concentrations of the drug were then back calculated (from AUC) using the equation of standard calibration curve. The variations between the observed concentrations were determined by calculating the % RSD (Rifino 2003).

In order to determine the accuracy and precision of the developed method, known amounts of ATZ at low, medium and high concentration (12, 24 and 36 µg/ml) were subjected to recovery studies as per the procedure described earlier. All standard samples were also performed for intraday and interday variability. The results obtained are tabulated in table 3.2 and 3.3.

Intraday precision of the assay

Primary stock solutions were appropriately diluted using suitable solvent to obtain final concentration of 12 (LQC), 24 (MQC) and 36 µg/ml (HQC). Three different sets of primary stock solutions were prepared and diluted in the similar manner. The AUC was

calculated three times on the same day. The solutions were prepared freshly each time. The % relative error was calculated and the results recorded.

The % relative error was calculated using the formula, (Rifino 2003)

$$\% \text{ Relative error} = \frac{[\text{Observed value} - \text{True value}]}{\text{True value}} * 100$$

Interday precision of the assay

Primary stock solutions were appropriately diluted using suitable solvent to obtain final concentration of 12 (LQC), 24 (MQC) and 36 µg/ml (HQC). Three different sets of primary stock solutions were prepared and diluted in the similar manner and AUC was calculated on three consecutive days. The solutions were prepared freshly on each time. The % relative error was calculated and the results recorded.

Table 3.2 Accuracy of ATZ measurement using mobile phase at 215 nm.

Conc. of ATZ (µg/ml)	RT	Area (mV.s)	Conc. of ATZ obtained (µg/ml)	Accuracy	Precision (RSD %)
12	5.12	196.82 ± 6.64	12.68	99.23	0.769
24	5.11	398.64 ± 7.79	24.90	100.49	0.489
36	5.15	599.80 ± 3.75	37.08	100.79	0.799

Table 3.3 Precision of ATZ measurement using mobile phase at 215 nm, Data presented as Mean, n=3.

Conc. (µg/ml)	Measured conc. (µg/ml)		Accuracy (%)	
	Intra day	Inter day	Intra day	Inter day
12	12.68	12.71	99.23	100.22
24	24.90	25.01	100.49	100.40
36	37.08	36.88	100.79	99.45

3.2.5 Estimation of ATZ in nanoparticulate formulations

Nanoparticulate formulations (PLGA, PLGA PEG, PCL and PCL PEG NPs) were dissolved in required amount of acetonitrile to obtain the final concentration of ATZ ranging between 8 to 40 µg/ml. The contents were gently mixed to ensure uniform mixing and

kept aside for 30 min at room temperature. The samples were filtered using 0.2 μ membrane filter and the filtrate was collected and estimated by RP HPLC at UV detection wavelength 215 nm and mobile phase flow rate 1.5 ml/min. Results were processed using data processing software Spinchrom CFR.

3.3 Estimation method for Exemestane

3.3.1 High Performance Liquid Chromatography (HPLC)

Quantitative estimation of exemestane (EXE) was done by HPLC as reported by Breda et al. with slight modifications [Breda et al. 1993]. The HPLC system (Shimadzu, Japan) composed of a UV-visible spectrophotometric detector. The separation was performed on a reversed phase C-18 HPLC column (Lichro Cart – RP8, 250 mm × 4.6 mm, 5 μ). Column temperature was maintained at 25 °C throughout the experiment using column oven. A filtered and degassed mixture of acetonitrile:0.02M phosphate buffer (pH 4.0) (75:25) was used as mobile phase. The run time was 10 min and the retention time of EXE was 5.0 min. The mobile phase was delivered at a flow rate of 1.0 ml/min, the injection volume was 20 μl and the effluent was monitored at ultraviolet detection at 247 nm. Data processing was done using Spinchrom CFR (Spinchotech, Japan).

3.3.2 Preparation of standard stock solutions

Stock solution of EXE was prepared in acetonitrile by accurately weighing 10 mg of EXE in 10 ml (1000 μg/ml) acetonitrile. Further dilution was performed using mobile phase.

3.3.3 Preparation of calibration curve

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml volumetric flask to prepare a working stock solution of EXE (100 μg/ml). Suitable aliquots of working stock solution were accurately measured and transferred to 10 ml volumetric flasks. The final volume was made up to 10 ml with the mobile phase to give final concentrations of 8, 16, 24, 32 and 40 μg/ml. Standards were analyzed by RP HPLC at UV detection wavelength 247 nm and mobile phase flow rate 1.0 ml/min. After 10 min elution, results were processed using data processing software Spinchrom CFR. The above procedure was repeated three times and results recorded in the table 3.4 and shown in figure 3.3 and 3.4. All the estimations were carried out at 25 °C, and care was taken to prevent solvent evaporation at every stage of estimation. Calibration plot was constructed for the measured area against drug concentration.

Accuracy and precision of the method was determined by performing recovery studies after addition of known concentration of EXE.

Table 3.4 Calibration curve of EXE by HPLC at 247 nm, Data presented as Mean ± SD, n=3.

Concentration (µg/ml)	Retention time (min)	Area (mV.s)
8	5.02	174.34 ± 7.52
16	5.01	344.44 ± 6.74
24	5.03	532.47 ± 7.51
32	5.02	708.61 ± 9.23
40	5.01	917.14 ± 10.06

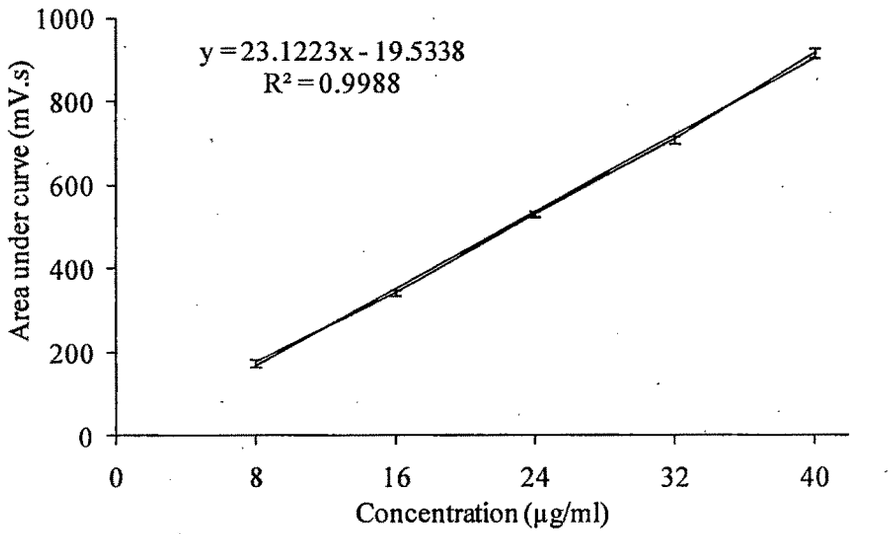

Figure 3.3 Regressed calibration curve of EXE by HPLC at 247 nm. Data presented as Mean ± SD, n = 3 (some error bars are too small to be shown).

3.3.4 Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of EXE were subjected to recovery studies at low medium and high concentration (12, 24, and 36 µg/ml) as per the procedure described earlier. All standard samples were also performed for intraday and interday variability. The results obtained are tabulated in table 3.5 and 3.6.

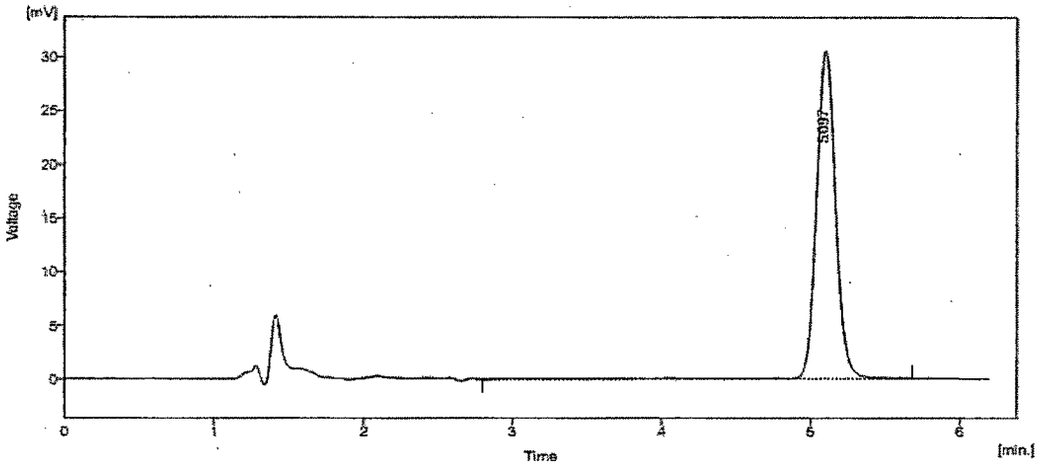

Figure 3.4 Chromatogram of EXE solution by HPLC at 247 nm.

Table 3.5 Accuracy of EXE measurement using mobile phase at 247 nm.

Conc. of EXE (µg/ml)	RT	Area (mV.s)	Conc. of EXE obtained (µg/ml)	Accuracy	Precision (RSD %)
12	5.02	264.55 ± 3.17	12.28	99.37	0.633
24	5.02	536.43 ± 5.51	24.04	100.74	0.744
36	5.01	796.99 ± 7.24	35.31	99.78	0.214

Table 3.6 Precision of EXE measurement using mobile phase at 247 nm, Data presented as Mean, n=3.

Conc (µg/ml)	Measured conc (µg/ml)		Accuracy (%)	
	Intra day	Inter day	Intra day	Inter day
12	12.28	12.30178	99.37	100.1376
24	24.04	23.87716	100.74	99.27881
36	35.31	35.17743	99.78	99.60535

3.3.5 Estimation of EXE in nanoparticulate formulations

Nanoparticulate formulations (PLGA, PLGA PEG, PCL and PCL PEG NPs) were dissolved in required amount of acetonitrile to obtain the final concentration of ATZ ranging between 8 to 40 µg/ml. The contents were gently mixed to ensure uniform mixing and

kept aside for 30 min at room temperature. The samples were filtered and the filtrate was collected and estimated by RP HPLC at UV detection wavelength 247 nm and mobile phase flow rate 1.0 ml/min. Results were processed using data processing software Spinchrom CFR.

3.4 References

Rifino CB. 2003. Pharmaceutical Process Validation. In: Robert AN, Wachter AH (Eds.) Process Validation and Quality Assurance. Switzerland: Marcel Dekker, Inc., 1-43.

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