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Development and Validation of Simultaneous Detrmination of Anastrozole and Temozolomide in Pharmaceutical Dosage Forms

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ABSTRACT: A fast, sensitive and accurate reverse phase liquid chromatographic method was developed and validated for the simultaneous determination of Anastrozole and Temozolomide in an anticancer dosage form. Chromatographic separation was achieved on Inertsil C8 column (250 x 4.6 mm, 5 µm particle size) with a separate gradient mobile phase consisting of Ammonium acetate buffer and Acetonitrile with at a flow rate of 1.0 mL/ min. The analytes were detected at 215 nm for Anastrozole and Temozolomide by PDA detector. The retention time of Temozolomide and Anastrozole were found to be at 7.663 ± 0.1 and 23.158 ± 0.1 mins respectively. The linearity of the method ranged between 0.0165 and 15.0 µg/ mL for both the Anastrozole and Temozolomide with correlation coefficient 0.999 for both the drugs in a binary mixture. The LOD was found to be 0.00825 µg/ mL and 0.0085 µg/ mL for Anastrozole and Temozolomide respectively and LOQ was found to be 0.0165 µg/ mL and 0.017 µg/ mL for Anastrozole and Temozolomide respectively.

Key words: RP-HPLC, Gradient, Anastrozole, Temozolomide, Anticancer.

INTRODUCTION

Temozolomide. Temozolomide (referred to as TMZ) medication is used to treat certain types of brain cancer [1]. It is a chemotherapy drug that works by slowing cancer cell growth. In some patients, Temozolomide decreases the size of brain tumors. The therapeutic benefit of temozolomide depends on its ability to alkylate/methylate DNA, which most often occurs at the N-7 or O-6 positions of guanine residues.

Fig. 1. (4-methyl-5-oxo- 2, 3, 4, 6, 8-pentazabicyclo [4.3.0] nona-2, 7, 9-triene- 9-carboxamide).

This methylation damages the DNA and triggers the death of tumor cells. However, some tumor cells are able to repair this type of DNA damage, and therefore diminish the therapeutic efficacy of temozolomide, by expressing an enzyme called O-6-methylguanine-DNA methyltransferase (MGMT) or O-6-alkylguanine-DNA alkyltransferase (AGT or AGAT) [1, 4].

Anastrozole. Anastrozole (an-ASS-troh-zole) [2] is a medicine, used extensively in the treatment of breast cancer. Anastrozole is a potent and selective non-steroidal aromatase inhibitor indicated for the treatment of advanced breast cancer in post-menopausal women with disease progression following tamoxifen therapy. Many breast cancers have estrogen receptors and growth of these tumors can be stimulated by estrogens. In post-menopausal women, the principal source of circulating estrogen (primarily estradiol) is conversion of adrenally-generated androstenedione to estrone by aromatase in peripheral tissues, such as adipose tissue, with further conversion of estrone to estradiol. Many breast cancers also contain aromatase; the importance of tumor-generated estrogens is uncertain. Treatment of breast cancer has included efforts to decrease estrogen levels by ovariectomy premenopausally and

by use of anti-estrogens and progestational agents both pre- and post-menopausally, and these interventions lead to decreased tumor mass or delayed progression of tumor growth in some women. Anastrozole is a potent and selective non-steroidal aromatase inhibitor. It significantly lowers serum estradiol concentrations and has no detectable effect on formation of adrenal corticosteroids or aldosterone [2, 3, 6, 7].

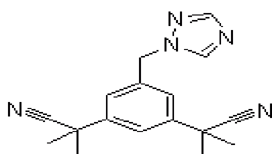

Fig.2.1,3Benzenediacetonitrile,alpha,alpha,alpha',alpha'-tetramethyl-5-(1H-1,2,4-triazol-1-ylmethyl)-2-[3-(2-Cyanopropan-2-yl)-5-(1,2,4-triazol-1-ylmethyl)phenyl]-2-methylpropanenitrile.

Reagents and Materials. Reference standards of Anastrozole and Temozolomide were supplied by Anant laboratories with purity of 99.9% and 99.95% respectively. Anastrozole Bromo compound, Anastro zole impurity A and Anastrozole impurity B and AIC HCL (5amino imidazole-4-carboxamidehydrochloride) impurity, 2-Azahypoxanthine impurity and TMZ II impurity for Temozolomide [4] were supplied by Anant pharmaceuticals India Pvt. Ltd. HPLC grade Acetonitrile (Merck), Ammonium acetate (S.D Fine Chemicals), o-phosphoric acid and Milli Q water were used throughout the analysis. All other reagents were of analytical grade and were used without further purification. Mobile phase was filtered using 0.45µ membrane filters by Millipore (USA).

Instrumentation. The HPLC system is a binary gradient HPLC with Dionex Ultimate 3000 (Dionex Corporation,) with PDA Detector. Data acquisition was performed by the Chromoleon software operated on a Pentium® IV microprocessor. The experimental conditions were optimized with an Inertsil C8 column (250 x 4.0 mm, 5 µm particle size) at room temperature [5, 9, 12].

Chromatographic conditions. The mobile phase A consists of Ammonium acetate buffer (0.1%) and mobile phase B consists of Acetonitrile. The Ammonium acetate buffer was prepared by dissolving 1.0 grams of Ammonium acetate in 1000ml o f water. A gradient programme with mobile phase A and Mobile phase B was pumped at a flow rate of 1 mL/min. The proportion of Acetonitrile was kept constant at 5% for a period of 7 minutes. A gradient programme was followed [4] 1-7min Ammonium acetate buffer (0.1%): Acetonitrile (95:05). 7.1-12 min Ammonium acetate buffer (0.1%): Acetonitrile (93:07). 12.1-17 min Ammonium acetate buffer (0.1%): Acetonitrile (50:50). 17.1-40 min Ammonium acetate buffer (0.1%): Acetonitrile (30:70). 40.1-45 min Ammonium acetate buffer (0.1%): Acetonitrile (95:05). 45.1-55 min Ammonium acetate buffer (0.1%): Acetonitrile (95:05). The elution was monitored at 215 nm and the injection volume was 10 µL.

Preparation of Standard solution. Standard stock solution was prepared by dissolving accurately 50 mg Anastrozole and 50 mg Temozolomide in 100 mL of equal volumes of Acetonitrile and water. 2 ml of standard stock solution was diluted to 100 ml with Acetonitrile: water 50:50 to obtain each of 10 µg/mL of Anastrozole and Temozolomide. The solution was filtered through 0.45µ nylon filter before analysis. A series of solutions containing Anastrozole and Temozolomide were prepared with mobile phase A and 10 µl of these solutions were injected in to the HPLC system.

Preparation of Resolution solution. Transferred 2.0 mg of each of AIC HCL (5amino imidazole-4-carboxamidehydrochloride) impurity, 2-Azahypoxanthine impurity and TMZ II impurity and Anastrozole Bromo compound, Anastrozole impurity A and Anastrozole impurity B dissolved in 200 mL of equal volumes of Acetonitrile: water. Further dilute 1 mL of this solution and 2 mL of standard stock solution into 100 mL of Acetonitrile: water (50:50).

Preparation of sample solution. Commercial Anstrogold tablets (Anastrozole tablets 1 mg) and Temozol 20 (Temozolomide capsules 20 mg) were purchased from the local market. For the analysis of Anstrogold tablets, 20 tablets were weighed and powdered. A quantity equivalent 10 mg of Anastrozole transferred into 100 mL volumetric flask and for the analysis of Temozol 20 capsules, blend of 20 capsules mixed and equivalent to 10 mg of Temozolomide was transferred into separate 100 mL volumetric flask, to these flask added 60 mL of equal volumes of Acetonitrile and water respectively and

the mixture was subjected to vigorous shaking for 10 min for complete extraction of drugs and diluted up to volume with same solvent. Further diluted 10 mL of these solutions to 100 ml with Acetonitrile: water 50:50. Concentration of this solution was 10µg/mL for Anastrozole and10µg/mL for Temozolomide. The solution was filtered through 0.45µ nylon filter before analysis. 10 µl of these solutions were injected in to the HPLC system.

Method Validation. The validation of the method was done following the ICH guidelines Q2B [8, 10, 11].

System suitability study. For this study first upon a 10 µl of blank solution (Acetonitrile: water 50:50) and placebo was injected and ran for 60 minutes with the gradient programme. After this 10 µl of Resolution solution was injected followed by standard solutions in 6 replicate and the % relative standard deviation (% r.s.d) of the response peak areas was calculated.

Precision (Repeatability). Precision of the assay was demonstrated by injecting six different sample solutions containing Anastrozole equivalent to 10µg/mL and Temozolomide equivalent to 10µg/mL and % r.s.d was calculated.

Intermediate Precision (Reproducibility). Intermediate Precision of the method was demonstrated by carrying out the experiment on different day, by different analyst and on different instrument using a different C-8 column.

Linearity. Linearity of the method was evaluated at different concentration levels by diluting the standard Anastrozole and Temozolomide solutions to give solutions over the range 0.165-15.0 µg/mL for both Anastrozole and Temozolomide. The first level and last level were injected in six replicates and % r.s.d was calculated for these runs and rests of levels were injected in duplicate. concentration were inputted into a Microsoft Excel® spreadsheet program to plot calibration curves .Limit of detection and limit of quantitation to calculate Limits of Detection (LOD) and Limits of Quantification (LOQ) values, sequential dilutions prepared and analyzed by the proposed method. The LOD and LOQ established by evaluating the level (signal to noise ratio of 3:1 and 10:1 respectively) at which the analytes can be readily detected and quantified with accuracy.

Accuracy. To demonstrate the accuracy of the proposed method was ascertained by carrying out recovery studies employed by Placebo spiking method. Known quantities of Anastrozole and Temozolomide (10 µg/mL) were supplemented to pre-quantified placebo solution and then experimental and true values compared i.e. freshly prepared placebo of the pharmaceutical formulation were spiked with various amounts of pure Anastrozole and Temozolomide at 50, 100 and 150%. Each solution was injected in triplicate and the peak area response of Anastrozole and Temozolomide were calculated.

Solution stability. The stability of standard and sample in solution was checked by determining the percentage deviation of the amounts present in solution after 72h at room temperature in comparison with the amount at zero time.

Robustness. Robustness of the method was demonstrated by deliberately changing the chromatographic conditions. The flow rate of the mobile phase changed from 1.0 to 0.9 and 1.1mL/min. The temperature of the column was changed from 25°C to 22°C and 27°C. The solutions described for robustness study were applied on the column in triplicate and the responses were determined

RESULTS AND DISCUSSION

Reverse phase liquid chromatography using silica-base column is successfully applied in many separations of pharmaceuticals. Asymmetric peaks, irreproducible retention and non-robust separation methods can be obtained. In this work we proposed a simple, sensitive and accurate HPLC method for simultaneous determination of Anastrozole and Temozolomide. To obtain symmetrical peaks with better resolution the chromatographic conditions i.e. eluent optimization (pH, silanol blockers) were optimized. Various chromatographic conditions such as mobile phase composition, analytical columns with different packing materials (C8, C18, phenyl, cyano), and configurations (10, 15, 25cm columns) tested to obtain sharp peaks with reduced tailing and better resolution. Finally an Inertsil C8 column (250 x 4.6mm 5 µm particle size) column selected which provided reduced peak tailing. Mobile phase selected from peak parameters (symmetry, tailing etc.), run time, ease of preparation and cost. The most suitable mobile phase composition found to Ammonium acetate buffer (0.1%) and Acetonitrile. Typical chromatograms of Anastrozole (215 nm) was shown in Fig. 3, Temozolomide (215 nm) shown in Fig. 4, Mix Standard chromatogram of Anastrozole &

Temozolomide (215 nm) shown in Fig. 5 and System suitability chromatogram of Mix Standard of Anastrozole & Temozolomide (215 nm) shown in Fig. 6 under the above mentioned conditions.

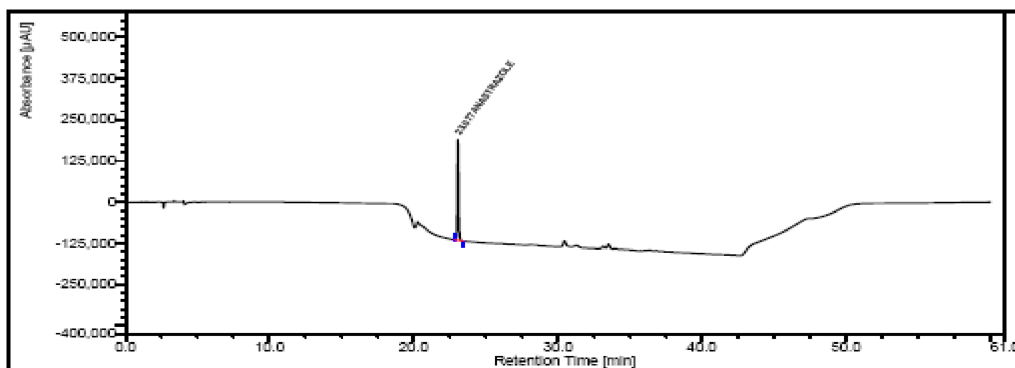

Fig. 3. Typical chromatograms of Anastrozole (215 nm).

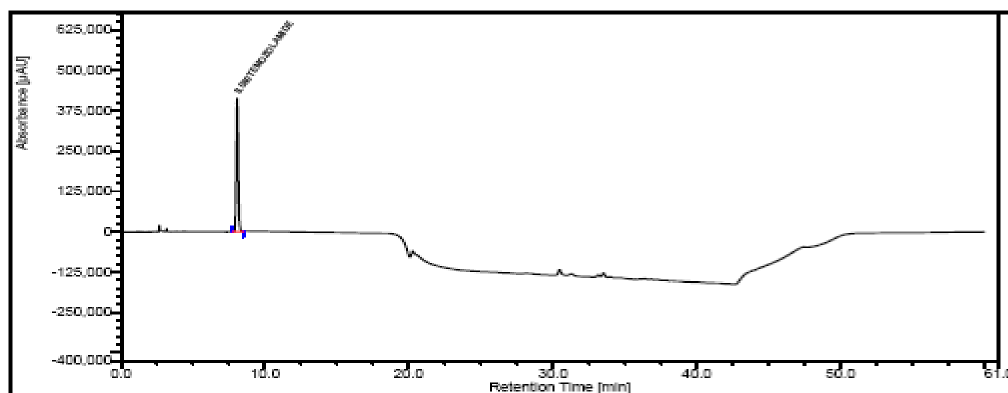

Fig. 4. Typical chromatograms of Temozolomide (215 nm).

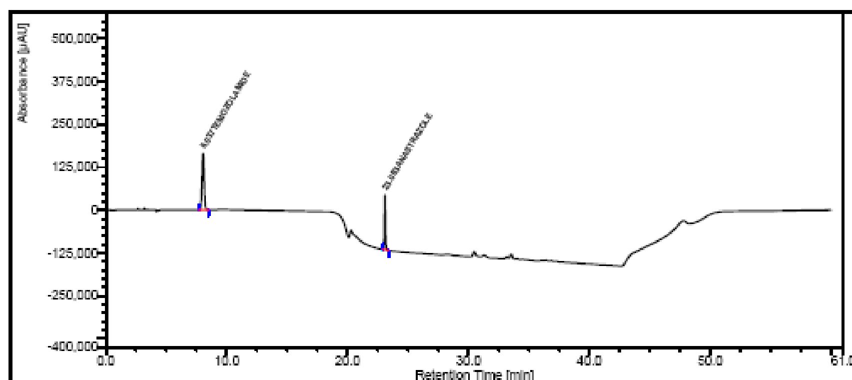

Fig. 5. Typical chromatogram of Mix Standard of Anastrozole & Temozolomide (215 nm).

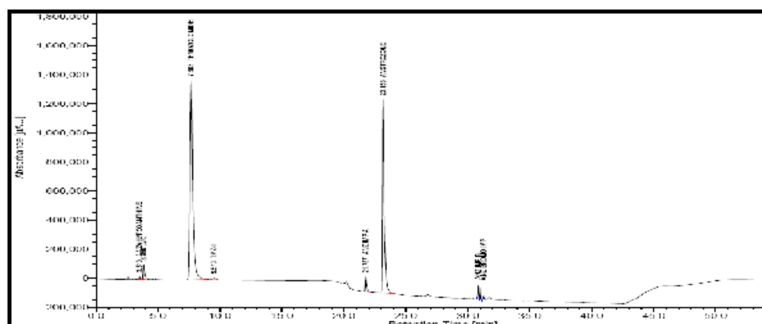

Fig. 6. System suitability chromatogram of Mix Standard of Anastrozole & Temozolomide (215 nm).

Specificity and system suitability. Specificity of the method is demonstrated in terms of spectral as well as peak purity data of the drug. Peak passed the peak purity test. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solutions. The parameters obtained are shown in Table 1.

Linearity. The proposed chromatographic method validated using ICH guidelines. Validation parameters performed include Linearity, accuracy, precision, limit of detection and quantitation, Ruggedness and robustness. Linear calibration plots for the proposed method were obtained in the concentration range of 0.17-15 µg/mL for Anastrozole and 0.17 to 15 µg/ mL for Temozolomide. The linear regression equation for Anastrozole was found to be y = 997514x + 7103.2 with correlation coefficient 0.999 and the linear regression equation for Temozolomide found to be y = 1,984,462.43x - 1,121.41 with correlation coefficient 0.999. The parameters obtained are shown in Table 2.and linearity graph for Anastrozole & Temozolomide are as shown in Fig. 6 and Fig. 7 respectively.

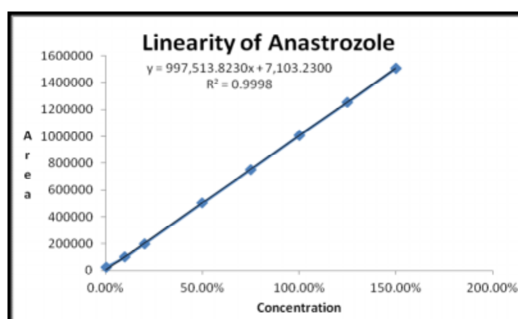

Fig. 7. Linearity graph for Anastrozole.

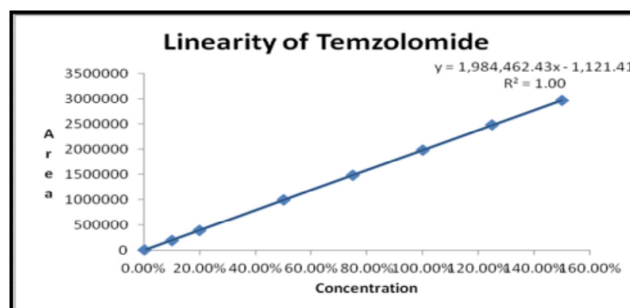

Fig. 8. Linearity graph for Temozolomide.

Limit of Detection and Limit of Quantification. LOD found to be 0.092µg/ mL, 0.075 µg/mL for Anastrozole & Temozolomide respectively (signal to noise ratio of 3:1). LOQ found to be 0.17 µg/ mL, 0.16 µg/mL for Anastrozole & Temozolomide respectively (signal to noise ratio of 10 : 1).

Precision and Intermediate Precision (Reproducibility). Precision determined by injecting five standard solutions on the same day and six individual sample preparation (n = 6). Relative standard deviation (r.s.d. %) of the % assay calculated to represent precision. The results of precision and intermediate Precision (Ruggedness) presented in Table 3.

Table 1. System suitability parameters.

<table>
<tr><td>Parameter</td><td colspan="2">Temozolomide</td><td colspan="2">Anastrozole</td></tr>
<tr><td>Tailing factor</td><td colspan="2">1.6</td><td colspan="2">1.9</td></tr>
<tr><td>Theoretical Plates</td><td colspan="2">7239</td><td colspan="2">172812</td></tr>
<tr><td colspan="5">Resolution</td></tr>
<tr><td>2-Azahypoxanthine and AIC Impurity</td><td>2.00</td><td colspan="2">ANZ Impurity A and Anastrozole</td><td>7.04</td></tr>
<tr><td>Temozolomide and TMZ-II impurity</td><td>4.46</td><td colspan="2">ANZ impurity B and ANZ Bromo Impurity</td><td>2.24</td></tr>
</table>

Table 2. Linearity parameters.

		Area of Temozolomide		Area of Anastrozole
1	0.17 %	3273	0.16 %	24152
2	10 %	198599	10 %	100016
3	20 %	392978	20 %	200224
4	50 %	990627	50 %	501898
5	75 %	1488360	75 %	752937
6	100 %	1982557	100 %	1005739
7	125 %	2480228	125 %	1253672
8	150 %	2975332	150 %	1506657

Table 3.

Precision	**Label Claim of Anastrozole**	**Label Claim of Temozolomide**	**% assay of Anastrozole**	**% assay of Temozolomide**
Mean	1.00	19.13	99.98	95.67
% RSD			0.57	1.21

Intermediate Precision (Reproducibility)	Label Claim of Anastrozole	Label Claim of Temozolomide	% assay of Anastrozole	% assay of Temozolomide
Mean	1.01	19.24	100.52	96.19
% RSD			1.58	1.21
% VARIATION			0.54	0.55

Accuracy and Recovery

Table 4. % Recovery of Anastrozole.

	Amount added	Amount found	% Recovery	
50%	5.10	5.18	101.55	
		5.19	101.74	Mean=101.67
		5.19	101.73	% R.S.D. =0.11
100%	10.20	10.24	100.35	
		10.28	100.75	Mean=100.50
		10.24	100.40	% R.S.D. =0.22
150%	15.30	15.03	98.25	
		15.11	98.73	Mean=98.41
		15.03	98.25	% R.S.D. =0.28

Table 5. % Recovery of Temozolomide.

	Amount added	Amount found	% Recovery	
50%	4.95	4.95	99.91	
		4.94	99.77	Mean=99.98
		4.96	100.27	% R.S.D. =0.26
100%	9.90	9.90	99.99	
		9.90	99.99	Mean=99.82
		9.85	99.49	% R.S.D. =0.29
150%	14.85	14.79	99.63	
		14.79	99.59	Mean=99.62
		14.79	99.63	% R.S.D. =0.02

Accuracy and recovery. The recovery percentage and % r.s.d. were calculated for each analyte. Recovery of Anastrozole and Temozolomide ranged from 98.28-101.74% and 99.59-100.27% respectively and % r.s.d. Anastrozole and Temozolomide for The results are shown in Table 4 and 5 respectively. The acceptance criteria for recovery of Anastrozole and Temozolomide at a concentration level of 50%, 100 % and 150% is between 98 and 102%.

Stability. The stability of Anastrozole and Temozolomide in solution was checked by determining the percentage deviation of the amounts present in solution after 72h at room temperature in comparison with the amount at zero time. The results obtained after 72h showed no significant variation; the percentage deviation was less than 2% of the initial amount. This is indicative of good stability of each component in the mixture over a period of 72hr.

Robustness. Robustness studies signified that the results of the method remained unaffected by small, deliberate changes in the flow rate and column temperature. The RSD of mean assay values was found to be 0.81% for Anastrozole and 0.32% for Temozolomide with a flow rate of 0.9 mL/min. The r.s.d. of mean assay values was found to be 0.27% for Anastrozole and 0.98% for Temozolomide with a flow rate of 1.1 mL/min. Also, r.s.d. of mean assay values was found to be 0.81 and 0.27% for Anastrozole and Temozolomide, respectively, at 22°C and 0.50 and 1.13% for Anastrozole and Temozolomide, respectively, at 27°C.

CONCLUSIONS

The advantages of the proposed method involve a simple procedure for sample preparation and relatively short time of analysis. Apart from this, it

can be used for assays of Anastrozole and Temozolomide in biological fluids or in pharmacokinetic investigations. The proposed method was validated by testing its linearity, accuracy, precision, limits of detection, and limit of quantitation. Robustness and stability of solutions. The results of the analysis of pharmaceutical dosage forms by the proposed methods are highly reproducible, reliable, and are in good agreement with the label claims of the drug. The additives usually present in the pharmaceutical formulations of the assayed samples did not interfere with Anastrozole and Temozolomide. It may be said that the proposed methods are precise, sensitive, and accurate, so that these can be used as standard Pharmacopeial methods for the simultaneous determination of Anastrozole and Temozolomide using the HPLC systems with PDA detector.

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