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Development and validation of a simple and sensitive high performance liquid chromatographic method for the simultaneous determination of anastrozole, bicalutamide, tamoxifen, and their synthetic impurities

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ABSTRACT

A simple and sensitive analytical method for simultaneous determination of anastrozole, bicalutamide, and tamoxifen as well as their synthetic impurities, anastrozole pentamethyl, bicalutamide 3-fluoro-isomer, and tamoxifen e-isomer, was developed and validated by using high performance liquid chromatography (HPLC). The separation was achieved on a Symmetry® C-8 column (100 × 4.6 mm i.d., 3.5 μm) at room temperature (± 24 °C), with a mobile phase consisting of acetonitrile/water containing 0.18% *N*,*N* dimethyloctylamine and pH adjusted to 3.0 with orthophosphoric acid (46.5/53.5, v/v) at a flow rate of 1.0 mL min^{-1} within 20 min. The detection was made at a wavelength of 270 nm by using ultraviolet (UV) detector. No interference peaks from excipients and relative retention time indicated the specificity of the method. The calibration curve showed correlation coefficients (r) > 0.99 calculated by linear regression and analysis of variance (ANOVA). The limit of detection (LOD) and limit of quantitation (LOQ), respectively, were 2.2 and 6.7 μg mL^{-1} for anastrozole, 2.61 and 8.72 μg mL^{-1} for bicalutamide, 2.0 and 6.7 μg mL^{-1} for tamoxifen, 0.06 and 0.22 μg mL^{-1} for anastrozole pentamethyl, 0.02 and 0.07 μg mL^{-1} for bicalutamide 3-fluoro-isomer, and 0.002 and 0.007 μg mL^{-1} for tamoxifen e-isomer. Intraday and interday relative standard deviations (RSDs) were < 2.0% (drugs) and < 10% (degradation products) as well as the comparison between two different analysts, which were calculated by f test.

1. Introduction

Anastrozole (ATZ) (Fig. 1a) is a selective non-steroidal aromatase inhibitor widely used for the treatment of breast cancer in post-menopausal women. This drug has been shown to inhibit the enzyme aromatase, which is responsible for converting androgens (produced by the women in the adrenal glands) to estrogens. It significantly reduces serum estradiol concentration. In addition, there is detectable effect on formation of aldosterone or corticosteroids [1–4]. Anastrozole pentamethyl (ANP) (Fig. 1b) is a synthetic impurity from ATZ, which was selected for this research. Another drug used in treatment of breast cancer is tamoxifen (TMF) (Fig. 1c). TMF is a non-steroidal anti-estrogen compound, which belongs to a class of drugs called selective receptor modulators. It has an additional side chain (trans isomer) that accounts for its antiestrogenic activity. TMF also interacts with other corepressors or coativactors in the tissue and binds with different estrogen receptors, producing both antiestrogenic and estrogenic effects [1–4]. Tamoxifen e-isomer (TEI) (Fig. 1d) is a synthetic degradation product from TMF, the properties of which have not been fully investigated. Bicalutamide (BCM) (Fig. 1e) was also selected in this research due to its efficacy in the treatment of prostate cancer; it is a non-steroidal anti-androgen drug. Thus, BCM competes with androgen for the binding of androgen receptors, blocking the action of androgens from testicular and adrenal origin which stimulate the growth of malignant and normal prostatic tissue [1–4]. Bicalutamide 3-fluoro-isomer (BFI) (Fig. 1f) is a synthetic impurity from BCM, and its pharmacological properties have not been fully investigated. Therefore, it is very important to develop and validate a suitable analytical method for this degradation product in tablets.

Currently, all degradation products are determined by chromatography or related techniques; however, for over decade high performance liquid chromatography (HPLC) has been the most important in pharmaceutical dosages. New attention has been given to the toxicological issues, which brought a greater sensitivity to the significance of impurities at trace levels. HPLC is the first choice for the analysis of pharmaceuticals and their

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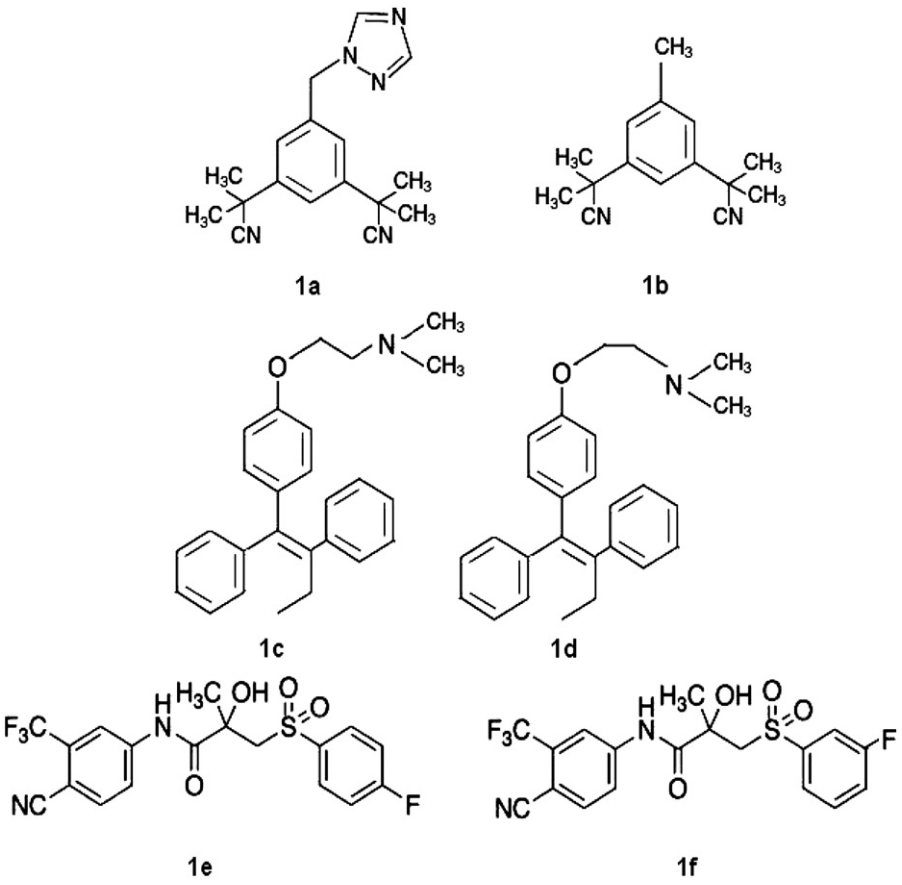

Fig. 1. Chemical structures of investigated compounds (1a——ATZ, 1b—ANP, 1c—TMF, 1d—TEI, 1e—BCM, and 1f—BFI).

formulations because sample preparation is substantially simple and derivatization of the drug prior to analysis is not required. It is also able to solve many problems posed by the pharmaceutical companies. Furthermore, the majority of related substances determinations are performed by HPLC; It offers the desired sensitivity and typical detection limits for degradation products of 0.1% or lower, which can be easily achieved by using conventional ultraviolet (UV) detectors. HPLC methods must be able to separate all the degradation products from each other; these methods should also be optimized to separate and quantify in the dosage forms. Moreover, these methods need to be validated demonstrating specificity, linearity, limit of detection, limit of quantitation, precision, accuracy, and robustness. The validation of analytical methods is very important in the registration of new drugs. In order to assure the quality and safety of a medicine, it is necessary to effectively monitor and control the impurities. Therefore, reliable HPLC methods to determine degradation products in drugs must be developed and validated. Related substances are one of the most important issues in modern pharmaceutical analysis [5–8].

A small number of analytical methods based on different techniques have been reported for analysis of ATZ, BCM, and TMF and their related substances or metabolites by liquid chromatography mass spectrometry detection (LC-MS) or liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) [9–14], liquid chromatography UV or photodiode array (PDA) detections (LC-UV or LC-PDA) [15–33], capillary gas chromatography and gas chromatography [34,35], capillary electrophoresis [36,37], and spectrophotometry [38,39].

LC-MS methods are very sensitive and specific; nevertheless, they are not very popular in laboratories from Latin America because they are expensive and time-consuming, also require complicated sample preparation techniques. Complicated pretreatment methods may introduce errors, and the use of large volumes of organic solvents poses a health hazard to those performing analyses and contributes to environmental pollution. No published study is available on the simultaneous HPLC determination and validation of ATZ, BCM, and TMF as well as their main related substances (ANP, BFI, and TEI). Consequently, the aim of the present study was to develop and validate a simple and sensitive reversed-phase HPLC method that would be capable of simultaneously determining ATZ, BCM, TMF and their impurities in tablets, and be able to be successfully applied by pharmaceutical companies.

2. Materials

2.1. Materials

The HPLC-grade acetonitrile and orthophosphoric acid were purchased from Merck (Darmstadt, Germany). *N*,*N* dimethyloctylamine was purchased from Sigma-Aldrich (St. Louis, USA). High-purity water was prepared by using a Millipore Milli-Q system (Milford, USA).

2.1.1. Reference substances

The reference substances (ATZ (98%), TMF (99%), BCM (98%), ANP (100%), TEI (98%), and BFI (100.00%)) were purchased from Sigma-Aldrich and United States Pharmacopeia.

2.1.2. Samples

The samples were obtained from local pharmaceutical companies and drugstores (tablets containing 1.0 mg ATZ/tablet, 50.0 mg BCM/tablet, and 20.0 mg TMF/tablet).

2.2. Instrumentation and analytical conditions

An Agilent 1200 series HPLC (Agilent, Palo Alto, CA, USA) was employed, consisting of an on-line degasser, binary pump, autosampler, thermostated column oven and UV detector. Data were acquired and processed with ChemStation (Agilent, Palo Alto, CA, U.S.A.). Many columns were evaluated such as Symmetry® C-18 and C-8 (5 μm, Waters) with different lengths (250 × 4.6, 150 × 4.6, 100 × 4.6, 50 × 4.6, 150 × 3.9, 50 × 3.9, 250 × 3.0, 150 × 2.1, and 100 × 2.1 mm). However, a C-8 reverse-phase column (Symmetry® C-8, 150 × 4.6 mm, 3.5 μm, Waters) was used for the separation and validation of oncologic drugs and their degradation products. An isocratic elution was achieved by using a mobile phase which consisted of acetonitrile/water containing 0.18% *N*,*N* dimethyloctylamine and pH adjusted to 3.0 with orthophosphoric acid (46.5/53.5, v/v). The flow-rate was 1.0 mL min^{-1} and the injection volume was 20 μL. The absorbance detection wavelength was 270 nm. The column temperature was set at room temperature (± 24 °C) in all experiments performed.

2.3. Preparation of standard, sample, and placebo

2.3.1. Standard solution

A stock solution was prepared in dilution phase containing acetonitrile/water (20:80, v/v) at a concentration of 200 μg mL^{-1} of ATZ, 100 μg mL^{-1} of BCM, 40 μg mL^{-1} of TMF, 1 μg mL^{-1} of ANP, 0.5 μg mL^{-1} of BFI, and 0.24 μg mL^{-1} of TEI. This stock solution was sonicated for 15 min. It was then serially diluted and completed with water to obtain working standard solutions from 10.0 to 150.0 μg mL^{-1} to ATZ, from 5.0 to 75.0 μg mL^{-1} to BCM, from 2.0 to 30.0 μg mL^{-1} to TMF, from 0.050 to 0.750 μg mL^{-1} to ANP, from 0.0250 to 0.3750 μg mL^{-1} to BFI, and from 0.010 to 0.150 μg mL^{-1} to TEI.

2.3.2. Sample solution

Twenty tablets of each drug sample were powdered and weighed. Equivalent portions of 1 mg of ATZ, 50 mg of BCM, and 20 mg of TMF were accurately weighed and diluted with 20 mL of acetonitrile in 100 mL volumetric flask. This solution was sonicated for 30 min and filtrated. An aliquot of 0.1 mL was transferred to 10 mL volumetric flask and the volume was completed with water. The final sample concentrations were 100 μg mL^{-1} of ATZ, 50 μg mL^{-1} of BCM, and 20 μg mL^{-1} of TMF.

2.3.3. Placebo solution

Identical pharmaceutical formulations of the samples without their active ingredients were weighed. Equivalent portions of 1 mg of ATZ, 50 mg of BCM, and 20 mg of TMF were accurately weighed and diluted with 20 mL of acetonitrile in 100 mL volumetric flask. This solution was sonicated for 30 min and filtrated. An aliquot of 0.1 mL was transferred to 10 mL volumetric flask and the volume was completed with water. The final sample concentrations were equivalent to 100 μg mL^{-1} of ATZ, 50 μg mL^{-1} of BCM, and 20 μg mL^{-1} of TMF.

2.4. Procedures

2.4.1. Method validation

The method was validated according to the ICH Q3A Guidance for Industry: Impurities in New Drug Substances (International Conference on Harmonization) [7], United States Pharmacopeia, Thirty-fifth Edition (2012) [40], and ICH Guidance for Industry (International Conference on Harmonization 2005) [41].

2.4.2. System suitability

A standard solution containing 100.0 μg mL^{-1} (ATZ), 50.0 μg mL^{-1} (BCM), 20.0 μg mL^{-1} (TMF), 0.50 μg mL^{-1} (ANP), 0.25 μg mL^{-1} (BFI), and 0.10 μg mL^{-1} (TEI) was prepared by dilution with dilution phase. System suitability was determined from six replicate injections of standard solution.

2.4.3. Specificity

The specificity of the method was assessed by comparing the chromatogram obtained with placebo solution and relative retention time of each impurity. Injections were made in triplicate.

2.4.4. Calibration curve

The linearity was determined by the calibration curve obtained using seven standard solutions in the concentration range of 10.0 to 150.0 μg mL^{-1} (ATZ), 5.0 to 60.0 μg mL^{-1} (BCM), 2.0 to 36.0 μg mL^{-1} (TMF), 0.05 to 0.60 μg mL^{-1} (ANP), 0.025 to 0.300 μg mL^{-1} (BFI), and 0.01 to 0.15 μg mL^{-1} (TEI), respectively. Triplicate determinations at each concentration level were performed. The range (interval between lower and upper concentrations in the sample) of the appropriate amount of samples was determined. The slope and other statistics of calibration curves were calculated by linear regression and analysis of variance (ANOVA) [40–45].

2.4.5. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were determined based on standard deviation among responses and slopes of the regression equation of the calibration curve [45].

2.4.6. Precision

The intra-day and inter-day precisions were determined by analyzing the standard and sample solutions. For this evaluation, six standard solution and ten sample solution replicates at 100% of the test concentration were prepared in different days by different analysts. The statistical data were obtained from *F* test [45,46].

2.4.7. Accuracy

The accuracy was evaluated by adding known amounts of standard solution in the sample solution. The accuracy was evaluated at three concentration levels, which were from 80 to 120% for each drug as well as their synthetic impurities. The recovery experiments were performed in triplicate and data were determined by dividing the value obtained for the sample prepared with the added standard, by the amount added, and then multiplied by 100%.

2.4.8. Robustness

Robustness was assessed by testing the susceptibility of measurements to deliberate variation of the analytical parameters. It was evaluated as to the influence of operational conditions (factors), which can affect the overall separation of the proposed analytical method. The analyses were performed in duplicate and introduced a phantom factor in order to evaluate the random error through an experimental design of Placket and Burman [47,48]. The pH of mobile phase, % of organic modifier, flow rate, wavelength, % of *N*,*N* dimethyloctylamine, and injection volume on the column were evaluated.

2.4.9. Stability

Standard and sample solutions were prepared separately at 100% as described to obtain solutions containing 100.0 μg mL^{-1} (ATZ), 50.0 μg mL^{-1} (BCM), 20.0 μg mL^{-1} (TMF), 0.50 μg mL^{-1} (ANP), 0.25 μg mL^{-1} (BFI), and 0.10 μg mL^{-1} (TEI). These solutions were stored in the refrigerator at (± 4 °C), and triplicate measurements were made on three consecutive days.

3. Results and discussions

3.1. Development of HPLC method

The development of this analytical method was a challenging task due to the very similar chemical characteristics between oncologic drugs and their respective synthetic impurities, especially BCM and BFI. It was critical to select adequate analytical conditions which could be fitted according to development of the method. In order to achieve this goal, several columns C-18 and C-8 have been tested. Based on the chemical structures of compounds, there are some chromophore groups, which is very useful to maximize detection and minimize background noise [49]. Therefore, UV detection was selected. In addition, there are no functional groups which can be easily ionized and also the aromatic rings of these compounds provide them with hydrophobic characteristics (Fig. 1); thus, they should be easily retained in the column. Each column was evaluated by focusing on separation of the critical pair (BCM and BFI), as well as on their capability of overall separation of the six compounds. Limited or partial separations were achieved by using columns C-18 with 5.0 μm size of particle in different lengths; nonetheless, the molecules were strongly retained on the column and the separations were still poor due to the high hydrophobicity of the critical pair and other compounds. A satisfactory separation was achieved with a C-8 column with 3.5 particle size; it also provided faster and better separation and higher efficiency. The particle size substantially improved the performance over the C-8 column with 5.0 μm particle size for the same column length. The column performances showed that the C-8 with 3.5 μm particle size provided the best overall separation. Due to that, some compounds showed less interaction with this stationary phase; thus, it can be deduced that these substances eluted with more facility, and also that its particle size has influenced the overall separation and critical pair because the pressure of equipment and number of theoretical plates on the column were higher. Therefore, this column was selected for further method validation because it was able to separate the molecules from each other under appropriate mobile phase conditions. Acetonitrile was chosen as organic modifier because it showed better separation characteristics than methanol and also has less viscosity [49], resulting in low column pressure. When methanol was used as an organic modifier at pH 3.0, it was not able to provide a suitable overall separation of the compounds in the chromatographic profile. The peak of TMF and TEI became broadened even at very low level; thus, this phenomenon made the use methanol inappropriate as sample diluent or organic modifier. At different pH values of the mobile phase, these substances showed different retention behaviours based on whether they are ionized or neutral at specific pH. In order to determine the pH value that should provide the best overall separation, different pH values of aqueous solution in the mobile phases were tested. However, the best separation was achieved when the pH was established to 3.0; at this pH, the compounds were stable in their molecular forms. Even though the molecules cannot be easily ionized, the pH and organic modifier strength were very sensitive. They can substantially affect the separation and retention time of these substances under the conditions of reversed phase chromatography. Therefore, acetonitrile–water as mobile phase at pH 3.0 was established for validation of the analytical method.

When *N,N* dimethyloctylamine was added to the mobile phase, it strongly affected the separation and significantly improved peak shapes due to blockage of ionized silanols; thus, it was conclusive to excellent changes in the selectivity and desired peak tailing. It substantially reduced the peak tailing of the compounds without any significant negative impact on the overall separation. The unacceptable peak tailing of TMF and TEI peaks is most probably due to molecules adsorbed on the stationary phase and eluted in the mobile phase. Mobile phase additive ions, such as *N,N* dimethyloctylamine, can compete for the stationary phase adsorption and also impact the retention behaviours of the compounds. Based on the overall separation, it is clear that *N,N* dimethyloctylamine improved the peak tailing factor of the six compounds of interest. Moreover, it slightly decreased the retention of molecules and reduced the interaction of these compounds with the stationary phase.

3.2. Method validation

3.2.1. System suitability

A standard solution was injected six times into the HPLC. The system was deemed to be suitable because the resolution between oncologic drugs and their respective synthetic impurities was greater than 0.9, the reproducibility of 6 injections of each compound peak area produced a relative standard deviation (RSD) lower than 2%, and the tailing factors were higher than 0.98 (Table 1). With these results, it can be concluded that the chromatographic system is perfectly adjusted to perform analysis.

3.2.2. Specificity

Specificity of the current method was demonstrated by good separation of oncologic drugs as well as their synthetic impurities with adequate resolution (Table 1 and Fig. 2). Furthermore, it was investigated by observing potential interferences between the investigated compounds and excipients from the placebo sample; no interfering peaks were observed in the placebo (Fig. 3) and

Table 1
Results obtained for system suitability.

Parameters	ATZ	ANP	BCM	BFI	TMF	TEI
Retention time (min)	4.743	15.978	9.368	9.928	5.950	4.061
Relative retention time	–	0.29	–	0.94	–	1.46
Area	6.522	8.127	1,524.971	53.658	450.120	67.307
Theoretical plates	70,899	10,3684	10,5207	11,0555	70.309	87,697
Capacity factor	2.171	9.683	5.263	5.637	2.978	1.715
Symmetry	0.751	0.933	0.960	0.924	0.764	0.934
Resolution	4.190	0.933	12.903	1.844	5.810	–
Tailing factor	0.995	0.843	1.034	0.952	1.272	1.040

Relative retention time = ratio between the retention time of a standard and degradation product.

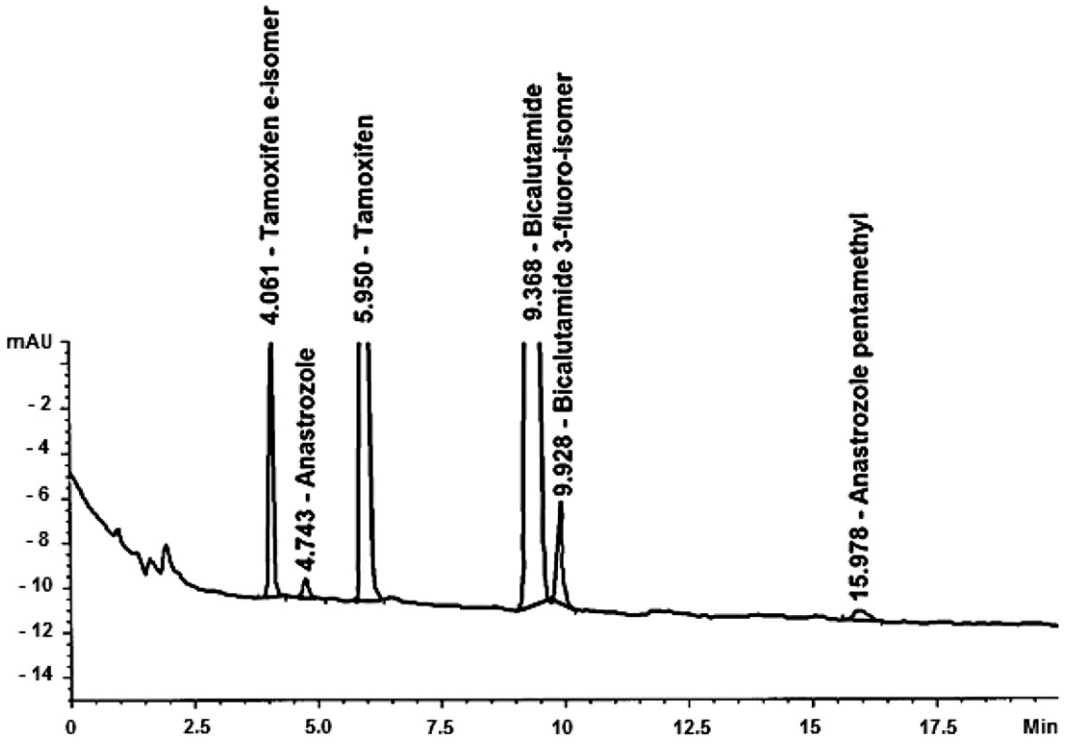

Fig. 2. Representative chromatogram of drugs and their synthetic impurities. Concentrations: 0.1 μg mL^{-1} of TEI, 100.0 μg mL^{-1} of ATZ, 20.0 μg mL^{-1} of TMF, 50.0 μg mL^{-1} of BCM, 0.25 μg mL^{-1} of BFI, and 0.5 μg mL^{-1} of ANP. Chromatographic conditions: Isocratic separation, Symmetry® C-8 column (100 × 4.6 mm i.d., 3.5 μm) at room temperature (±24 °C), with a mobile phase consisting of acetonitrile/water containing 0.18% *N*,*N* dimethyloctylamine and pH adjusted to 3.0 with orthophosphoric acid (46.5/53.5, v/v), at a flow rate of 1.0 mL min^{-1} within 20 min. The detection was made at a wavelength of 270 nm by using UV detector.

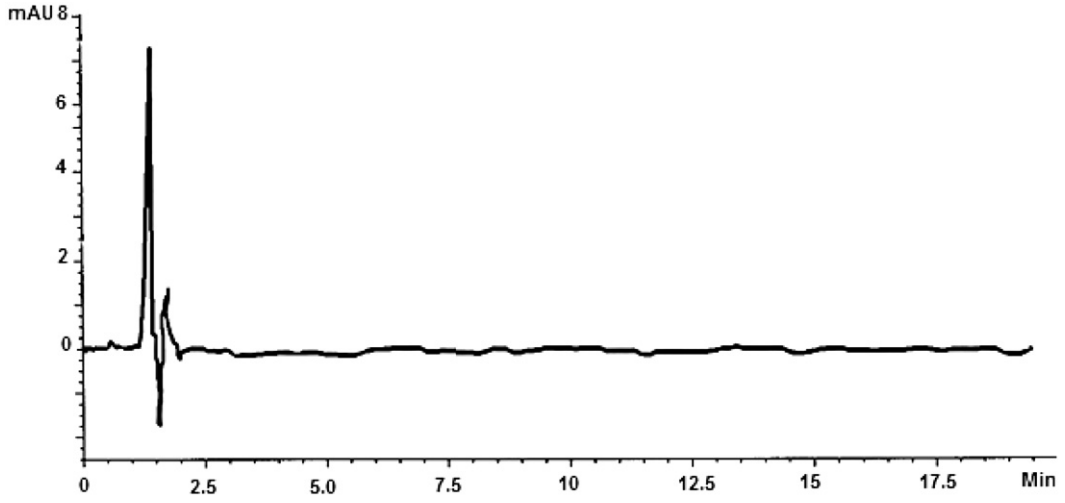

Fig. 3. Representative chromatogram of placebo. Concentrations: 100.0 μg mL^{-1} of ATZ, 50.0 μg mL^{-1} of BCM, and 20.0 μg mL^{-1} of TMF. Chromatographic conditions: Isocratic separation, Symmetry® C-8 column (100 × 4.6 mm i.d., 3.5 μm) at room temperature (±24 °C), with a mobile phase consisting of acetonitrile/water containing 0.18% *N*,*N* dimethyloctylamine and pH adjusted to 3.0 with orthophosphoric acid (46.5/53.5, v/v), at a flow rate of 1.0 mL min^{-1} within 20 min. The detection was made at a wavelength of 270 nm by using UV detector.

sample (Fig. 4) chromatograms. Therefore, the specificity of the analytical method was proved.

3.2.3. Calibration curve

The calibration curve was linear by analyzing standard solutions at seven concentration levels. The correlation coefficients (r) were >0.99 (Tables 2 and 3). Therefore, the drugs and their synthetic impurities presented good linearity. The ANOVA for linearity of the drugs and their degradation products is also presented in Tables 2 and 3, respectively. The distribution variable (F) value for lack of fit was smaller than the tabulated F value for 95% confidence, according to the ANOVA test; the HPLC method showed no lack of fit.

3.2.4. Limit of detection (LOD) and limit of quantitation (LOQ)

According to the International Conference on Harmonization (ICH) recommendations [41], the approach based on the standard deviation (SD) of the response and the slope was used for determining the detection and quantitation limits. The theoretical values are showed in Tables 2 and 3, respectively; these results also proved the sensitivity of the proposed analytical method.

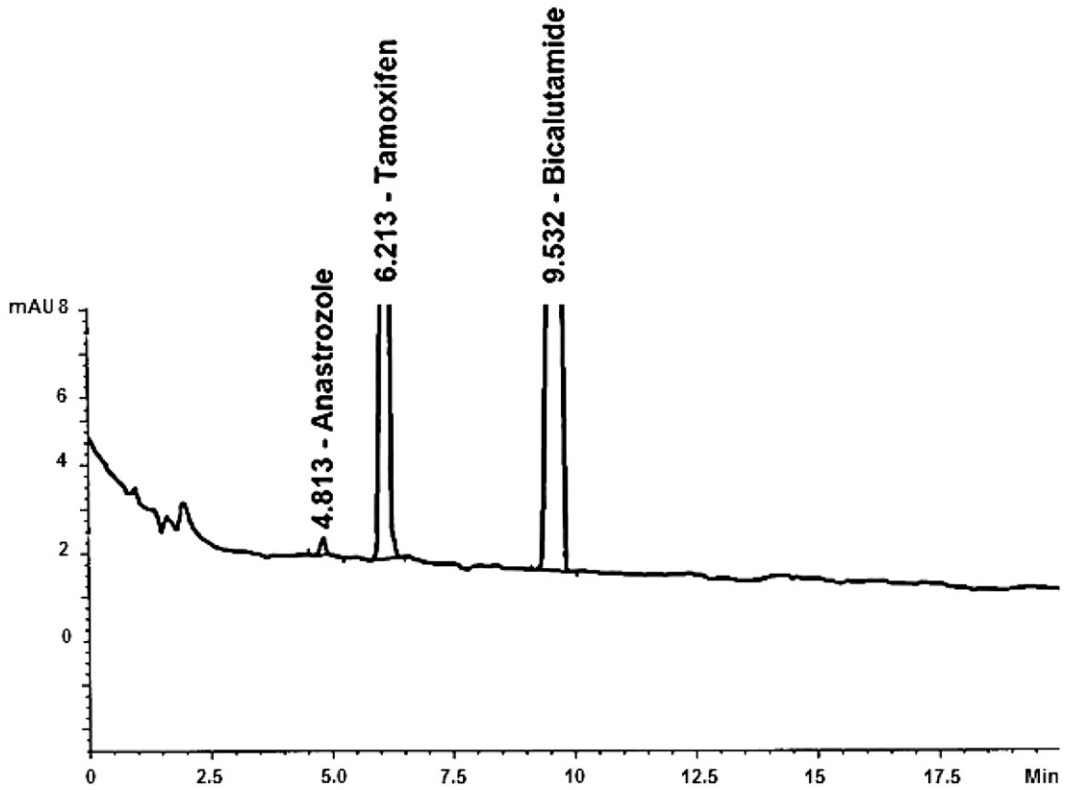

Fig. 4. Representative chromatogram of samples. Concentrations: 100.0 μg/mL of ATZ, 20.0 μg mL^{-1-1} of TMF, and 50.0 μg mL^{-1} of BCM. Chromatographic conditions: Isocratic separation, Symmetry® C-8 column (100 × 4.6 mm i.d., 3.5 μm) at room temperature (±24 °C), with a mobile phase consisting of acetonitrile/water containing 0.18% *N*,*N* dimethyloctylamine and pH adjusted to 3.0 with orthophosphoric acid (46.5/53.5, v/v), at a flow rate of 1.0 mL min^{-1} within 20 min. The detection was made at a wavelength of 270 nm by using UV detector.

Table 2
Linear regression and ANOVA statistical data in the analysis of ATZ, ANP, and BCM.

Statistical Parameters	ATZ	ANP	BCM
Concentration range (μg mL^{-1})	10.0–150.0	0.05–0.60	5.0–60.0
Regression equation	$y=0.064\times+0.016$	$y=16.44\times-0.298$	$y=30.87\times-4.149$
Correlation coefficient (*r*)	0.9999	0.9970	0.9995
Limit of detection (μg mL^{-1})	2.2	0.06	2.61
Limit of quantitation (μg mL^{-1})	6.7	0.22	8.72
Standard error	0.0489	0.3786	26.9384
F	27,370.1799	737.5485	5,134.7244
SS (residual)	0.0119	0.7168	3,628.4122
MS (residual)	0.0023	0.1433	725.6825
SS (regression)	65.6621	105.7423	3726,179.4230
MS (regression)	65.6621	105.7423	3726,179
Lower 95%	−0.0740	−0.9978	−53.9138
Upper 95%	0.1069	0.4010	45.6145

F=distribution variable.
SS=sum of squares.
MS=mean square.

3.2.5. Precision

The precision was determined by repeatability and intermediate precision levels. For repeatability and intermediate precisions, 10 independent samples containing the synthetic impurities were performed on two different days by two different analysts. The RSD was $<2\%$ (drugs) and $<10\%$ (degradation products), respectively (Table 4); these results indicated the precision of the proposed analytical method. The *F* test was also applied in order to compare the variability between the analysts and sample variations; the computed *F* value for all compounds did not exceed the *F* critical value (4.02) (Tables 5 and 6). Thus, it can be concluded that there is no difference between the variances obtained. A chromatogram of samples contaminated with synthetic impurities is shown in Fig. 5.

3.2.6. Accuracy

According to ICH guidelines, the standard solution addition should be done in a range from 80 to 120% of the nominal concentration. The accuracy of the method was evaluated at three concentration levels. Triplicate determinations were made at each concentration level. The accuracy was expressed as percentage of standard recovered from sample matrix. The mean recoveries of investigated oncologic drugs and their degradation products were

Table 3
Linear regression and ANOVA statistical data in the analysis of BFI, TMF, and TEI.

Statistical parameters	BFI	TMF	TEI
Concentration range ($\mu g\ mL^{-1}$)	0.025–0.300	2.0–36.0	0.01–0.15
Regression equation	$y=225.0\times-1.287$	$y=22.52\times+1.858$	$y=684.0\times-0.597$
Correlation coefficient (r)	0.9999	0.9994	0.9988
Limit of detection ($\mu g\ mL^{-1}$)	0.02	2	0.002
Limit of quantitation ($\mu g\ mL^{-1}$)	0.07	6.7	0.007
Standard deviation	1.6237	0.0489	0.0270
F	1876.769	27,370.1799	2192.3416
SS (residual)	13.1824	0.0119	16.6877
MS (residual)	2.6364	0.0023	3.3375
SS (regression)	4948.0970	65.6621	7317.0607
MS (regression)	4948.0970	65.6621	7317.0610
Lower 95%	−4.2873	−0.0740	−3.9727
Upper 95%	1.7117	0.1069	2.7770

F=distribution variable.
SS=sum of squares.
MS=mean square.

Table 4
Average of precision results performed by two analysts.

Area (20)	ATZ	ANP	BCM	BFI	TMF	TEI
Average	7.985	8.789	1531.935	71.489	483.177	71.419
SD	0.155	0.253	16.835	1.248	2.07139	1.608
RSD%	1.944	2.888	1.098	1.746	0.428	2.252

SD=standard deviation.
RSD%=relative standard deviation.

Table 5
F-test two-sample for variances of ATZ, ANP, and BCM.

ATZ	Variable 1	Variable 2	ANP	Variable 1	Variable 2	BCM	Variable 1	Variable 2
Mean	7.877	8.093	–	8.604	8.974	–	1534.706	1529.165
Variance	0.014	0.010	–	0.039	0.020	–	428.366	152.920
Observations	10	10	–	10	10	–	10	10
Df	9	9	–	9	9	–	9	9
F	1.360	–	–	1.936	–	–	2.801	–
$P(F<=f)$ one-tail	0.327	–	–	0.169	–	–	0.070	–
F critical one-tail	4.025	–	–	4.025	–	–	4.025	–

df=degree of freedom.
F=distribution variable.

Table 6
F-test two-sample for variances of BFI, TFC, and TEI.

BFI	Variable 1	Variable 2	TMF	Variable 1	Variable 2	TEI	Variable 1	Variable 2
Mean	72.202	70.775	–	481.274	485.081	–	70.332	72.505
Variance	1.085	1.072	–	0.635	0.370	–	1.708	1.129
Observations	10	10	–	10	10	–	10	10
Df	9	9	–	9	9	–	9	9
F	1.011	–	–	1.714	–	–	1.512	–
$P(F<=f)$ one-tail	0.493	–	–	0.216	–	–	0.273	–
F critical one-tail	4.025	–	–	4.025	–	–	4.025	–

df=degree of freedom.
F=distribution variable.

found to be in the range of 98.00 and 102.00%, indicating good accuracy for the chromatographic method (Table 7).

3.2.7. Robustness

In order to simultaneously investigate a number of factors in a predefined number of experiments, an experimental design from Plackett and Burman of eight analytical runs was successfully applied. After selecting the possible critical factors, nominal parameters were established with lower and upper limits. In the majority of experiments, an overall separation among the peaks was performed, and it was very clear that the percentage of acetonitrile and pH in the mobile phase were the most important factors for the separation and selectivity of these compounds; however, neither pH in the mobile phase nor organic modifier values exceeded the limits of the critical reference value (18.51) (Table 8). Therefore, the proposed method can be considered reliable and robust.

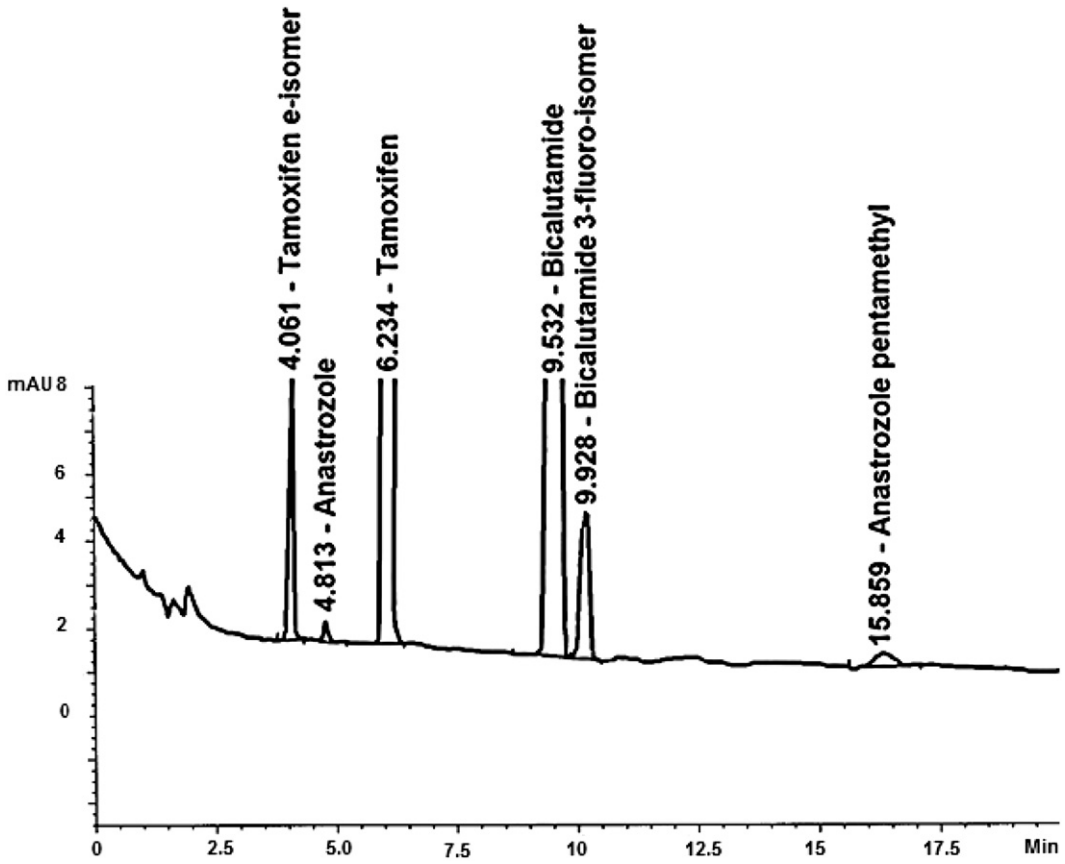

Fig. 5. Representative chromatogram of samples contaminated with impurities. Concentrations: 0.1 μg mL^{-1} of TEI, 100.0 μg mL^{-1} of ATZ, 20.0 μg mL^{-1} of TMF, 50.0 μg mL^{-1} of BCM, 0.25 μg mL^{-1} of BFI, and 0.5 μg mL^{-1} of ANP. Chromatographic conditions: Isocratic separation, Symmetry® C-8 column (100 × 4.6 mm i.d., 3.5 μm) at room temperature (±24 °C), with a mobile phase consisting of acetonitrile/water containing 0.18% *N*,*N* dimethyloctylamine and pH adjusted to 3.0 with orthophosphoric acid (46.5/53.5, v/v), at a flow rate of 1.0 mL min^{-1} within 20 min. The detection was made at a wavelength of 270 nm by using UV detector.

Table 7
Accuracy of the validation method for the determination of oncologic drugs and their respective synthetic impurities in tablets, which was evaluated in a range from 80 to 120% of the nominal concentration. Results obtained in the recovery of ATZ, ANP, BCM, BFI, TMF, and TEI standard solutions added to the sample solutions.

Compound	Amount added (mL)	Amount found (mL)	Standard deviation	Relative standard deviation (%)	Recovery (%)
ATZ (80%)	4.00	4.012	0.23	0.22	100.30
ATZ (100%)	5.00	5.080	0.15	0.14	101.34
ATZ (120%)	6.00	6.876	0.77	0.78	98.45
ANP (80%)	0.20	0.199	0.67	0.67	99.54
ANP (100%)	0.30	0.304	0.35	0.34	101.57
ANP (120%)	0.40	0.403	0.57	0.56	100.77
BCM (80%)	2.00	1.974	0.99	1.00	98.74
BCM (100%)	3.00	2.993	0.87	0.87	99.78
BCM (120%)	4.00	4.013	0.92	0.91	100.34
BFI (80%)	0.10	0.101	0.47	0.46	101.97
BFI (100%)	0.15	0.151	0.53	0.52	101.23
BFI (120%)	0.20	0.203	0.49	0.48	101.79
TMF (80%)	0.80	0.790	0.59	0.59	98.77
TMF (100%)	1.20	1.176	0.24	0.24	98.01
TMF (120%)	1.60	1.631	0.72	0.70	101.98
TEI (80%)	0.04	0.040	0.45	0.44	101.43
TEI (100%)	0.06	0.061	0.95	0.93	101.75
TEI (120%)	0.08	0.081	0.89	0.87	101.49

3.2.8. *Stability solutions*

To demonstrate the stability of standard working and sample solutions during analysis, both solutions were analyzed over a period of 12 h while being stored at refrigerator (±4 °C). The results showed that the retention times and peak areas of the drugs as well as their synthetic impurities remained almost unchanged and no significant degradation was observed during this period, suggesting that these solutions were stable for at least 3 days when stored in a refrigerator at ±4 °C, which was sufficient for the whole analytical process.

4. Conclusion

The proposed method described in this paper is the first known reverse-phase HPLC method that can simultaneously separate and

Table 8
Robustness of the validation method for the determination of oncologic drugs and their respective synthetic impurities in tablets.

Factor	Value of critical *F*	ATZ calculated *F*	ANP calculated *F*	BCM calculated *F*	BFI calculated *F*	TMF calculated *F*	TEI calculated *F*
A	18.51	0.322	0.246	1.018	0.040	0.144	1.896
B	18.51	0.029	6.400	0.959	0.090	1.391	0.069
C	18.51	0.127	3.104	0.982	0.010	0.001	0.001
D	18.51	0.063	1.487	1.108	1.960	0.608	0.007
E	18.51	5.592	10.875	1.016	2.890	0.144	1.738
F	18.51	0.001	1.600	1.043	0.040	0.608	1.896
G	18.51	1.998	0.400	0.956	1.960	1.391	0.103

A=pH of mobile phase.
B=% of organic modifier.
C=flow rate.
D=wavelength.
E=% of *N*,*N* dimethyloctylamine.
F=injection volume.
G=Phantom factor.

quantitate the oncologic drugs and their synthetic impurities in tablets. It was found to be efficient, accurate, and sensitive. The excipients had no interference in the HPLC analysis. The method is simple, using a minimum number of reagents. The speed of analysis and its low cost make the method suitable for routine quality control analysis.

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