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Article

A Stability-Indicating HPLC Method for the Determination of Bazedoxifene Acetate and its Related Substances in Active Pharmaceutical Ingredient

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A simple, cost effective, stability-indicating reversed-phase high-performance liquid chromatography method was developed for the quantitative determination of bazedoxifene acetate (BAZ) drug substance in the presence of its impurities and degradation products. The method was developed using an X-terra RP-18, 150 × 4.6 mm, 3.5 μm column with a mobile phase containing solvent A, a mixture of 10 mM K_2HPO_4 (pH 8.3) and acetonitrile in the ratio of 70:30 (v/v); and solvent B, a mixture of water and acetonitrile in the ratio 10:90 (v/v). The eluted compounds were monitored at 220 nm, and within a short run time of 18 min, BAZ and its impurities were satisfactorily separated with resolution more than 2.0. BAZ was subjected to stress degradation and found to be sensitive towards acidic, basic, oxidative, thermal and hydrolytic stress conditions and stable in photo degradation conditions. The degradation products were well resolved from BAZ peak and its impurities; the mass balance in each case was more than 99.5%, proving the stability-indicating power of the method. The developed method was validated as per International Conference on Harmonization guidelines with respect to specificity, linearity (correlation coefficient > 0.9994), limit of detection, limit of quantification, accuracy (recovery range 96.3 to 102.1%), precision (relative standard deviation < 2.8%) and robustness.

Introduction

Bazedoxifene acetate (BAZ) {1H-Indol-5-ol,1-[[4-[2-(hexahydro-1H-azepin-1-yl) ethoxy] phenyl] methyl]-2-(4-hydroxyphenyl)-3-methyl-monoacetate} is a third generation selective estrogen receptor modulator (SERM), which is under development by Pfizer following the completion of their takeover of Wyeth Pharmaceuticals. Pfizer are seeking approval for BAZ in the prevention and treatment of postmenopausal osteoporosis. BAZ is the result of an exclusive research collaboration between Wyeth Pharmaceuticals and Ligand Pharmaceuticals.

BAZ is approved in the European Union (marketed in Italy and Spain), and is currently in the late phases of review by the United States Food and Drug Administration. When approved, BAZ is to be sold by Pfizer under the tradename Viviant in the US and Conbriza in the EU. BAZ in combination with conjugated estrogens, Aprela, is currently undergoing Phase III studies for the treatment of postmenopausal symptoms (including the prevention of postmenopausal osteoporosis/treatment of osteopenia).

SERMs are a class of agents that provide favorable therapeutic effects on the bone while minimizing undesirable effects of estrogens on other tissues by acting differently at the various estrogen receptors throughout the body (1). Osteoporosis is a chronic and progressive skeletal disorder that is common in elderly individuals, characterized by low bone mass and weak bone strength, and leading to increased risk of fractures. It affects approximately 40% of postmenopausal women as a result of declining levels of estrogen (2–3). BAZ has been shown to prevent bone loss, increase bone mineral density and reduce bone turnover in postmenopausal women. Both preclinical and clinical data indicate that BAZ has a unique combination of attributes, making it an attractive option for the treatment and prevention of osteoporosis (4–7).

Currently, no stability-indicating high-performance liquid chromatography (HPLC) method is available in the literature or the Pharmacopoeia. A stability-indicating method for the quantitative estimation of BAZ and its related substances in active pharmaceutical ingredients may be useful for characterizing the properties of BAZ.The determination of impurities with low run time is one of the most difficult tasks for pharmaceutical analysis during method development. Hence, a rugged and robust stability-indicating reversed-phase (RP)-HPLC method was developed for the quantitative determination of BAZ and its impurities (A–E) (Figures 1A–1F) with a short run time of 18 min. The impurities were supplied by Dr Reddy's Laboratories (Bachupally, India). This method was validated according to the International Conference on Harmonization (ICH) guidelines (9–10).

Experimental

Materials and reagents

All impurities (Imp B–E) are identified during synthetic process development and Imp-A is formed as a degradent during initial method development; therefore, all of these impurities are considered for analytical method development. All of the impurities, active pharmaceutical ingredient standards and samples were supplied by Dr. Reddy's Laboratories. The HPLC-grade acetonitrile and analytical grade orthophosphoric acid and dipotassium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). Water was prepared by using a Millipore Milli-Q Plus water purification system.

Chromatographic conditions and equipment

LC was conducted on a Waters HPLC with photodiode array (PDA) detector. The output signal was monitored and processed using Empower software. The chromatographic column

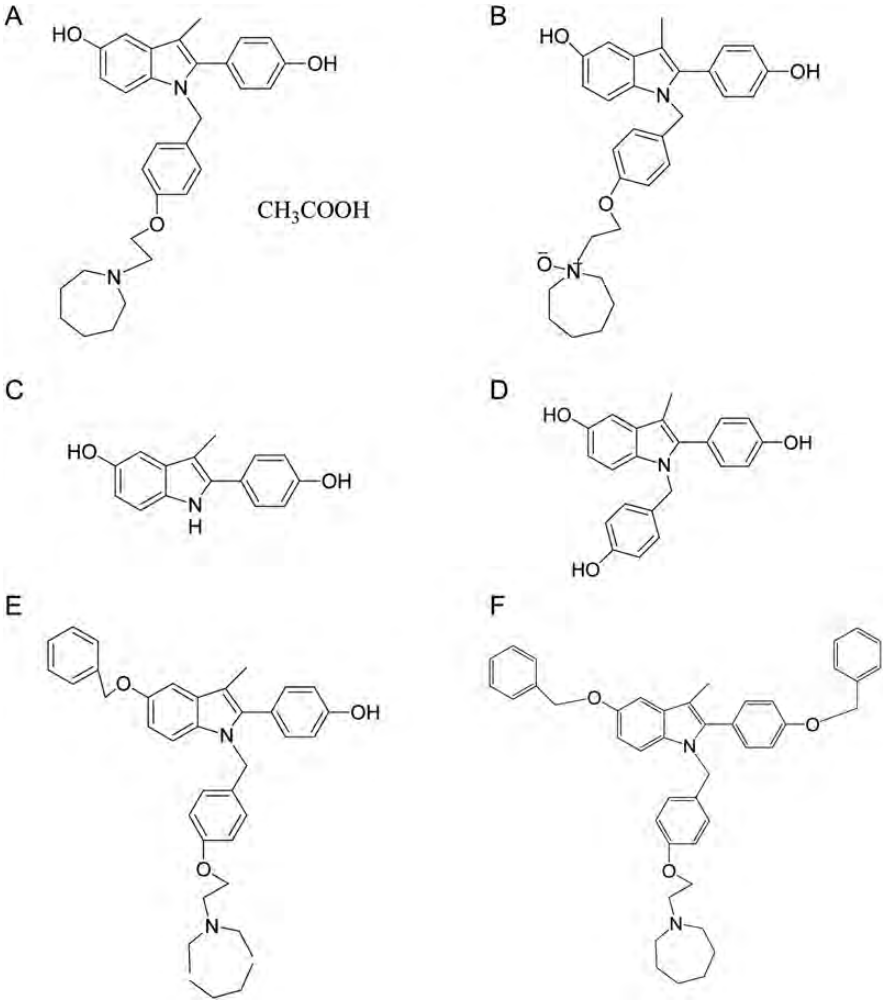

Figure 1. Chemical structures and names: BAZ, 1H-Indol-5-ol,1-[[4-[2-(hexahydro-H-azepin-1-yl) ethoxy] phenyl]methyl]-2-(4-hydroxyphenyl)-3-methyl-monoacetate (A); Impurity A, 2-(4-Hydroxy-phenyl)-3-methyl-1-{4-[2-(1-oxy-azepan-1-yl)-ethoxy]-benzyl}-iH-Indole-5-ol (B); Impurity B, 2-(4-Hydroxy-phenyl)-3-methyl-1H-indole-5-ol (C); Impurity C, 1-(4-Hydroxy-benzyl)-2-(4-hydroxy-phenyl)-3-methyl-1H-indole-5ol (D); Impurity D, 4-{1-[4-(2-Azepane-1-yl-ethoxy)-benzyl]-5-benzyloxy-3-methyl-1H-indole-2-yl}-Phenol (E); Impurity E 1-[4-(2-Azepane-1-yl-ethoxy)-benzyl]-5-benzyloxy-2-(4-benzyloxy-phenyl)3-Methyl-1H-indolel (F).

was an X-Terra RP-18 150 × 4.6 mm, with 3.5 μm particle size. The separation was achieved by using a gradient method. Solvent A contained a mixture of 10 mM K_2HPO_4 (pH adjusted to 8.3 by using orthophosphoric acid) and acetonitrile in the ratio of 70:30 (v/v); and solvent B contained a mixture of water and acetonitrile in the ratio of 10:90 (v/v).

The flow rate of the mobile phase was 1.0 mL/min. The HPLC gradient program was set as: time (min)/%B: 0.01/32, 5/32, 7/100, 15/100, 16/32 and 18/32. The column temperature was maintained at 40°C and the detection was monitored at a wavelength of 220 nm. The selected diluent was solvent B. The injection volume was 5 μL.

LC–MS-MS conditions

An LC–tandem mass spectrometry (MS-MS) system (Agilent 1200 series liquid chromatograph coupled with Applied Biosystems 4000 Q Trap triple quadruple mass spectrometer with Analyst 1.4 software; MDS SCIEX, Foster City, CA) was used for the unknown compounds formed during forced degradation studies. An X-Terra RP-18 150 × 4.6 mm, 3.5 μm particle size column was used as the stationary phase. A 0.01M solution of ammonium acetate (pH adjusted to 8.3 by using ammonia solution) was used as buffer. Buffer and acetonitrile in the ratio of 70:30 (v/v) was used as solvent A and water and acetonitrile in the ratio of 10:90 (v/v) were used as solvent B. The gradient program was set as: time (min)/%B: 0.01/10, 10/10, 40/60, 43/60, 55/90, 64/100, 65/10 and 70/10. Solvent B was used as the diluent. The flow rate was 1.0 mL/min. The analysis was performed in positive electrospray ionization mode, the ion source voltage was 5,000 V and the source temperature was 450°C. GS1 and GS2 were optimized to 30 and 35 psi, respectively. Curtain gas flow was 20 psi.

Preparation of stock solutions

A solution of BAZ standard (500 μg/mL) was prepared by dissolving an appropriate amount of the drug in solvent B as the diluent [acetonitrile–water 90:10 (v/v)]. An individual stock

solution (50 μg/mL) of all the impurities (Imp-A to Imp-E) was prepared in diluent (Figures 1A–1F).

Preparation of sample solution

BAZ working solutions containing 500 and 100 μg/mL were prepared from the previously described stock solution for the determination of related substances and assay determination.

Stress studies

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the developed LC method for BAZ was conducted in the presence of its five impurities. Stress studies were performed at an initial concentration of 500 μg/mL of BAZ to determine the stability-indicating property and specificity of the proposed method. Intentional degradation was attempted under stress conditions of ultraviolet (UV) light (254 nm), heat (105°C), acid (0.5N HCl at 70°C), base (0.5N NaOH at room temperature [RT]), hydrolysis (70°C) and oxidation (1.0% H_2O_2 at RT) to evaluate the ability of the proposed method to separate BAZ from its degradation products. For heat and light studies, the study period was 10 days, whereas the study periods were 30 h for neutral hydrolysis, 3 h for base hydrolysis, 5 h for acid hydrolysis and 1 h for oxidation.

The degraded samples were analyzed using a PDA detector. The purity angle was found to be less than the purity threshold for BAZ and impurities in all stressed samples, demonstrating peak homogeneity. Assay of the stressed samples was performed by comparison with reference standards, and the mass balance (% assay + % impurities + % degradation products) was calculated for stressed samples. Assay was also calculated for the BAZ sample by spiking all impurities at the specification level (i.e., 0.15%).

Method Validation

The described method has been extensively validated (9–10).

Precision

The repeatability of the related-substance method was checked by a six-fold analysis of 500 μg/mL of BAZ spiked with 0.15% of specification limit (0.75 μg/mL) of each of the five impurities. The relative standard deviation (RSD) of the percent area of each impurity was calculated. The repeatability of the method was evaluated by conducting six independent assays of BAZ at 100 μg/mL against qualified reference standards.

The intermediate precision (ruggedness) of the method was evaluated by performing the analysis with different analysts using a different column and different instrument on different days.

Limit of detection and quantification The limit of detection (LOD) and limit of quantification (LOQ) for BAZ and its impurities were determined at signal-to noise ratios of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. The precision study was also determined at the LOQ level by injecting six ($n = 6$) individual preparations and calculating the RSD of the area for each impurity and for BAZ.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the found value. Accuracy of the related substances by the HPLC method was established by standard addition and recovery experiments. Recovery was calculated for each added concentration. The study was conducted for impurities in triplicate using four concentration levels from LOQ, 0.075, 0.15 and 0.225% of the analyte concentration (500 μg/mL) and recovery of the impurities was calculated.

Accuracy of the assay method was evaluated in triplicate using three concentration levels, 50, 100 and 150 μg/mL of BAZ, and recovery was calculated for each added concentration.

Linearity of response

The linearity of the detector response to different concentrations was evaluated for all impurities and BAZ by injecting separately prepared solutions covering the range of LOQ to 200% (LOQ, 0.0375, 0.075, 0.1125, 0.15, 0.1875, 0.225 and 0.30% of the normal sample concentration). The correlation coefficients, slopes and *Y*-intercepts of the calibration curve were determined.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication of its reliability during normal usage.

To determine the robustness of the method, the experimental conditions were deliberately changed. The resolution of BAZ and all impurities was evaluated. The mobile phase flow rate was 1.0 mL/min; to study the effect of flow rate on resolution, it was changed to 0.8 and 1.2 mL/min, and the effect of column temperature was studied at 35 and 45°C (instead of 40°C).

Solution stability and mobile phase stability

The solution stability of BAZ and its impurities was conducted by leaving spiked sample solutions in tightly capped volumetric flasks at room temperature for 48 h. The content of impurities was determined at 12-h intervals for 48 h. The stability of the mobile phase was determined by analysis of freshly prepared sample solutions at 12-h intervals for 48 h. The mobile phase was prepared at the beginning of the study period and not changed during the experiment.

Results and Discussion

Method development and optimization

The primary objective of this work was to develop a stability-indicating HPLC method for the determination of BAZ and its impurities (degradants, intermediates, starting material

and impurities from the synthetic process for BAZ) within a short run time. The pKa of BAZ drug substances is 11.0, with a UV spectral maximum response at 220 nm, and this wavelength was used for LC detection.

Initially, attempts were made by using different C18 and C-8 HPLC columns (Inertsil ODS-3V, Symmetry shield RP-18, Zorbax XDB C-18, ACE3 C-18 and X-Terra RP-8) with gradient elution using different buffers (K_2HPO_4, Na_2HPO_4 and CH_3COONH_4) at different pH (2–7) (8). In all of the preceding columns and intended experimental conditions, separation of impurities was not satisfactory. The resolution between Imp-A and Imp-B was poor, imp-D and imp-E were late-eluting and the peak shape for BAZ was not good (USP tailing $>$ 2.0) (Figures 2A and 2B). Attempts were made with gradient and mobile phase pH optimization. At acidic mobile phase pH, all impurities eluted earlier and the separation of all impurities (Imp-A to Imp-E) and BAZ was found to be inadequate.

Buffer pH was found to be critical in the analyte separation and was extensively studied in method optimization. Buffer pH should be selected $\pm$1.5 units from the pKa of the analytes. This ensures that the analytes are either 100% ionized or non-ionized and should help control run-run reproducibility. In reverse-phase HPLC, the retention of analyte increases with increasing hydrophobicity. BAZ, Imp-D and Imp-E are basic in nature, so decreasing the pH increases ionization and decreases hydrophobicity, resulting in decreased retention time. It was found that after decreasing the buffer pH from 9.0 to 5.0 while keeping the other chromatographic parameters unchanged, the retention times of BAZ, Imp-D and Imp-E were decreased. The remaining impurities were relatively insensitive to buffer pH (Figure 3). Overall, a buffer pH of 8.3 was found to be satisfactory.

Further development was conducted with a basic mobile phase using an X-Terra RP-18 150 $\times$ 4.6 mm, 3.5 µm particle size column with hydride-based embedded polar group technology, providing superior pH stability. Mobile phase A contained a mixture of 10 mM K_2HPO_4 buffer (pH adjusted to 8.3 by using orthophosphoric acid) and acetonitrile in a ratio of 70:30 (v/v), while mobile phase B contained a mixture of water and acetonitrile in a ratio of 10:90 (v/v) with the gradient elution set as: time (min)/%B: 0.01/38, 5/38, 7/100, 15/100, 16/38 and 18/38, flowing at a rate of 1.0 mL/min, with a column oven temperature of 40°C. This provided greater separation (resolution $>$ 2.0) between BAZ and its impurities, within a short run time of 18 min (Figure 2C).

System suitability parameters were evaluated for BAZ and its five impurities. The tailing factor for all five impurities and BAZ was found to be less than 1.4. The USP resolution of BAZ and its five potential impurities was greater than 2.0 for all pairs of compounds in the finalized method chromatographic conditions. These values are presented in Table I.

Validation of the method

Precision

The RSD in the study of the repeatability of six independent assays of BAZ was within 0.14%. In the related substances method repeatability study, the RSD of the percent of the five impurities, A–E, was within 3.0%. The RSD of the assay results obtained in the intermediate precision study was 0.8%, and in the related substances intermediate precision study, the RSD for the percent of the five impurities, A–E, is well within 2.0%. The RSD values are presented in Table II.

LOD and LOQ

The determined LOD, LOQ and precision at LOQ values for BAZ and its five impurities are reported in Table II.

Linearity

A linearity calibration plot for the assay method was obtained over the calibration ranges tested; i.e., 25 to 200 µg/mL, and the correlation coefficient obtained was greater than 0.999. A linearity calibration plot for the related substances method was obtained over the calibration ranges tested; i.e., LOQ to 0.3% (LOQ, 25, 50, 75, 100, 125, 150 and 200% of the specification limit, i.e., 0.15%). The correlation coefficients, slopes and *Y*-intercepts of the calibration curve were determined.

Accuracy

The recovery of BAZ from the drug substance was ranged from 99.4 to 99.9%. The recovery of the five impurities in BAZ active pharmaceutical ingredients ranged from 97.6 to 102.7%. The percentage recovery of the impurities and BAZ is listed in Table III.

Robustness

In all of the deliberately varied chromatographic conditions (different flow rate and different column temperature), the resolution between all pairs of compounds was greater than 2.0. The resolution values are presented in Table IV.

Stability in solution and in the mobile phase

RSD for the assay of BAZ during solution stability and mobile phase stability experiments was within 1.0%. No significant changes in the amounts of the five impurities were observed during solution stability and mobile phase experiments when performed using the related substances method. The results from the solution stability and mobile phase stability experiments confirmed that standard solutions and solutions in the mobile phase were stable for up to 48 h during the assay and determination of related substances.

Results from forced degradation studies

All forced degradation samples were analyzed at an initial concentration of 500 µg/mL of BAZ, with previously described HPLC conditions, using a PDA detector to ensure the homogeneity and purity of the BAZ peak. Degradation was not observed when BAZ was subjected to light, and slight degradation was observed when the drug was subjected to heat and water conditions. Significant degradation was observed when the drug was subjected to oxidative hydrolysis (1.0% H_2O_2 at RT for 1 h), leading to the formation of Imp-A, acid (0.5N HCl at 70°C for 5 h), leading to the formation of Imp-B and base

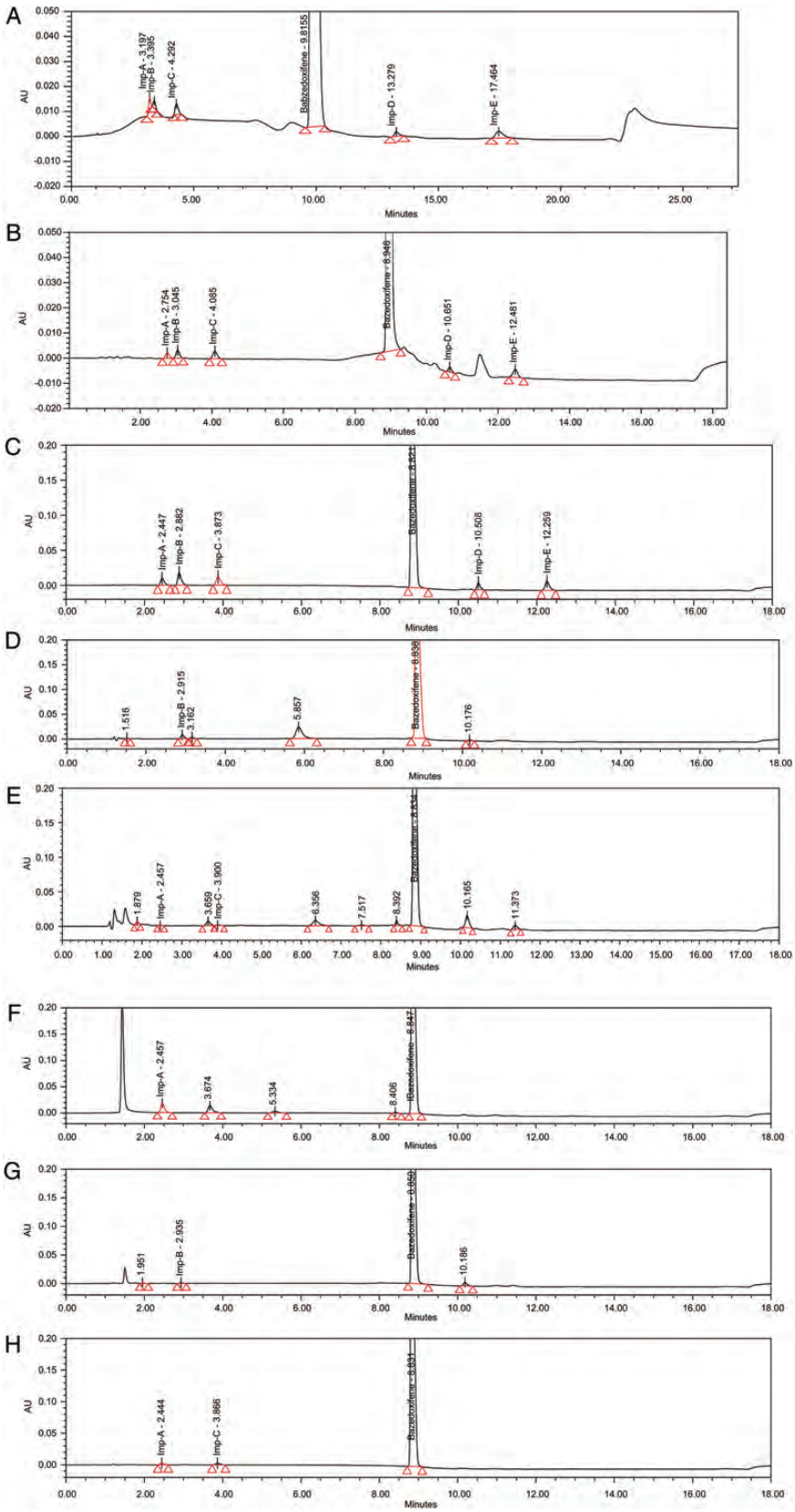

Figure 2. Different column trials: BAZ spiked chromatogram with an Inertsil ODS-3V column (150 × 4.6 mm, 5.0 μm) (A); BAZ spiked chromatogram with a Symmetry shield RP-18 column (150 × 4.6 mm, 3.5 μm) (B); chromatogram representing BAZ spiked with impurities A, B, C, D and E (finalized conditions) (C); acid degradation chromatogram (D); base degradation chromatogram (E); oxidative degradation chromatogram (F); water hydrolysis degradation chromatogram (G); thermal degradation chromatogram (H).

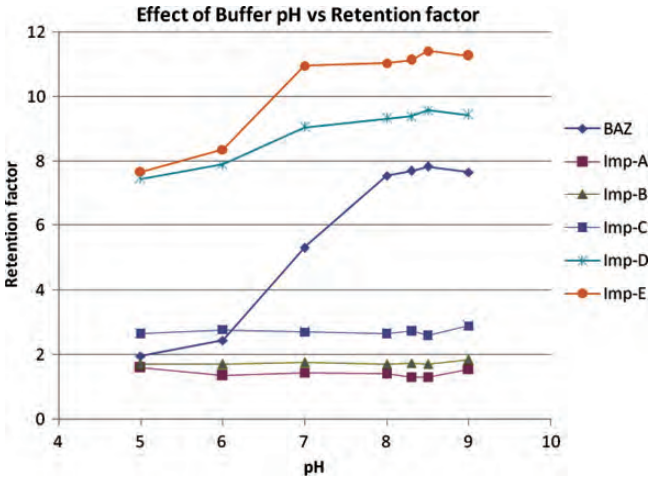

Figure 3. Effect of mobile phase buffer pH on retention factors of BAZ and Impurities A–E.

Table I
System Suitability Results (Retention Time, Relative Retention Time, USP Resolution and USP Tailing)

Sample	Name	RT (min)*	RRT† ($n = 6$)*	USP resolution‡ ($n = 6$)*	USP tailing ($n = 6$)*
1	Imp-A	2.50 + 2.19	0.28 + 1.79	—	1.11 + 3.74
2	Imp-B	2.93 + 1.76	0.33 + 1.41	2.23 + 2.16	1.12 + 4.77
3	Imp-C	3.91 + 2.43	0.44 + 2.26	4.72 + 5.45	1.09 + 3.60
4	BAZ	8.95 + 0.69	1.00 + 0.69	25.38 + 2.73	1.25 + 2.19
5	Imp-D	10.70 + 0.90	1.20 + 0.21	9.83 + 4.02	1.20 + 5.36
6	Imp-E	12.53 + 1.07	1.40 + 0.7	8.77 + 4.56	1.13 + 4.97

*Mean + RSD% ($n = 6$).
†Relative retention times (RRT) were calculated against the retention time (RT) of BAZ.
‡USP resolutions were calculated between two adjacent peaks.

Table II
Method Validation Results: LOD, LOQ, Regression, Repeatability and Intermediate Precision*

Parameter	BAZ	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E
LOD (μg/mL)	0.03	0.04	0.03	0.03	0.04	0.04
LOQ (μg/mL)	0.07	0.13	0.07	0.09	0.12	0.11
Regression equation (y)						
Slope (b)	14408417	9682723	16447537	14190867	8619166	12827930
Intercept (a)	57	29	−553	−116	−7	−106
Correlation coefficient	0.9997	0.9994	0.9998	0.9999	0.9998	0.9998
Repeatability (RSD)	0.3	0.3	1.98	0.2	0.5	2.81
Intermediate precision (RSD)	0.5	0.4	1.5	0.4	1	1.5

*Note: Linearity range is LOQ to 200% with respect to 500 μg/mL BAZ for impurities.

Table IV
Method Validation: Robustness Data (USP Resolution)*

Parameter	Imp-A	Imp-B	Imp-C	BAZ	Imp-D	Imp-E
Actual flow and temperature (1.0 mL/min and 40°C)	—	2.23	4.72	25.38	9.83	8.77
Different flow, 0.8 mL/min	—	2.43	5.02	21.35	9.58	8.7
Different flow, 1.2 mL/min	—	2.1	3.93	20.86	7.39	5.76
Different column temperature, 35°C	—	2.8	4.9	21.5	9.3	8.1
Different column temperature, 45°C	—	2.03	4.7	23.44	8.81	7.58

*Note: USP resolutions were calculated between two adjacent peaks. Resolution is how far apart the peaks are, relative to how broad they are.

Table V
Method Validation: Summary of Forced Degradation Results*

	Impurity formed (%)						Assay	Mass balance†
	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Total degradation		
Acid hydrolysis	ND	0.51	ND	ND	ND	5.44	94.27	99.71
Base hydrolysis	0.09	ND	0.05	ND	ND	9.21	90.3	99.51
Water hydrolysis	ND	0.09	ND	ND	ND	1.1	98.71	99.81
Oxidative degradation	2.96	ND	ND	ND	ND	5.87	93.79	99.66
Thermal degradation	0.45	ND	0.29	ND	ND	0.75	98.87	99.62
Photolytic degradation	0.08	ND	0.04	ND	ND	0.13	99.76	99.89

*Note: ND, not detected.
†Mass balance: (% assay + % sum of all degradants).

(0.5N NaOH at RT for 3 h), leading to the formation of Imp-C and Imp-A (Figures 2D–2H). This was confirmed by by LC–MS-MS analysis and by co-injecting Imp-A, Imp-B and Imp-C standards with these degraded samples. LC–MS-MS analysis was performed as per the previously described experimental conditions. The masses of impurities were 239.2, corresponding to Imp-B in acid degradation; 345.4, corresponding to Imp-C in base degradation; and 486.4, corresponding to Imp-A in oxidative hydrolysis. Results from force degradation studies are presented in Table V.

Assay studies were conducted for stress samples (at 100 μg/mL) against a qualified reference standard of BAZ. The mass balance results (% assay + % sum of all impurities + % sum of all degradants) were calculated for all stressed samples and found to be more than 99%. The purity and assay of BAZ was unaffected by the presence of its impurities and degradation products, which thus confirms the stability-indicating power of the developed method.

Table III
Method Validation: Accuracy (Recovery) Data

Amount spiked*	Recovery† (%)					
	BAZ	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E
LOQ	—	101 + 1.04	100.9 + 1.56	98.2 + 1.64	96.3 + 0.87	102.1 + 2.07
50%	99.4 + 0.66	100.7 + 1.19	97.8 + 1.13	97.6 + 0.51	99.3 + 1.27	99.8 + 0.55
100%	99.9 + 0.14	100.1 + 0.21	100.4 + 1.93	99.0 + 0.41	100.3 + 0.15	99.4 + 1.8
150%	99.7 + 0.27	100.5 + 0.58	100.1 + 1.57	101.1 + 0.86	100.4 + 1.59	99.3 + 1.61

*Amount of five impurities spiked with respect to specification limit, 0.15%.
†Mean + RSD%.

Conclusion

The rapid gradient RP-HPLC method developed for the quantitative analysis of BAZ and related substances in active pharmaceutical ingredients is precise, accurate, linear, robust and specific. Satisfactory results were obtained during validation of the method. This method exhibited excellent performance in terms of sensitivity and speed. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of samples.

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