Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 962–968

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Determination and characterization of degradation products of Anastrozole by LC–MS/MS and NMR spectroscopy

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ARTICLE INFO

Article history:
Received 25 March 2011
Received in revised form 7 August 2011
Accepted 13 August 2011
Available online 24 August 2011

Keywords:
Development
Validation
Stability indicating method
Anastrozole
Forced degradation

ABSTRACT

Two new degradation products for Anastrozole active pharmaceutical ingredient (ANZ) have been identified and reported in this paper. The ANZ was subjected to thermal, photolytic, oxidative and base stress conditions prescribed by ICH guidelines. Separation of ANZ from its existing impurities and the two new impurities was achieved by using on Oyster ODS-3 (100 mm × 4.6 mm × 3.0 μm) column with an isocratic mixture of 10 mM ammonium formate and acetonitrile in the ratio 60:40 (v/v). The flow rate was 0.5 ml min^{-1}. The elution was monitored at 215 nm. An isocratic stability indicating reverse phase liquid chromatographic (RP-LC) and LC–MS/MS method was developed for the determination of purity and assay of ANZ through forced degradation studies. The two new impurities detected were further subjected to spectroscopic studies. Based on the results obtained from the different spectroscopic studies, these impurities have been characterized as 2,2′-(5-((1H-1,2,4-triazol-1-yl)methyl)-1,3-phenylene)bis(2-methylpropanoicacid) (Diacid) and 2-(3-((1H-1,2,4-triazol-1-yl)methyl)-5-(2-cyanopropan-2-yl)phenyl)-2-methylpropanoicacid (Monoacid). ANZ was found to degrade in base, slightly in oxidative degradation conditions. The degradation products were well resolved from main peak and its impurities thus proved the stability, indicating power of the method. The developed method was validated as per International Conference on Harmonization (ICH) guidelines with respect to specificity, limit of detection, limit of quantitation, precision, linearity, accuracy, robustness and system suitability.

1. Introduction

Organic impurities in Active Pharmaceutical Ingredients (APIs) mainly arise during the synthesis [1] and a stability indicating RP-LC method can be used to determine the API in the presence of its impurities and degradation products. Anastrozole (ANZ), chemically known as 2,2′-[5-(1H-1,2,4-triazol-1-ylmethyl)-1,3-phenylene]di(2-methyl propionitrile) [2] is used in the treatment of breast cancer in post-menopausal women. Impurities in API are very undesirable. A review of literature revealed two chromatographic methods on ANZ. The method [3] focused on the assay development while method [4] on isolation and characterization of only three process related impurities. So far only one analytical method on degradants and related compounds in ANZ tablets are reported in the literature [5] and the methods [6–14] revealed the pharmacokinetics and biological activities. In the present developed method two new degradants in significant levels were identified, characterized and determined in ANZ by LC–MS/MS. The two new impurities arise during forced degradation studies under variety of conditions recommended by the International Conference on Harmonization (ICH). Since the selective determination of API in the presence of as many impurities as possible is beneficial in assessing its final purity and assay, an isocratic, stability indicating liquid chromatographic method that can selectively determine ANZ in the presence of process related impurities and its degradation products has been developed and validated [15].

2. Materials and methods

2.1. Materials

The investigated samples of ANZ were obtained from Ogene Systems (I) Pvt. Ltd., Hyderabad, India. Ammonium formate, acetonitrile, HCl, NaOH and H_2O_2 (30%) were obtained from Merck Specialty Chemicals, Mumbai, India. All the reagents were of analytical grade. All the solutions were prepared in Milli Q water (Millipore, Billerica, MA, USA).

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doi:10.1016/j.jpba.2011.08.022

2.2. Experimental

2.2.1. RP-LC and LC–MS/MS method

ANZ and its impurities were determined by HPLC prominence with PDA detector (Shimadzu Corporation, Kyoto, Japan) instrument with LC solution software. An Oyster ODS-3 (100 mm × 4.6 mm × 3.0 μm, Merck, Darmstadt, Germany) column was employed for the separation of impurities from ANZ. Separation was achieved using a mobile phase in an isocratic mixture of 10 mM ammonium formate in water and acetonitrile in the ratio of 60:40 (v/v). The flow rate of the mobile phase was kept at 0.5 ml min^{-1}. The detection wavelength was 215 nm. The injection volume was 20 μl. Mobile phase was used as diluent.

2.2.2. Mass spectrometry (LC–MS/MS)

The LC–MS/MS analysis was performed on Quattro Micro™ API mass spectrophotometer (Waters, Seoul, Korea). The analysis was performed in the SCAN mode with electrospray ionization source (ES^+) and triple quadrapole mass analyzer. The analysis parameters for capillary, cone voltage were 3.50 kVand 25 V, respectively. Source, dessolvation gas temperatures were 95 °C and 350 °C, dessolvation gas flow fixed at 450 l h^{-1}. The mass spectrum data was processed by using Mass Lynx software.

2.2.3. NMR spectroscopy

The ^1H and ^{13}C NMR experiments were performed in DMSO at 25 °C temperature using mercury plug 300 MHz FT NMR spectrometer, Bruker, Bio Spin Corporation, Billerica, MA, USA. The ^1H and ^{13}C chemical shifts were reported on the δ scale in ppm relative to tetramethyl silane and DMSO, respectively.

2.2.4. Preparation of stock solutions

0.5 mg ml^{-1} and 0.1 mg ml^{-1} stock solutions of ANZ were prepared by dissolving 5.0 mg in 10 ml of diluent for purity and 2.0 mg in 20 ml of diluent for assay methods, respectively. 0.15% and 1% of blend by spiking the impurities to 0.5 mg ml^{-1} ANZ was prepared in diluent (Mobile phase used as the diluent).

2.2.5. Forced degradation conditions

To perform the forced degradation study the drug was subjected to acidic, alkaline, oxidative, photolytic and thermal stress conditions. The analysis was carried out by HPLC with a PDA detector. 20 μl of each forced degradation samples were injected at regular intervals and the final stress conditions were established in such a way that 10–20% degradation of ANZ and the degradants were identified by using LC–MS/MS system.

2.2.6. Oxidative degradation

50 mg of ANZ sample was taken into a 100 ml round bottom flask, 10 ml of 10% H_2O_2 solution was added, contents were mixed well and kept for constant stirring for 2 h at 100 °C. 1.0 ml of this solution was diluted to 10 ml with diluent.

2.2.7. Acid degradation

50 mg of ANZ sample was taken into a 100 ml round bottom flask, 10 ml of 0.5 N HCl solution was added, contents were mixed well and kept for constant stirring for 2 h at 100 °C and the mixture was neutralized. 1.0 ml of this solution was diluted to 10 ml with diluent.

2.2.8. Base degradation

50 mg of ANZ sample was taken into a 100 ml round bottom flask, 10 ml of 0.5 N NaOH solutions was added and the contents were mixed well for 10 min at 100 °C.

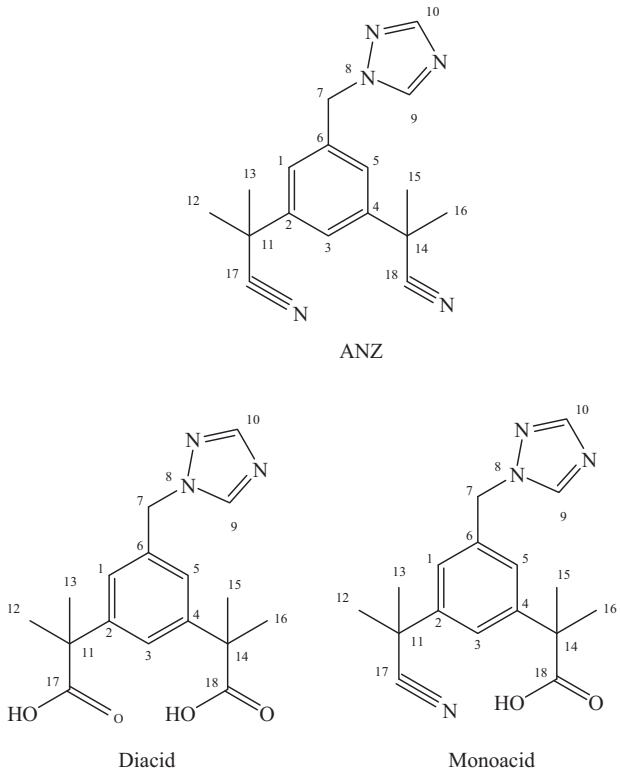

Fig. 1. Structure of ANZ and its two new impurities.

2.2.9. Thermal degradation

ANZ sample was exposed to heat and dried at 100 °C temperature in a convention oven for 12 days.

2.2.10. Photolytic degradation

ANZ sample for photo stability testing was placed in a UV chamber (Vision lab equipments, India) and exposed to light in an overall illumination of ≥200 Wh m^{-2} at 25 °C with UV radiation at 200–400 nm. Control samples which were protected from light with aluminum foil were also placed in the light cabinet and exposed concurrently. Following removal from the light cabinet, the samples were analyzed.

3. Results and discussion

3.1. Mass spectrometry (LC–MS/MS analysis)

LC–MS/MS analysis of ANZ degradants revealed that the new degradants were identified as Monoacid and Diacid (Fig. 1). Monoacid exhibits molecular ion peak as sodium adduct (M+Na) in positive ionization mode at m/z 334 amu. Diacid exhibits molecular ion peak as sodium adduct (M+Na) at m/z 353 amu, and disodium adduct (M+2Na) at m/z 375 amu (Fig. 2). Based on this mass spectral data these impurities are identified during the base degradation products of ANZ.

3.2. Degradation behavior of ANZ

HPLC studies of samples obtained on forced decomposition of ANZ under different conditions suggested the following degradation behavior.

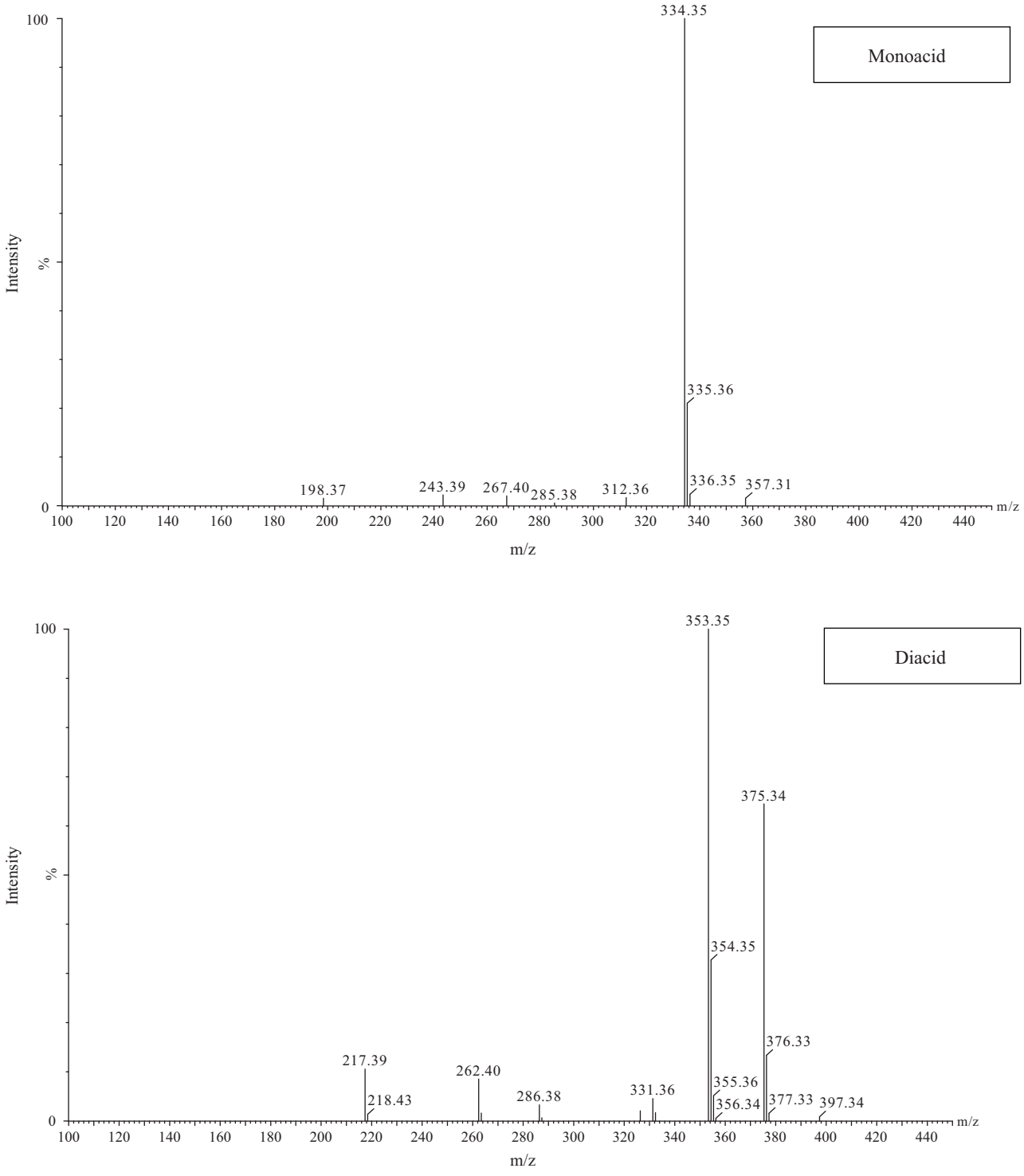

Fig. 2. Mass spectrum of new base degradants.

3.2.1. Photo stability and thermal stress studies

The drug was found to be stable and no major degradants were observed in thermal and photo stability conditions. The level of impurities in all these samples were found to be <0.10%.

3.2.2. Basic, acidic and oxidative degradation studies

ANZ was degraded and resulted in the formation of the two new degradants, when it was exposed to base degradation in 0.5 M NaOH at 100 °C for 10 min at retention times 3.0 min and 4.06 min. The drug was found to be mildly degraded in 0.5 M HCl and 10% H_2O_2. No major degradants were observed in both the cases and the percentage impurities in all these samples were found to be <0.20%. A typical chromatogram of ANZ subjected to acid, base and oxidative degradation conditions is shown in Fig. 3. The chromatographic peak purity for ANZ and its impurities peaks demonstrated that all the peaks were pure in all cases, confirming the absence of other impurities co-eluting in the same retention time and the mass balance of stressed samples was close to 99.7%.

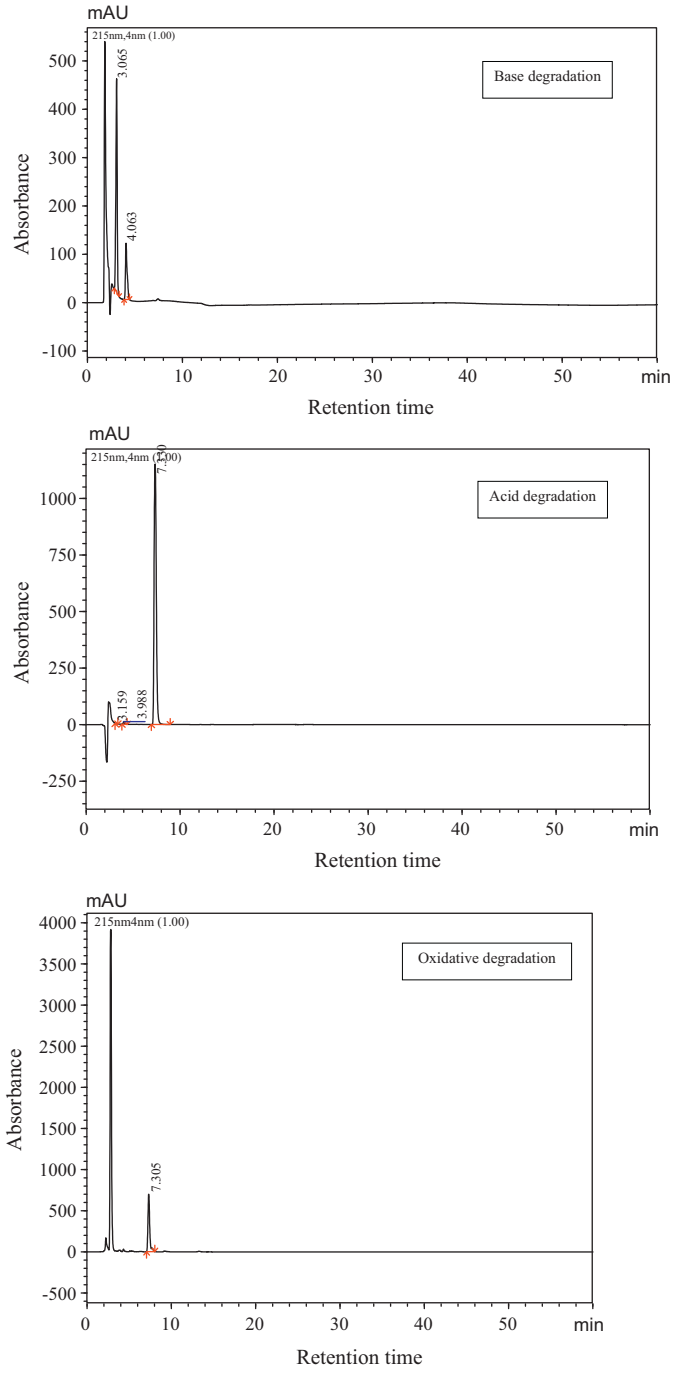

Fig. 3. HPLC chromatogram for base, acid and oxidative degradation of ANZ.

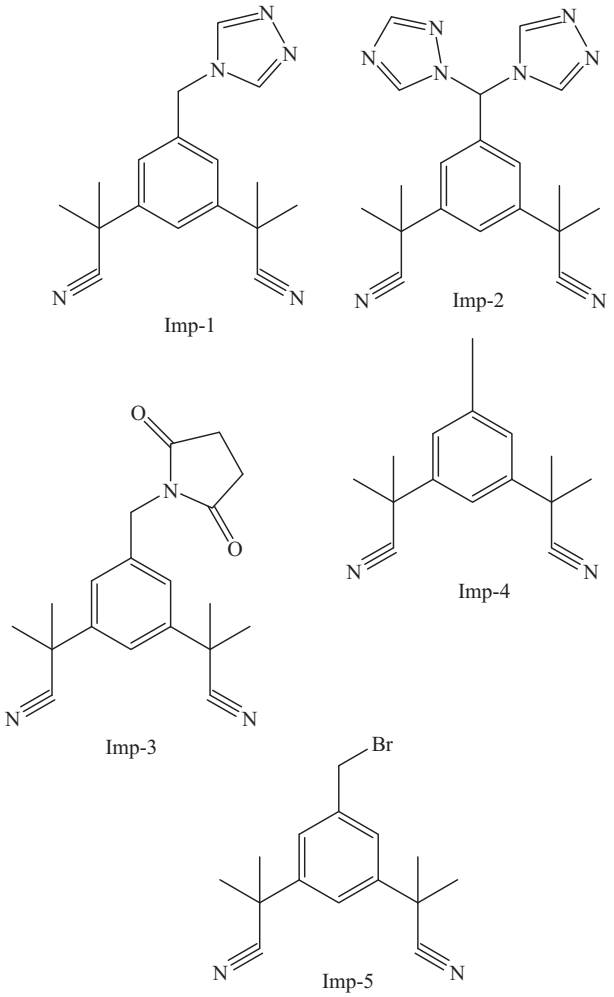

Fig. 4. Existing process related impurities in ANZ.

3.3. NMR spectroscopy

The ^{1}H NMR spectrum of ANZ base degradant Monoacid exhibited separate broad peak at 12.0 ppm for the proton corresponding to the presence of carboxylic acid group. Whereas Diacid, which is in the form of disodium salt of carboxylate anion does not exhibit any peak at 12.0 ppm in ^{1}H NMR. The ^{1}H NMR and ^{13}C chemical shift assignment was reported in Table 1. The differentiation of signal at 121.34 ppm and 178.26 ppm for Monoacid was observed in ^{13}C corresponding to carbon numbers C-17 and C-18 and its absence in DEPT confirmed the presence of cyano group which is further supported by IR spectrum which showed a peak at 2160 cm^{-1} The signal 178.34 ppm and 179.16 ppm corresponds to carbon number C-18 and C-17 in ^{13}C NMR and its presence in DEPT in the case of Diacid.

3.4. Method development and method validation

The main target of the method is to identify the possible degradants and get well resolutions between ANZ and its process related impurities and degradation products. The blend solution of ANZ base degradants were prepared by spiking 1% of existing impurities (Fig. 4) and degradation product to 0.5 mg ml^{-1} ANZ test solution and it was run through C_{18} column with phosphate buffer in the pH range of 3.0–6.0 along with acetonitrile or methanol in different combinations. In most cases, one or two among the four problems *viz.* poor resolutions, bad peak shapes, blank interference or late elution between degradants and process related impurities in methanol were observed. Hence phosphate buffer was replaced by formate buffer and acetonitrile was selected as organic solvent. Best results were achieved using Oyster ODS-3 (100 mm × 4.6 mm × 3.0 μm) column using an isocratic mixture of 10 mM ammonium formate and acetonitrile (60:40, v/v) as mobile phase. The flow rate of the mobile phase was kept at 0.5 ml min^{-1} and the injection volume was 20 μl. The detection wavelength was 215 nm and temperature 30 °C is adequate for the analysis. The proposed method was validated as per ICH guidelines and the data is presented.

Table 1
^{1}H NMR and ^{13}C NMR chemical shift assignments for ANZ (API) base degradants.

Monoacid			Sodium salt of Diacid		
Carbon no.	^{1}H/ppm	^{13}C	Carbon no.	^{1}H/ppm	^{13}C
1,5,(2H)	m/7.24–7.4	124.53	1,5,(2H)	m/7.1–7.2	125.21
3(1H)	t/7.1	124.12	3(1H)	br s/7.25	131.42
6	–	136.29	6	–	137.18
7(2H)	dd/5.4	55.36	7(2H)	t/5.4	53.17
12,13,15,16 (12H)	s/1.39, 2 × s/1.4 s/1.45	25.38	12,13,15,16 (12H)	2 × s/1.38 2 × s/1.42	26.42
11	–	32.42	11	–	58.25
14	–	58.14	14	–	57.64
9(1H)	m/7.9–8.0	144.18	9	s/8.1	144.23
10(1H)	m/8.6–8.7	153.27	10	s/8.9	153.11
17	–	121.34	17,(1H)	–	179.16
18(1H)	br s/12.0	178.26	18(1H)	–	178.34

Note: For carbon numbers refer Fig. 1.

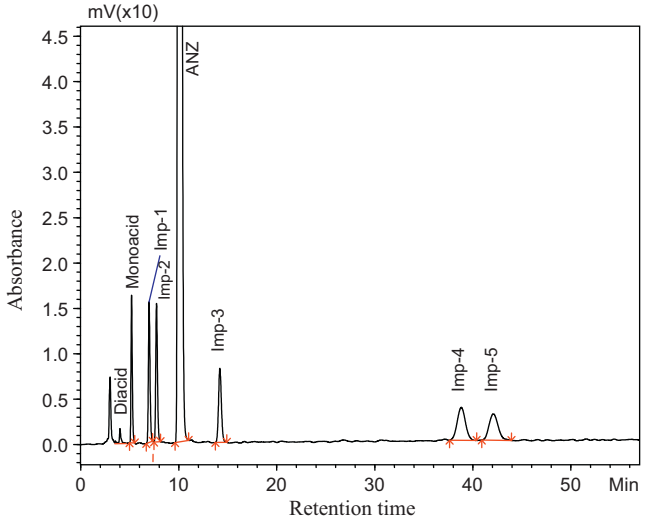

Fig. 5. Blend chromatogram of existing impurities and base degradants in ANZ.

3.4.1. Specificity

The specificity of the developed LC method was checked in the presence of its process related impurities and degradants. For this purpose, all the stressed samples of ANZ were spiked with its impurities (0.15% with respect to ANZ concentration). All the impurities and degradants were well resolved from one another and ANZ peak indicating the specificity of the proposed method to quantify ANZ and its seven impurities (Fig. 5).

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

Initially 1000 ppm of both Monoacid and Diacid stock solution was prepared with respect to 0.5 mg ml^{-1} ANZ. This solution was further diluted to 0.01 μg ml^{-1} for LOD, 0.03 μg ml^{-1} for LOQ and the signal-to-noise (S/N) ratio maintained 3:1 for LOD and 10:1 for LOQ for Monoacid and Diacid, respectively.

3.4.3. System precision, method precision and intermediate precision

System precision for ANZ at 0.2 μg ml^{-1} was determined by analyzing six replicate injections on different systems and different days and the relative standard deviation was found to be 0.56% and 0.92%, respectively. The precision of the method was checked by injecting 0.15% of impurities from individual preparations with respect to 0.5 mg ml^{-1} ANZ. % RSD of area for each impurity was calculated. Precision at LOQ also was determined by injecting individual preparations of ANZ spiked at LOQ level of its impurities. The intermediate precision of the method was also verified on six different days in the same laboratory using the specification and LOQ levels. Assay method precision was evaluated by carrying out independent assays of test sample of ANZ at 0.1 mg ml^{-1} level against qualified reference standard. The intermediate precision of the assay method was evaluated by a different analyst. The % RSD values for precision and intermediate precision were found to be 1.1 and 1.8, respectively for the assay of ANZ.

3.4.4. Linearity

Linearity test solutions for impurities were prepared individually at six concentration levels in the range of LOQ to 200% of the specification level *viz.* 0.15%. Linearity test solutions for ANZ assay were prepared from stock solution at five concentration levels from 50 to 150% of assay analyte concentration (0.1 mg ml^{-1} of ANZ). The peak area versus concentration data was subjected to least-squares linear regression analysis (Table 2).

3.4.5. Accuracy

Standard addition and recovery experiments were conducted to determine the accuracy of ANZ impurities for their quantification. The study was carried out in triplicate at LOQ, 100% and 150% with respect to specification level *viz.* 0.15% (Table 3). The accuracy of the ANZ assay was evaluated in the concentration levels *viz.* 50, 100 and 150% with respect to 0.1 mg ml^{-1} of ANZ test concentration. The assay ranged from 99.8 to 100.1%

3.4.6. Robustness

The robustness was illustrated by getting the resolution between any two compounds to be greater than 2.0, when mobile phase flow rate (±0.2 ml min^{-1}), organic solvent ratio (±5%) and column temperature (±2 °C) were deliberately varied (Table 4).

3.4.7. Solution stability

The solution stability of ANZ and its impurities in diluent was determined by leaving 0.15% spiked sample solution in a tightly capped volumetric flask at room temperature for 36 h and measuring the amounts of the compounds for every 12 h. The solution stability of ANZ assay (in diluent) was determined by leaving 0.1 mg ml^{-1} ANZ solution in tightly capped volumetric flasks at room temperature for 24 h during which they were assayed at 12 h intervals and comparing the results with those obtained from freshly prepared solution. The mobile phase was prepared at the beginning of the study period and was not changed during the

Table 2
Regression weights for Monoacid and Diacid.

Conc. (μg ml^{-1})	Peak area for Monoacid	Peak area for Diacid	r^2 value for Monoacid	r^2 value for Diacid
0.03 (LOQ)	145	202	0.993	0.996
0.145	232,465	316,526		
0.217	348,697	474,789		
0.290	464,930	633,052		
0.363	581,162	791,315		
0.435	697,395	949,578		
0.580	929,860	1,266,104		

Table 3
% Recovery study for ANZ (API).

Parameter	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Monoacid	Diacid
LOD	0.010	0.011	0.014	0.015	0.015	0.011	0.011
LOQ	0.030	0.033	0.042	0.045	0.045	0.033	0.033
Precision (% RSD) at LOQ	0.72	0.32	0.41	0.65	0.98	0.89	0.89
Intermediate precision at LOQ (% RSD)	1.75	1.24	1.36	1.22	1.59	1.36	1.63
Accuracy (% recovery) at							
LOQ	99.5 ± 0.12	98.8 ± 0.19	99.9 ± 0.34	99.5 ± 0.13	99.8 ± 0.76	99.8 ± 0.98	99.8 ± 0.98
100%	99.6 ± 0.17	99.2 ± 0.24	99.8 ± 0.65	99.7 ± 0.33	99.4 ± 0.35	98.3 ± 0.96	98.3 ± 0.96
150%	99.3 ± 0.14	98.7 ± 0.33	98.8 ± 0.48	99.9 ± 0.11	99.5 ± 0.44	99.0 ± 0.51	99.0 ± 0.51

Table 4
System suitability parameters for robustness study.

Parameter	Variation	Monoacid			Diacid		
		RSD	Theoretical plates	Asymmetry	RSD	Theoretical plates	Asymmetry
Flow	0.3 ml min^{-1}	0.63	15,041	0.92	0.52	14,625	1.04
	0.5 ml min^{-1}	0.72	15,234	1.02	0.42	14,320	1.06
	0.7 ml min^{-1}	0.56	14,986	1.04	0.51	15,082	1.07
Temperature	23 °C	0.92	14,657	1.02	0.44	17,063	1.14
	25 °C	0.86	15,022	1.01	0.53	16,854	0.98
	27 °C	0.94	14,457	1.02	0.41	17,293	1.05
Organic solvent	55:45	0.27	18,644	1.04	0.63	16,327	1.03
	60:40	0.34	17,942	1.02	0.61	16,853	1.02
	65:35	0.42	18,214	1.01	0.58	16,097	0.99

Table 5
Peak areas of ANZ (API) and degradants observed in solution stability.

Compound	Peak area			
	Initial	12 h	24 h	36 h
Monoacid	525,140	513,849	510,407	511,864
Diacid	727,654	723,491	719,486	718,943
ANZ	454,981	450,891	450,124	449,862

experiment. The % RSD values for solution stability experiments were calculated and found to be 1.11 and 1.42 for purity and assay methods, respectively. All the samples were found to be stable up to 36 h (Table 5).

3.4.8. Results of forced degradation studies

ANZ was degraded significantly in basic conditions and the two new degradants were formed under these conditions. This was confirmed by co-injecting the two impurities to these degraded samples and determined by LC–MS analysis. The two new degradants formed when photodiode array detector was employed to check and ensure the homogeneity and purity of ANZ peak in all the stressed sample solutions. Assay studies were carried out for stress samples against ANZ qualified working standard and the mass balance (% assay + % sum of all degradants) results were presented in Table 6. The purity and assay of ANZ was unaffected by the presence of its impurities and degradation product and thus confirms the stability-indicating power of the developed method.

Table 6
Results of forced degradation studies.

Stress conditions	% Degradants formed								% of assay	Mass balance
	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Monoacid	Diacid	Total impurities		
Unstressed	N/D	N/D	N/D	N/D	N/D	N/D	N/D	0.03	–	–
Acid	N/D	N/D	N/D	N/D	N/D	0.04	0.07	0.12	99.73	99.85
Oxidative	N/D	N/D	N/D	N/D	N/D	0.07	0.06	0.13	99.62	99.75
Base	N/D	N/D	N/D	N/D	N/D	5.24	4.32	9.56	90.32	99.88

4. Conclusion

This proposed method enables us to report two new significant degradant impurities under basic conditions and also establish a method on ANZ to quantitate the related substances and degradants in active pharmaceutical ingredient. The proposed RP-LC and LC–MS/MS method developed for determination of ANZ and its impurities were found to be specific, precise, accurate, robust and stability-indicating and validated for the routine analysis in the active pharmaceutical form.

Acknowledgement

The authors wish to thank Dr. B.M. Choudary, Managing director, Ogene System (I) Pvt. Ltd., Hyderabad for providing facilities.

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