Separation, Characterization, and Quantitation of Process-Related Substances of the Anti-Hypertensive Drug Doxazosin Mesylate by Reversed-Phase LC with PDA and ESI-MS as Detectors

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Abstract

A simple and rapid reversed-phase liquid chromatography (LC) method with photodiode array (PDA) and electrospray ionization (ESI)–mass spectrometry (MS) as detectors was developed and validated to separate, identify, and quantitate the related substances of Doxazosin mesylate (DXZN) for monitoring the reactions involved during process development. The high-performance liquid chromatography profiles of related-substances of DXZN are used as fingerprints to follow the procedures used in the manufacturing units. The separation is accomplished on an Inertsil ODS-3 column with acetonitrile–ammonium acetate (10mM, pH 4.0) as the mobile phase, using a gradient elution mode and monitoring the eluents by a photodiode array detector at 265 nm at ambient temperature. LC–ESI-MS–MS is used to identify the additional impurities formed during the synthesis. The identified impurities were synthesized and characterized by UV, Fourier transform-IR, ^{1}H NMR, and MS data. The detection limits for the impurities are 0.74 – 4.14 × 10^{-9}g , and the method is found to be suitable not only for the monitoring of synthetic reactions, but also for quality assurance of DXZN in bulk drugs and formulations.

Introduction

Doxazosin mesylate (DXZN), (±) 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-[(2,3-dihydro-l,4-benzodioxin-2-yl)carbonyl]piperazine monomethane sulfonate, is a selective α_1-adrenergic blocker belonging to the family of quinazoline drugs (1). It is used in the treatment of hypertension either alone or in combination of diuretics and β-adrenergic-receptor-antagonists (2). More recently, it was found to be effective in the treatment of benign prostatic hyperplasia (BPH) (3). Assurance of its quality is quite important, not only to prove the efficacy, but also to ensure the safety of the patients who ultimately receive it during the treatment of hypertension.

A thorough literature search has revealed that differential pulse-polarographic (4,5), square-wave voltametric (6,7), high-performance liquid chromatographic (HPLC) (8–11), and UV-spectrophotometric (12) methods have been reported for the determination of DXZN in biological fluids. However, for the analysis of pharmaceuticals, only a limited number of HPLC (13), HP thin-layer chromatography (TLC) (14,15), and voltametric (16) methods were reported. The degradation behavior of DXZN was studied under a variety of stress conditions using UV and HPLC (17,18), but none of these methods addressed the separation of the process-related substances of DXZN. Thus, the reported methods are not suitable for monitoring the synthetic procedures of DXZN. To the best of our knowledge, methods for the determination of process-related impurities of DXZN are not available in the literature. HPLC is a powerful analytical technique to separate, identify, and determine all the components of complex materials. It satisfies most of the requirements demanded by the modern pharmaceutical industry. It is the leading analytical technique used in all phases of drug discovery, development, and quality control. In this present investigation, a simple and rapid reversed-phase (RP)-LC method for the separation and determination of process-related substances of DXZN is developed. The separation was achieved on an Inertsil ODS-3 column, using ammonium acetate (10mM, pH 4.0)–aceonitrile as a mobile phase, under a gradient elution monitored at UV 265 nm, using a photodiode array detector (PDA). Further, LC–electrospray ionization (ESI)-mass spectrometry (MS)–MS was used to identify the side reaction products that originated during the synthesis. The method was applied for the determination of related substances of DXZN, not only in reaction mixtures, but also in bulk drugs and pharmaceuticals. It is quite useful for process control and to optimize the quality of the finished products of DXZN.

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Experimental

Materials and reagents

All reagents were of analytical-reagent grade, unless stated otherwise. Glass-distilled and deionized water (Nanopure, Barnsted, MA), HPLC-grade acetonitrile, ammonium acetate, chloroform, sodium hydroxide, hydrochloric acid (Ranbaxy, SAS Nagar, Mumbai, India) and acetic acid (S.D. Fine Chem, Mumbai, India), piperazine, ethyl 2,3-dihydro-1, 4-benzodioxine-2-carboxylate, and catechol (Sigma-Aldrich, St. Louis, MO) were used. Samples of DXZN and process related substances, specifically catechol (CTHL), ethyl 2,3-dihydro-1,4-benzodioxin-2-carboxylate (EDBC), 1-(2,3-dihydro-1,4-benzodioxine-2-carbonyl) piperazine (DCPZ), and 4-amino-2-chloro-6,7-dimethoxyquinazoline (ACDQ) were collected during the process development and used. Two side-reaction products, bis-amide or [(1-(2,3-dihydro-1,4-benzodioxin-2-ylcarbonyl)-4-(2,3-dihydro-1,4-benzodioxin-2-ylcarbonyl)]piperazine (Impurity-V) and 6,7-dimethoxy-2-(piperazin-1-yl)quinazolin-4-amine (Impurity-VI), of DXZN were synthesized in the laboratory by known methods (19–20) and used.

Synthesis of impurity-V

Piperazine (1.027 g) (0.962 moles) and 2-carboethoxy-1-4-benzadioxane (2.00 g) (1.152 moles) were taken in to a 250-mL R.B. flask, provided with a stirrer, thermo well, condenser, and nitrogen blanket. The reaction mixture was heated for 4 h to maintain the internal temperature between 75°C and 80°C and stirred under the absence of nitrogen. The reaction mixture was cooled to room temperature and dissolved in 50–100 mL of $CHCl_3$. Then it was washed with 50-mL volumes of saturated $NaHCO_3$ solution for three times, followed by water and acidified with 10% HCl (~ 15–20 mL) to adjust the pH to 2. The aqueous layer was separated, extracted into $CHCl_3$, and evaporated to form a pasty liquid. To this, 100 mL of hexane was added and boiled for 2 h. Brown colored bis-amide crystals of 98.3% purity were formed.

Synthesis of impurity-VI

A 250-mL round bottom flask fitted with a stirrer, condenser, and thermowell was charged with 100 mL of isoamylalcohol, 0.5 g (0.33 mole) of 4-amino-2-chloro-6,7-dimethoxyquinazoline, and 0.7 g (0.4 mole) of piperazine. The reaction mixture was stirred and refluxed for 72 h, and it was cooled to 80°C. The precipitated white color solid that was obtained was recrystallized from 2-propanol, and the pure white colored crystals of 6,7-dimethoxy-2-(piperazin-1-yl)quinazolin-4-amine (impurity-VI) (97.80%) were dried.

Apparatus

LC-PDA

The LC system was composed of two LC-10ATVP pumps, a SPD-M10AVP diode array detector and a SIL-10ADVP auto injector, a DGU-12A degasser, and a SCL-10AVP system controller (Shimadzu, Kyoto, Japan). A RP Inertsil ODS-3 (GL Sciences, Tokyo, Japan) column (25 cm × 4.6-mm i.d; particle size 5 µm) was used for the separation. The chromatographic and integrated data were recorded using an HP-Vectra (Hewlett Packard, Waldbronn, Germany) computer system.

LC–ESI-MS–MS

LC–ESI-MS–MS analyses were performed with an Agilent Series 1100 LC (Agilent Technologies, Palo Alto, CA) and a LC–MSD Trap SL mass spectrometer (Agilent, Waldborn, Germany) equipped with ESI and atmospheric pressure chemical ionization interfaces. The chromatographic separation was carried out using the conditions developed during the LC-PDA analysis. Analyses were performed with an ESI interface in the positive-ion mode using nitrogen as a nebulizer and disolvation gas. The operating parameters were: nebulizer gas flow, 0.9 L/min; dry gas flow, 7.0 L/min; nebulizer pressure, 50 psi; ICC target, 30,000; capillary voltage, –3500 V; maximum accumulation time, 300 ms; skimmer, 40 V; and dry temperature, 325°C. MS measurements were performed in the full-scan mode over the mass range of *m/z* 50 to 800 with 0.21 scans/s.

Chromatographic conditions

The mobile phase was acetonitrile–ammonium acetate (10mM, 33:67, v/v), and the buffer pH was adjusted to 4.0 with acetic acid. Before delivering into the system, it was filtered through a 0.45-µm PTFE filter and degassed using a vacuum. The analysis was carried out under linear gradient conditions as shown in Table I, at a flow rate of 1.0 mL/min at room temperature (28°C). Chromatograms were recorded at 265 nm using a SPDM-10AVP as a PDA.

Analytical procedures

Solutions of DXZN (0.5 mg/mL) and impurities (0.5 mg/mL) were prepared in the mobile phase, and 20 µL of each solution was injected and chromatographed under the previous conditions. The system suitability was conducted by using 0.1% of ACDQ spiked to the DXZN and evaluated by making five replicate injections. The system was deemed suitable for use if the tailing factor for ACDQ and DXZN were less than or equal to 1.2, and the resolution was greater than 2.0. Synthetic mixtures and process samples were analyzed under identical conditions. The quantities of impurities were calculated from their respective peak areas.

Table I. Gradient Elution Program for Separation of DXZN and Its Process-Related Substances

Time (min)	10mM Ammonium acetate (%)	Acetonitrile (%)	Rate of change (%)
0.01	67	33	Isocratic
8	67	33	Isocratic
15	40	60	3.85
25	35	65	0.50
28	67	33	–10.66

Results and Discussion

Optimization of the chromatographic conditions

Figure 1 shows chemical reactions involved in the synthesis of DXZN in a chemical laboratory. CTHL was reacted with 2,3-dibromopropionate to give EDBC and refluxed further with piperazine to afford DCPZ, which was then condensed with ACDQ to obtain DXZN (19). As shown in Figure 1, there are five compounds, including the starting material and side product, bis-amide (impurity-V), present as impurities in DXZN. Bis-amide generally formed as a side product during the second stage of reactions.

The present study was aimed at developing a chromatographic system capable of eluting and resolving DXZN and its process-related substances that originated from the synthesis. In the preliminary experiments, all the impurities of DXZN were subjected to separation by RP-HPLC on an Inertsil ODS-3 column with water–methanol as the eluent. Three compounds (DCPZ, ACDQ, and CTHL) were merged when the concentration of methanol was kept above 40%. However, after decreasing its concentration, all three compounds were well separated, except EDBC and impurity-V, but in methanol, DXZN eluted as a broad peak. When methanol was replaced by acetonitrile, the peak shape of DXZN was improved, but it was not yet satisfactory. Because DXZN is basic in nature (pK_a 8.63), analysis by LC represented a challenge to the pharmaceutical analysts. It interacted with the residual silanol groups of bonded-silica phases, leading to peak tailing. At pH 7, the neutral DXZN adsorbed on the column for a long time (> 30 min) because of the interaction with the free silanol groups of bonded silica, causing the peak to become broad. Buffering the aqueous mobile phase to suppress the silanol ionization (ion suppression) generally reduced the band tailing. Because of this, in another attempt, the water was replaced with 10mM ammonium acetate. The effect of the buffer pH on separation was studied (Figure 2). Upon decreasing the pH of the buffer by acidifying it with acetic acid, DXZN was protonated (ionized) and became more hydrophilic in character because its retention was decreased. It is clearly seen in Figure 2 that when increasing the pH, the retention of DXZN and DCPZ also increased, and the peaks became broad as the retention of the remaining compounds were unaffected. The optimum pH was found to be 4.0, where the separation of DXZN and its related-substances was good. The effect of acetonitrile on the separation was studied (Figure 3) in the range of 30% to 60% of the mobile phase. As shown in Figure 3, when the concentration of acetonitrile was 33%, all four impurities and DXZN were separated with good resolution, but EDBC and impurity-V were retained in the column for a longer time. On increasing the acetonitrile concentration, these two were eluted, but the remaining compounds (DXZN, CTHL, and ACDQ) were not separated. To shorten the retention times and sharpen the peak shapes of EDBC and ACDQ, a gradient elution (as shown in Table I) was attempted. All the compounds were well resolved from DXZN with good peak shapes and resolution (R_s > 2.50). The LC chromatogram of a synthetic mixture containing DXZN and its related substances is shown in Figure 4. The peaks

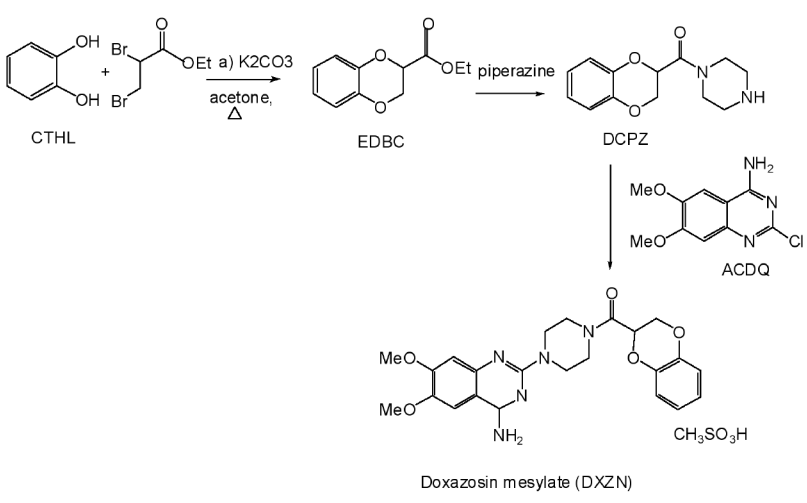

Figure 1. Synthetic reactions involved in the preparation of DXZN.

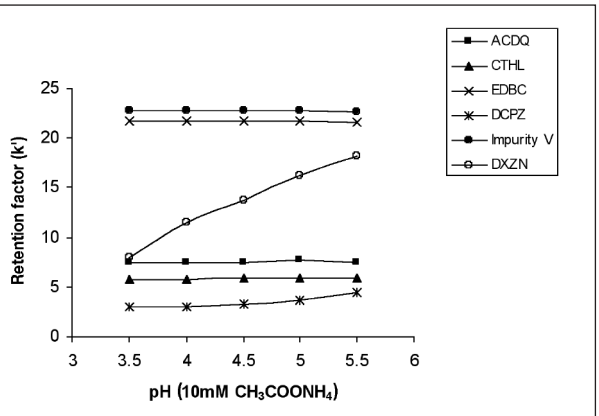

Figure 2. Effect of buffer pH on the retention of DXZN and its process-related substances.

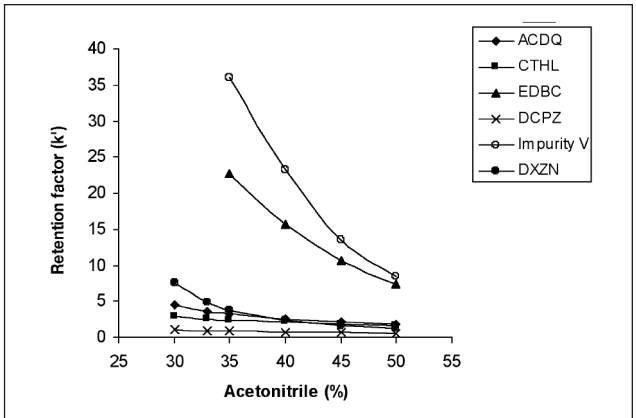

Figure 3. Effect of acetonitrile on the retention of DXZN and its process-related substances.

were identified and compared with the retention times and online UV-absorption spectra of the individual compounds. The chromatographic parameters, retention time (t_R), retention factor (k'), and resolution (R_s) among successive peaks were determined under optimized conditions and are given in Table II.

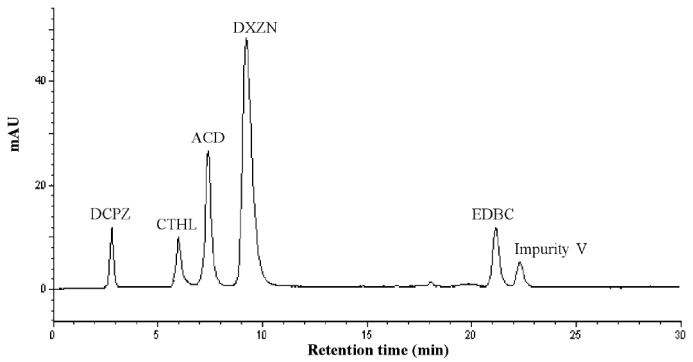

Figure 4. HPLC chromatogram of synthetic mixture of DXZN and its process-related substances (10 µg/mL each).

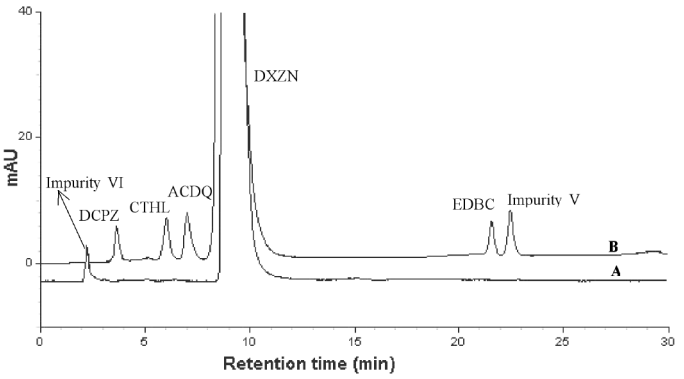

Figure 5. Overlay HPLC chromatograms of DXZN as a bulk drug (A) and spiked with 0.1% of all process-related substances (B).

Analysis of bulk drugs

The proposed HPLC method has been adopted to determine potential impurities in different batches of DXZN, synthesized as per the scheme shown in Figure 1. Different batches of DXZN were analyzed, and the total amount of impurities was found to be in the range of 0.09–0.12% in all the batches. The typical chromatograms of a bulk drug of DXZN, spiked with 0.1% of all impurities, are shown in Figures 5A and 5B. It can be seen in Figure 5A that the unknown impurity-VI, at t_R 2.35 min, was around 0.1% and the remaining were insignificant. However, it did not match with any of the process-related substances when comparing with the retention times. It was investigated by LC–ESI-MS–MS, followed by its synthesis and characterization using UV, IR, NMR, and MS data.

LC–ESI-MS–MS

The LC–MS analysis was carried out in positive-ion mode using the chromatographic conditions described in the experimental section. When the batch sample was analyzed, impurity-VI and DXZN were eluted at t_R 2.35 and 8.93 min, respectively. The ESI–MS data of impurity-VI and DXZN have shown protonated molecular ions [M+H] at 290 and 452 amu indicating that the molecular masses were 289 and 451 amu, respectively. Impurity-VI had 162 amu less mass units compared with DXZN. A MS–MS analysis was carried out for further characterization. The MS–MS spectra of DXZN and impurity-VI are shown in Figures 6A and 6B, respectively. It can be seen from Figures 6A and 6B that the

Table II. Retention Data of DXZN and its Process-Related Substances

Serial number	Compound	Abbreviation	Retention time (min)	Retention factor	Resolution
1	1-(2,3-Dihydro-1,4-benzodioxin-2-carbonyl)-piperazine	DCPZ	3.12	0.95	0.00
2	Catechol (DCPZ and CTHL)	CTHL	5.34	2.60	9.24
3	4-Amino-2-chloro-6,7-dimethoxyquinazoline (CTHL and ACDQ)	ACDQ	6.98	3.66	4.08
4	Doxazosin mesylate (ACDQ and DXZN)	DXZN	8.90	4.94	2.78
5	Ethyl 2,3-dihydro-1,4-benzodioxin-2-carboxylate (DXZN and EDBC)	EDBC	21.16	13.11	17.72
6	Bis amide (EDBC and Impurity-V)	Impurity-V	22.22	13.81	2.37

MS–MS analysis of the $[M+H]^+$ ion at *m/z* 452 of DXZN gave daughter ions at *m/z* 344.1, 290.1, and 247. In the case of impurity-VI, the $[M+H]^+$ ion at *m/z* 290 gave daughter ions at *m/z* 274, 247, 231, and 221. The ion at *m/z* 344 was formed from the loss of $-C_6H_4O_2$ from *m/z* 452, and it further lost a neutral fragment $-C_3H_2O$ and formed a daughter ion at *m/z* 290. The ion observed at *m/z* 247 with the elemental formula $C_{12}H_{15}N_3O_2$ was formed in two different ways: (*i*) because of the fission in the piperazine ring of the main ion *m/z* 452 and (*ii*) by loss of *N*-methylenemethanamine ($-C_2H_5N$) as a neutral fragment from *m/z* 290. The impurity-VI *m/z* 290 was fragmented in three different ways: (*i*) it lost an *N*-methylenemethanamine ($-C_2H_5N$) and gave an ion at *m/z* 247, and it also lost an $-NH_2$ free radical and gave an ion at *m/z* 231; (*ii*) the ion at *m/z* 290 lost an $-NH_2$ free radical and gave a stable ion at *m/z* 274, and it also lost *N*-methylenemethanamine ($-C_2H_5N$) and gave a stable ion *m/z* 231; and (*iii*) the ion at *m/z* 221 was formed because of the loss of $-C_4H_7N$ from *m/z* 290. The proposed MS–MS fragmentation for DXZN and impurity-VI are shown schematically in Figure 7. Here, the impurity was characterized using one of the daughter ions of the main compound, having the same molecular mass (i.e., *m/z* 290 amu) as it is closely related to the DXZN daughter ion. The fragments at *m/z* 290 and 247 were identical in both the DXZN and impurity-VI. This indicated that the 6,7-dimethoxy-2-(piperazin-1yl)-4-amine moiety (viz., *m/z* 290) was present in both DXZN and impurity-VI. Based on these observations, it was assumed that the impurity could be similar to the *m/z* 290 daughter ion of DXZN. The impurity was predicted as 6,7-dimethoxy-2-(piperazin-1-yl)quinazolin-4-amine. Later, it was synthesized and characterized by UV, FT-IR, ^{1}HNMR, and MS. It was analyzed by HPLC, and its retention time matched well with the impurity detected in the batch samples of DXZN. Finally it was confirmed as 6,7-dimethoxy-2-(piperazin-1-yl) quinazolin-4-amine (impurity-VI).

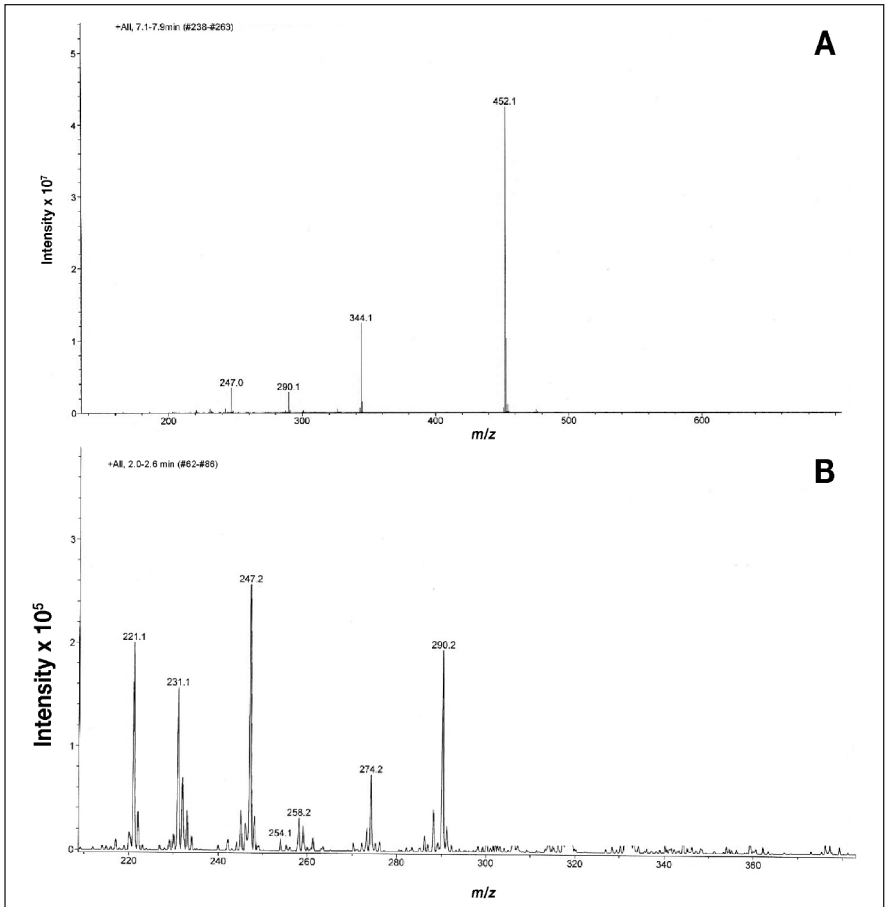

Figure 6. MS–MS spectra of [M+H]+ ions of DXZN, *m/z* 452 (A), and impurity-VI, *m/z* 290 (B).

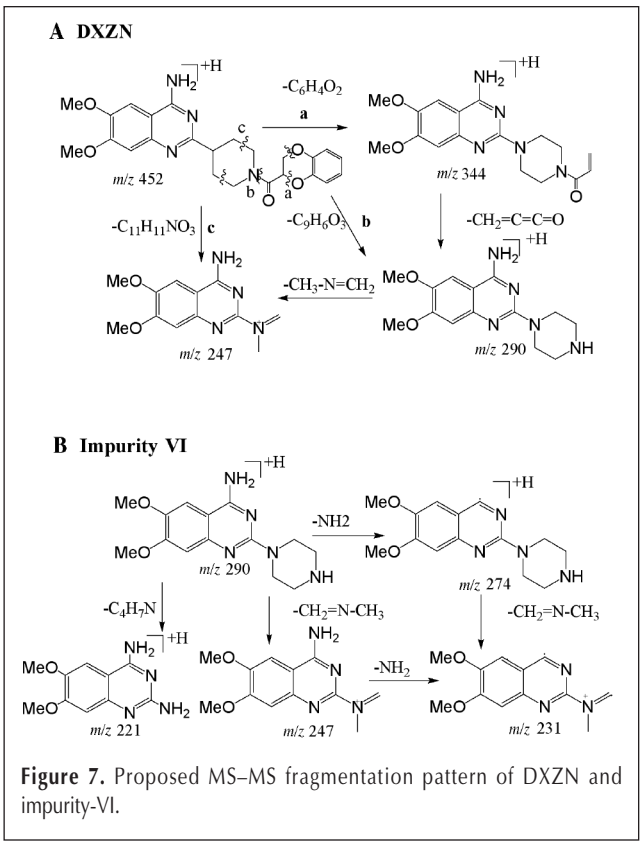

Figure 7. Proposed MS–MS fragmentation pattern of DXZN and impurity-VI.

Synthesis and characterization

The two side products were synthesized (Figure 8) and characterized using UV, IR, ^{1}H NMR, and MS. Impurity-VI was characterized by UV λ_{max} 215, 240 nm, IR (KBr) cm^{-1} 3328–3214 (N-H str., $-NH_2$), 2922 (C-H str., arom), 2831, 1487 cm^{-1}, ^{1}HNMR ($CDCl_3$ + 2 drops DMSO-d_6): δ 2.82 (m, 4H, piperazine ring); 3.75 (m, 4H, piperazine ring); 3.80 (s, 3H, $-OCH_3$); 3.85 (s, 3H, $-OCH_3$); 6.40 (broad s, 2H, NH_2); 6.71 (s, 1H, arom.); and 7.30 (s, 1H, arom.). MS (EI) was characterized by: *m/z* 289 (M^+) and 247 ($M^+-C_2H_4N$). The bis-amide (Impurity-V) was characterized by UV λ_{max} 224, 276 nm, IR (KBr) cm^{-1} 2934 (C-H str. aliphatic), 1657 (C=O str.), 1494, and 1254

(C-O str., cyclic ether). ^{1}HNMR ($CDCl_3$) was characterized by: δ 3.3–4.25 (m, 8H), 4.35–4.50 (m, 4H), 4.82 (m, 2H), and 6.70–6.85 (m, 8H). MS (ESI: M+H) was characterized by: *m/z* 411, 410 (M^+), 353, and 249.

Rationalization

The formation of impurity-VI was explained by the presence of any excess amount of unreacted piperazine present in DCPZ, reacting with ACDQ during the third step, as shown in Figure 1. It gave impurity-VI as a potential impurity in the bulk drug of DXZN. Ojha et al. (18) observed that the impurity was a degradation product of DXZN, and it was formed under forcible acid and base degradation. The impurity-V (bis-amide) was formed because two molecules of EDBC condensed with one molecule of piperazine to form bis-amide (impurity-V) as a side product. The synthesis and formation of these two side products are shown in Figure 8.

Validation

The method was validated with respect to the precision, accuracy, linearity, and limits of detection (LOD) and quantitation (LOQ).

Precision

The precision of the method was checked by injecting six (n = 6) times of 0.1% of each related-substance spiked to DXZN. The relative standard deviation (RSD) of the t_R and peak area were calculated. The ranges of RSD were found to be 0.07% to 0.33% for t_R and 0.55% to 2.22% for peak areas of DXZN, respectively. The results are recorded in Table III.

Accuracy

DXZN was spiked with each related-substance at five different levels of 50%, 75%, 100%, 125%, and 150%, with respect to the nominal concentration of the main compound, and the recoveries were determined. The recoveries were in the range of 93.56–101.57% with RSDs between 0.27–3.35%, respectively. The results are shown in Table IV.

Linearity

The linearity of a peak area versus its concentration was studied for all related-substances at seven different levels (i.e., 50%, 75%, 100%, 125%, 150%, 175%, and 200%) with respect to the main compound. The data were subjected to linear-regression analysis, and the results are shown in Table V. Table V shows that the calibration curves were linear, with good correlation coefficients of 0.999 for all the compounds.

LOD and LOQ

LOD and LOQ represent the concentration of an analyte that would yield a signal-to-noise ratio of 3 for the LOD and 10 for the LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of the analytical background by injecting blank samples and calculating the signal-to-noise ratio for each compound by injecting a series of solutions until the signal-to-noise ratio of 3 for LOD and 10 for LOQ were achieved. The LOD and LOQ values for DXZN were found to be 3.2×10^{-9} g and 9.6×10^{-9} g, respectively. The results are recorded in Table V.

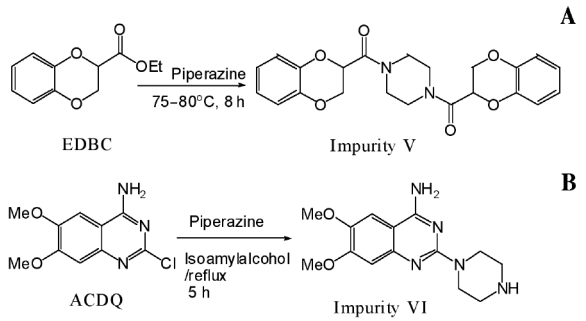

Figure 8. Synthesis of impurity-V (A) and impurity-VI (B) as side reaction products of DXZN.

Table III. Precision Data of DXZN Spiked with 0.1% of all Process-Related Substances

Compound	Peak area	RSD (%)*	t_R	RSD (%)*
CTHL	8315	0.89	5.46	0.21
EDBC	7568	2.29	21.57	0.25
DCPZ	10343	0.93	3.05	0.33
ACDQ	17294	2.05	7.02	0.16
Impurity-V	4177	1.11	22.15	0.07
Impurity-VI	13788	0.67	2.35	0.25
DXZN	23466461	0.55	8.96	0.24

* The RSD was an average of six determinations (n = 6).

Table IV. Recovery of Process-Related Substances from DXZN as Determined by HPLC

	Nominal 0.1% of impurity spiked to DXZN				
	50	75	100	125	150
Amount added (µg/mL)	0.250	0.375	0.500	0.625	0.750
% Recovery (± RSD %)*					
CTHL	97.35 ± 0.85	96.36 ± 1.05	99.92 ± 0.93	95.60 ± 0.95	98.20 ± 0.55
EDBC	90.57 ± 1.10	97.78 ± 1.53	94.86 ± 2.59	94.39 ± 3.35	95.40 ± 2.09
DCPZ	97.89 ± 2.60	95.55 ± 1.35	96.55 ± 1.23	93.79 ± 2.72	95.55 ± 0.48
ACDQ	100.26 ± 0.27	101.57 ± 0.91	100.09 ± 2.09	101.49 ± 0.45	99.24 ± 0.50
Impurity-V	98.43 ± 1.23	100.65 ± 0.38	101.14 ± 2.15	99.85 ± 1.20	100.08 ± 0.75
Impurity-VI	93.56 ± 1.23	97.73 ± 1.91	101.20 ± 2.25	96.85 ± 0.42	100.5 ± 1.15

* n = 3.

Table V. Linearity, LOD, and LOQ

Compound	Range (µg/mL)	Regression equation	r^2	LOD ($\times 10^{-9}$ g)	LOQ ($\times 10^{-9}$ g)
CTHL	0.25–1.00	$y = 16630x + 82$	0.9995	2.82	8.56
EDBC	0.25–1.00	$y = 15022x + 184$	0.9986	3.12	9.48
DCPZ	0.25–1.00	$y = 20943x + 22$	0.9987	4.14	12.56
ACDQ	0.25–1.00	$y = 35501x - 724$	0.9996	1.70	5.96
Impurity-V	0.25–1.00	$y = 8331x + 83$	0.9985	1.62	4.88
Impurity-VI	0.25–1.00	$y = 27833x - 54$	0.9999	0.74	2.24

Conclusion

A comprehensive gradient elution RP-LC procedure for the monitoring of impurities that originated during the synthesis of DXZN was developed and validated. The method was found to be linear, accurate, reproducible, and capable of separating all impurities associated with the synthetic process of DXZN. It is useful for the process development and quality assurance of DXZN. The side products formed during the synthesis were separated, identified by LC–ESI-MS–MS, synthesized, and characterized by UV, FT-IR, ^{1}HNMR, and MS data.

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