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ORIGINAL ARTICLE

Validated gradient stability indicating HPLC method for determining Diltiazem Hydrochloride and related substances in bulk drug and novel tablet formulation

Vivekanand A. Chatpalliwar[a,*], Pawan K. Porwal[a], Neeraj Upmanyu[b]

[a]*Department of Pharmaceutical Chemistry, SSDJ College of Pharmacy, Chandwad, India*
[b]*Department of Pharmaceutical Science, Pharmaceutical Chemistry Division, University of Sagar, Sagar, India*

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Abstract A stability-indicating liquid chromatographic method has been developed and validated for the determination of Diltiazem Hydrochloride (DTZ) together with its six related substances (Diltiazem sulphoxide, Imp-A, Imp-B, Imp-D, Imp-E, and Imp-F) in a laboratory mixture as well as in a novel tablet formulation developed in-house. Efficient chromatographic separation was achieved on a Hypersil BDS C_{18} (150 mm × 4.6 mm, 5.0 μm) with mobile phase containing 0.2% Triethylamine (TEA) in gradient combination with acetonitrile (ACN) at a flow rate of 1.0 mL/min and the eluent was monitored at 240 nm. In the developed method, the resolution of DTZ from any pair of impurities was found to be greater than 2.0. The test solution and related substances were found to be stable in the diluent for 24 h. The developed method resolved the drug from its known impurities, stated above, and also from additional impurities generated when the formulation was subjected to forced degradation; the mass balance was found close to 99.9%.

Regression analyses indicate correlation coefficient value greater than 0.997 for DTZ and its six known impurities. The LOD for DTZ and the known impurities was at a level below 0.02%. The method has shown good, consistent recoveries for DTZ (99.8–101.2%) and also for its six known impurities (97.2–101.3%). The method was found to be accurate, precise, linear, specific, sensitive, rugged, robust, and stability-indicating.

*Corresponding author. Tel.: +91 2556 252529;
fax: +91 2556 253179.
E-mail address: vchatpalliwar@yahoo.co.in (V.A. Chatpalliwar)

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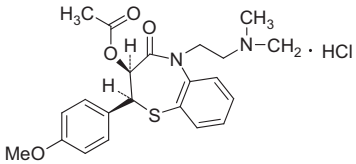
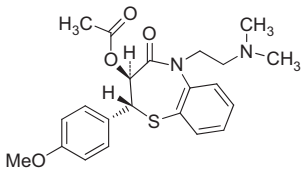
1. Introduction

Diltiazem Hydrochloride (DTZ), 3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1, 5-benzothiazepin-4(5H)-one monohydrochloride (Fig. 1) [1] – a calcium channel blocker which inhibits influx of calcium (Ca^{2+}) ions – is used for treatment of several cardiovascular disorders [2], viz. essential hypertension [3] and supraventricular tachyarythmias [4].

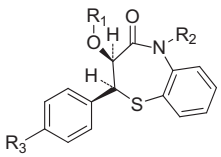
Regulatory requirements for the identification, qualification, and control of impurities in drug substances and their formulated products are now being explicitly defined, particularly through the



(2S,3S)-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate hydrochloride; Ditiazem Hydrochloride



A. (2R,3S)-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate; Diltiazem impurity-A



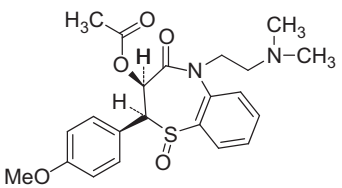
B. R1 = CO-CH 3 , R2 = H, R3 = OCH 3 :
(2S,3S)-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate; Diltiazem impurity-B

C. R1 = CO-CH 3 , R2 = CH 2 -CH 2 -N(CH 3) 2 , R3 = OH:
(2S,3S)-5-[2-(dimethylamino)ethyl]-2-(4-hydroxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate; Diltiazem impurity-C

D. R1 = CO-CH 3 , R2 = CH 2 -CH 2 -NH-CH 3 , R3 = OCH 3 :
(2S,3S)-2-(4-methoxyphenyl)-5-[2-(methylamino)ethyl]-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate; Diltiazem impurity-D

E. R1 = R2 = H, R3 = OCH 3 :
(2S,3S)-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydro-1,5-benzothiazepin-4(5H)-one; Diltiazem impurity-E

F. R1 = H, R2 = CH 2 -CH 2 -N(CH 3) 2 , R3 = OCH 3 :
(2S,3S)-5-[2-(dimethylamino)ethyl]-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydro-1,5-benzothiazepin-4(5H)-one; Diltiazem impurity-F



(2S,3S)-5-[2-(dimethylamino)ethyl]-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydro-1,5-benzothiazepin-4(5H)-one 1-Oxide; Diltiazem sulphoxide

Figure 1 Chemical structures of Diltiazem Hydrochloride and its related substances.

International Conference on Harmonization (ICH). It is also recommended by ICH that all routine impurities at or above 0.1% level, should be identified through appropriate analytical methods [5–7]. DTZ is cited in the British Pharmacopoeia to have contamination by compounds A, B, C, D, E, and F (Fig. 1) [8]. Therefore, it was thought worth determining the impurities of DTZ to ensure the quality, efficacy and safety of the final pharmaceutical formulation. Of the six official impurities, a method for analyzing DTZ in the presence of five impurities, namely A, B, D, E, and F, was required to be developed. Diltiazem sulphoxide [9], a reported impurity, is likely to be present in formulations of DTZ.

Numerous analytical methods for the determination of DTZ in bulk drug as well as in formulations have been reported in

literature viz. spectrophotometry [10,11], gas chromatography [12], HPTLC [13], HPLC [14–18]. Recently, HPLC–MS and CE methods have been reported to characterize the DTZ metabolites [19–22]. A RP-HPLC method using monolithic silica support for separation of DTZ and its impurities has been published [23]. Two validated stability indicating HPLC methods have also been reported for DTZ in bulk drug [24] and in tablets [25]. These stability indicating analytical methods are validated for assay of DTZ, and not for analyzing the drug in the presence of its known impurities.

An HPLC method for assay of Diltiazem Hydrochloride and its related substances (RS) in bulk drug and finished tablets is reported [26] without any comment on the stability indicating potential of the method.

From preceding details of relevant literature it was apparent that a validated method is required to be developed which would be capable for simultaneous determination of DTZ in the presence of its reported impurities, and also serve as stability-indicating. Thus, the aim of current study was to develop and validate an LC method for the determination of DTZ and its known impurities (Diltiazem sulphoxide, Imp-A, Imp-B, Imp-D, Imp-E, and Imp-F) along with degradation products, in a novel tablet dosage form, in accordance with the ICH guidance document [27].

2. Experimental

2.1. Reagents and chemicals

Qualified standards of DTZ and Diltiazem sulphoxide were gifted by Torrent Research Center (Ahmadabad, India). Following authenticated impurity standards were obtained from Stride Arcolab Limited (Bangalore, India): Imp-A, Imp-B, Imp-D, Imp-E, and Imp-F. Novel sustained release tablets of DTZ and a placebo were formulated in Department of Pharmaceutics, S.S.D.J. Coll. Pharm., Neminagar, Chandwad, India. The excipients used to formulate the tablets were aerosol 200 and eudragit and magnesium stearate which were procured from local supplier. Analytical/HPLC grade chemicals and solvents used were obtained from Ranbaxy Fine Chemicals Limited (Delhi, India).

2.2. Chromatography apparatus and conditions

The chromatograph consisted of an HP-Agilant 1100 HPLC system with G1311A quaternary pump, G1315A diode array detector and variable wavelength detector, a G1313A auto-sampler, and a G1322A vacuum degasser. The data were evaluated by HP Chemstation Software.

DTZ, pKa 7.7 [28], was freely soluble in selected analytical solvents like water, acetonitrile (ACN) and methanol (MeOH). The chromatographic conditions were optimized by different means (using different columns, different buffers and different organic phases). Early chromatographic work was performed with different brands of C_8 and C_{18} columns as stationary phase and various combinations of buffered (pH 4.5–5.0) organic phases (ACN and/or methanol). The flow rate of mobile phase was varied within 1.0–1.5 mL/min. Wavelength for monitoring the eluent was selected by scanning standard solution of drug within 200–400 nm using double beam UV–visible spectrophotometer (Shimadzu 1800, Japan).

All noted measurements were performed with an injection volume of 10 μL and UV detection at 240 nm of samples dissolved in a diluent; Mobile phase-A [0.2% triethylamine (TEA) pH adjusted to 4.5 with *o*-phosphoric acid (*o*-PA)]: Mobile phase-B [ACN] in 3:2 (v/v).

2.3. Preparation of solutions

2.3.1. Preparation of resolution solution

On lines with official procedures [8], impurity stock solutions of Diltiazem impurity-A and Diltiazem impurity-D (50 μg/mL, each) were individually prepared by dissolving their appropriate amounts in the diluent. DTZ (25 mg) was dissolved in 60 mL of diluent in a 100 mL volumetric flask. To this solution, 2.5 mL of each of above impurity solutions was added and sonicated in cool condition (10 °C ± 2) for 10 min. The volume of thus obtained clear solution was made up to 100 mL with the diluent to give the resolution solution containing 250 μg/mL DTZ, and 1.25 μg/mL each of Imp-A and Imp-D.

Diluted standard of DTZ (1.25 μg/mL) was prepared by dissolving appropriate amount of the drug in the diluent. Similar method was employed to prepare diluted solutions of Diltiazem sulphoxide, Imp-B, Imp-E and Imp-F to contain 1.25 μg/mL.

2.3.2. Preparation of laboratory mixture solutions

Appropriate amounts of active pharmaceutical ingredient (DTZ), Diltiazem suphoxide, Imp-A, Imp-B, Imp-D, Imp-E, and Imp-F, and amount of excipients equivalent to average weight of tablet was transferred to a 200 mL volumetric flask, 100 mL of diluent was added and sonicated for 15 min with intermittent shaking and diluted to volume with the diluent to contain 250 μg/mL DTZ, and 1.25 μg/mL each of known impurities. This solution was filtered through a 0.45 μm Nylon 66-membrane filter and used for the analysis.

2.3.3. Preparation of sample solution

An amount of powdered tablet (In-house DTZ SR, 60 mg) equivalent to 50 mg of the active pharmaceutical ingredient (DTZ) was transferred to a 200 mL volumetric flask. Diluent (100 mL) was added to it and sonicated for 15 min with intermittent shaking and diluted to volume with the diluent. This solution was filtered through a 0.45 μm Nylon 66-membrane filter and used for the analysis. Similar method was employed to prepare placebo solution.

2.4. System suitability

System suitability parameters were evaluated to verify that the analytical system is working properly and can give accurate and precise results. Parameters such as peak asymmetry factor, tailing factor, resolution between Imp-A and Imp-D, resolution between Imp-D and DTZ, and % RSD of theoretical area obtained from two diluted standard solutions of DTZ (in triplicate), were evaluated.

2.5. Filter-compatibility studies

Laboratory mixture solution was subjected to filter-compatibility studies. The solution was filtered using Whatman filter paper no. 42 and 0.45 μm Nylon 66-membrane filter.

Table 1 Linearity parameters of the calibration curves for DTZ and its RS.

Compound	Linearity range (μg/mL)	R^2	Slope	Intercept	Standard error	*t-Stat*	*P-value*	F_R
DTZ	0.35–1.50	0.999	34.62	−0.80	0.39	−2.05	0.06	
Diltiazem sulphoxide	0.20–1.50	0.998	32.76	−0.33	0.32	−1.01	0.33	0.95
Diltiazem Impurity-F	0.12–7.50	0.998	40.78	−2.73	2.31	−1.35	0.20	1.17
Diltiazem Impurity-A	0.30–1.50	0.997	25.19	−0.62	0.32	−1.90	0.08	0.73
Diltiazem Impurity-E	0.30–1.50	0.998	60.15	−1.00	0.65	−1.53	0.15	1.74
Diltiazem Impurity-B	0.20–1.50	0.999	53.87	−0.67	0.49	−1.37	0.20	1.56
Diltiazem Impurity-D	0.27–1.50	0.998	33.44	−0.65	0.38	−1.74	0.10	0.97

Another laboratory mixture solution was centrifuged (unfiltered). Chromatography was performed on these three solutions, in triplicate, and difference between concentrations of each component in filtered and unfiltered sample solutions was calculated.

2.6. *Analytical method validation*

2.6.1. *Specificity*

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and degradation products. These studies were performed in two parts, Specificity part-A and Specificity part-B.

In Specificity part-A, separation and resolution were observed between DTZ standard solution, placebo solution, and its six impurities, namely Diltiazem sulphoxide, Imp-A, Imp-B, Imp-D, Imp-E, and Imp-F (known impurities). In Specificity part-B, sample was subjected to various stress conditions [29], viz. different levels of acidic hydrolysis, alkaline hydrolysis, neutral hydrolysis, and oxidation conditions at a concentration of 1 mg/mL. Samples were also subjected to thermal and photo-degradation in dry state. Chromatography was performed for stressed sample solutions and calculations were done using area normalization method. The mass balance studies were done for each type of stress study.

2.6.2. *Linearity*

Linearity test for the method was performed according to the guidelines laid by ICH. Appropriate aliquots of DTZ stock solution were spiked with appropriate volumes of stock solutions of known impurities (related substances) and diluted with the diluent to get solutions containing required concentrations. Linearity of DTZ and its RS was determined over a range of obtained limit of quantification (mentioned in Table 1) to 300% of specification limit (range was inclusive of concentrations at LOQ, 50, 80, 100, 120, 150, 200 and 300%).

Calibration curve was drawn by plotting the peak areas of DTZ and RS versus its corresponding concentration. The process was repeated for three consecutive days (twice each day) in the same concentration range. Values of coefficient of regression, slope and *Y*-intercept of the calibration curve were calculated. The relative response factors (F_R) of all RS were calculated and concentrations were adjusted accordingly.

2.6.3. *Precision*

Six solutions containing DTZ (250 μg/mL) were spiked with RS solutions 1.25 μg/mL (a 0.15% of DTZ concentration). Chromatography was performed and value of % RSD was calculated considering peak area for DTZ and each RS. Similarly, intermediate precision of the method was also evaluated by another analyst, on a different day in the same laboratory.

2.6.4. *Limit of detection (LOD) and limit of quantification (LOQ)*

The LOD and LOQ for DTZ and all RS were estimated by signal-to-noise ratio, 3:1 and 10:1, respectively, injecting a series of six diluted solutions with known concentrations.

2.6.5. *Accuracy*

Recovery studies were performed in triplicate at concentration levels of 50, 100, 200 and 300% of DTZ (250 μg/mL) to evaluate the accuracy of the proposed method. Solutions for the purpose were prepared by standard addition of DTZ stock solution to laboratory mixture solution.

2.6.6. *Stability of laboratory mixture solution*

The stability of DTZ stock solution (250 μg/mL) and laboratory mixture solution was evaluated at regular intervals for 24 h. The difference in areas of respective peaks in the obtained chromatograms was calculated.

2.6.7. *Robustness*

The method was performed with little variations like changing the pH (±0.2 unit) of mobile phase, changing the mobile phase flow rate (±0.2 mL/min), and increasing the temperature from normal (±5 °C). Chromatograms of six replicas of laboratory mixture solution were obtained and effect of each deliberate change was evaluated by applying system suitability parameters and calculating value of % RSD for each deliberate change.

3. Results and discussion

3.1. *Development of the stability-indicating chromatographic method*

Official HPLC method [8] to analyze DTZ and its RS (Imp-A) was not found to be stability-indicating. Following these methods to analyze stability-indicating samples of DTZ tablets

– produced by acid, alkali, hydrogen peroxide, heat and light treatment and spiked with laboratory mixture solution – did not yield satisfactory results. The methods were not able to produce sufficient resolution between degradation products with the RS. The chromatogram of sample containing degradation products generated by oxidative stress showed elution of Diltiazem sulphoxide (relative retention time 0.35) which did not meet the acceptance criteria for peak purity, assessed by a PDA detector. Additionally, the method applied to stability-indicating samples of alkaline degradation yielded a chromatogram displaying co-elution of degradation products at RRT 0.68 with Impurity-F.

Therefore, the reported method [8] was modified to get better peak shape and resolution amongst peaks of all degradation products, RS and DTZ.

Another official method [1] required longer saturation time of HPLC-system, probably due to the use of an ion-pair reagent in the mobile phase. Although, some other reported methods [18,25] were also considered, in that the degradation products were either not well resolved or co-eluted with DTZ related impurities.

The method which was thought to be developed was envisaged to be capable of eluting wide range of compounds of different polarities, with excellent efficiency and sufficient band spacing. During development of chromatography, elution was performed using C_{18} columns. Mobile phase consisting of ACN, MeOH and 50 mM potassium phosphate buffer (25:25:50) with pH 5.5 was used preliminary in isocratic elution. The chromatogram showed co-elution of Imp-A and Imp-D with DTZ. Further, increasing the proportion of ACN in the mobile phase resulted in rapid elution of Diltiazem sulphoxide. Replacement of the potassium phosphate component with *o*-PA yielded a mobile phase ACN: MeOH: *o*-PA (30:15:55) with pH 5.0 and flow rate 1.0 mL/min gave optimum resolution in separate peaks of DTZ and RS, although tailing was observed in few peaks. This tailing was gradually removed to some extent by addition of aqueous TEA (0.2%, v/v). However, proper resolution amongst the degradation products in stability samples was not obtained.

Therefore, a gradient mode of elution was tried for greater chance of success in the context. The gradient mobile phase consisted of two major components: Mobile Phase A containing aqueous TEA (0.2%, v/v) whose pH was adjusted to 5.0 with *o*-PA, and Mobile Phase B was ACN. The finally developed gradient method was consist of % change in mobile phase B with respect to time (0.01 → 34.99 min: 22% B; 35.00 → 44.99 min: 33% B; 45.00 → 60.00 min: 38% B). The mobile phase was mixed and eluted at 1.0 mL/min by the system and column temperature was maintained at 25 °C.

Optimum separation conditions were obtained with a BDS (Thermo Hypersil BDS, 150 mm × 4.6 mm i.d. with 5.0 µm particles) column, injection volume 10 µL, column oven temperature maintained at 25 °C, monitoring the elution by a UV detector at 240 nm.

3.2. *System suitability*

Chromatographic separation was performed with C_{18} column (Thermo Hypersil BDS, 150 mm × 4.6 mm i.d., 5.0 µm particles) with the above mentioned gradient mobile phase and a representative chromatogram is shown in Fig. 2, which display a tailing factor less than 1.5 for all the peaks, a resolution of 4.5 and 2.2 for Imp-A and Imp-D with respect to DTZ, respectively. The ratio of the peak areas of diluted DTZ standard solution and % RSD of six injections were 0.995 and 1.6, respectively.

Tailing factor, a parameter that ICH guidelines consider as a factor to be controlled, was within the established limits. The resolution factor between two consecutive peaks approximately represents twice the minimum request to be considered.

3.3. *Filter compatibility studies*

The results of filter compatibility studies performed and compared for unfiltered and filtered methods are tabulated in Table 2, and indicate that either 0.45 µm filter or Whatman filter can be used for regular analysis.

3.4. *Specificity*

The HPLC chromatograms recorded separately for DTZ alone and with its RS, blank and placebo preparations displayed a single, non-overlapped, peak for DTZ, as shown in Figs. 3, 4, 5 and 6, respectively. The resolution factor obtained between peak for DTZ and other peaks was more than 2.1 and the tailing factor of peak for DTZ and the RS was always in the range of 1.03–1.50. Thus, the HPLC method presented in this study is selective for DTZ and also for the other six related compounds, which might co-exist as impurities. HPLC results of specificity part-B (forced degradation studies) of DTZ, suggested the following degradation behavior; results are tabulated in Table 3.

3.4.1. *Degradation in acidic conditions*

DTZ was observed to be degraded to about 76% in acidic conditions, when treated with 0.1 M HCl for 1 h at 80 °C. Impurity-F was obtained as single degradation product

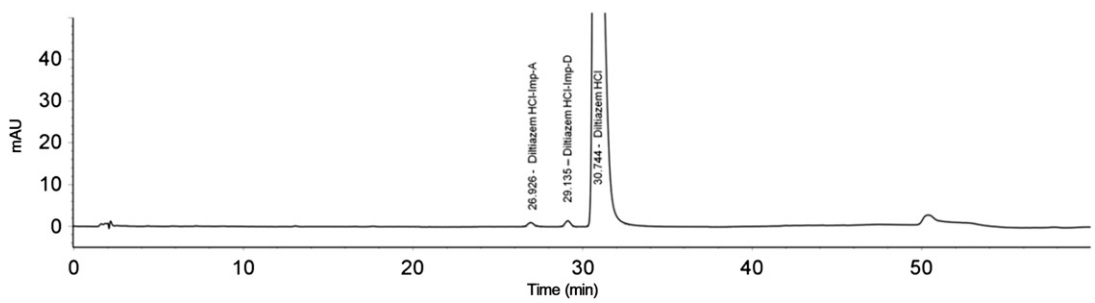


Figure 2 Chromatogram of resolution solution.

Table 2 Peak area difference of filtered sample solutions with unfiltered sample.

Compound	Difference with unfiltered sample (%)			
	Set-1		Set-2	
	0.45 μm filter	Whatman filter	0.45 μm filter	Whatman filter
Diltiazem sulphoxide	1.5	−1.7	−0.4	3.7
Dilitazem Impurity-F	−1.7	−2.7	−0.5	−0.1
Dilitazem Impurity-A	−0.8	−2.6	0.1	−0.2
Dilitazem Impurity-D	1.5	−0.4	−1.4	−3.7
Dilitazem Impurity-E	−1.8	4.2	−1.0	−0.2
Dilitazem Impurity-B	−1.6	6.6	−0.9	1.0
Total impurity	−1.0	−0.7	−0.8	0.0

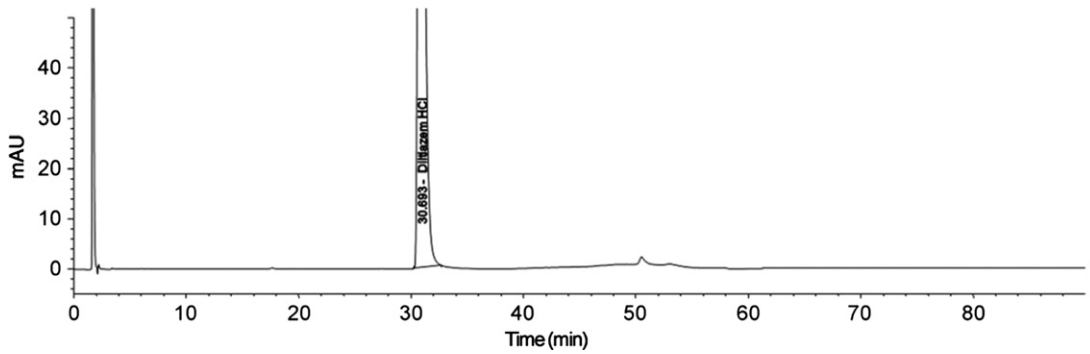


Figure 3 Chromatogram of DTZ standard solution.

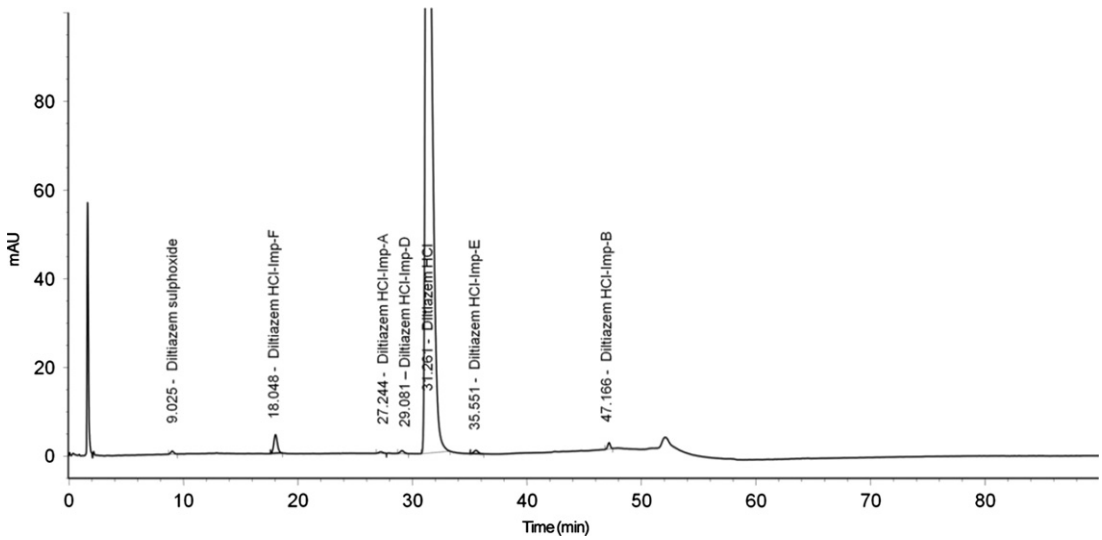


Figure 4 Chromatogram of DTZ and its RS.

(23.52%), eluted at 0.58 RRT as shown in Fig. 7A. DTZ was found to be stable (≈98.0% remaining) at reduced stress conditions (0.01 M HCl for 1 h at 80 °C) with Imp-F as the only degradation product (Fig. 7B).

3.4.2. Degradation in basic conditions

DTZ was found to be degraded to 72.7% under basic conditions, when treated with 0.1 M NaOH for 1 h at 80 °C. The chromatogram obtained on analyzing the stability sample (Fig. 8A) displayed more than seven peaks for degradation products; Impurity-F being obtained as major degradation product (22.06%), eluted at 0.58 RRT. DTZ was found stable (≈98.5% remaining) at reduced stress conditions (0.01 M NaOH for 1 h at 80 °C) with Imp-F as the only degradation product as shown in Fig. 8B.

Results of degradation studies suggest that long term storage of the drug leads to degradation, with fall in the content of DTZ and corresponding rise in Imp-F.

3.4.3. Degradation under oxidative conditions

The drug was reduced to 43% on peroxide degradation (3% H_2O_2 at 80 °C for 1 h) with Diltiazem sulfoxide as a major

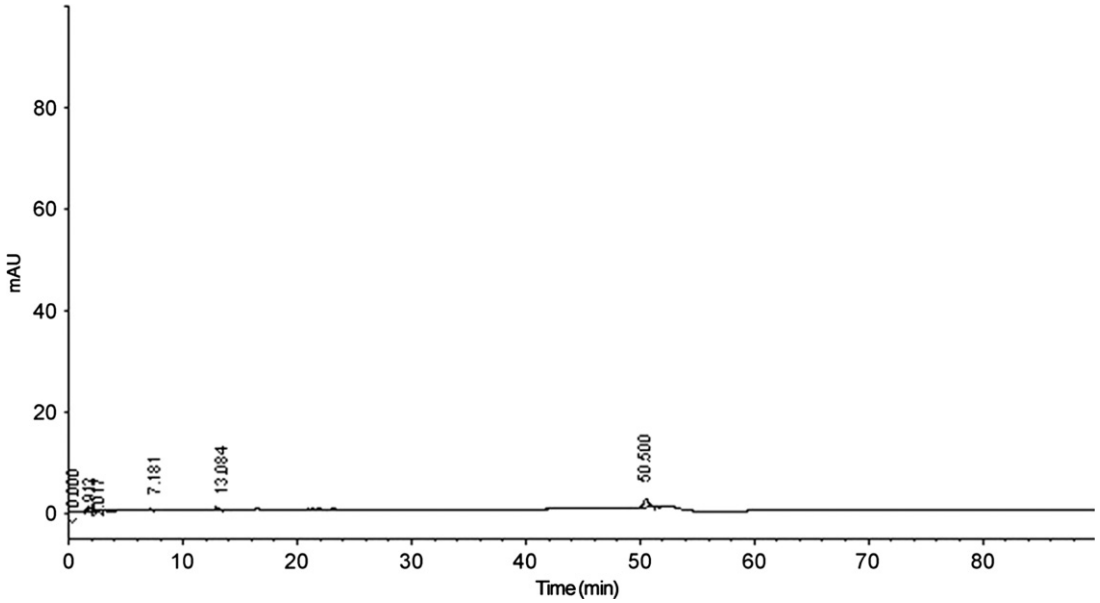


Figure 5 Chromatogram of diluent (blank) used in the study.

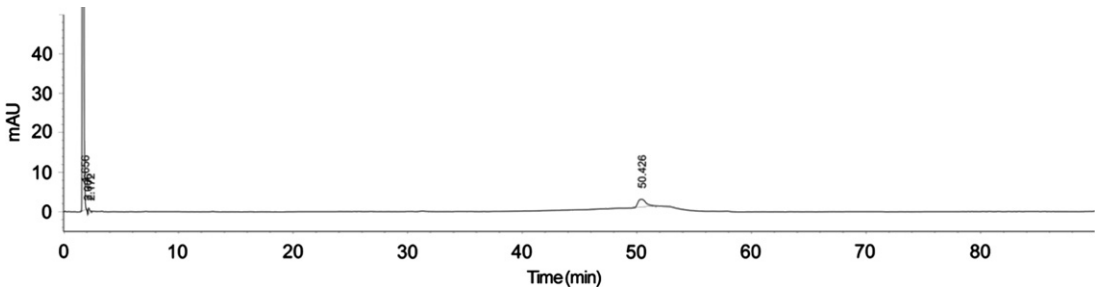


Figure 6 Chromatogram of placebo preparation used in tablet formulation.

Table 3 Specificity part-B (stress) studies representing degradation in various parameters.

Degradation stages	Condition	DTZ (remaining % by area normalization)	SKMI (% by area normalization/ RRT/name)	MUDP (by area %/RRT)	No. of degradation products	Mass balance (%)
As such		100.00	0	–	–	–
Acid degradation	a	76.19	23.52/0.58/Imp-F	–	1	99.71
	b	98.35	1.64/0.58/Imp-F	–	1	99.99
Base degradation	a	72.71	22.06/0.58/Imp-F	2.20/0.39	7	99.90
	b	98.43	1.57/0.58/Imp-F	No peak	1	99.90
Peroxide degradation	a	43.31	29.86/0.28/ Diltiazem sulphoxide	12.53/0.11	14	99.91
	b	98.02	0.58/0.29/Diltiazem sulphoxide	–	2	99.90
Neutral degradation		99.72	0.28/0.58/Imp-F	–	1	100.00
Thermal stress		100.00	–	–	–	100.00
UV light exposed		100.00	–	–	–	100.00

'**As such**'=no stress condition applied, **RRT**=relative retention time with respect to DTZ peak, **a**=high stress, **b**=moderate stress (as explained in text), **SKMI**=single known maximum impurity, **MUDP**=major unknown degradation product, '–'=no peak observed.

degradation product (29.86%) eluting at 0.28 RRT. The chromatogram obtained on analyzing the stability sample (Fig. 9A) displayed more than 14 degradation products, of which, a major unknown degradation product (12.53%) was observed at 0.11 RRT. DTZ was relatively stable (98.02% remaining) at milder oxidative degradation conditions (3% H_2O_2 at 80 °C for 10 min) with Imp-D and Imp-F as the degradation products (Fig. 9B).

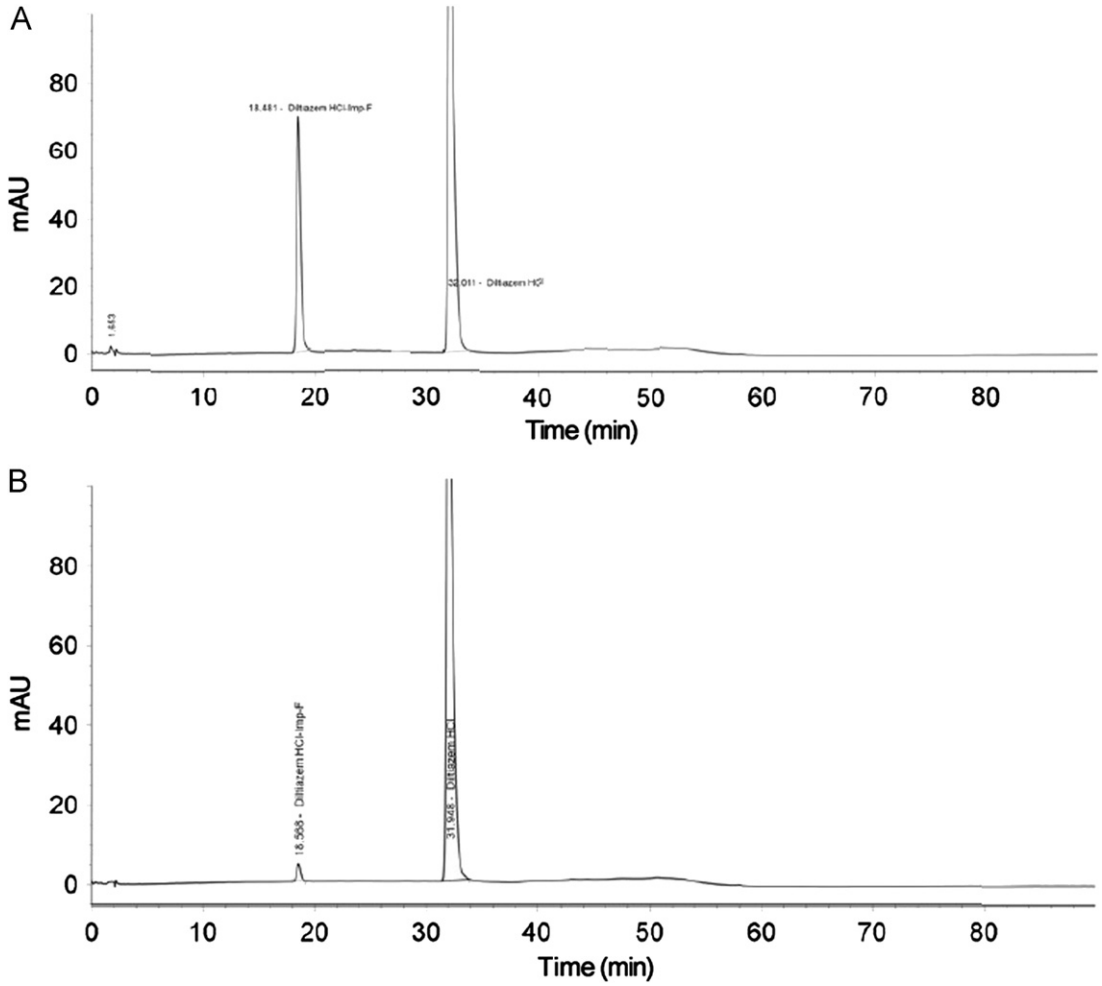


Figure 7 Chromatograms of acid stressed samples treated with 0.1 M HCl at 80 °C for 1 h (A) and 0.01 M HCl at 80 °C for 1 h (B).

3.4.4. Degradation in photolytic conditions

DTZ was found to be practically stable under the exposed conditions with an overall illumination of 1.2 million lx h with near-UV energy ≥ 200 Wh/m^2; the chromatogram is given in Fig. 10. This suggests that the drug was stable under photolytic conditions exposed for the period of study.

3.4.5. Thermal degradation

DTZ was found to be practically stable with dry heat as no degradation was observed when exposed to thermal heat at 80 °C for 8 h; the chromatogram is given in Fig. 11.

3.4.6. Degradation under neutral conditions

DTZ was found to have a negligible degradation of about 0.25% under neutral conditions (refluxed in water for 2 h at 80 °C) as only two degradation products were formed under the conditions studied; the chromatogram is given in Fig. 12.

3.5. Linearity

Calibration curves for DTZ and its RS, examined in pure solutions as well as in the laboratory mixture solutions, were found to be linear; correlation coefficients ≥ 0.997 in all the cases. Table 1 enlists the linearity parameters of the calibration curves for DTZ and its RS in laboratory mixture. UV-relative response factors (F_R) were calculated for each impurity using the following equation: $F_R = S_{impurity}/S_{DTZ}$.

Where, $S_{impurity}$ is slope of regression line for a given impurity and S_{DTZ} is the slope of the regression line for DTZ. Concentrations of DTZ and impurity were corrected. Statistical treatment of the linearity data of DTZ shows a linear response between lower levels to highest level. In addition, the analysis of residuals shows values randomly scattered around zero, which fits well within the linear model. The origin of linearity curve was within the lower and the upper limit of 95% that gives high degree of confidence to the value obtained for intercept.

3.6. LOD and LOQ

LOD and LOQ, as a measure of method sensitivity, were provided for degradation products and impurity calculated by means of signal-to-noise ratio. The LOD and LOQ for DTZ and its RS are tabulated in Table 4. From the results, it can be concluded that the proposed method can quantify small quantity of impurities in DTZ samples.

3.7. Precision and repeatability

The results obtained for repeatability studies and for intermediate precision are presented in Table 5. Values of % RSD

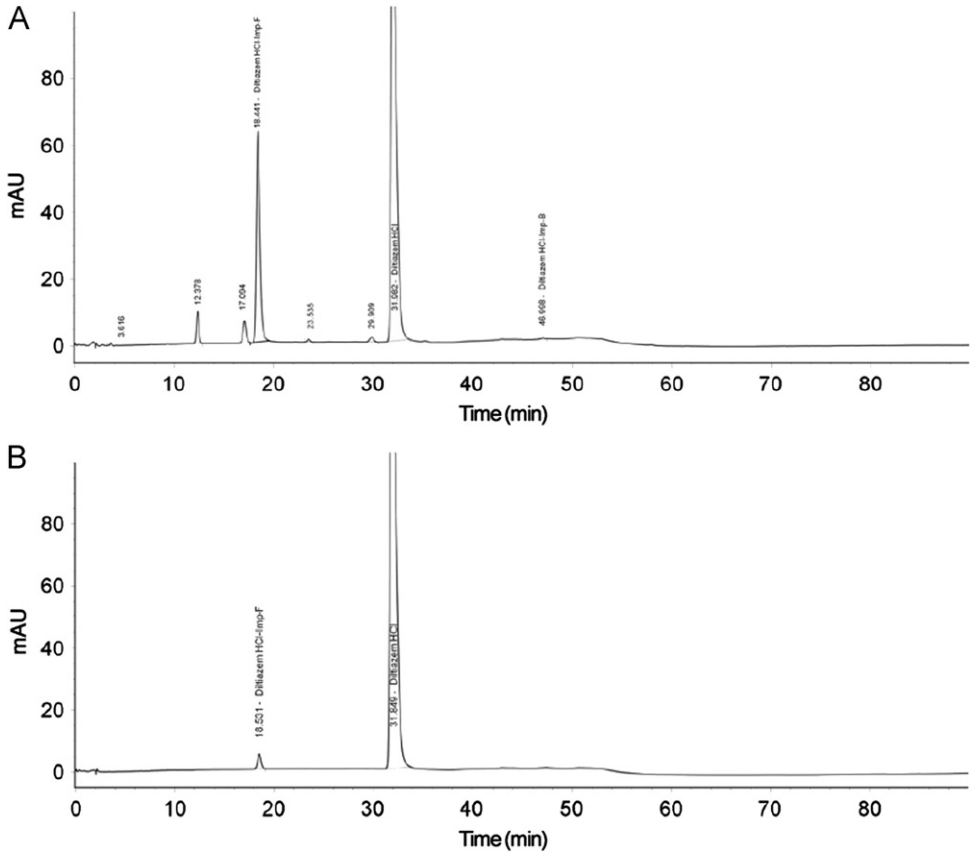


Figure 8 Chromatograms of alkali stressed samples treated with 0.1 M NaOH at 80 °C for 1 h (A) and 0.01 M NaOH at 80 °C for 1 h (B).

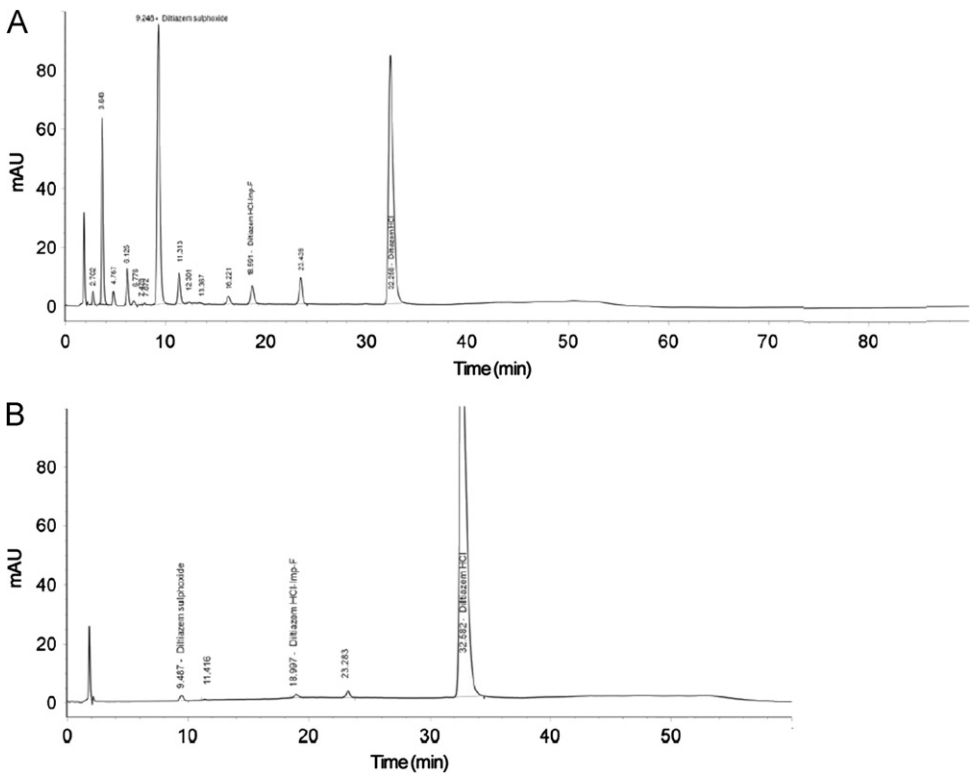


Figure 9 Chromatograms of peroxide stressed samples treated with 3% H_2O_2 refluxed at 80 °C for 1 h (A) and 3% H_2O_2 refluxed at 80 °C for 10 min (B).

for system precision of DTZ and total impurities were 0.3 and 0.8, respectively. Method precision has a % RSD below 1.9 for repeatability and 1.4 for intermediate precision, which comply with the acceptance criteria.

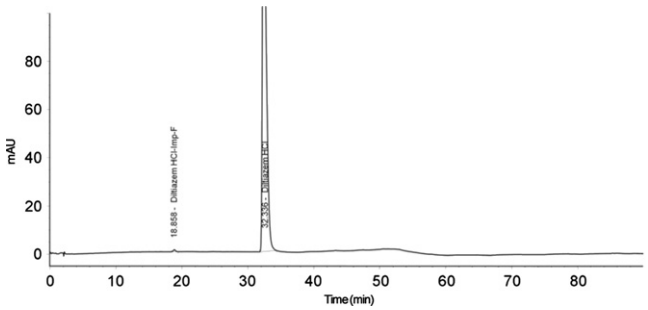


Figure 10 Chromatogram of photo stressed sample.

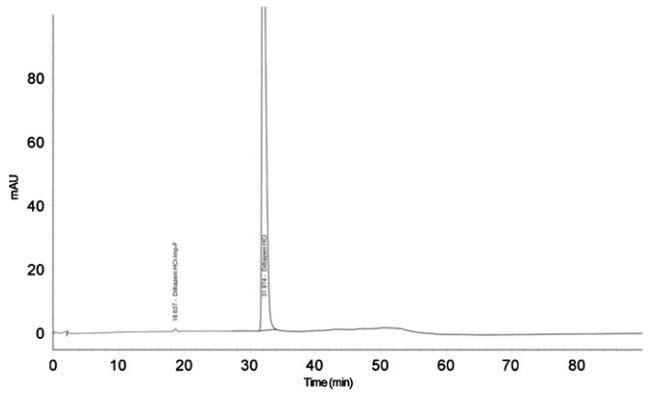


Figure 11 Chromatogram of thermal stressed sample.

3.8. *Accuracy*

The results are expressed as percent recoveries of the particular components in the samples. Table 6 shows that the overall percent recoveries of DTZ and its six RS at 50, 100, 200 and 300% of the test concentration. The method has shown good, consistent recoveries for DTZ (99.8–101.2%). However, the related compounds showed overall percent recoveries ranging from 97.9 to 102.8 with % RSD ranging from 1.2 to 3.1.

3.9. *Stability in analytical solution*

The % area change in peaks of DTZ and all impurities was less than 2.0% and 5.0%, respectively. From the data tabulated in Table 7, it was concluded that standard and sample solutions may be used up to 24 after preparation.

3.10. *Robustness*

Method robustness checked after deliberate alterations of mobile phase composition, flow, pH and temperature shows that the changes of the operational parameters do not lead to essential changes of the performance of the chromatographic system; results are displayed in Table 8. Tailing factor for DTZ and its RS always ranged from 1 to 1.5 and the components were well separated. The percent recoveries of DTZ and RS were good and did not show a significant change when the critical parameters were modified. Considering the results of modifications in the system suitability parameters and the specificity of the method, it would be concluded that the method conditions are robust.

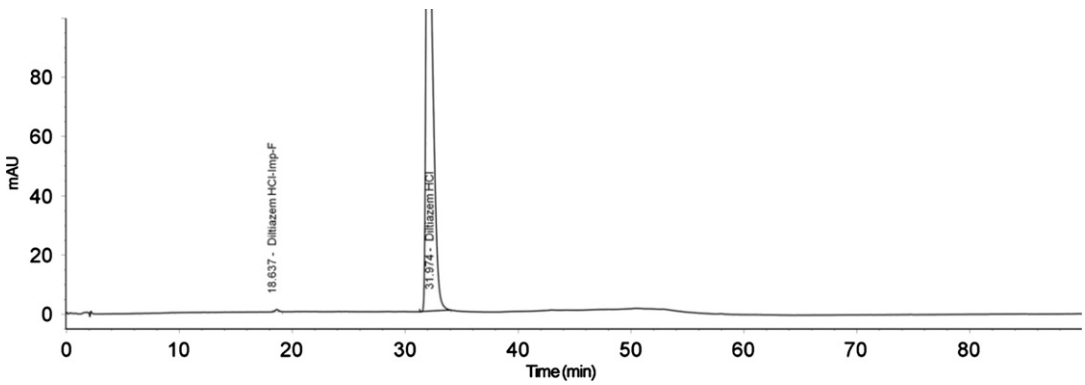


Figure 12 Chromatogram of neutral stressed sample.

Table 4 LOD and LOQ results for DTZ and its RS.

Compound	LOD		LOQ	
	Concentration (μg/mL)	% RSD of injection ($n=6$)	Concentration (μg/mL)	% RSD of injection ($n=6$)
DTZ	0.12	11.40	0.35	6.90
Diltiazem sulphoxide	0.07	9.80	0.20	4.30
Diltiazem Impurity-F	0.05	19.30	0.12	4.00
Diltiazem Impurity-A	0.10	14.20	0.30	3.30
Diltiazem Impurity-E	0.10	12.60	0.30	6.10
Diltiazem Impurity-B	0.07	10.90	0.20	5.30
Diltiazem Impurity-D	0.10	16.80	0.27	7.70

Table 5 Intra-day and intermediate precision of DTZ and its RS (% RSD of $n=6$ injections of test concentration).

Compound	Intra-day precision		Intermediate precision
	System precision	Method precision	Different day
DTZ	0.3	1.0	0.9
Diltiazem sulphoxide	0.8	1.7	0.7
Impurity-F	0.9	1.2	1.0
Impurity-A	0.4	1.9	1.7
Impurity-D	0.7	1.8	1.9
Impurity-E	0.8	1.5	0.8
Impurity-B	1.0	1.8	1.1
Total impurity	0.8	1.9	1.4

Table 6 Accuracy results of DTZ and its RS in the term of RSD (%) of mean recovery (%).

Added (%)	DTZ		Diltiazem sulphoxide		Impurity-F		Impurity-A		Impurity-D		Impurity-E		Impurity-B	
	MR (%)	RSD (%)	MR (%)	RSD (%)	MR (%)	RSD (%)	MR (%)	RSD (%)	MR (%)	RSD (%)	MR (%)	RSD (%)	MR (%)	RSD (%)
50	99.8	1.9	98.2	1.5	99.6	2.2	97.5	3.1	100.8	1.2	102.1	2.8	100.9	2.1
100	101.2	2.0	99.5	2.1	102.8	2.4	100.2	1.9	99.6	1.8	100.8	1.2	97.9	1.6
200	100.9	1.4	100.5	2.4	98.4	1.5	100.9	2.1	100.2	1.4	98.5	1.9	102.5	2.2
300	101.7	1.9	98.9	1.6	99.1	2.1	99.0	1.3	101.6	1.8	99.7	2.0	100.1	1.1

MR: mean recovery, $n=3$.

Table 7 Stability of DTZ and its RS in analytical solution (one day study).

Compound	Initial area	12 h		18 h		24 h	
		Area	Difference (%)	Area	Difference (%)	Area	Difference (%)
DTZ	36.15969	37.01846	2.4	37.59764	4.0	36.86213	1.9
Diltiazem sulphoxide	13.71313	14.14156	3.1	13.67648	−0.3	13.71130	0.0
Impurity-F	89.97086	89.69975	−0.3	91.34973	1.5	92.72081	3.1
Impurity-A	9.40208	9.34010	−0.7	9.03424	−3.9	9.74495	3.6
Impurity-E	24.32384	24.34320	0.1	24.27540	−0.2	24.36614	0.2
Impurity-B	21.66869	21.68923	0.1	20.83593	−3.8	21.72877	0.3
Impurity-D	13.51299	13.41356	−0.7	13.20469	−2.3	14.12792	4.6
Total impurity	172.59161	172.62739	0.0	172.37648	−0.1	176.39990	2.2

Table 8 Effect of various deliberated changes on the system suitability parameters.

System suitability conditions		Resolution between Diltiazem Imp-A and Imp-D	Resolution between Imp-D and DTZ	Ratio between the duplicate injection	% RSD for DTZ standard replicate injections
Initial	1.0 mL	3.5	2.2	0.97	1.06
Change in flow	1.2 mL	3.1	2.3	0.99	1.54
	0.8 mL	2.7	2.4	1.01	1.06
Change in column temperature	+5 °C	2.7	2.4	1.00	0.62
	−5 °C	3.0	2.2	1.02	1.78
Change in pH units	−0.2	2.5	2.8	0.99	1.63
	+0.2	1.5	4.8	1.04	1.61

4. Conclusion

The proposed HPLC method for estimation of related substances for Diltiazem Hydrochloride, is analyzed in bulk drug and DTZ SR tablets as per ICH guidelines. The method is found to be specific for the estimation of known, unknown impurities and degradation products. The method is also stability indicating as evident from results obtained when method applied to stability samples. The assay utilized a previously unreported set of conditions, including a gradient ramp and simple mobile phases, to effect the separation without using an ion-pair reagent. LOD and LOQ, established by this method, are lesser than earlier reported methods. The method is found to be linear in the specified range, precise and robust. Accuracy of the method is also established for the formulation. Hence, the proposed method stands validated and may be used for routine and stability sample analysis.

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