Development and validation of a stability-indicating RP-HPLC-FLD method for determination of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol; A novel drug candidate

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Abstract: The present study describes the development and validation of a simple high performance liquid chromatographic method for the determination of a novel drug candidate, 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol. The stability-indicating capacity of the method was evaluated by subjecting the compound's solution to hydrolytic, oxidative, photolytic, transition metal- and thermal- stress. The chromatographic separation was achieved over a C_{18} column (Promosil, 5 µm, 4.60 × 250 mm), maintained at 25°C, using an isocratic mobile phase comprising a mixture of acetonitrile and acidified water of pH 2.67 (1:1, v/v), at a flow rate of 1.00 mL/min and detection using a fluorescent light detector (excitation at 250 nm and emission at 410 nm). The Beer's law was followed over the concentration range 2.50-50.00 µg/mL. The recovery (98.56-100.19%, SD <5%), intraday accuracy and precision (97.31-100.81%, RSD <5%), inter-day accuracy and precision (97.50-100.75%, RSD <5%) and intermediate accuracy and precision (98.10-99.91%, RSD <5%) indicated that the method was reliable, repeatable, reproducible and rugged. The resolution and selectivity factors of the compound's peak from the nearest resolving peak, particularly in case of dry heat and copper metal stress, were found to be greater than 2 and 1, respectively, which indicated specificity and selectivity. The compound was extensively decomposed in alkaline-hydrolytic, oxidative, metal- and dry heat- stress. However, the compound in acidic and neutral conditions was resistant to photolysis. The results of the present study indicate that the developed method is specific, selective, sensitive and suitable, hence, may be used for quality control, stability testing and preformulation studies.

Keywords: Stability-indicating method, RP-HPLC-FLD, 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol, quality control analysis, Forced degradation studies

INTRODUCTION

5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM) is a 2, 5-disubstituted oxadiazole (fig. 1). The synthesis and use of the compound as a precursor of a number of less cytotoxic anti-microbial and anti-enzymatic derivatives was reported by Siddiqui *et al.* (2014). In 2016, Shehzadi *et al.* reported the drug-likeliness of the compound using a variety of computational tools. This study revealed that the compound could be an ideal candidate for the drug development owing to its excellent bioactivity as ion-channel modulator and enzyme inhibitor, pharmacokinetics (bioavailability >90% and renal clearance, -0.088 log mL/min/kg) and toxicity profile. Despite tremendous pharmacological potential of the compound, no analytical method was reported for its quality control analysis in the vast literature reviewed. As in the drug-development programs, time and expense are attributable cause of the delayed FDA approval, hence, simple and selective analytical methods are needed for routine use. The present study aimed at development and validation of a stability-indicating chromatographic method for the determination of OXCPM.

Stability-indicating methods are the validated protocols that can detect the changes in the physicochemical properties of a drug; hence, can accurately quantify and distinguish the analyte in the presence of its degradation products, process impurities and excipients. International Conference on Harmonization (ICH) guidelines strongly recommend undertaking of forced degradation studies on the new drugs, active ingredient and formulations to establish suitability and applicability of the proposed analytical methods in stability testing and routine quality control analysis (Bakshi and Singh, 2002; ICH, 2003;

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Kovaříková *et al*., 2004). Moreover, the data generated by such studies provide a significant information on the inherent stability characteristics of a molecule which may guide the identification of possible degradation pathways/mechanisms and potential degradation products, particularly when no/little information is available regarding such behavior, for example in case of a new drug molecule.

MATERIALS AND METHODS

Chemicals, reagents and other supplies

Analytical grade chemicals/reagents included sodium hydroxide (NaOH), copper (III) sulphate pentahydrate, iron (II) sulphate heptahydrate, magnesium sulphate heptahydrate, hydrogen peroxide (H_2O_2, 35%), hydrochloric acid (HCl, 37%) and sodium dihydrogen phosphate (E. Merck/Merck Germany), trifluoroacetic acid (Scharlab, Spain) and ortho phosphoric acid (BDH Chemicals, England). HPLC grade methanol (Duksan Pure Chemicals, Korea) and, acetonitrile and tetrahydrofuran (Tedia Company, Fairfield, USA) were purchased from M/S Qureshi Sons, Lahore, Pakistan. Polytetrafluoroethylene membrane syringe filters (0.45 µm, Minisart SRP 15, Sartorius Stedim Biotech, Germany) and nylon-H filters (0.45 µm, Micropore) were procured from the local market. OXCPM was gifted by Dr. Sabahat Zahra Siddiqui, Govt. College University, Lahore, Pakistan. The in-house prepared ultrapure water filtered through 0.45 µm nylon membrane was used throughout the study.

Instruments

The analysis was performed using HPLC system; 1200 series, Agilent Technologies, Waldron, Germany, having isocratic pump (G1310 A), auto-sampler (G1329 A), column thermostat (G1316 A), fluorescent light detector (G 1321 A) and ChemStation LC/LCMS for Windows, Rev. B.01.03 [204]. Other instruments included UV lamp (UVGL-58, USA), ultrasonicator (DSA50-SK1-1.8L, Germany), oven (U10 Memmert, Germany), pH Meter (Hanna Instruments, Romania) and Curio stability study chamber (SC-0709), incubator and Ultra-low chiller (MOF-U32V) of Sanyo Electric Corporation, Japan.

Development of the method

Mobile phase

Water, acidified to pH 2.67 with 10% phosphoric acid, was mixed in equal volume of acetonitrile, filtered and degassed.

Standard solutions

The standard stock solution (1.00 mg/mL) was prepared in methanol, which was then diluted appropriately with the mobile phase to prepare a range (2.50-50.00 µg/mL) of working standard solutions.

Chromatography conditions

Aliquots (20 µL) of the working standard solutions were eluted through a C_{18} column (Promosil, 5 µm, 4.60 × 250 mm), maintained at 25°C, with an isocratic mobile phase comprising a mixture of acetonitrile and acidified water (1:1, v/v), at a flow rate 1.00 mL/min and detection using FLD operated at 250 nm excitation and 410 nm emission.

System suitability

The system suitability parameters such as number of theoretical plates (N), height equivalent to theoretical plate (HETP), reduced plate height, peak repeatability, capacity factor ($k^{/}$) and tailing factor were calculated from the chromatogram to ensure the accuracy of the system during the analyses.

Method validation

The method validation was carried out as per standard guidelines (CDER, 1994; ICH, 2005) and is briefed as follows:

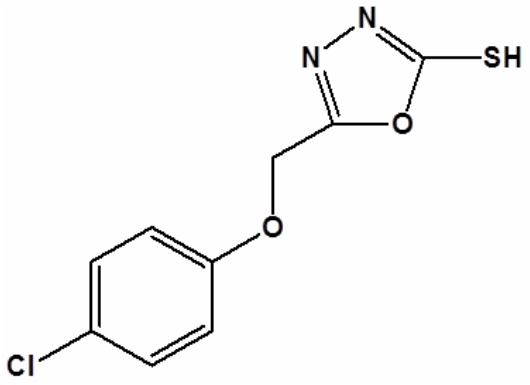

Fig. 1: Chemical structure of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM)

Linearity and Beer's range

The working standard solutions (2.50-50.00 µg/mL) were analyzed in triplicates, independent repetitions and not the repetitions at the same readings, using the chromatographic conditions mentioned above. The linearity was evaluated by visual inspection of the plot of concentration versus peak area (mAU*s) and validated from the linear regression equation (I) and Pearson's correlation coefficient (R^2, 0.990-1.000). The Beer's range was derived from the linearity studies.

$$Y=a + bX \quad \textbf{(I)}$$

Where X, Y, a and b represent explanatory variable (concentration), dependent variable (peak area), intercept and slope, respectively.

Sensitivity: Limit of detection (LOD) and limit of quantification (LOQ)

The sensitivity parameters, LOD at a signal to noise (S/N) ratio 3:1 and LOQ at S/N ratio 10:1, were determined statistically from standard deviation (SD) of the intercepts and mean of slope (S) of the standard curves (n=5).

Recovery

The recovery of the method was estimated at lower, middle and high concentration levels (2.50, 25.00 and 50.00 µg/mL), taken as unknown and quantifying them from the calibration curve. The amounts thus calculated were compared to the respective true values to determine percent recovery.

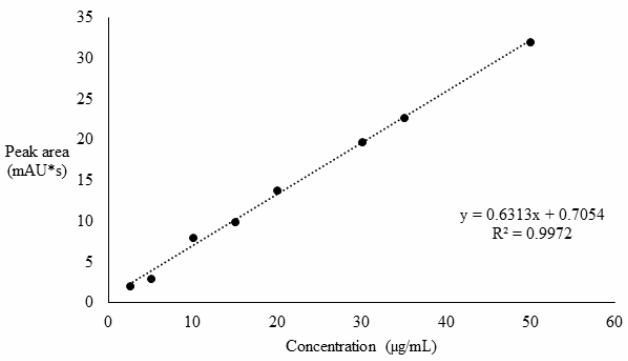

Fig. 2: Calibration curve of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM) at excitation/emission wavelength 250/410 nm

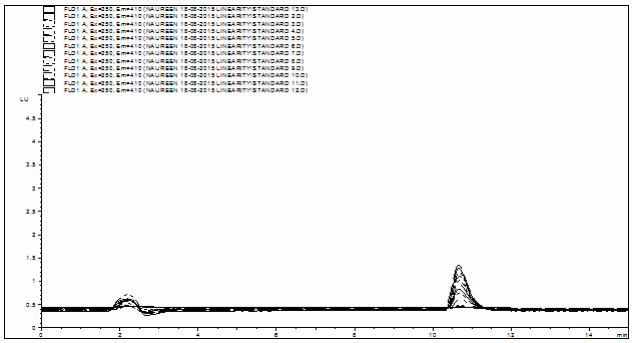

Fig. 3: Overlay of the chromatogram of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM) analyzed in concentration ranges (2.50-50.00 µg/mL)

Accuracy and precision

Accuracy and precision (repeatability, reproducibility and ruggedness) of the method was determined at the same concentration levels as taken in recovery studies. Repeatability and reproducibility (intra- and inter- day accuracy and precision) were assessed by analyzing the solutions 6 times in a single day and once daily for six consecutive days, respectively. Ruggedness (intermediate accuracy and precision) was determined by performing the analyses on the same instrument by two different analysts. Percent recovery and relative standard deviation (RSD) were taken as the accuracy and precision measures, respectively.

Specificity and selectivity

Specificity of the method was established by determining resolution and selectivity factor of the compound's peak from the nearest resolving peak, if any.

Robustness

Robustness of the method was determined by deliberately changing the sample preparation, column temperature, detection wavelengths, mobile phase composition and pH, and observing their effect on the percent recovery of the compound.

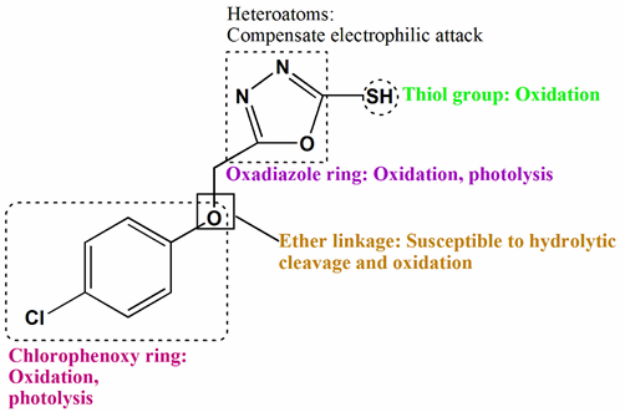

Fig. 4: Functional groups/hetero rings in 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM) susceptible to hydrolysis, oxidation and photodecomposition

Sample-solution stability

The compound's solution in screw-capped test tubes, protected from light, was kept at room temperature, refrigerator and freezer for 48 h and the recovery was compared with the freshly prepared sample.

Stability-indicating capacity (forced degradation studies)

The stability-indicating capacity of the method was established through forced degradation studies. The experimental conditions/protocol were adopted from standard guidelines (ICH, 2003). Briefly, the working standard solution at a concentration 200.00 µg/mL was subjected to hydrolytic, oxidative, photolytic, transition metal- and thermal- stress. For each degradation condition, four samples were generated as, compound's solution (real time/fresh and stressed) and blank (real time/fresh and stressed). The aliquots, withdrawn after a suitable time period, were neutralized (if needed), filtered and analyzed. The decrease in concentration of the compound in the stressed solution was considered a direct measure of the extent of degradation. A brief procedure of the stress is given as follows:

pH stress

For acidic, alkaline and neutral pH stress, equal volumes the working standard solution were separately mixed with 0.10, 1.00 and 5.00 N HCl, 0.10, 1.00 and 5.00 N NaOH and DDW, respectively. Two such sets were prepared and heated at 37±5°C and 121±5°C for 24 h and 1 h, respectively. The resulting mixtures were cooled to room temperature and neutralized before analysis.

Table 1: System suitability parameters calculated from the chromatogram of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM)

Parameters	Values
Retention time	11.040±0.326 min
Capacity factor (k')	4.29
Peak repeatability response	0.766
Tailing factor	1.83
Number of theoretical plate (N)	10655.15
Height equivalent to theoretical plate (HETP)	23.46 µm
Reduced plate height	4.69 µm

Table 2: Result of calibration, limit of detection (LOD) and limit of quantification (LOQ) of the RP-HPLC-FLD method for the determination of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM)

Standard curve	Concentration (µg/mL)	Slope	Intercept
1	2.50-50.00	0.6147	0.7566
2	2.50-50.00	0.7515	1.0202
3	2.50-50.00	0.6316	0.7054
4	2.50-50.00	0.6813	0.5311
5	2.50-50.00	0.6148	0.7541
Mean (n=5)		0.6588	0.7535
Standard deviation (SD)		0.059	0.175
LOD (3.3 * SD/S)			0.878 µg/mL
LOQ (10 * SD/S)			2.661 µg/mL

Table 3: Recovery, intra- and inter-day and intermediate accuracy and precision of the RP-HPLC-FLD method for the determination of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM)

Concentration (µg/mL)	% Recovery±SD (n=3)	Intraday accuracy; %RSD (n=6)	Inter-day accuracy; %RSD (n=6)	Intermediate accuracy; %RSD (n=6)
2.50	100.00±0.118	100.81; 2.111	97.50; 4.739	99.91; 1.584
25.00	98.56±0.385	97.31; 1.440	97.60; 2.969	98.10; 1.053
50.00	100.19±0.685	99.87; 1.162	100.75; 3.439	99.34; 1.102

Oxidative stress

Oxidative stress was induced by treating the compound's solution separately with different strengths of H_2O_2 (1, 3, 10 and 20%) for 28 h at room temperature.

Photolytic stress

The photolytic stress was applied to acidic (0.10 N HCl), alkaline (0.10 N NaOH) and neutral (DDW) solutions of the compound by storing them separately in UV (254 nm) and visible light (sunlight; 60,000 to 70,000 lux and tungsten lamp; 100 Watt for 12 h alternatively) for 48 h. The control consisting of the compound's solution was stored in dark for the same time period.

Transition metal stress

For transition metal stress, 4 mL of compound's solution was separately treated with equal volume of 1.50 mM copper, iron and magnesium solutions for 28 h at room temperature.

Thermal stress

The working standard solutions were subjected to dry- and moist- heat stress in a hot air oven (150±10°C) and in a stability chamber [60±5°C and relative humidity (RH), 90%)] for 48 h, respectively. The solutions placed at 2-8°C (refrigerator), -52±5°C (chiller) and 25±5°C (room temperature) for 48 h served as controls.

RESULTS

System suitability

The system suitability parameters, calculated from the eluted peak of the compound (50.00 µg/mL) are shown in table 1. These results verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis of the compound (CDER, 1994).

Method validation

Linearity and Beer's range

The plot of concentrations (2.50-50.00 µg/mL) versus peak area (mAU*s) is shown in fig. 2. A high value of correlation coefficient (0.9972±0.019) with a slope of 0.6313 (±0.81) and y-intercept approaching to zero (0.7054±0.16) indicated that the method was linear in the studied concentration range.

Table 4: Percentage of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM) recovered after exposure to different stress conditions

Stress condition	Stressor	% Recovery±SD (n=3)
Hydrolytic stress		
Acidic solution	5.00 N HCl, 37±5°C	98.12±0.132
Alkaline solution	5.00 N NaOH, 37±5°C	-
Neutral solution	DDW, 37±5°C	99.98±0.199
Acidic solution	5.00 N HCl, 121±5°C	-
Alkaline solution	5.00 N NaOH, 121±5°C	-
Neutral solution	DDW, 121±5°C	-
Oxidative stress	20% H_2O_2	-
Photolytic stress		
UV light		
Acidic solution	0.10 N HCl	100.05±0.431
Alkaline solution	0.10 N NaOH	-
Neutral solution	DDW	95.19±0.321
Visible light		
Acidic solution	0.10 N HCl	85.87±0.342
Alkaline solution	0.10 N NaOH	-
Neutral solution	DDW	100.11±0.221
Dark (control)	DDW	100.06±0.111
Transition metal stress		
Copper	1.50 mM copper	30.28±0.118
Iron	1.50 mM iron	-
Magnesium	1.50 mM magnesium	-
Thermal stress		
Dry heat	150±10°C	23.52±0.776
Moist heat	60±5°C and 90% RH	88.29±0.098
Room temperature (control)	-	101.55±0.119
Chiller (control)	-	100.11±0.276
Refrigeration (control)	-	100.11±0.341

** Data are presented as mean (n=3) ± standard deviation SD (<5%). Decrease in the peak area was taken as a direct measure of extent of degradation, - indicates no peak was observed, HCl: hydrochloric acid; NaOH: sodium hydroxide; DDW: double distilled water; H_2O_2: hydrogen peroxide; RH: relative humidity*

Sensitivity: Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were found to be 0.878 and 2.661 µg/mL, respectively, which indicated that the method was quite sensitive (table 2).

Recovery

The recovery at lower, middle and higher concentration was found to be ranging from 98.56 to 100.19% with SD <5%. These results indicated that the method was reliable.

Accuracy and precision

Intraday, inter-day, and intermediate accuracy was found to be 97.31-100.81%, 97.50-100.75% and 98.10-99.91%, respectively. In all such values, RSD was less than 5%, which indicated that the data were precise (table 3).

Specificity and selectivity

The resolution and selectivity factor of the compound's peak from the nearest resolving peak, in case of dry heat- and copper metal- stress, were greater than 2 and 1, respectively which indicated that the method was specific and selective.

Robustness

The method was found to be robust since the slight changes in sample solvent composition (methanol or mobile phase), pH of the mobile phase (±0.20), detection wavelength (±5 nm) and column temperature (±2°C) did not affect the recovery and chromatographic resolution.

Sample-solution stability

The solutions stored in the laboratory at room temperature, in refrigerator and in freezer were found to be stable for 48 h and % of the compound recovered was comparable to the freshly prepared solution.

Stability-indicating capacity (Forced degradation studies)

For evaluating the stability-indicating capacity of the developed method, stressed samples were run individually using the optimized chromatographic conditions. The

percentage of the analyte recovered from the stressed solution is shown in table 4. These results clearly indicated that the developed method could estimate unequivocally the compound in the presence of degradants/degradation products, hence was stability-indicating.

DISCUSSION

For the development of the analytical method, three types of columns, used in the study included Promosil C_{18} (5 µm, 4.60 × 250 mm), Eclipse X DB-C_{18} (5 µm, 4.60 × 150 mm) and Zorbax stablebond analytical SB-C_{18} (5 µm, 4.60 × 150 mm). The temperature of the column was changed by ±5°C to optimize the response of the compound. The best results were provided by the former column maintained at 25°C.

For the selection of the excitation and emission wavelengths, no data on the fluorescent behavior of the compound were available. Hence, the experiments were run by operating the detector at various excitation/emission wavelengths; 250/365, 258/365, 365/410 and 250/410 nm. The response of the detector was found to be good at 250/410 nm and all the peaks obtained were symmetrical.

For optimum resolution, selectivity and retention time, different mobile phases were used, which included methanol (100%), acetonitrile (100%), water (100%), acetonitrile: sodium hydrogen phosphate (1:4), acetonitrile: sodium dihydrogen phosphate (1:1), acetonitrile: water: trifluoroacetic acid (50:48:2), acetonitrile: water: phosphoric acid (50:48:2), acetonitrile: methanol: phosphoric acid (50:48:2), acetonitrile: water: phosphoric acid: tetrahydrofuran (50:42:2:6) and acetonitrile: methanol: water: phosphoric acid (50:38:10:2). However, the compound was detected using water (100%) and acetonitrile: methanol: phosphoric acid (50:48:2) with retention time at 2.373 and 3.631 min, respectively. This early elution, near the dead time (t_M) was not suitable. Finally, the optimum separation was achieved with mobile phase consisting acetonitrile: acidified water (1:1, v/v) at 11.040±0.326 min. The overlay of chromatograms of the compound obtained using the optimized conditions is shown in fig. 3, which shows that the peaks are Gaussian and have the same retention time.

The developed method complied with the acceptance criteria of the standard guidelines and protocols. The validation and system suitability parameters were within the range, hence the method was evaluated for the stability-indicating capacity. Milestone in the establishment of stability-indicating methods is the critical study of compound's structure to assess the likely decomposition routes/products. Our extensive literature survey did not reveal any data regarding the decomposition route/behavior and degradation products of the compound under investigation. Since, most of the drugs are congeners of pre-existing drugs, so attempt was made to investigate the degradation behavior of the compound on the basis of already reported data of the drugs/leads having similar functional groups/hetero rings. The compound contains thiol, hetero oxygen, ether, diazo and aryl halide linkages/groups that are susceptible to oxidative, hydrolytic, thermal and photo degradation (Bakshi and Singh, 2002). Various functional groups in the compound that are susceptible to degradation are highlighted in fig. 4.

In the forced degradation studies, a complete degradation of the compound was observed in all pH solutions upon heating at 121±5°C for 1 h. The degradation pattern indicated by appearance of additional peaks was similar in all acidic stress solutions whereby only one fluorescent degradation peak was observed at 7.557±0.375 min. The peak area and peak height of the degradation products decreased with increase in the strength of acid and did not result in formation of other peaks except for 5.00 N HCl where a new peak was also observed at 9.318±0.100 min. In alkaline conditions, the products being highly polar eluted through the column in 7.000±0.135 min. At neutral pH, the compound upon degradation gave rise to one peak at 6.519±0.098 min. No decomposition was indicated in the acidic (0.10-5.00 N HCl) and neutral (DDW) solutions of the compound stored at ambient temperature (37±5°C) except for 0.10 N NaOH - where degradation was 50.41±0.165%, and a similar degradation pattern as indicated at high temperatures, was observed.

The compound was found to be completely oxidized by H_2O_2 and resulted in formation of one degradation peak at 7.482±0.004 min. In photolytic stress, the acidic and neutral solutions remained stable, however, the former was decomposed to 14.13±0.342% without any additional peak formation in case of visible light. The alkaline solutions experienced the same decomposition behavior as in pH stress which indicate that the pH may the attributable cause of degradation and not the light. The stability profile of the compound in UV and visible light was found to be similar in FLD which indicated that the type or intensity of radiation did not affect the chromatographic behavior of the compound. Except for the copper, the compound underwent complete degradation in transition metal solutions and produced one degradation peak at 8.670±0.045 min. Under thermal stress in dry heat, the compound was degraded to 76.48±0.776% and resulted in formation of one fluorescent peak at 5.260±0.188 min, however, no degradation was observed in moist heat.

The nitrogen, oxygen (heteroatom and exocyclic) and sulphur atoms of the compound owing to their electron-

rich nature are susceptible to degradation due to the pH conditions. Our extensive literature survey indicated the degradation of oxadiazole ring in acidic, basic and neutral conditions due to electrophilic attack at the nitrogen (N_3), exocyclic heteroatom (sulphur) and N_3 or N_4, respectively (Kumar and Khokara, 2005). The oxidative degradation of the compound may be due to the electron-transfer oxidation caused by H_2O_2 resulting in conversion of thiol group to sulfinic acid and sulfonic acid (Okram *et al*., 2012). Moreover, the chlorophenoxy group and oxadiazole rings are reported to be susceptible to oxidation and photo-decomposition (Pignatello, 1992; Maciolek *et al*., 2011). The possible mechanism behind the observed hydrolytic and oxidative decomposition might be the same as reported earlier (Pignatello, 1992; Kumar and Khokara, 2005; Maciolek *et al*., 2011; Okram *et al*., 2012). However, detail investigation of the stressed solution using mass spectrophotometer is necessary to characterize the degradation products.

CONCLUSION

The results of the present study revealed that the method developed for analysis of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM) is simple, specific, reliable, repeatable and reproducible. ICH prescribed forced degradation studies indicate that the compound is extensively decomposed in hydrolytic, oxidative, dry heat- and metal- stress, however, remains stable/intact in photolytic conditions. The degradation products formed under stress conditions don't interfere in determination of the compound. Hence, the developed method is stability-indicating and may be applied for routine analysis, stability testing and pre-formulation studies of the compound.

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