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Original Article

Synthesis, Characterization, RP-HPLC Method Development and Validation for Qualitative Estimation of (4Z) 3 Methyl 1 (4 Nitrobenzoyl) 1H Pyrazole 4,5 Dione 4[(4fluorophenyl) Hydrazone]

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ABSTRACT

Aim: A new simple, accurate, precise, Reverse Phase High-performance Liquid Chromatography method was developed and validated for the estimation of newly synthesized (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone] derivative of pyrazolone. **Materials and Methods:** The chromatogram was run through Luna 5µ C_{18}(2). 250 × 4.80 mm. 272817-7 HPLC column; mobile phase consisting of acetonitrile and water in the ratio 90:10 was pumped through the column at a flow rate of 0.8mL/min. **Results:** The optimized wavelength was 237 nm. The retention time of (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone] was found to be 7.3 min. The percentage relative standard deviation was obtained as 0.3%. **Conclusion:** This method was accurate, precise, and sensitive; therefore, could be used for the quality control and bioanalytical evaluation of (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone] in drug testing laboratory.

Keywords: Column Chromatography, HPLC, Pyrazolone derivative.

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INTRODUCTION

Pyrazole-3-one commonly known as pyrazolone it is a derivative of pyrazole with an extra ketone ring.[1,2] Pyrazolone derivatives are known for their broad spectrum of activities including antipyretic, antifungal, antimicrobial, anticancer, analgesic, anti-inflammatory, antioxidant, SARS-coronavirus 3C-like protease inhibitors and are also used in amyotropic lateral sclerosis (Lou Gehrig's disease) and ischemic-reperfusion.[3-8] Several pyrazolone-containing drugs are approved by the food and drug administration (FDA) and are being marketed as therapeutics such as antipyrine (phenazone)[2,3-dimethyl-1-phenyl-3-pyrazolin-5-one] formerly used in the 19th century, dipyrone (metamizole), aminophenazone, morazone, propyphenazone for analgesic, antipyretic and anti-inflammatory effect. Further butazolidine is used as a potent anti-inflammatory medication to treat rheumatic diseases, Edavarone has been used as prevention and therapy for arterial wall injury, further it is also prescribed in ischaemic reperfusion injury based on its hydroxyl radical scavenging mechanism. This provides ample opportunity for continuous research and analysis of new analogs. Therefore, we have attempted to develop a novel pyrazolone moiety for the neuromodulatory effect of Parkinson's disease.

For qualitative analysis of newly synthesized derivatives a suitable analytical method that is easy, rapid, sensitive, accurate, precise, and economic is necessary to develop and validate by International Council on Harmonisation (ICH) $Q_2(R_1)$ guideline.[9] Few authors

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have quantified glucose by 1-phenyl-3-methyl-5-pyrazol with HPLC DAD.[10] The other reported method for the derivatization of carbohydrates using 1-phenyl-3-methyl-5-pyrazolone and separation using HPLC is optimized. Some of the pyrazolones are also used as labeling for the detection of carbohydrates by using DAD-HPLC.[11] Edaravone (3-methyl-1-phenyl-2-pyrazolin-5 -one) a derivative of pyrazolone has been estimated in pure and in pharmaceutical dosage form by liquid chromatography (LC) method.[12] Though there are several methods reported for substituted pyrazolone and formulations, it is extremely difficult for a newly synthesized molecule to develop a suitable qualitative and quantitative estimation method. We synthesized and characterized (4Z) 3-methyl 1-(4-nitrobenzoyl) 1H pyrazole-4,5 dione 4[(4fluorophenyl)hydrazone] and have developed and validated a new RP-HPLC method for its estimation. The newly developed method was found to be accurate, linear, precise, reproducible, sensitive, and stable from 0.5-50ppm.

MATERIALS AND METHODS

Chemicals, and Instrumentation

Chemicals and reagents were procured from RANKEM, Hi-media, India. HPLC-grade water and Acetonitrile were used in a ratio of 1:9 for the mobile phase. 0.22 µm nylon syringe filters. AR grade NaOH, HCl, and H_2O_2 were used for the forced degradation study. High-performance liquid chromatography (LC 20 AD Prominence) photo-diode-array detector with quadruplet solvent system, instinctive column thermostat, and sample injecting port (Make-Shimadzu, Japan) was used for performing method development and validation analysis. Brukers alpha ATR FT-IR was used to analyze infrared spectra (NGSM Institute of Pharmaceutical Sciences, Advance research center). ^{1}H NMR was analysed by JEOL spectrometer and DMSO D6 as a solvent. Shimadzu LCMS in binary gradient used to analyze mass spectra (DST-PURSE lab, Mangalore University).

Synthesis of (4Z) 3 methyl 1 (4-nitrobenzoyl) 1H pyrazole 4,5 dione 4 [(4 fluorophenyl)hydrazone]

The modified method of (B. C. Revana Sidhappa 2018) was used for synthesis. It is illustrated in the scheme (Figure 1). The compound was synthesized in two steps.

Step I: Synthesis of ethyl 2 arylhydrazono 3 oxobutyrate

0.01 mole of 4 fluroaniline was dissolved in a mixture of 8 mL HCl, 6mL of water, and cool in an ice bath at 0-5°C. Then 0.03mole of sodium nitrate cold solution was added. The formed solution of diazonium salt was filtered into 0.01mole of cold ethyl acetate solution and 0.12mole of sodium acetate in 50mL ethanol.[13]

Step II: Synthesis of substituted Pyrazolone

The reaction mixture containing 0.01 N of Ethyl 2 arylhydrazono 3 oxybutyrate with 0.01 N 4-nitrophenoxyacetic acid hydrazide was dissolved in 20mL, and the reaction mixture was refluxed for around 30 hr. The resulting mixture was poured into an ice-water solution. The mixture was agitated, the resultant precipitate was collected through filtration, was rinsed with water, recrystallized from ethanol, and then eluted by affinity chromatography using n-hexane and ethyl acetate (9:1) as the eluent.[14]

Selection of wavelength

The wavelength of compound (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone] was selected using a UV-visible spectrophotometer. The UV Visible spectra so obtained are depicted in Figure 2.[15]

Preparation of sample solution

An accurately weighed (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone] (Figure 3) (10mg) taken in a 10mL graduated flask containing acetonitrile

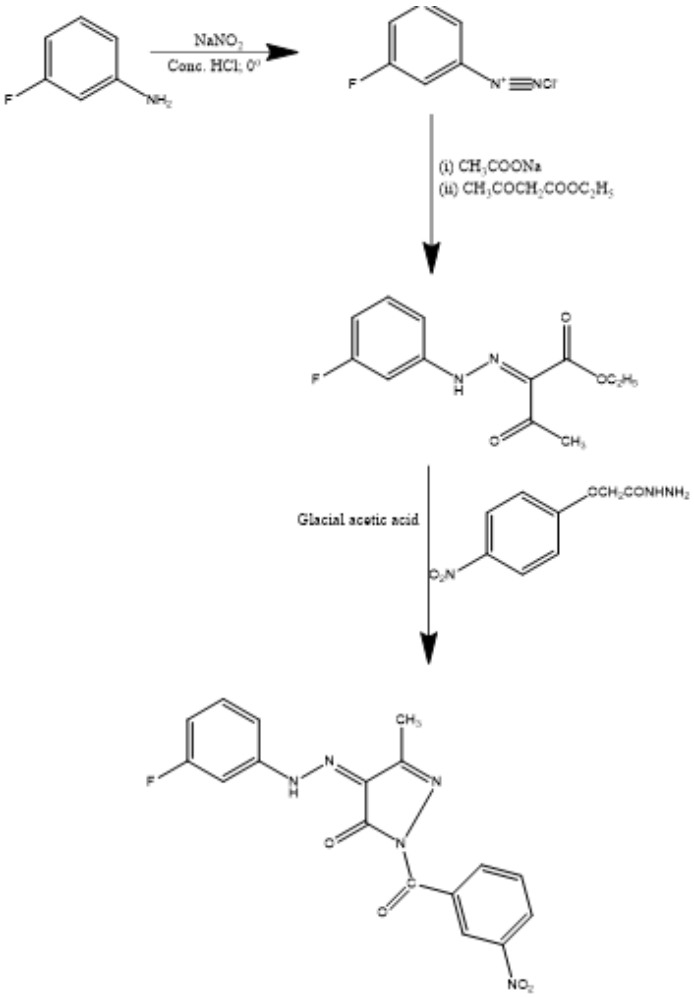

Figure 1: Scheme of Synthesis for (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone].

and dissolved using 20K/Hz high-frequency sound waves for 15 min. 1mL of the standard solution was transferred to a graduated flask and made up the volume using acetonitrile, which was further diluted to obtain a final concentration of 0.5-50 ppm. The prepared solutions are filtered through 0.22 µm syringe filters.

Chromatographic condition

The variable (mobile) phase was filtered through a 0.45 µm vacuum filter unit. The chromatographic separation of the prepared stock solution of (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone] was performed on Luna 5µm C_{18}(2). 250 × 4.80 mm. 272817-7 HPLC column. The flow rate and column temperature were maintained at 0.8 mL and 40°C respectively. The solvent system consisting acetonitrile and water in a ratio of 9:1. At the beginning of the experiment purging of the HPLC system was performed. Further, the system is equilibrated with the solvent system to get a smooth baseline. Data acquisition and processing were accomplished using lab solution software.[16]

Method validation

Validation of the technique was performed according to ICH procedures in terms of Accuracy, and detection limit (LOD). Quantification limit (LOQ), Linearity, Precision, Robustness, and Stability indicating capability.[17]

System suitability

The investigation was executed by injecting five replications of the sample. The relative Standard Deviation (SD) for Peak Area (PA), retention Time (RT), Tailing Factor (TF), and Theoretical Plate (TP) were evaluated. The sample solution was injected into the HPLC system and an Area Under the Curve (AUC) for each analytical peak was recorded. The amount was calculated in % assay.[18]

Linearity and range

To estimate the linearity of the technique, diverse standard solutions were attenuated by standard stock solution with a specific solvent with successive nine concentrations, and each concentration of test samples was injected in triplicates and analysed. Linear regression analysis was used to evaluate the linearity of the concentration.[19]

Specificity

There are two main parameters in specificity. They are selectivity and Forced degradation.

Selectivity

Selectivity is an important feature of liquid chromatography, it provides the ability of the analytical technique to differentiate between the analytical sample and adjuvants or impurities of the test mixture. Selectivity was analyzed by administering a 10µL solution of the test sample, blank and dummy independently.

Forced degradation

Forced degradation is mainly carried out for Stress testing. Forced degradation was performed by using different parameters like thermal degradation, humidity, acidic, basic, and oxidative degradation. Thermal degradation was performed by keeping samples at 60°C for upto 24hr. The moisture degradation of the samples was kept at 35±5°C for 7 days. For acid degradation, the samples were reacted with 0.1N HCl for 5 hr and neutralized with 0.1N NaOH whereas for basic degradation the samples were treated with 0.1N NaOH and neutralized with 0.1N HCl. For oxidative degradation H_2O_2 is commonly used. The samples were treated with 3% H_2O_2 and maintained at room temperature for 24 hr. The test samples of the forced degradation study were run in HPLC as per the method given.[20]

Sensitivity

It is performed by detection limit (LOD) and Quantification limit (LOQ). LOD is the least concentration of a test sample detected. LOQ is the least concentration of the test sample determined by satisfactory accuracy and precision. LOD; LOQ of the sample were evaluated by analyzing different solutions in a range of 0.5-50ppm concentrations. LOD is measured by signal-to-noise fraction of about 3/4:1/4 while LOQ was measured by 10:1 respectively.[21]

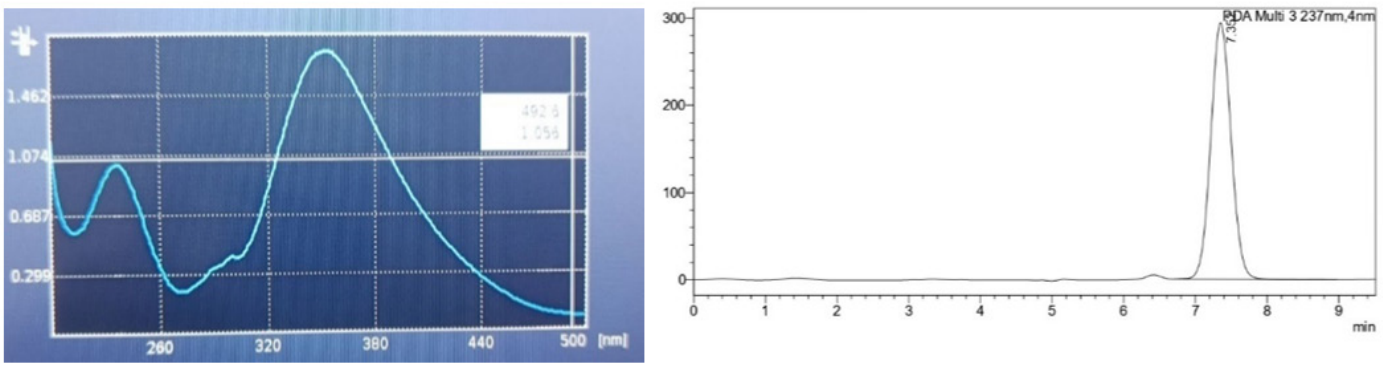

Figure 2: UV-visible spectra and pure synthesized HPLC spectra.

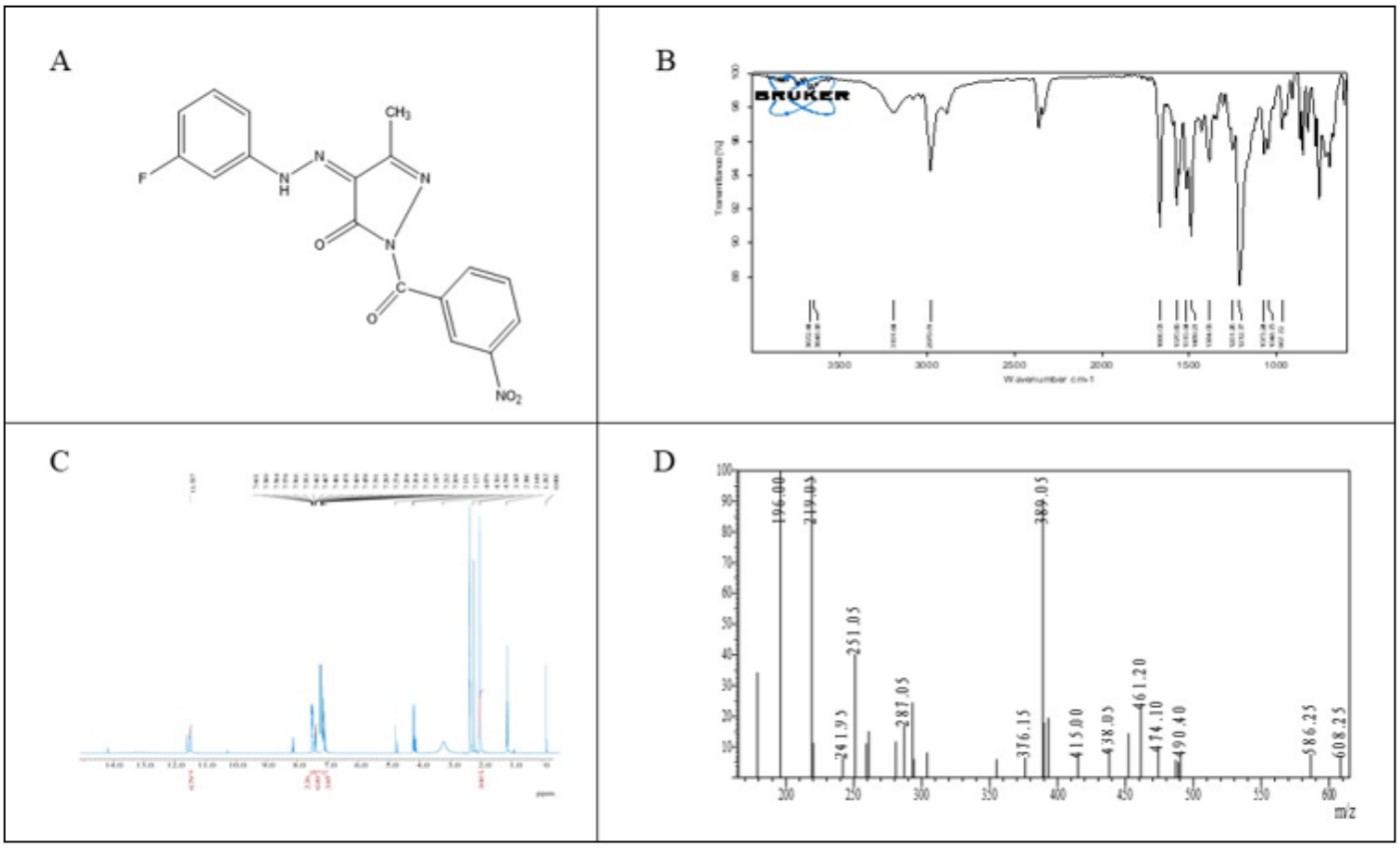

Figure 3: A) Chemical structure of (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone], B) IR, C) NMR and D) MASS SPECTRA.

Precision

Precision is of two types, system precision, and method precision. The area of peaks was considered to calculate SD, % RSD, and % assay. Technique precision was established by sextuplicate of sample solution on the same day at 100% concentration level. Technique precision (intraday/repeatability) was performed by preparing 6 working samples of a single batch. Intermediate precision (interday) It is performed in 6 working samples for 3 different days and by different operators.[22]

Accuracy

It is typically performed by calculating a known quantity of standard material with variable conditions. For percentage recovery, samples are prepared in triplicates at a range of 50-150% of the target concentration. The analyzed percent recovery must be in the range of 98–102% and % RSD should be less than 2.0% as per ICH guidelines.[23]

Filter interference

This is done to estimate the appropriateness of the filter for aliquot preparation. Executed on 0.45µ nylon filter against centrifuged sample at 5000 rpm.[24]

Stability of analytical solution

The stability of the sample is executed by keeping the samples for 0, 24, and 48 hr at room temperature 37°C ± 2°C and evaluated for the changes (Table 1).[25]

Robustness

It measures the volume of an analytical method to persist unaffected by minor thoughtful deviations in process parameters. variable phase ratios, temperature, and flow rate; these are performed one factor at a time.[26]

RESULTS

Synthesis and optimization of (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone].

Yellow crystal (ethanol), IR (ATR, cm^{-1}): 1048.73(C-N str), 1570.85 (N-O str), 1666.03 (Ar C=O str), 2979.81 (Ar C-H str), 3191.64 (N-H str). ^{1}H NMR (DMSO-d6, 0.6 Hz): δ 2.3 (3H, s), 7.28 (4H, m),7.15(2H, m), 7.8(2H, m), 11.15(1H, d) MS (ESI) *m/z*: 376.15 $[M]^{+1}$; Calculated exact mass: 369.09 Figure 2. UV-visible spectrum method was optimized at 237 nm 354nm.

System suitability

The test was accomplished by injecting a quintet of the sample. The relative SD for the area of peak and Retention Time (RT)

Table 1: System suitability study result.

Injection Number	Area	Height	Tailing Factor	Hetp(Usp)
1	11670576	437045	1691	88.696
2	11667612	447792	1787	83.941
3	11664603	459322	1887	79.492
4	11621897	470251	1986	75.528
5	11655029	481957	2083	72.006
Average	11655943	459273.4	1886.8	79.9326
STDEV	19907.61	17755.14	155.4290835	6.619857763
% RSD	0.170794	3.86592	8.237708475	8.28179962

Table 2: Forced degradation study.

Sl. No.	Sample/ Degradant	Degradation Condition	% Absolute Degradation in Assay (Mean ± SD)
1	Control sample	Not applicable	100
2	Thermal degradation	60° for 24 hr	57.00903 ± 9.06
3	Humidity degradation	7 Days at room temperature	51.30158 ± 0.28
4	Acid hydrolysis	0.1N HCl	26.35548 ± 0.07
5	Alkali hydrolysis	0.1N NaOH	21.46151 ± 0.12
6	Oxidative degradation	3% H_2O_2	42.72969 ± 0.62

Table 3: Interday and intraday precision study.

Sample	% Assay	
	Intra-Day	Inter-Day
1	100	100
2	100.2521	100.0363
3	100.3819	99.97117
4	100.2359	99.48226
5	99.91694	99.17157
6	99.31637	99.95631
Average	100.0172	99.76961
STDEV	0.384056	0.357746
%RSD	0.38399	27888.41

was recorded as less than 1.0%, tailing factor was less than 1.2. Theoretical plates for test samples were 1992.2. The system suitability report is depicted in Table 1. The sample solution was injected into the chromatography system and an AUC for each peak was documented. The quantity was calculated as % assay.

Linearity

The linear standard curve for calibration was assessed using linear regression (R^2) analysis. For the test sample, R^2 was Y = 579609x + 8687.8. The observed co-relation coefficient (r) was found to be 1.0000 for equality. The linearity of the technique was detected over the concentration series of 2.00-20.00 µg/mL with an *R*-value of 1.000 under the experimental environments. The linearity reading is depicted in Figure 4.

Specificity (Selectivity, forced degradation)

During the specificity analysis, the diluent did not interfere with test sample RT. Figure 3 depicts the diluent and sample chromatogram. The stress environments and absolute % degradation was determined against the control sample (Table 2). The thermal and humidity degradation has shown 57.00 to 51.30% of degradation whereas oxidative degradation has shown 42.72%. Hydrolysis with acid and alkali degraded the sample by 26.35% and 21.46% respectively. Table 2 depicts the outcomes found during the study.

Sensitivity (LOD and LOQ determination)

A calibration line from 2.0-20 µg/mL was plotted. The statistically obtained LOD and LOQ of PPA-6 were found to be 0.119059 µg/mL and 0.396864 µg/mL. The method was capable to calculate 2 µg/mL concentrations with adequate accurateness and precision. Based on the quantification of recovery at LOQ, the % average recovery for the test sample was estimated at 100.1% with 1.04% precision RSD. The signal-to-noise ratio at LOQ aliquot level for the test sample was found to be 74.0 respectively. Hence, the LOQ was found to be 5 µg/mL.

Precision

The intraday and interday precision evaluation was executed on a sextuplet taken on the same day (intraday precision) and three successive days (interday precision). The obtained complete % RSD for the test compound was 0.384. The effects are stated in Table 3.

Table 4: Relative standard deviation at different levels of accuracy.

Levels	% RSD
50	1.71695 ± 0.02
100	0.924544 ± 0.08
150	0.604701 ± 0.01

Table 5: Solution stability study.

Time point	% Assay of Drug	Cumulative		
		Average	STDEV	%RSD
Day 0	100	NA	NA	NA
Day 1	99.33689323	99.66844661	0.234443647	0.235224
Day 2	98.58389279	99.30692867	0.511263575	0.514832

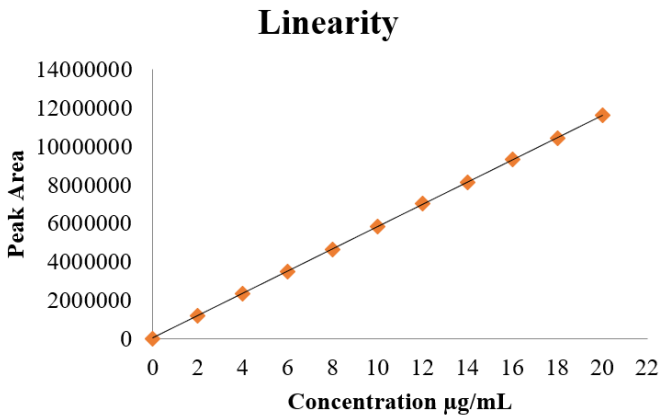

Figure 4: Linearity.

Accuracy (recovery)

The recovery of the sample solution was found to be within the given limits and the % RSD was found to be in the range of 1.7% to 0.6% which is depicted in Table 4.

Filter interference and Stability of analytical solution

There was no change observed in the data of filter interference as well as no noteworthy variation in the assay report for 2 days for the aliquote. The results are described in Table 5.

Robustness

Peak area percentage RSD, Tailing Factor (TF), Theoretical Plates (TP), and Retention Time (RT) were examined during the experiment and noted in the acceptance range i.e., 2%.

DISCUSSION

The present research has been described with the purpose to synthesize (4Z) 3 Methyl 1 (4 Nitrobenzoyl) 1H Pyrazole 4,5 Dione 4[(4fluorophenyl)Hydrazone] and develop a new validated method for its estimation by RP-HPLC technique (method) utilizing photodiode array sensor. Literature discloses that there are no analytical approaches reported for the assessment of (4Z) 3 Methyl 1 (4 Nitrobenzoyl) 1H Pyrazole 4,5 Dione 4[(4fluorophenyl)Hydrazone]by RP-HPLC method. Due to the utmost importance of analytical methods in the quality control of drugs, the development of an analytical/investigative method for the determination of novel ligands by RP-HPLC has established substantial attention in the current era.

The IR spectra of the compound depicted the absorption bands at 1048.73(C-N str), 1570.85 (N-O str), 3191.64 for (N-H stretching), 2979.81 for aromatic (CH), 1666.03 for aromatic (C=O stretching), 1570.85 for (N-O) and 1048.73 for (C-N), respectively. In the proton NMR of the compound, revealed triplicate single methyl at δ 2.3 regions. The aromatic protons of flurobenzene were observed as multiplets in the region δ 7.28-7.46. The presence of other protons in the pyrazolone moiety was observed as multiplets of nitrobenzene showed in the region of δ 7.15-7.8 and 11.53 of hydrazide doublet. The mass spectrum of the compound showed a molecular ion peak at M/z = 376.15 (M^+).

The developed method was validated for accuracy, precision, reproducibility, specificity, and robustness in agreement with ICH guiding principle. The UV is method optimized in the 237 and 354 nm wavelength but the prominent peak in the HPLC is at 237nm and hence this wavelength is designated.

A satisfactory symmetric peak was attained using the C_{18} Luna column (250 × 4.80 mm, 5µm) since is a polar drug.

The most optimum ratio for the mobile phase containing acetonitrile and water was found to be 10:90.

An RT of 7.35 min. was detected where the column oven temperature was set at 40°C and the sample temperature was 8°C. The run time of the method was fixed at 10 min.

After the optimization of the method parameters, forced degradation studies were performed to determine the stability of the method. Degradation via acid, base, hydrogen peroxide, water, humidity, and the heat was performed. Degradation of test molecules for validation of chromatographic assays is performed and the observed values of degradation were within this limit suggesting that the developed method was stable. After the establishment of the stability of the method, validation parameters such as accuracy, linearity, precision, system suitability, and robustness were evaluated. With the flow rate of 0.8mL/min, the method was found to be accurate, precise, and linear within a range of 0.5ppm-50ppm. Robustness studies were accomplished specifying that the developed technique is rugged. Therefore, the purpose of synthesizing (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone] and developing a steady process for its analysis along with its validation was accomplished.

CONCLUSION

The current work was designed at developing a simple, rapid, economical RP-HPLC assay technique for qualitative analysis of the novel synthesized compound. The RP-HPLC with PDA sensor assay method was successfully developed on the (Shimadzu) C_{18} column in gradient elution mode. The developed method does not contain toxic reagents. Due to the newly synthesized molecule, the wavelength was selected by the offline UV detector of the new molecule. The λ_{max} for PPA6 was selected as 237 nm. The gradient selected for separation consisted of 9:1 acetonitrile (eluent A) and HPLC-grade water (eluent B). This system provided a retention time of 7.3 with good resolution. The purpose of this study includes establishing a qualitative method that is capable of separating and evaluating (4Z) 3 methyl 1 (4 nitrobenzoyl) 1H pyrazole 4,5 dione 4[(4fluorophenyl)hydrazone], efficiently in the shortest feasible run time with reasonable accuracy and reliability.

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ABBREVIATIONS

AUC: Area under the curve; **FDA:** Food and Drug Administration; **HCl:** Hydrochloric acid; **HPLC-DAD:** High-pressure liquid chromatography-diode array detector; **H_2O_2:** Hydrogen peroxide; **ICH Q_2 (R1):** International council of harmonization Q2; **LC:** Liquid chromatography; **LOD:** Detection limit; **LOQ:** Detection quantity; **NaOH:** Sodium hydroxide; **PA:** Peak area; **R^2:** Linear regression; **RSD:** Relative standard deviation; **RT:** Retention time; **TF:** Tailing factor; **TP:** Theoretical plates; **SD:** Standard deviation; **UV:** Ultraviolet.

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