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Development and validation of stability indicating HPTLC method for estimation of pirfenidone and characterization of degradation product by using mass spectroscopy

Pirfenidone is used as a novel antifibrotic agent approved for mild-to-moderate idiopathic pulmonary fibrosis. An extensive literature search revealed that, method validation by high-performance thin-layer chromatography (HPTLC) and structural determination by tandem mass spectrometry (MS/MS) method was not reported till date. Precoated silica gels plates were used as a stationary phase. Methanol: ethyl acetate: toluene (1:2:7 v/v) was delivered best separation at 315 nm (Rf 0.49±0.03) by densitometry analysis. Degradation analysis was performed as per ICH guidelines Q2 (R1). Isolation of degradation product was done by the HPTLC method and characterized by using MS/MS method. All the validation parameters were found within the range. Moreover, its possible degradation pathway was also proposed. The Proposed developed and validated HPTLC method was found to be more sensitive, simple, precise, accurate, cost-effective and robust. This method could be applied for the analysis of bulk drug and tablet formulation, degradation study. This degradation pathway of the drug will further help to identify the degradation products of Pirfenidone which may be used for the impurity profiling of the drug.

Keywords: high-performance thin-layer chromatography (HPTLC), pirfenidone, tandem mass spectrometry (MS-MS) studies, degradation mechanism.

Introduction

Pirfenidone is a mini non-peptide molecule of low molecular weight 185.2 g/mol, with a chemical name 5-methyl-1-phenyl-2-(1H) pyridine (Fig. 1). It is an agent that combines anti-inflammatory and anti-fibrotic activity, acting through the control of tumor necrosis factor-α (TNFα) and tumor necrosis factor-β (TNFβ), the pathway, as well as through the modulation of cellular oxidation[1]. Idiopathic pulmonary fibrosis (IPF) is the most common form of incurable and often fatal idiopathic interstitial lung disease [4–6]. The purpose of the ASCEND (A Study of Cardiovascular Events in Diabetes) study was to evaluate and confirm the efficacy and safety of pirfenidone [7].

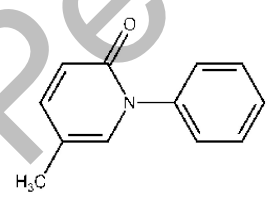

Figure 1. Structure of Pirfenidone

The creation and testing of the analytical method include a sequence of operations that are continuous throughout the life cycle of the product and the substances. It is important to isolate drugs from impurities, degradants, and formulating excipients and analyze them separately for the accurate quantitative determination of drug of interest in a dosage form.

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Considering the quality of the substance and carrying out the content analysis without separating or extracting it became important today, as these extraction or separation processes are found to be time-consuming, repetitive and often costlier [8–9].

A comprehensive literature search revealed that HPTLC system validation and structural determination for Pirfenidone by MS/MS system has not been documented to date. The production and validation of a simple, reliable, accurate and economical, stability-indicating HPTLC method and the identification and characterization of degradation products using MS/MS were therefore considered worthwhile.

Consequently, this research aims to develop simple, precise, cost-effective methods using various analytical tools such as:

- design and validation of the HPTLC method for estimating pirfenidone in a pharmaceutical dosage form;
- MS-MS studies to isolate the potential product for degradation and classify its likely fragmentation pattern.

Experimental

2.1 Chemical and reagents: Pirfenidone was received from Intas Pharmaceutical Ltd, Gujarat as a gift sample. All the chemical used were of analytical grades.

2.2 Preparation of Standard and sample solution: For sample solution 10 mg of pirfenidone was weighed, transferred to a 10 ml volumetric flask, then added 5 ml of methanol and sonicated for 10 minutes. For standard stock solution, twenty tablets of pirfenidone samples were weighed and crushed to obtain a fine powder. The average tablet weight was measured. Through this accurately weighed the tablet powder equivalent to around 10 mg of Pirfenidone was transferred to a 10 ml volumetric flask, added 5 ml of methanol and 10 min ultra sonicated, then made up the methanol amount. 1 ml of this solution was further diluted to 100 μg/ml concentration by using of 10 ml of methanol to form the resulting solution. To remove any particulate matter both the solutions were filtered using Whatman filter paper no. 42.

2.3 Calibration curve preparation: The normal stock solution containing Pirfenidone has been prepared in methanol. Six concentration levels over a range of 200–1200 ng/band in pirfenidone were analyzed for a linear relationship between peaks and concentration.

2.4 Method validations for HPTLC

Proposed process has been validated according to the Criteria for Methodological Validation of the International Conference on Harmonization ICH Q2 (R1).The process was validated using different parameters such as accuracy, precision, specificity and linearity [8–11].

2.5 Accuracy (recovery test): This analysis was performed at three stages, i.e. 80 %, 100 %, and 120 % recovery point, respectively, as shown in Table 2. The solution was done in accordance with the procedure stated in the preparation of the sample solution on and the amount of drug recovered was calculated [12–13].

2.6 Precision: Tablet sample solutions were tested intraday and interday precision at various time intervals on the same day and three different days, respectively. The tablet sample solution was prepared and analyzed like the one defined in the market formulating analysis.

2.7 Linearity: Accurately measured 10 mg of Pirfenidone was transferred to 10 ml volumetric flask, 5ml of methanol was added, and 10 minutes of ultra-sonic pressure was applied, then methanol was removed. Diluted 1 ml of the resulting solution with methanol to 10ml (Pirfenidone concentration 100 μg/ml). The above solution of Pirfenidone was applied to the TLC plates in the range 0.1–0.6 μl using a microsyringe with the help of LINOMAT-V automated sample applicator. The plate was created and scanned under the optimized conditions of chromatography. The peaks achieved for Pirfenidonewere incorporated after scanning. Peak area was recorded for each drug concentration and the calibration curve of Concentration Vs Peak area was constructed for Pirfenidone.

2.8 Robustness: Robustness studies were performed through the shift in mobile step, saturation time of chamber. Most mobile phase and chamber saturation period composition varied within the range of ±0.1 ml and ±2 min, respectively, of the optimized conditions used. Mobile phase volume had varied by ±1 ml. The effect of these changes was investigated on both the Rf values and peak area.

2.9 Detection limit and quantification limit: The limit of detection (LOD) and limit of quantification (LOQ) were calculated separately, based on the standard calibration curve response deviation. To measure the LOD and LOQ the standard deviation of Y-intercept and slope of the calibration curves was used by using formula.

LOD – 3.3 SD/S and LOQ – 10 SD/S,

there SD — is the standard deviation of the response; S — is the slope of the calibration curve.

2.10 Forced degradation study of pirfenidone: In the test of forced degradation, the six samples were prepared by injecting 10 mg of pirfenidone into the product in a 10 ml volumetric flask and adding 3 ml of 0.1 N HCl, 0.1 NaOH, 3 percent H_2O_2 and first 4 flasks of distilled water, respectively. Heat the flask at 80 ºC for acidic and basic conditions for 2 hrs in the water bath and for oxidative condition for 1 hr, and 3 hrs at 80 ºC for distilled water respectively. As per guidelines for force degradation, the photolytic degradation was carried out in UV light (254 nm) for 48 hrs and the drug was kept at 100 ºC for 1 hr for thermal degradation. The remaining two flasks are then moved respectively. All the flasks were removed after the respective time intervals and allowed to cool. The samples were then similarly analyzed as defined under a tablet analysis [14–18].

2.11 Isolation and identification of degradation products by HPTLC-MS/MS: The degradation study was conducted on six conditions of sample stress, but alkaline and acidic these are two conditions of sample stress used in HPTLC-MS/MS studies due to the percentage of degradation rather than other stress. The degraded products are isolated and classified by applying a degraded sample solution to the TLC plate (9 µl-bands) and under optimized chromatographic conditions, the plate was created. It was under the observed 254 nm UV cabinet after drying the plate, and it defined and labeled the degraded band. When the damaged band was found, it was scraped out into the Eppendorf tube and held in methanol overnight. The sample was filtered through Whatman filter paper and subjected to MS/MS on the next day. Two forms of spectra were obtained by the MS/MS studies: Q1 (used for parent compound identification), and Q3 (used fragmentation pattern identification). The MS/MS spectra are shown in Figure 5 and Table 4.

Results and Discussion

3.1 Optimization of Mobile Phase

The mobile phase used for HPTLC analysis was methanol: ethyl acetate: toluene (1:2:7 v/v). They shows good resolution at Rf value of 0.49±0.3. The Pirfenidone HPTLC densitogram shown in Figure 2.

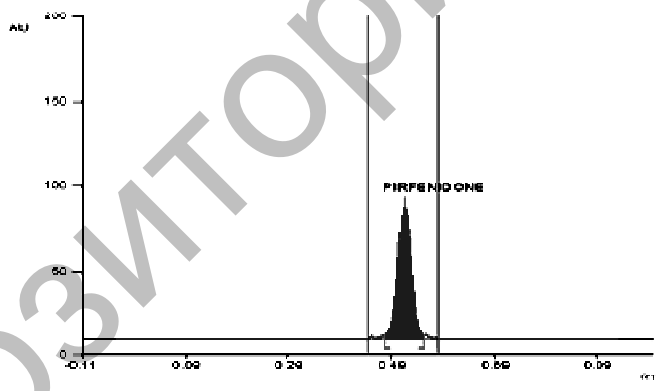


Figure 2.Typical densitogram of Pirfenidone

3.2 Linearity: The pirfenidone linearity equation is $y = 303.11 + 36.612$ with a correlation coefficient (R2) of 0.997; the Peak area for each concentration of drugs was registered, and for Pirfenidone the calibration curve of the Concentration Vs Peak area was constructed. The standard data for the calibration is given in the Table 1. The linearity for Pirfenidone is depicted in Figure 3.

Table 1

Standard calibration data for Pirfenidone

Sr. no	Concentration ng/band	Peak area
1	200	321.79
2	400	653.39
3	600	989.28
4	800	1224.61
5	1000	1518.29
6	1200	1877.52

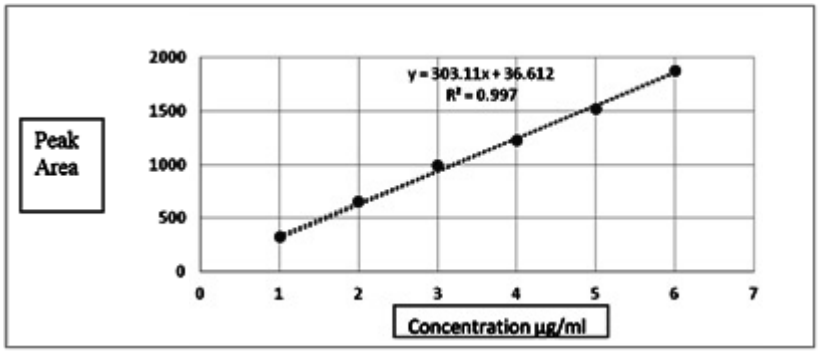


Figure 3. Linearity of the pirfenidone

3.3 Precision and Accuracy: Precision and accuracy tests are shown in Table 2 and Table 3 respectively. It was found to comply with the guidelines of ICH Q2 (R1).

Table 2

Result of precision

Drug	Intraday precision			Inter day precision		
	% label claim	S.D	R.S.D	%label claim	S.D	R.S.D
Pirfenidone	99.46	1.81	1.82	99.70	0.43	0.43

Table 3

Result of accuracy

Level of recovery	Recovery (%)	S.D	% R.S.D
80 %	98.29	0.85	0.86
100 %	100.55	0.20	0.20
120 %	98.62	0.93	0.93

3.4 Robustness: The effect of saturation time, mobile phase shift on both the Rf values and peak area was investigated. Table 4 displays the findings of robustness studies and its statistical validity.

Table 4

Result of robustness studies

Factor	**Level**	**Area**	**Rf**
Saturation time			
5 min	–5	1957.55	0.51
10 min	0	1975.40	0.49
15 min	+5	1986.49	0.47
	S. D ± R. S. D		1.9 ± 0.34
Total mobile phase	**Level**	**Area**	**Rf**
9 ml	–1	1975.44	0.47
10 ml	0	1975.90	0.49
11 ml	+1	1975.23	0.46
	S. D ± R. S. D		1.5 ± 0.26
Time from developing to scanning	**Level**	**Area**	**Rf**
9 ml	5 min	1975.44	0.53
10 ml	30 min	1975.90	0.49
11 ml	60 min	1975.80	0.52
	S. D ± R. S. D		1.1 ± 0.20

3.5 LOD AND LOQ

The sensitivity of the proposed method has been calculated in terms of a detection limit (LOD) and quantification limit (LOQ) 0.034 and 0.104 ng band respectively.

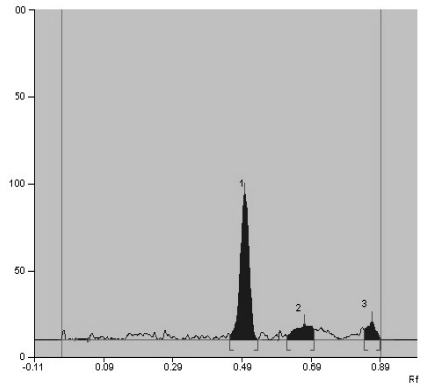
3.6 Forced Degradation Study of Pirfenidone

Forced pirfenidone degradation experiments were performed under various stress conditions such as acidic, alkaline, oxidation, neutral, photolytic, and thermal. Table 5 shows the percent assay of the active material and its Rf values of the degradation products. Acid, alkaline, oxidation, acidic, photolytic, and thermally treated Pirfenidone densitograms are shown in Figure 4, *a–f*, respectively.

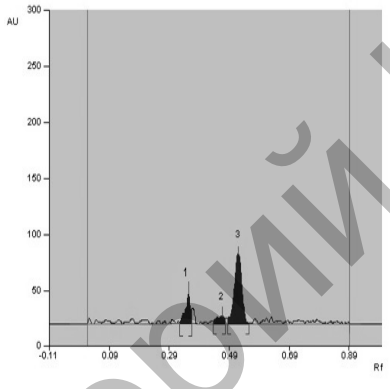
Table 5

Results of forced degradation study

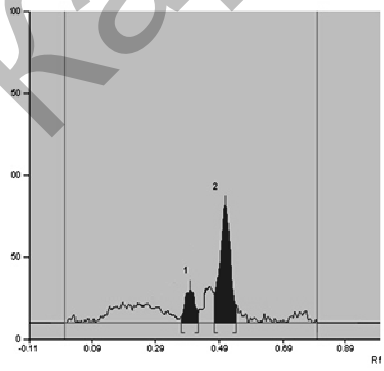
Sr. no.	Stress condition	Temperature and time	% assay of active substance	Rf value of degradaned product
1	Acid (0.1 N HCl)	80 ºC for 2 hrs.	80.36 %	0.67, 0.87
2	Alkaline (0.1 N NaOH)	80 ºC for 2hrs.	81.9 %	0.33, 0.47
3	Oxidative (3 % H_2O_2)	80 ºC for 1 hrs.	96.72 %	0.40
4	Neutral	80 ºC for 3 hrs.	91 %	0.12, 0.40, 0.42
5	UV degradation	48 hrs.	97.9 %	0.75
6	Thermal	80 ºC for 1 hr	99.16 %	0.76



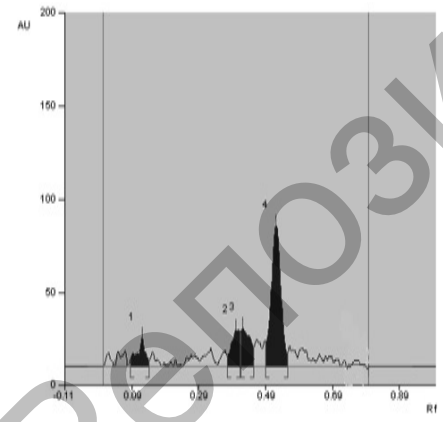
a) densitogram of sample treated with (0.1 N HCl)



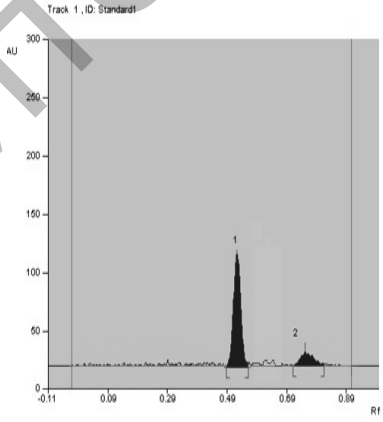
b) densitogram of sample treated with (0.1 N NaOH)



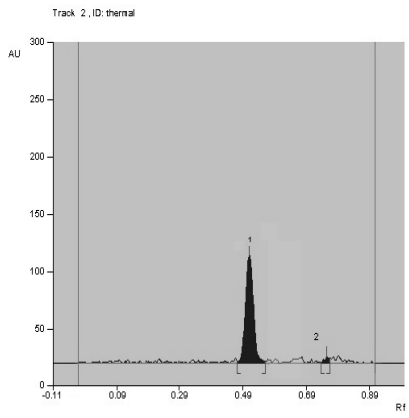
c) densitogram of sample treated with (3 % H_2O_2)



d) densitogram of sample treated with Neutral



e) densitogram of sample treated with Photo Degradation



f) densitogram of sample treated with Thermal

Figure 4. Degradation of Pirfenidone

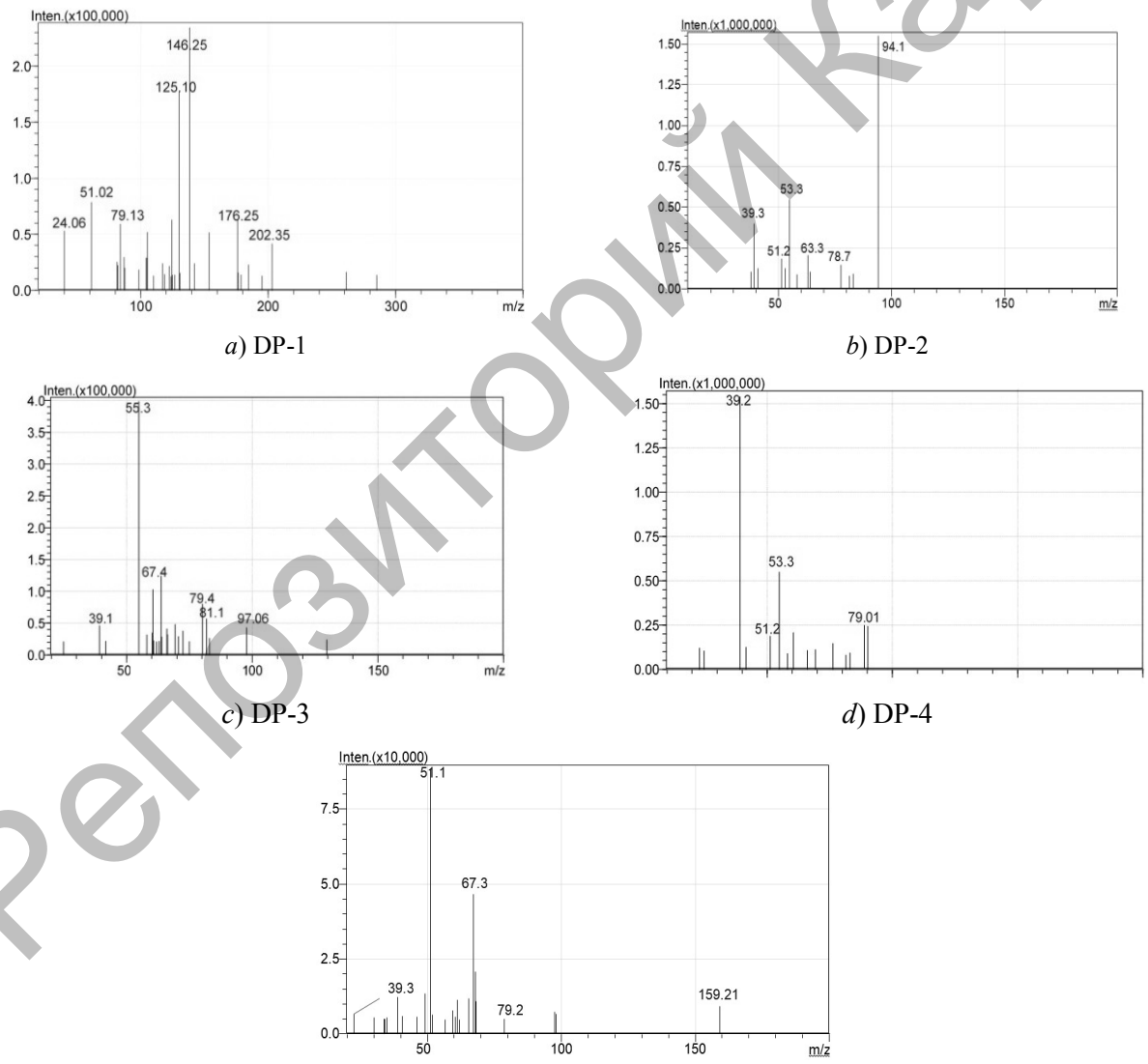
3.7 Isolation and identification of degrade product by HPTLC — MS/MS (Tandum mass spectroscopy) method

The degradation product has been extracted using the HPTLC method and the degradation product structure was calculated using MS/MS studies. The fragmentation drug pattern was developed by conducting positive electro spray ionization (ESI) mode MS-MS studies in the mass range of 50–1500 g/mol to maximize the mass parameter that specifically informs of the drug's molecular ion peak. Those were further changed to get the medication completely separated.

The MS/MS spectroscopy was primary help to disclose the drug degradation into five degradation products (referred to as DPs I-V, according to the structure in which the peak appeared in the chromatogram from left to right). After degradation five fragmented products was defined based on IR spectra. The five components for the degradation were DP-1, DP-2, DP-3, and DP-4 and DP-5. All result given in the HPTLC-MS/MS range was shown below (Fig. 5).

One oxidation product creates an oxidative stress state. H_2O_2 was oxidized by pirfenidone. The oxidation of ring-A will be postulated. That occurs in the form of DP-1 at nitrogen (m/z = 202.09), as shown in Figure 5, *a*. In mass spectra, the parent ion of m/z 202.09 was observed. Figure 6 and Table 6 outlined the potential degradation pathway for DP-1 to DP-5. Degradation product-2 and degradation product-3 form acidic stress state. The acid splits the bond between N–C and DP-2 shape. Besides, acidic cleavage occurs on an A-ring to form DP-3. The DP-2 was shown in Figure 5, *b* (m/z 93.06). As shown in Figure 5, *c* the parent ion m/z 93.6 is observed in the mass spectrum and DP-3 (m/z 96.06). The parent ion observed for m/z 96.06 is mass spectrum. DP-4 was formed by breaking of C–N bond of ring A cleavage of C=O to DP-4 in alkaline stress condition two degradation product was observed. The DP-4, as shown in Figure 5, *d* (m/z 78.05). The mass spectrum observes the parent ion into m/z 78.05.

Degradation product (DP-5) was formed by cleavage of C-C bond in ring B of pirfenidone, followed by loss of C=O (Carbonyl group) (m/z 158.1 Fig. 5, *e*).



a) DP-1 *b*) DP-2

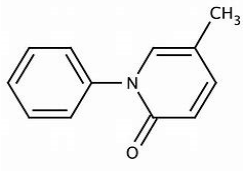
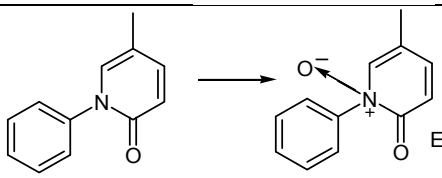
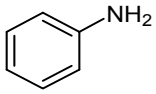
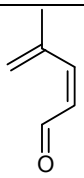
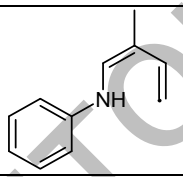
c) DP-3 *d*) DP-4

e) DP-5

Figure 5. MS/MS spectra of Pirfenidone

Table 6

Summary of degraded product

Parameter	Structure of the degraded product	Possible mechanism
Drug		Pirfenidone
DP-1	 Exact Mass: 202.09	H_2O_2 Oxidation at nitrogen
DP-2	 Exact Mass: 93.06	Acidic breakdown of bond
DP-3	 Exact Mass: 96.06	Acidic breakdown of two bond
DP-4	 Exact Mass:78.05	Nitrogen carbon bond break in base and benzene ring separate
DP-5		The breakdown of C=O removed in NaOH alkaline condition

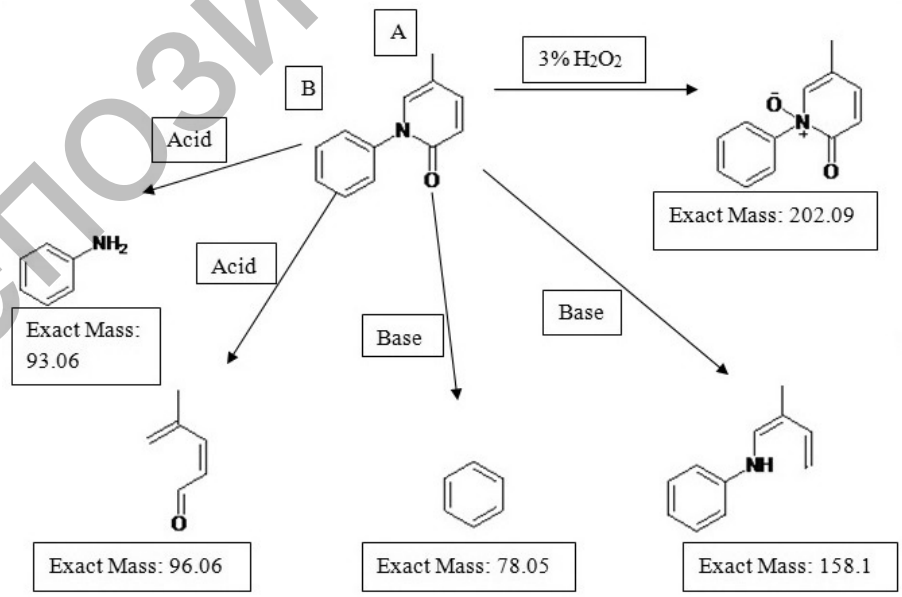


Figure 6. Formation of DP-1, DP-2, DP-3, DP-4, and DP-5 after degradation study

Conclusions

The proposed HPTLC method for estimation of Pirfenidone in pharmaceutical dosage form was found to be accurate, precise, specific and less time consuming. The method was validated as per guidelines of ICH Q2R1. The developed HPTLC method was able to quantitative Pirfenidone in presence of its degradation products. Thus, it can represent good method for analysis of Pirfenidone as there are no reported methods for the same. The fragmentation patterns obtained from HPTLC-MS / MS methods were predicted to show two degradation products in acid and base hydrolysis, while hydrogen peroxide oxidation resulted in one degradation product. Comparison the IR spectrum of pure drugs and finding m /z ratios with various peaks in MS spectra with degradation materials was performed. Thus the present research may be used for routine quality control of pirfenidone-containing pharmaceutical formulations.

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Пирфенидонды анықтаудың ӨЖСХ әдістемесін әзірлеу мен валидациялау және масс-спектроскопия көмегімен алынған ыдырау өнімдерінің сипаттамасы

Пирфенидон өкпенің жеңіл және орташа идиопатикалық фиброзын емдеу үшін мақұлданған жаңа антифиброзды препарат ретінде қолданылады. Әдебиеттерді талдау көрсеткендей, пирфенидонды өнімділігі жоғары сұйық хроматография (ӨЖСХ) көмегімен анықтау әдістемесін валидациялау, сондай-ақ тандемдік масс-спектрометрия (ТМС) әдісінің көмегімен деградация өнімдерін сипаттау осы уақытқа дейін ұсынылмаған. Стационарлық фаза ретінде силикагельмен алдын-ала жабылған пластиналар пайдаланылды. Пирфенидонның ең жақсы бөлінуі (Rf = 0,49±0,03) 315 нм кезінде жылжымалы фаза ретінде метанол:этилацетат:толуол (1:2:7 көлем) қоспасын қолданғанда денситометриялық талдау арқылы анықталды. Деградация талдауы ICH Q2 (R1) әдістемесіне сәйкес жүргізілді. Ыдырау өнімінің бөлінуі ӨЖСХ әдісімен жүргізілді және ТМС әдісі арқылы сипатталды. Барлық валидация параметрлері диапазон шегінде табылды. Сонымен қатар, пирфенидонның ықтимал деградация жолы ұсынылды. Ұсынылып отырған пирфенидонды анықтайтын ӨЖСХ әдісі сезімтал, қарапайым, дәл, экономикалық тиімді және сенімді болып шықты. Бұл әдіс дәрі-дәрмектер мен таблеткалардың массасын талдау, деградацияны зерттеу үшін қолданыла алады. Дәрілік заттың деградацияға ұшырау механизмі дәрілік зат қоспаларының таралу бейінін анықтау үшін пайдаланылуы мүмкін пирфенидонның ыдырау өнімдерін идентификациялауға қосымша көмектеседі.

Кілт сөздер: өнімділігі жоғары сұйық хроматография, пирфенидон, тандемдік масс-спектрометрия әдісімен зерттеулер, деградация механизмі.

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Разработка и валидация методики ВЭЖХ определения пирфенидона и характеристика продуктов разложения с помощью масс-спектроскопии

Пирфенидон используется в качестве нового антифиброзного средства, одобренного для лечения легкого и умеренного идиопатического фиброза легких. Анализ литературы показал, что валидация методики определения пирфенидона с помощью высокоэффективной жидкостной хроматографии (ВЭЖХ), а также описание продуктов деградации с помощью метода тандемной масс-спектрометрии (ТМС) до настоящего времени не представлены. В качестве стационарной фазы были использованы пластины с предварительным покрытием силикагелем. Наилучшее разделение пирфенидона (Rf = 0,49±0,03) было отмечено при использовании в качестве подвижной фазы метанол: этилацетат: толуол (1:2:7 об.) смеси при 315 нм с помощью денситометрического анализа. Анализ деградации проводился в соответствии с методологией ICH Q2 (R1). Выделение продукта разложения проводили методом ВЭЖХ и характеризовали с помощью метода ТМС. Все параметры валидации были найдены в пределах диапазона. Также был предложен возможный путь деградации пирфенидона. Предложенный метод ВЭЖХ определения пирфенидона оказался более чувствительным, простым, точным, достоверным, экономически эффективным и надежным. Он может быть применен для анализа массы лекарств и таблеток, исследования деградации. Механизм деградации лекарственного средства дополнительно поможет идентифицировать продукты разложения пирфенидона, которые могут использоваться для определения профиля распределения примесей лекарственного средства.

Ключевые слова: высокоэффективная жидкостная хроматография, пирфенидон, исследования методом тандемной масс-спектрометрии, механизм деградации.

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