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Research Article

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ABSTRACT

Defactinib is an orally bioavailable and selective inhibitor of focal adhesion kinase (FAK), which is involved in multiple carcinogenic signaling pathways. The present study was undertaken to develop and validate a simple, rapid, and reliable high-performance thin-layer chromatography (HPTLC) method for the estimation of defactinib in bulk drug. Defactinib API was used as the working standard, and chromatographic separation was achieved on pre-coated silica gel 60 F254 HPTLC plates using toluene: methanol: glacial acetic acid in the optimized ratio of 8:2:0.5 (v/v/v) as the mobile phase. The chamber was saturated for 30 min, and the plate was developed to a solvent-front distance of 80 mm. The developed method was validated in accordance with the principles of ICH Q2(R2) guidelines with respect to linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness. The method exhibited good linearity over the concentration range of 1–8 µg/band, with a correlation coefficient (R^2) of 0.99496. The intra-day and inter-day precision studies showed %RSD values below 2%, indicating satisfactory precision. Accuracy studies performed at 80%, 100%, and 120% concentration levels showed percentage recoveries within 98.5–99.5%. The calculated LOD and LOQ were 0.49 µg/band and 1.51 µg/band, respectively. Robustness studies demonstrated that minor deliberate variations in mobile-phase composition, chamber saturation time, and detection wavelength did not significantly affect the method performance. The developed HPTLC method provides a simple, accessible, and reliable approach for routine estimation and quality assessment of defactinib in bulk drug.

KEYWORDS: Defactinib, HPTLC, Method development, ICH guigelines(Q2R2), Linearity

1. Introduction

Defactinib chemically known as N methyl-4-({4-[({3-methyl(methylsulfonyl)aminopyrazin-2-yl}methyl)amino]-5-(trifluoromethyl)pyrimidin2-yl}amino)benzamide. Defactinib has a molecular weight of 510.49 g/mol and a molecular formula of $C_{22}H_{21}F_3N_3O_3S$. The structure of defactinib showed in the figure 1.

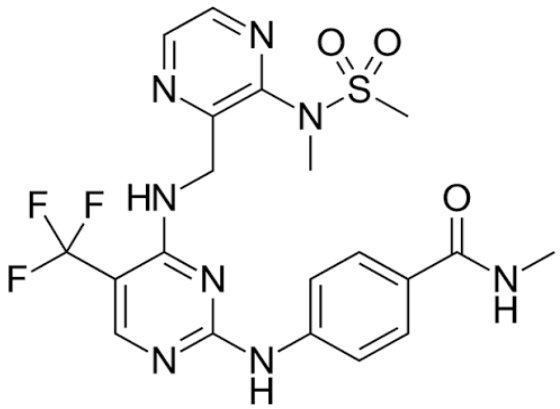


Figure 1: Structure of defactinib

Defactinib is a novel oral bioavailable selective inhibitor of focal adhesion kinase (FAK), a non-receptor tyrosine kinase implicated in multiple carcinogenic signaling pathways.[2] Many cancers, including breast, colon, and ovarian cancers, have elevated levels of FAK, and inhibiting it may make cancer cells more susceptible to chemotherapeutic drugs.[3] Defactinib is a potent and selective ATP-competitive small chemical inhibitor of proline-rich tyrosine kinase 2 (Pyk2) and FAK with an IC50 of less than 0.6 nM.[4] Clinically, the medication is well tolerated, with few drug-drug interactions, constant bioavailability regardless of food consumption, with the main toxicities being headache, weariness, low-grade nausea, and a temporary Gilbert's-type isolated unconjugated hyperbilirubinemia.[5] Focal adhesion kinase (FAK), commonly referred to as protein tyrosine kinase 2, is inhibited by the investigational oral bioavailable small molecule defactinib (DB). Tumor suppression would result from activating the NF2 gene, which codes for Merlin, by inhibiting FAK, which is typically active in malignancies.[6]

AVMAPKI FAKZYNJA CO-PACK, a first-in-class dual rapidly accelerated fibrosarcoma (RAF)/MEK clamp, was approved by the US Food and Drug Administration (FDA) in May 2025 to treat recurrent KRAS-mutated low-grade serous ovarian carcinoma when combined with defactinib, a selective focal adhesion kinase (FAK) inhibitor.[7]

Structure–activity relationship

The para-fluorophenyl ring, tertiary amine linker, and pyrimidine core of defactinib are put together by amide and aryl coupling.[12]

The pyrimidine core of defactinib preserves planarity for efficient binding and permits important hydrogen bonding. By fitting into a hydrophobic region, the para-fluorophenyl group increases metabolic stability and affinity. Its tertiary amine linker improves pharmacokinetic characteristics and guaranties correct alignment.[13]

2. Materials and methods

2.1. Chemicals and Reagents:

Defactinib API was obtained from Dhamtec Pharma and Consultants, Navi Mumbai, Maharashtra, India, and was used as the working standard for the development and validation of the HPTLC method. Toluene, methanol, and glacial acetic acid used for preparation and optimization of the mobile phase were of analytical/HPLC grade and were procured from reputed pharmaceutical or laboratory chemical suppliers. Pre-coated silica gel 60 F254 HPTLC plates were used as the stationary phase.

2.2. Sample preparation:

An accurately weighed quantity of 1 mg of defactinib API was transferred into a 1 mL volumetric flask and dissolved in methanol. The solution was sonicated for sufficient time to ensure complete dissolution and the volume was made up to the mark with methanol to obtain a standard stock solution of 1 mg/mL (1000 µg/mL). The prepared solution was used for HPTLC method development and validation studies.

2.3. Instrumentation:

The CAMAG Linomat V sample applicator, Twin Trough development chamber (10 x 10 cm), UV cabinet with dual wavelength UV lamp, Hamilton syringe (100 µL), CAMAG TLC Scanner 3, and WinCATS software version 1.4.4 were all part of the CAMAG HPTLC system. The Silica Gel 60 F254 plates were 20 cm by 20 cm and had a thickness of 0.25 mm.

2.4. Optimization of analytical factors:

The optimization of various analytical factors was carried out to obtain suitable chromatographic separation and reproducible results for the determination of Defactinib by HPTLC. Different chromatographic conditions were evaluated, including the composition of the mobile phase, sample application, chamber saturation, and development conditions.

Mobile Phase Composition:

Different ratios of toluene, methanol, and glacial acetic acid were investigated to obtain satisfactory migration and compact peak shape of defactinib. The mobile phase consisting of toluene: methanol: glacial acetic acid in the ratio of 8:2:0.5 (v/v/v) provided satisfactory chromatographic performance and was selected as the optimized mobile phase.

Application of Sample:

The defactinib standard solution was applied as 6mm bands on pre-coated silica gel 60 F254 HPTLC plates using a suitable sample applicator. The application conditions were optimized to obtain sharp and symmetrical bands with good reproducibility.

Development Conditions:

The development chamber was saturated with the optimized mobile phase for 30 mins before chromatographic development. The plate was developed to a suitable solvent-front distance maintained at 80 mm and subsequently dried to remove residual solvent before densitometric scanning.

3. Results and Discussion:

3.1. Method Validation:

The ICH Q2(R2) guidelines' guiding principles were followed in the validation of the analytical method. To determine the created method's applicability, accuracy, and dependability, a number of validation characteristics were evaluated. These characteristics included robustness, limit of quantification (LOQ), limit of detection (LOD), linearity, precision, and accuracy.

3.1.1. Working range and linearity:

The linearity of the developed HPTLC method for defactinib was assessed over a concentration range of 1–8 µg/band. Calibration curves were prepared by plotting the peak area against the corresponding concentrations of defactinib standards. The obtained correlation coefficient (R^2)

was 0.99496, demonstrating a good linear relationship between concentration and peak area over the selected working range.

3.1.2. Precision:

The precision of the developed HPTLC method was assessed by evaluating intra-day and inter-day precision. Intra-day precision was determined by analyzing the same sample solution in triplicate on the same day, whereas inter-day precision was evaluated by analyzing the sample solutions on three consecutive days. The percentage relative standard deviation (%RSD) values obtained for both intra-day and inter-day precision were below 2%, indicating satisfactory repeatability, reproducibility, and reliability of the developed HPTLC method.

3.1.3. Accuracy:

Recovery studies:

Accuracy of the proposed methods was evaluated by the standard addition method at three concentration levels, namely 80%, 100%, and 120%. A fixed quantity of defactinib API solution was used as the sample solution, and accurately weighed defactinib API was used as the standard for spiking. The required quantities of defactinib standard were added to the sample solution to obtain the 80%, 100%, and 120% concentration levels. Each concentration level was prepared and analyzed in triplicate. Peak areas were recorded for the HPTLC methods, respectively. The percentage recovery was calculated from the amount of defactinib recovered relative to the amount added. The percentage recovery of defactinib was found to be within the range of 98.5–99.5%, with low %RSD values.

3.1.4. Limit of detection (LOD) and limit of quantification (LOQ):

The LOD and LOQ of the developed HPTLC method were determined using the equations LOD = 3.3 × (SD/S) and LOQ = 10 × (SD/S), where SD represents the residual standard deviation and S denotes the slope of the calibration curve. The calculated LOD and LOQ values for defactinib were 0.49 µg/band and 1.51 µg/band, respectively. These results demonstrate the satisfactory sensitivity of the developed HPTLC method for the determination of defactinib.

3.1.5. Robustness:

The suggested method's robustness was assessed by introducing small, but intentional changes to the analytical parameters and measuring the impact in terms of %RSD. Six identical concentrations of typical defactinib solutions were examined. The detection wavelength was

adjusted by ±5 nm for the UV technique. Intentional modifications were made to the mobile-phase composition and chamber saturation time for the HPTLC technique. The optimal ratio of toluene: methanol: glacial acetic acid (8:2:0.5) was changed to 7.9:1.9:0.4 and 8.1:2.1:0.6, and the chamber saturation duration was changed by ±5 minutes. Based on the acquired %RSD values, the impact of these differences was assessed.

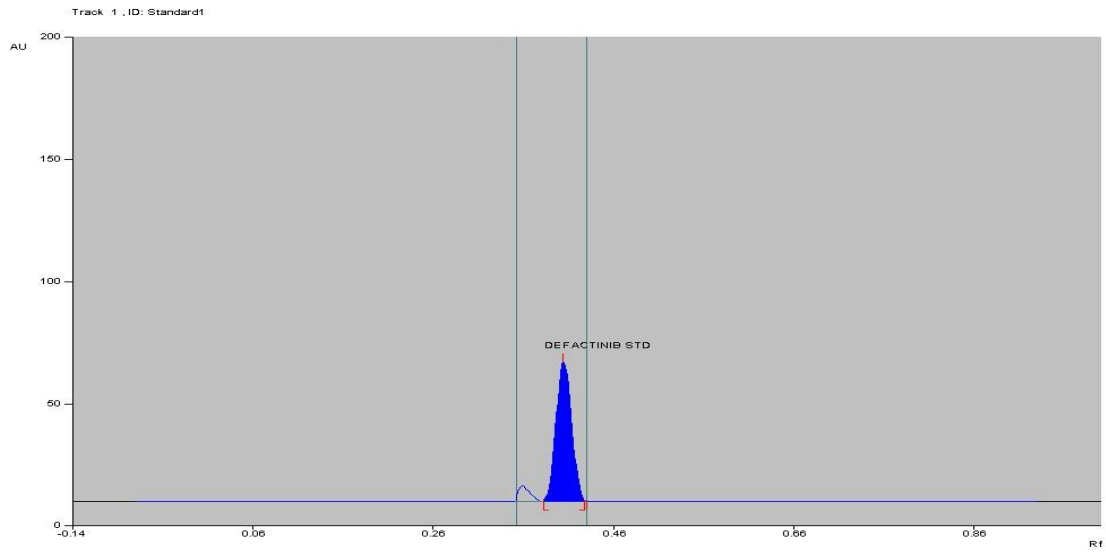


Fig no:2 Standard densitogram of defactinib API

Linearity and Calibration Curve

Increased area was directly proportional to concentration, according to the calibration curve. The regression coefficient was determined to be 0.99496 using the equation y=311.893+637.944*X, and the linearity curve was confirmed to be linear. In Figure 3, the linearity curve is displayed. Tables 1 through 3 presented the precision, accuracy, and robustness results.

S. No	Concentration (µg/µl)	Peak area (AU)
1	1	796.8
2	2	1558.2
3	4	3125.3
4	6	4240.7
5	8	5235.3

Table no:1 Linearity studies for defactinib API

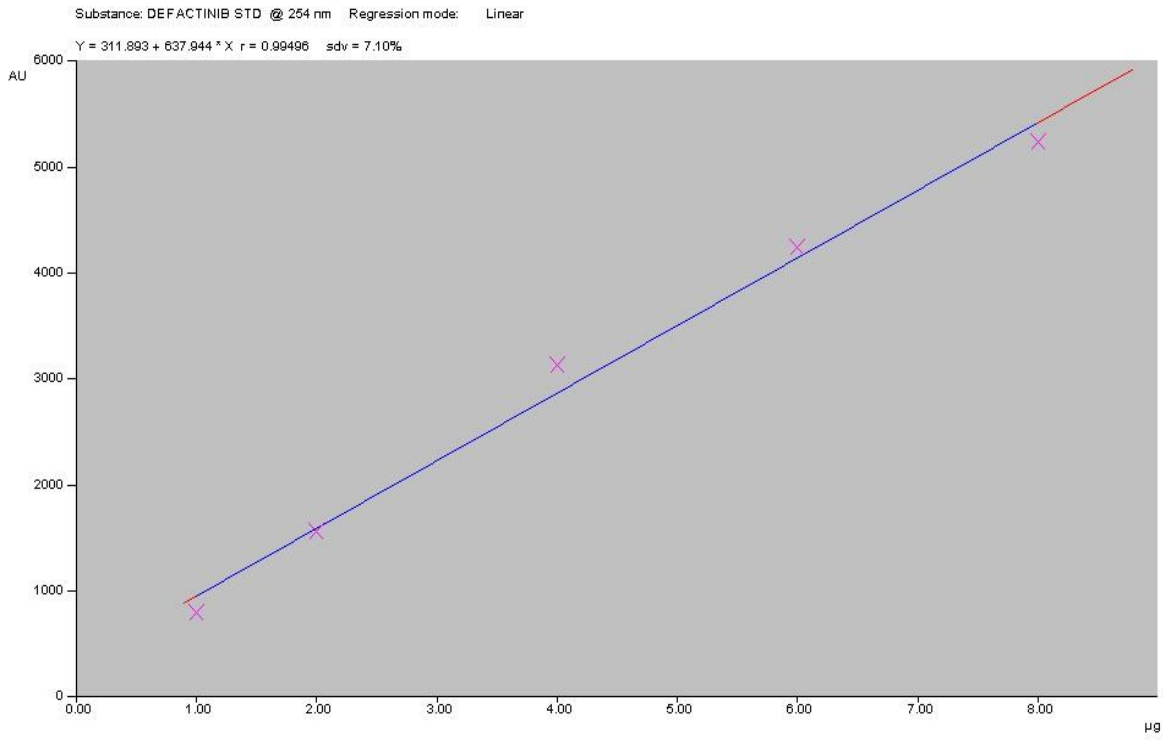


Fig no:3 Linearity curve of defactinib

PRECISION

CONCENTRATION (µg/ml)	PEAK AREA	MEAN AREA	SD	%RSD
INTER-DAY PRECISION				
1	1720.4			
1	1712.5	1702.53	24.42	1.43%
1	1674.7			
2	2020.5			
2	2019.1	2005.93	24.02	1.19%
2	1978.2			
4	3125.2			
4	3110.7	3130.07	22.20	0.70%
4	3154.3			

INTRA-DAY PRECISION

1	1734.4			
1	1728.2	1718.9	21.7	1.26%
1	1694.1			
2	2011.5			
2	2015.1	2004.9	14.60	0.72%
2	1988.2			
4	3065.2			
4	3145.7	3108.4	40.57	1.30
4	3114.3			

Table No:2 Precision results of the developed HPTLC method

ACCURACY

Concentration	**Amount added**	**Amount found**	**Recovery (%)**	**Mean**	**Standard deviation**	**%Rsd**
80%	5	4.97	99.4			
	5	4.98	99.6	99.53	0.11547	0.11%
	5	4.98	99.6			
100%	4	3.98	99.5			
	4	3.87	96.75	98.5	1.520691	1.54%
	4	3.97	99.25			
120%	6	5.98	99.66			
	6	5.97	99.5	99.55	0.096225	0.09%
	6	5.97	99.5			

Table no:3 Recovery studies for defactinib (API)

ROBUSTNESS

PARAMETERS	Variations	Rf (Defactinib)
Changes in mobile phase ratio	7.9:1.9:0.4	0.39
	8.1:2.1:0.6	0.40
Changes in chamber saturation time	25 mins	0.40
	35mins	0.41
Changes in detection wavelength	269nm	0.39
	279nm	0.40

Table no:4 Robustness evaluation of the developed HPTLC method

4. CONCLUSION

The present study demonstrates the applicability of HPTLC as a simpler and more accessible chromatographic platform for routine pharmaceutical analysis of defactinib API. The method is therefore particularly relevant for laboratories involved in bulk-drug quality assessment where rapid analysis, operational simplicity, and cost-effective instrumentation are important considerations. The developed method complements the existing LC–MS/MS approaches by providing a comparatively simple, accessible, and routine-quality-control-oriented strategy for defactinib estimation. Overall, this study expands the available analytical options for defactinib and provides a useful foundation for its routine pharmaceutical quality assessment.

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Ethics, Consent to Participate, and Consent to Publish declarations: Not applicable.

References:

1. MedChem Express. Defactinib (VS-6063) [Internet]. Monmouth Junction (NJ): MedChemExpress; [cited 2026 Sep 10]. Available from: https://www.medchemexpress.com/Defactinib.html
2. Banerjee SN, Van Nieuwenhuysen E, Aghajanian C, D'Hondt V, Monk BJ, Clamp A, Prendergast E, Oaknin A, Ring K, Colombo N, Holloway RW. Efficacy and safety of avutometinib±defactinib in recurrent low-grade serous ovarian cancer: primary analysis of ENGOT-OV60/GOG-3052/RAMP 201. Journal of Clinical Oncology. 2025 Sep 1;43(25):2782-92.
3. Gerber DE, Camidge DR, Morgensztern D, Cetnar J, Kelly RJ, Ramalingam SS, Spigel DR, Jeong W, Scaglioni PP, Zhang S, Li M. Phase 2 study of the focal adhesion kinase inhibitor defactinib (VS-6063) in previously treated advanced KRAS mutant non-small cell lung cancer. Lung Cancer. 2020 Jan 1;139:60-7.
4. Infante JR, Camidge DR, Mileshkin LR, Chen EX, Hicks RJ, Rischin D, Fingert H, Pierce KJ, Xu H, Roberts WG, Shreeve SM. Safety, pharmacokinetic, and pharmacodynamic phase I dose-escalation trial of PF-00562271, an inhibitor of focal adhesion kinase, in advanced solid tumors. Journal of Clinical Oncology. 2012 May 1;30(13):1527-33.
5. Kulkarni NS, Gupta V. Repurposing therapeutics for malignant pleural mesothelioma (MPM)–updates on clinical translations and future outlook. Life sciences. 2022 Sep 1;304:120716.
6. Del Valle MM, Colon EM, Kettimuthu K, Maner-Smith K, Olson JJ, Kucheryavykh LY. Window of opportunity study measuring defactinib and avutometinib delivery in glioblastomas. Cancer Chemotherapy and Pharmacology. 2026 Mar 6;96(1):22.
7. Arang N, Lubrano S, Rigiracciolo DC, Nachmanson D, Lippman SM, Mali P, Harismendy O, Gutkind JS. Whole-genome CRISPR screening identifies PI3K/AKT as a downstream component of the oncogenic GNAQ–focal adhesion kinase signaling circuitry. Journal of Biological Chemistry. 2023 Feb 1;299(2).
8. Zeng XQ, Li N, Ma LL, Tseng YJ, Zhao NQ, Chen SY. Prognostic value of focal adhesion kinase (FAK) in human solid carcinomas: a meta-analysis. PLoS One. 2016 Sep 16;11(9):e0162666.
9. Qiao W, Wang W, Liu H, Guo W, Li P, Deng M. Prognostic and clinical significance of focal adhesion kinase expression in breast cancer: a systematic review and meta-analysis. Translational Oncology. 2020 Nov 1;13(11):100835.
10. Hu HH, Wang SQ, Shang HL, Lv HF, Chen BB, Gao SG, Chen XB. Roles and inhibitors of FAK in cancer: current advances and future directions. Frontiers in pharmacology. 2024 Feb 12;15:1274209.
11. Aakriti J, Vithalkar MP, Maity S, Baby K, Nagareddy PR, Nayak Y. Focal adhesion kinase (FAK): emerging target for drug-resistant malignant tumors. Molecular Biology Reports. 2025 Dec;52(1):248.
12. Yang M, Xiang H, Luo G. Targeting focal adhesion kinase (FAK) for cancer therapy: FAK inhibitors, FAK-based dual-target inhibitors and PROTAC degraders. Biochemical pharmacology. 2024 Jun 1;224:116246.
13. Prabhu KS, Kuttikrishnan S, Mariyam Z, Habeeba U, Panicker AJ, Masoodi T, Junejo K, Uddin S. PI3 K/AKT/mTOR pathway and its role in breast cancer stem cells. Naunyn-schmiedeberg's Archives of Pharmacology. 2025 Dec;398(12):16779-95.
14. Sherma V. Review of HPTLC in drug analysis. J AOAC Int. 2010;93(3):754-764.

15. Jadaun GP, Dixit S, Saklani V, Mendiratta S, Jain R, Singh S. HPLC for proteins: principles, methods and applications. Pharm Methods. 2017;8(1)
16. Mahesh A, Mueen Ahmed K.K., Bandar E, Aldhubaib, Sree Harsha. High performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery. Pharmaceutical Methods. 2011:9(1):75.
17. Breaux J, Boulas P. Understanding and implement analytical methods development and validation. Pharm Technol Anal Chem. 2019:6-13.
18. David G. Watson. Pharmaceutical Analysis: A Text Book for Pharmacy Students and Pharmaceutical chemists. 2nd edition. Elsevier Publication; 2005:286.
19. International Conference on Harmonisation Q2(R2): validation of analytical procedures: text and methodology. 20051-13.