Hplc Method Development And Validation Of Ivacaftor And Lumacaftor, Characterization Of Its Degradants By Lc-Ms/Ms

Bhagya Kumar Tatavarti[1], Vijaya Bhaskar KPT[2] and Kiran Kumar Khatare[3]

[1,2,3]Department of Chemistry, K.B.N. College(A), Vijayawada-520 001., A.P.

ABSTRACT

Objective: An assay method was developed and validated for the simultaneous estimation of Ivacaftor and Lumacaftor using RP-HPLC.

Methods: An effective chromatographic separation was achieved using Hyper clone 5µ BDS C18 130°A column of dimensions 250X4.6mm, 5µm, as a stationary phase.HSA pH-2.5/OPAand acetonitrile in 60:40 v/v was used as a mobile process with a rate of 1 ml/min and UV detection was carried outat 260nm, respectively. Isocratic chromatography at ambient temperature was performed.

Results: Ivacaftor and Lumacaftor were separated by a running time of around 10min,at 3.152min. and 6.932min. respectively. By injecting the norm six times, device suitability parameters were studied and the outcomes were well under the acceptance criteria. The linearity analysis was performed at levels ranging from 25% to 150% and the R^2 value was found to be 0.999.

Conclusion: Assay method validation was performed by using the marketed formulation and found to be within the limit. Degradation tests were conducted and the degradants were characterized by using LC-MS/MS.

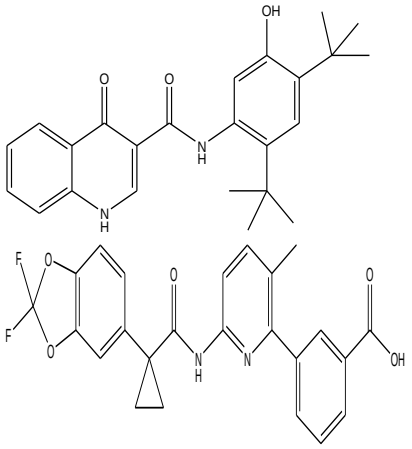
Key Words: Development, Validation, Ivacaftor, Lumacaftor, LC-MS/MS.

INTRODUCTION

1. Ivacaftor[Fig.1] is a medication used to treat cystic fibrosis[Shteinberg Michal et al, 2021; Warnock L, 2023; Shteinberg M, 2021] in people with certain mutations in the cystic fibrosis trans membrane conductance regulator (CFTR) gene (primarily the G551D mutation), who account for 4–5% cases of cystic fibrosis. It is also included in combination
   medications, lumacaftor/ivacaftor, tezacaftor/ivacaftor,
   and elexacaftor / tezacaftor / ivacaftor [Middleton PG, 2019] which were used to treat people with cystic fibrosis.Cystic fibrosis is caused by any one of several defects in the CFTR protein [Sharma S, 2018; Csanády L, 2019], which regulates fluid flow within cells and affects the components of sweat, digestive fluids, and mucus [Ohar JA, 2019; Dash S, 2018]. One such defect is the G551D mutation [Slocombe L, 2021; Monroe JG et al., 2022], in which the amino acid glycine (G) [Wang W, 2013] in position 551 is replaced with aspartic acid [Adelnia Hossein, 2019] (D). G551D is characterized by a dysfunctional CFTR protein on the cell surface. In the case of G551D, the protein is trafficked to the correct area, the epithelial cell surface [Gudipaty SA et al, 2017], but once there the protein cannot transport chloride through the channel. Ivacaftor, a CFTR potentiator, improves the transport of chloride through the ion channel by binding to the channels directly to induce a non-conventional mode of gating which in turn increases the probability that the channel is open.

2. **Lumacaftor** (**VX-809**)[Fig. 1] is a pharmaceutical drug that acts as a chaperone[Bascos NA, 2019; Sadigh-Eteghad S, 2015] during protein folding and increases the number of CFTR proteins that are trafficked to the cell surface. It is available in a single pill with ivacaftor; the combination, lumacaftor/ivacaftor (brand name Orkambi), is used to treat people with cystic fibrosis who are homozygous [Gabbett MT et al, 2019] for the F508del mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, the defective protein that causes the disease. Lumacaftor is a drug used in combination with Ivacaftor as the fixed dose combination product Orkambi for the management of Cystic Fibrosis (CF) in patients aged 6 years and older. Cystic Fibrosis is an autosomal recessive disorder caused by one of several

different mutations in the gene for the Cystic Fibrosis Trans membrane Conductance Regulator (CFTR) protein, a trans membrane ion channel involved in the transport of chloride and sodium ions across cell membranes [Mishra NN, 2011; Lombard J. 2014] of the lungs, pancreas [Banks PA, 2010; Wang Y et al, 2011], and other organs. Mutations in the CFTR gene result in altered production, misfolding, or function of the CFTR protein and consequently abnormal fluid and ion transport across cell membranes. As a result, CF patients produce thick, sticky mucus that clogs the ducts of organs where it is produced making patients more susceptible to infections [Riley LW, 2019; Negut Irina, 2018], lung damage, pancreatic insufficiency [Capurso Gabriele, 2019; RitivoiuMirela-Elena, 2023], and malnutrition [Trehan I, 2013; Mark HE, 2020]. Lumacaftor improves CF symptoms and underlying disease pathology by aiding the conformational stability of F508del-mutated CFTR proteins, preventing misfolding and resulting in increased processing and trafficking of mature protein to the cell surface.



Ivacaftor Lumacaftor

Figure 1: Chemical structures of Ivacaftor and Lumacaftor

There were number of HPLC methods [Karuppasamy C, 2020; Dr. Nagamallika Gorantla, 2019; Sravanthi B, 2016; AkramN. Md, 2017; Saniye Özcan, 2023]and one UPLC method [Balaswami B 2019] was reported in the literature, but these methods are developed only for routine analysis of Lumacaftor and Ivacaftor in bulk and formulation studies. In this present study we developed a new HPLC method for the estimation of Lumacaftor and Ivacaftor in the combined and dosage formsin vitromethod and also the characterization of its degradants carried out by LC-MS/MS.

MATERIAL AND METHODS

Chemicals

Acetonitrile,Ortho phosphoric acid, Hexane sulfonic acid and water all are of HPLC grade were purchased fromMerck India pvt ltd., Worli, Mumbai, India. APIs of Ivacaftor, Lumacaftor were purchased from Spectrum Parma research solutions pvt ltd., Hyderabad.

Equipment

HPLC

The chromatographic device of Waters quaternary pump alliance e-2695, PDA detector 2998 and chromatographic software Empower -2.0 were used.

LC-MS/MS

An HPLC system (waters alliance e2695 model) connected with mass spectrometer QTRAP 5500 triple quadrupole instrument (sciex) was used [Syed Rafi, 2021; Naveen V M K, 2021; Bhavani P, 2020]. By the Empower 2.0 software operation was performed. Working parameters of mass spectrometry after optimization as follows :Ion spray voltage 5500V [Naresh Kumar D, 2017]; temperature source 550°C ; Drying gas temperature 120-250°C; Collision gas –Nitrogen [Supriya T, 2018]; Pressure 55psi; Drying gas flow stream-5mL/min; Delustering potential 40V; Entrance potential 45V ; Exit potential 15V;Capillary voltage 5500V and Dwell time 1Sec respectively.

Preparation of buffer

In 1 It of HPLC Water, 1.8g of Hexane sulfonic acid was dissolved, adjust its pH-2.5 with OPAand filter through 0.45 µ filter paper.

Preparation of mobile phase

Mix Acetonitrile and buffer in 40:60 v/v ratio and sonicated to 5 min. After that filter it by using 0.45µm membrane filter paper.

Diluent

Mobile phase.

Preparation of standard solution

Standard stock solution of Ivacaftor andLumacaftor were prepared by appropriately estimating about25 mg of Ivacaftor and 40 mgof Lumacaftor drug in a 100 ml volumetric flask. Then

the drug was liquified in solvent and filter through a 0.45µ filter. Standard stock solution concentrations of 250µg/ml and 400µg/ml were obtained. Further pipette 5ml of the above solution into a 50ml volumetric flask and make up to the mark with diluents.

Preparation of the solution for samples

TenIvacaftor and Lumacaftor tablets were accurately weighed and triturated to get a fine powder. A 91.6 mg of Ivacaftor and Lumacaftorsample was transferred into a 100 ml volumetric flask and dissolved in diluent. The solution was ultra-sonicated for 10 min and made the volume with diluent. Further pipette 5ml of the above solution into a 50ml volumetric flask and make up to the mark with diluents. The tablet sample solution was then filtered through 0.45 micron syringe filter and utilized for preparing sample solution for the assay.

Optimization of chromatographic conditions

Various combinations of mobile phases were screened with respect to resolution, theoretical plate count, tailing and other system suitability parameters. Finally the separation was performed with freshly prepared the mobile step is composed of Acetonitrile and buffer in 40:60 v/v ratio with a 1 ml/min flow rate. Wave length of 260 nm with injection volume 10 µl and ambient temperature was maintained during the entire process to obtain symmetric peak of Ivacaftorand Lumacaftor.

Method Optimization:

The current study was designed to develop a simple, reliable and rapid analytical RP-HPLC system which can be used to evaluate assay method of current estimation of Ivacaftor and Lumacaftor pharmaceutical and bulk dosages forms. In order to have good results for the assay, the chromatographic conditions were optimized. Different combinations of Ivacaftor and Lumacaftor have been tried to optimize the mobile process. The final working mobile phase was acetonitrile: HSA pH-2.5/OPA at 40:60v/v. Based on its polarity, the mobile phase was selected for each drug. In order to achieve adequate sensitivity for the two smaller proportions of APIs (Ivacaftor and Lumacaftor), detection was carried out at several wavelengths. Finally, as a detection wavelength, the 260 nm wavelength at which the two drugs showed strong absorbance was chosen. The rate of flow was 1.0 ml/min, which is important as it affects the parameters of peak symmetry. The retention time for Ivacaftor and

Lumacaftor was 3.152 min and 6.932 min. respectively. The suggested approach is checked in compliance with the ICH guidelines [ICH Q2(R1); 2005] and found to be within the limit.

Method validation

In acquiescence with ICH recommendations [Shivani C P 2016; V L N Balaji Gupta T, 2021; Mukta D. Naykode et al, 2017; Mayanka Singh, 2011; Ashutosh Kumar S, 2016; Malathi S, 2020], the validity parameters were established.

RESULTS AND DISCUSSION

System suitability

In six replicates, system suitability was achieved by injecting a regular solution containing 25µg/ml Ivacaftor and 40µg/ml of Lumacaftor. The findings suggest that the criteria of system suitability were within the boundaries.

Table 1: Results of system suitability

System suitability parameter	Acceptance criteria	Ivacaftor			Lumacaftor		
		Mean	Std Dev	% RSD	Mean	Std Dev	% RSD
USP Plate count	NLT 2000	4649	19.201	0.41	12245	16.709	0.14
USP Tailing	NMT 2.0	1.05	0.025	1.26	0.96	0.029	1.31
USP Resolution	NLT 2.0	-	-	-	17.06	0.043	0.25
Retention time	NLT 2.0	3.155	0.003	0.10	6.934	0.003	0.04

Mean±SD (n=6)

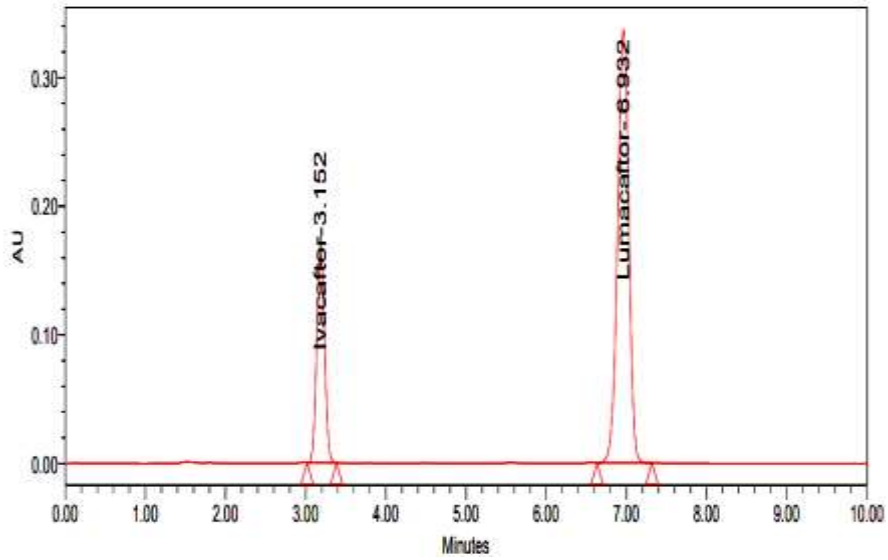


Figure 2: Chromatogram of system suitability

Specificity

At the retention time of Ivacaftor and Lumacaftor, no intervention [Potturi Ramadevi, 2021] from the blank occurred. The process is also unique.

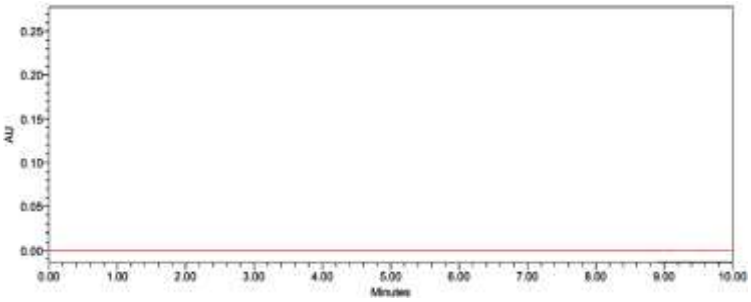


Figure 3: Chromatogram of blank

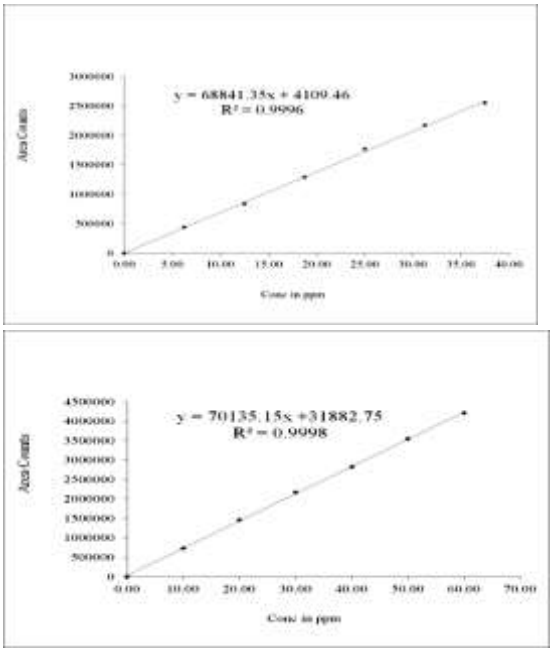
Linearity

Linearity was determined by plotting a curve between peak area to its respective concentration. From this calibration curve [Malak Y, 2020;Vijayakumari M, 2020], it was noticed that the curve was linear over the 6.25-37.5µg /ml Ivacaftor and 10-60µg /ml Lumacaftor concentration range. The calibration curve regression equations were Y=68841.35x + 4109.46 (R^2 =0.999) for Ivacaftor and Y=70135.15x + 31882.75 (R^2 = 0.999) for Lumacaftor.

Table 2: Linearity data

Linearity level	Ivacaftor		Lumacaftor	
	Conc. (µg/ml)	Peak area counts	Conc. (µg/ml)	Peak area counts
Linearity-1	6.25	442624	10.00	729383
Linearity-2	12.50	836984	20.00	1458638

Linearity-3	18.75	1291647	30.00	2174572
Linearity-4	25.00	1768351	40.00	2830368
Linearity-5	31.25	2172646	50.00	3550920
Linearity-6	37.50	2551942	60.00	4207679
Slope	68841.35		70135.15	
Intercept	4109.46		31882.75	
CC	0.99960		0.99984	



A **B**

Figure 4: Linearity plot of (A) Ivacaftor and (B) Lumacaftor

Precision

The precision of this approach was evaluated in terms of inter and intraday variations. The intraday studies were calculated by six repeated tests of the Ivacaftor and Lumacaftor sample solution under the same experimental conditions on the same day. In the same Laboratory, the intermediate precision of this approach was carried out by examining the analysis with various analyst and different instruments [Gomathy S, 2020; SubbaRaoYarlagadda, 2021; Ramachandran D, 2020]. As the percent RSD values were found to be < 2 percent, the method is highly accurate. At each added concentration, good recovery of the drug was achieved; suggesting that the procedure was successful. Below table represents the outcomes given.

Table 3: Results of precision

Parameter	Ivacaftor			Lumacaftor		
	Mean % Recovery	Std Dev	Conc. (µg/ ml)	Mean % Recovery	Std Dev	Conc. (µg/ ml)
Method precision	100.2	0.571	25.0	100.1	0.659	40.0
Intermediate precision	100.1	0.749	25.0	99.9	0.880	40.0

Mean±SD (n=6)

Accuracy

By measuring the recovery experiments at three stages (50 percent, 100 percent, and 150 percent), the precision [Gadhvi M.P, 2013; SenthilRajan D, 2020; Rajakumari R et.al., 2016] of the process was carried out. APIs were prepared at concentrations of 12.5, 25.0, 37.5µg/ ml of Ivacaftor and 20.0, 40.0, 60.0µg/ml of Lumacaftor. For each spike level, the test solution wasinjected three times and as per the test process, the assay was performed and the RSD values were less than 2 percent. Recovery percentage, mean and relative standard deviation have been determined. Recovery values showed that the approach within the desired range was specific.

Table 4: Results of accuracy

Accuracy level	Ivacaftor		Lumacaftor	
	% Recovery	**Std Dev**	**% Recovery**	**Std Dev**
50%	100.4	0.330	100.2	0.870
100%	100.3	0.320	100.1	0.890
150%	99.8	0.690	100.3	0.520

Mean±SD (n=3)

Robustness

By varying flow rate and mobile phase composition, the robustness of the chromatographic process was calculated. It was found that RSD was within the appropriate range.

Table 5: Results of robustness

Change in parameter	Ivacaftor (% RSD)	Lumacaftor (% RSD)
Flow plus (1.1 ml/min)	0.45	0.45
Flow minus (0.9 ml/min)	0.67	0.77

Organic plus (44:56)	0.65	0.31
Organic minus (36:64)	1.08	0.90

RSD- Relative standard deviation; All the values are presented as Mean±SD (n=3)

Forced degradation

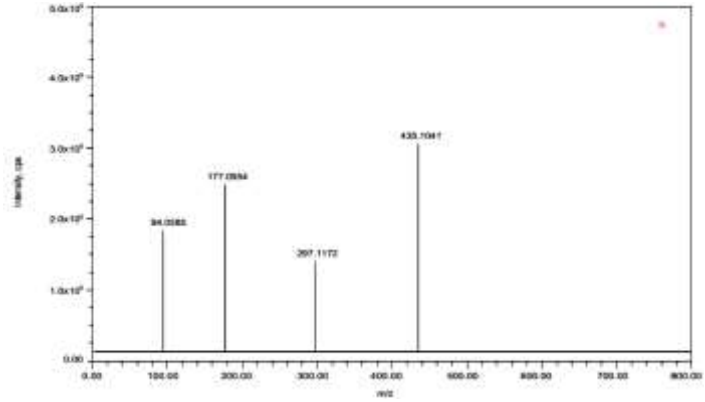
The forced degradation study [CharuPandya P, 2018; BirvaAthavia A et al., 2017; Swati K, 2020] was carried out according to ICH guidelines include acid, base, peroxide, reduction, thermal and hydrolysis degradation. From the chromatograms it is evident that selected drugs were stable under the applied stress conditions though the degradation peaks[Narasimha S. Lakka, 2020; Balasaheb B Chavan et al, 2018; Mukta D. Naykode et al, 2017] were obtained. The formed degradants were characterized by using LC-MS/MS.

Forced degradation:

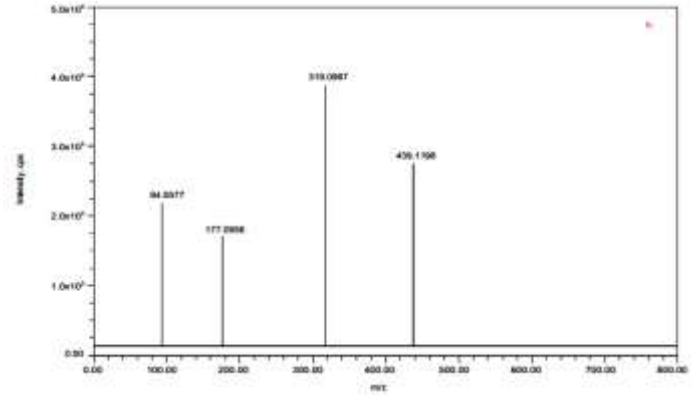
Table 6: Results of forced degradation

Stress condition	**Ivacaftor (%degradation)**	**Lumacaftor (%degradation)**
	Mean	**Mean**
Control degradation	0	0
Acid degradation	11.8	13.4
Alkali degradation	13.4	12.3
Peroxide degradation	14.3	2.2
Reduction degradation	1.8	10.9
Thermal degradation	0.8	10.0
Photolytic degradation	4.8	2.7
Hydrolysis degradation	2.6	4.5

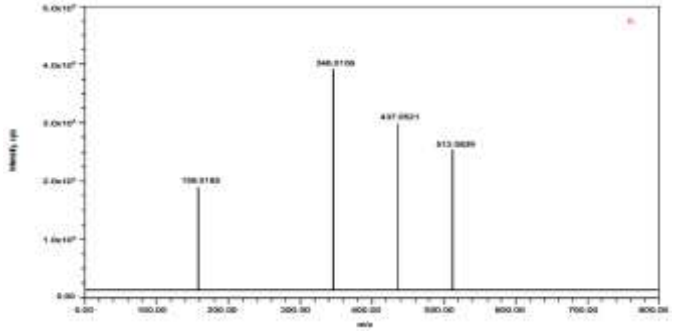
Data expressed as Mean±SD (n=3)



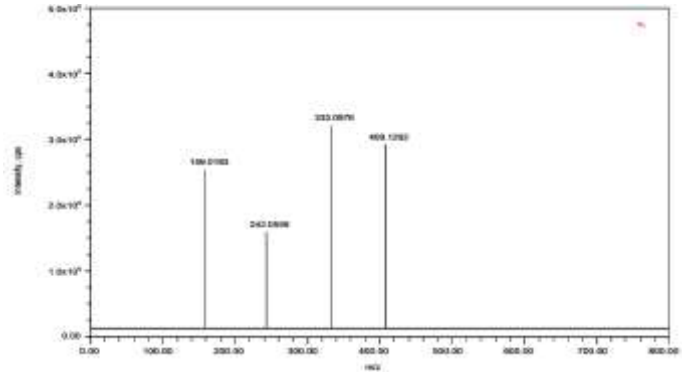
A



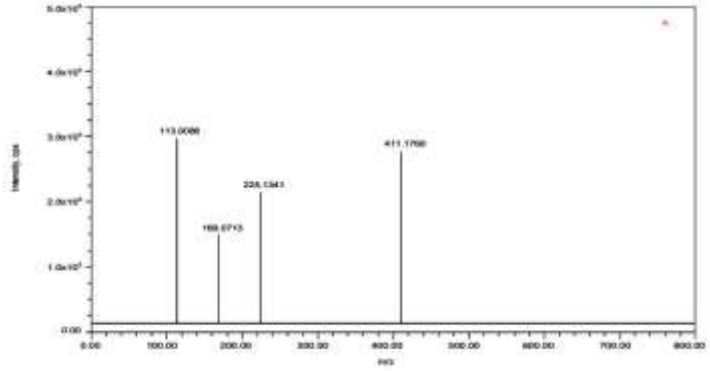
B



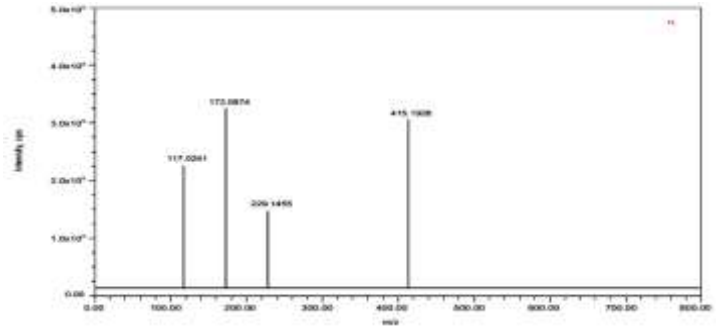
C



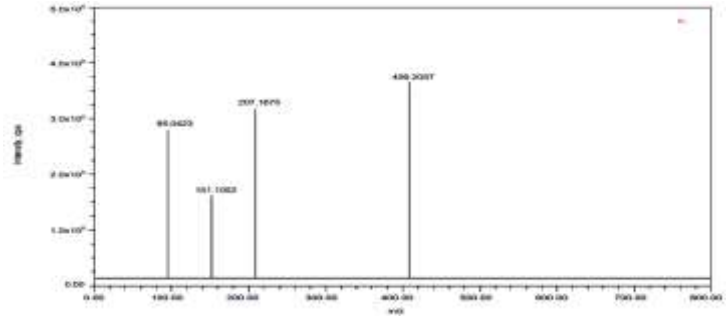
D



E



F

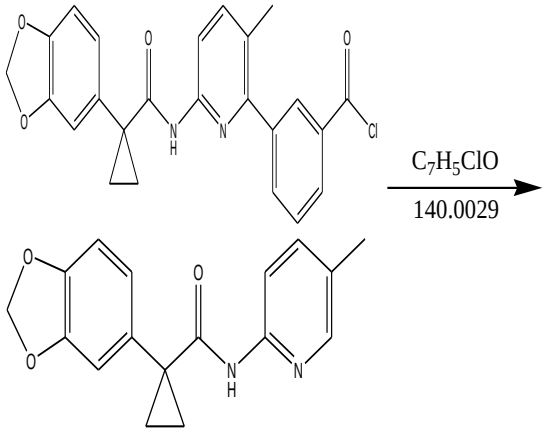


G

Figure 5: Mass spectras of (A) D_1 (B) D_2 (C) D_3 (D) D_4 (E) D_5 (F) D_6 (G) D_7

Collision induced dissociation of Ivacaftor and Lumacaftor

Scheme 1



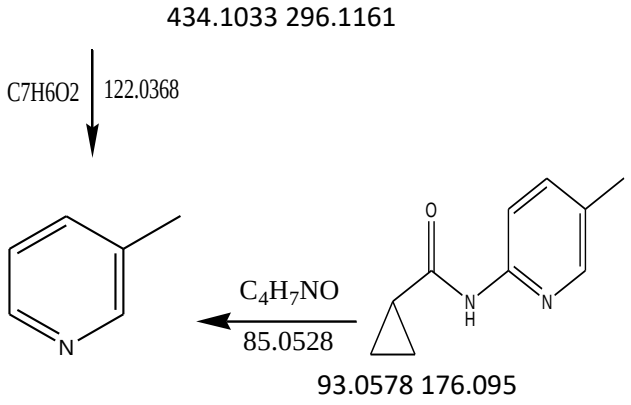
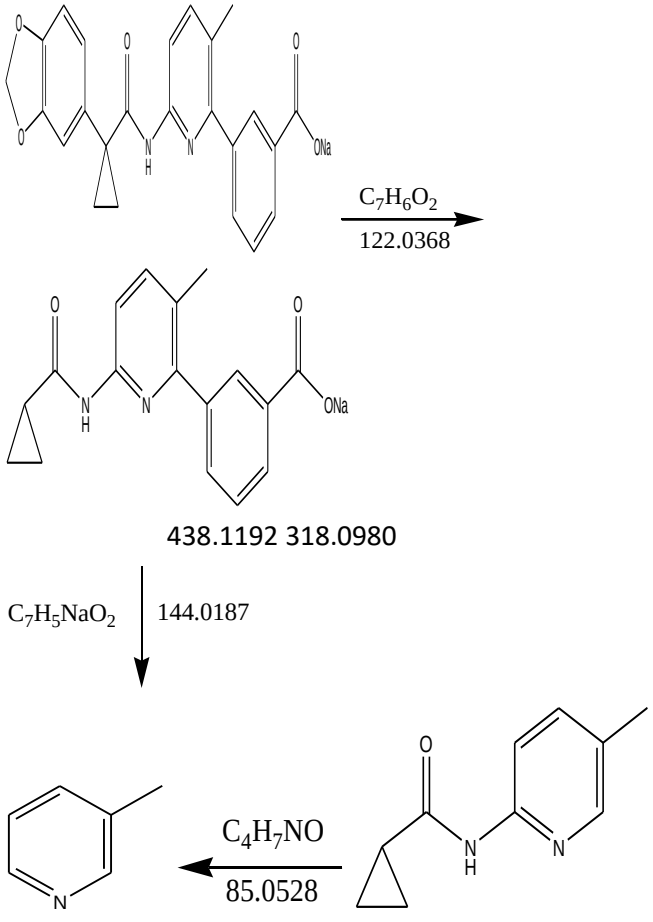


Figure 6: Mechanism for proposed fragmentation of DP_1 of m/z-434

MS/MS degradation product

The fragmentation mechanism of degradation product 1of m/z-434.1033 observed under acidic, degradation conditions is shown in figure 6. Abundant substance ions are seen on the spectrum at m/z-296.1161 (C_7H_5ClOloss), m/z-176.0950 ($C_7H_6O_2$ loss), m/z-93.0578(C_4H_7NO loss). The proposed structures were confirmed by the accurate mass measurements and MS / MS studies.

Scheme 2



93.0578 176.0950

Figure 7: Proposed fragmentation mechanism of DP_2 of m/z-438

MS/MS degradation product

Figure 7 shows the fragmentation process of degradation product 2of m/z-438, which was observed under conditions of Alkali degradation. Abundant product ions are seen on the spectrum at m/z-318.0980 ($C_7H_6O_2$ loss), m/z-176.0950 ($C_7H_5NaO_2$ loss), m/z-93.0578 (C_4H_7NOloss). The proposed structures were confirmed by the accurate mass measurements, MS/MS studies.

Scheme 3

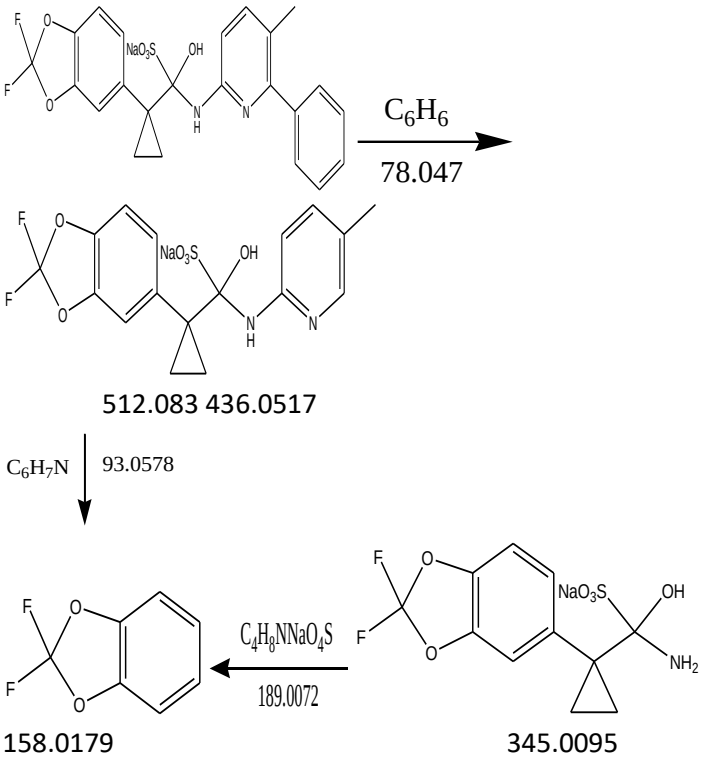


Figure 8: Proposed fragmentation mechanism of DP_3 of m/z-512

MS/MS degradation product

Figure 8 shows the fragmentation mechanism of degradation product 3 of m/z-512.0830, which has observed under conditions of reduction degradation. Abundant product ions are shown in the spectrum at m/z-436.0517 (C_6H_6 loss), m/z-345.0095(C_6H_7N loss), m/z-158.0179 ($C_4H_8NNaO_4S$ loss). The proposed structures were confirmed by the MS/ MS experiments in combination with accurate mass measurements.

Scheme 4

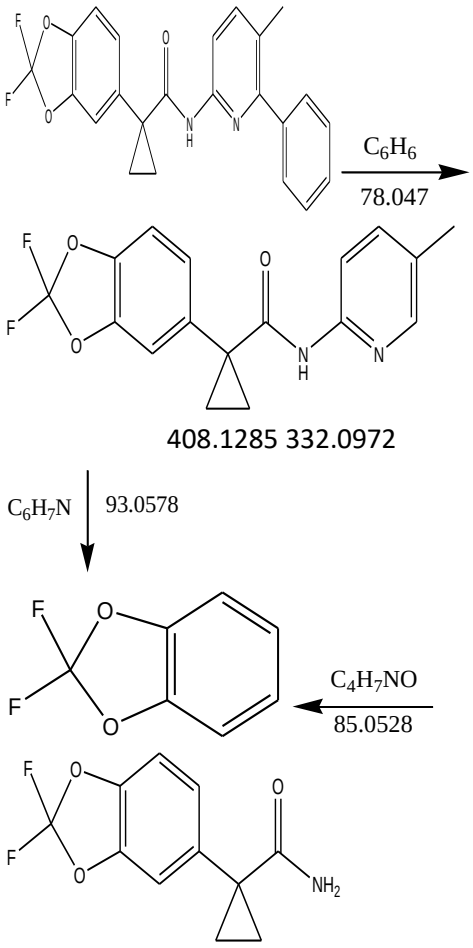


Figure 9: Proposed fragmentation mechanism of DP_4 of m/z-408

MS/MS degradation product

The fragmentation mechanism of degradation product 4 of m/z-408.1285 observed under Thermal degradation conditions is shown in Figure 9. Abundant substance ions shown on the spectrum at m/z-332.0972 (C_6H_6 loss), m/z-241.0550 (C_6H_7N loss), m/z-158.0179 (C_4H_7NO loss, from). The proposed structures wereconfirmed by the MS/MS experiments in combination with accurate mass measurements.

Scheme 5

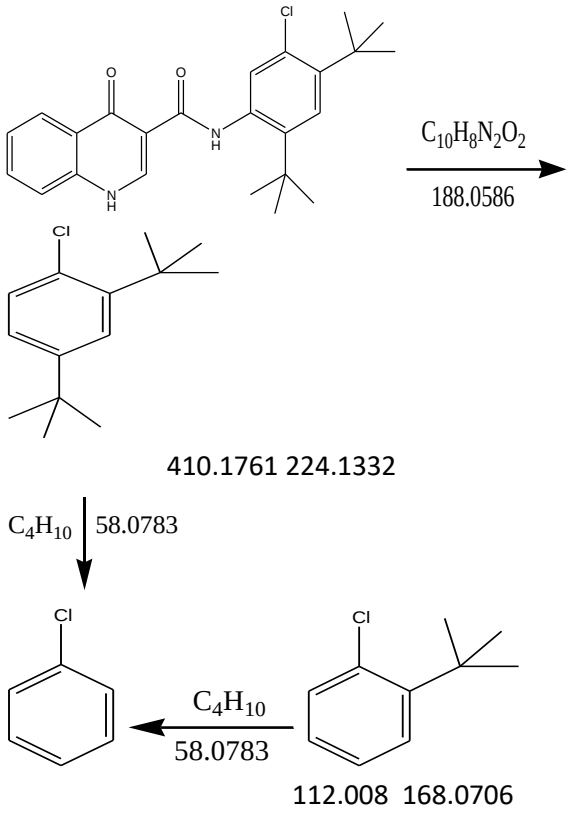
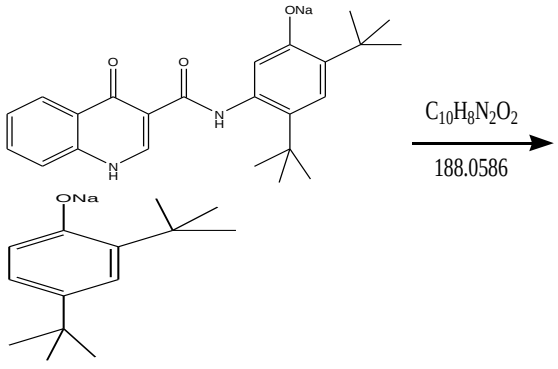


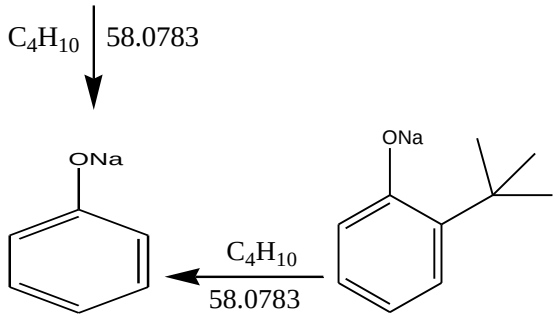
Figure 10: Proposed fragmentation mechanism of DP_5 of m/z-410

MS/MS degradation product

Figure 10shows the fragmentation process of degradation product 5 of m z-410.1761, that was observed under conditions of acid degradation. Abundant substance ions are seen on the spectrum at m/z-224.1332 ($C_{10}H_8N_2O_2$ loss), m/z-168.0706 (C_4H_{10} loss), m/z-112.0080 (C_4H_{10} loss). The proposed structures were confirmed by the MS/MS experiments in combination with accurate mass measurements.

Scheme 6





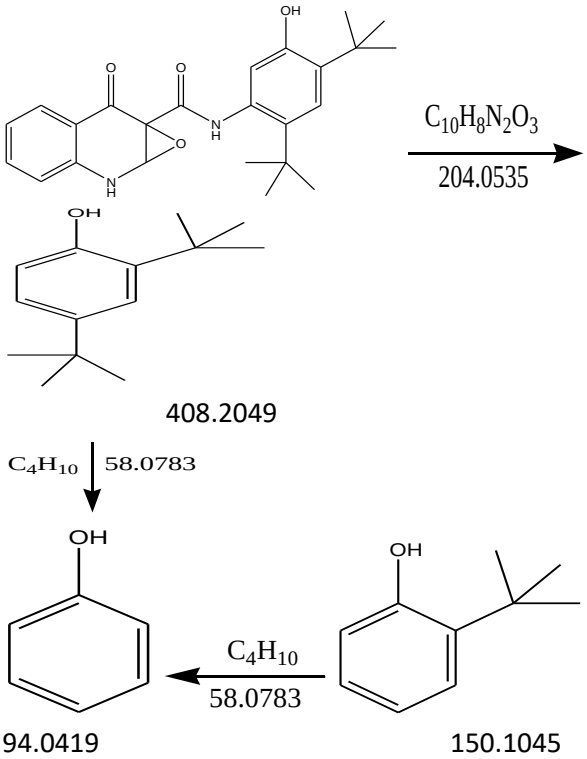
116.0238 172.0864

Figure 11: proposed fragmentation mechanism of $DP_6$of m/z-414

MS/MS degradation condition

Figure 11shows the possible fragmentation mechanism of degradation product 6 of m/z-414.1919, which was observed under conditions of Alkali degradation. Abundant substance ions on the spectrum at m/z-228.1490 ($C_{10}H_8N_2O_2$ loss), m/z-172.0864 (C_4H_{10} loss), m/z-116.0238 (C_4H_{10} loss). The proposed structures were confirmed by the MS/MS experiments in combination with accurate mass measurements.

Scheme 7



408.2049 206.1671

94.0419 150.1045

Figure 12: Proposed fragmentation mechanism for product degradation 7 of m/z-408

MS/MS degradation product

Figure 12 shows themechanism of fragmentation of degradation product 7 of m/z-408.2049, which was observed under peroxide degradation. The spectrum shows abundant ions of the substance at m/z-206.1671 (loss of $C_{10}H_8N_2O_3$), m/z-150.1045 (loss of C_4H_{10}), m/z-94.0419 (loss of C_4H_{10}).The proposed structures were confirmed by accurate mass measurements, MS/MS experiments.

Conclusion

In this study a fast novel, economical, sensitive and easily available method of HPLC has been produced for the simultaneous determination of Ivacaftor and Lumacaftor in bulk and a type of pharmaceutical dosage form. The advantage of this process was no HPLC methods were reported. This method consists of shorter run time, low price, accessibility, sensitivity, reliability and reproducibility. These properties are important when a large number of samples are to be analyzed. The validation of all the parameters like linearity, accuracy, specificity, robustness was done and found to be within the acceptance criteria. The RSD values were found to be less than 2.0 percent for all the parameters, which indicates the validity of the process and the results obtained by this process are seen to be in good agreement. So, the proposed method could be easily used for the routine analysis and pharmaceutical formulations of Ivacaftor and Lumacaftor in quality control laboratories without any preliminary separation.

References

1. AdelniaHossein, BlakeyIdriss, Little Peter J, Ta Hang T. Hydrogels Based on Poly(aspartic acid): Synthesis and Applications. Front Chem 2019; 7: 755. doi:10.3389/fchem.2019.00755.
2. AkramN. Md, Dr. Umamahesh M. A New Validated RP-HPLC Method for the Determination of Lumacaftor and Ivacaftor in its Bulk and Pharmaceutical Dosage Forms. Oriental Journal Of Chemistry 2017; 33: 1492-1501. DOI:10.13005/ojc/330354.
3. Ashutosh Kumar S, Manidipa Debnath, Dr. Seshagiri Rao J.V.L.N, Dr. Gowri Sankar D. Development and validation of a sensitive RP-HPLC method for simultaneous estimation of rosuvastatin and fenofibrate in tablet dosage form by using PDA detector in Gradient mode. Research J. Pharm. and Tech 2016; 9: 549-54.
4. Bala saheb B Chavan, Vijaya jyothi P, Pradip bhai D Kalariya, et al. Alcaftadine: Selective separation and characterization of

degradation products by LC-QTOF-MS/MS. Chromatographia 2018; 81: 631-8.

5. BalaswamiB and Venkata RamanaP. A New Stability-Indicating RP-UPLC Method Development and Validation for the Simultaneous Estimation of Ivacaftor and Tezacaftor in Pharmaceutical Dosage Form. Int J Pharm Biol Sci. 2019; 9: 1158-166.
6. Banks PA, Conwell DL, Toskes PP. The management of acute and chronic pancreatitis. GastroenterolHepatol (N Y) 2010; 6 (2 Suppl 3): 1–16.
7. Bascos NA, Landry SJ. A History of Molecular Chaperone Structures in the Protein Data Bank. Int J Mol Sci 2019; 20: 6195. doi:10.3390/ijms20246195.
8. Bhavani P, Prasada Rao K, Mohan S. Novel validated reversed-phase high-performance liquid chromatography method for determination of glucosamine, diacerein, and methyl sulfonyl methane in micro sample rat plasma and its application to pharmacokinetic and dissolution studies. Asian J Pharm Clin Res 2020; 13:50-63.
9. Birva Athavia A, Zarna Dedania R et.al., Stability indicating HPLC method for determination of Vilazodone hydrochloride. Int J Curr Pharm Res 2017; 9:123-9.
10. Capurso Gabriele, Traini Mariaemilia, Piciucchi Matteo, Signoretti Marianna, Arcidiacono Paolo Giorgio. Exocrine pancreatic insufficiency: prevalence, diagnosis, and management. Clin Exp Gastroenterol. 2019; 12: 129–39. doi:10.2147/CEG.S168266.
11. CharuPandya P, Sadhana Rajput J. Development and validation of stability indicating method RP-HPLC method of Acotiamide. Int J Pharm PharmSci 2018; 10:1-8.
12. Csanády L, Vergani P, Gadsby DC. Structure, Gating, and Regulation of the CFTR Anion Channel. Physiological Reviews 2019; 99: 707–38. doi:10.1152/physrev.00007.2018.
13. Dash S, Das SK, Samal J, Thatoi HN. Epidermal mucus, a major determinant in fish health: a review. Iran J Vet Res2018; 19: 72–81.
14. Dr. Naga mallika Gorantla, Jyothi Dodla pati, Sujatha Jadi. A New Validated RP-HPLC Method for Simultaneous Estimation of Lumacaftor and Ivacaftor in Pharmaceutical Dosage Form. Int. J. Pharm. Sci. Rev. Res. 2019; 56: 30-7.
15. Gabbett MT, Laporte J, Sekar R, et al. Molecular support for heterogonesis resulting in sesquizygotic twinning. N Engl J Med. 2019; 380:842-49. https://www.nejm.org/doi/full/10.1056/NEJMoa1701313.
16. Gadhvi M.P, Bhandari A, Suhagia B.N, Desai U.H. Development and validation of RP-HPLC method for simultaneous estimation of atazanavir and ritonavir in their combined tablet dosage form. Research J. Pharm. and Tech 2013; 6:200-203.

17. Gomathy S, Narenderan S.T, Meyyanathan S.N, Gowramma B. Development and validation of hplc method for the simultaneous estimation of apigenin and luteolin in commercial formulation. J Crit Rev 2020; 7: 4785-90.
18. Gudipaty SA, Lindblom J, Loftus PD, Redd MJ, Edes K, et al.Mechanical stretch triggers rapid epithelial cell division through Piezo1. Nature 2017; 543: 118–21. doi:10.1038/nature21407.
19. International conference on the harmonization. ICH harmonized tripartite guideline. Validation of analytical procedures:Text andmethodology Q2(R1); 2005.
20. Karuppasamy C, Sandhiya P, Rajakumar R. Method development and validation of lumacaftor and ivacaftor in pharmaceutical dosage forms in RP-HPLC. International Journal of Research in Pharmaceutical and Nano Sciences 2020; 9: 1-6. https://doi.org/10.36673/IJRPNS.2020.v09.i01.A01.
21. Lombard J. Once upon a time the cell membranes: 175 years of cell boundary research. Biology Direct 2014; 9: 32. doi:10.1186/s13062-014-0032-7.
22. Malak Y, Al-Bathish A. A, Gazy M. K. El-Jamal. Rp-hplc and chemometric methods for the determination of two anti-diabetic mixtures;metformin hydrochloride-canagliflozin and metformin hydrochloride-gliclazide in their pharmaceutical formulation. Int J Pharm PharmSci 2020; 12: 83-94.
23. Malathi S, Arunadevi N. Development and validation of stability-indicating simultaneous estimation of metformin and alogliptin in tablets by high-performance thin layer chromatography. Int J Pharm PharmSci 2020; 12: 68-73.
24. Mark HE, Dias da Costa G, Pagliari C, Unger SA. Malnutrition: the silent pandemic. BMJ 2020; 371: m4593. doi:10.1136/bmj.m4593.
25. Mayanka Singh, ManojCharde, Rajesh Shukla, Rita M.C. Determination of calcipotriene in calcipotriene cream 0.05% w/w by RP-HPLC method development and validation. Research J Pharm and Tech 2011; 4: 1219-23.
26. Middleton PG, Mall MA, Dřevínek P, Lands LC, McKone EF, et al. Elexacaftor-Tezacaftor-Ivacaftor for Cystic Fibrosis with a Single Phe508del Allele. N Engl J Med 2019; 381: 1809–819. doi:10.1056/NEJMoa1908639.
27. Mishra NN, Liu GY, Yeaman MR, Nast CC, Proctor RA, McKinnell J, Bayer AS. Carotenoid-related alteration of cell membrane fluidity impacts Staphylococcus aureus susceptibility to host defense peptides. Antimicrob Agents Chemother 2011, 55: 526–31. doi:10.1128/AAC.00680-10.
28. Monroe JG, Srikant T, Carbonell-Bejerano P, Becker C, Lensink M, et al. Mutation bias reflects natural selection in Arabidopsis thaliana. Nature 2022; 602: 101–05. doi:10.1038/s41586-021-04269-6.

29. Mukta D. Naykode, Durgacharan A. Bhagwat, Swapnil D. Jadhav, Harinath N, et al. Analytical and bio analytical method for quantification of pure azilsartan, not its salt by RP-HPLC. Research J Pharm and Tech 2017; 10: 708-14.
30. Mukta D. Naykode, Durgacharan A. Bhagwat, Swapnil D. Jadhav, Harinath N, et al. Analytical and bio analytical method for quantification of pure azilsartan, not its salt by RP-HPLC. Research J Pharm and Tech 2017; 10:708-14.
31. Narasimha S. Lakka, Chandra sekar Kuppan, Kona S. Srinivas, RavitejaYarra. Separation and characterization of new forced degradation products of Dasatinib in tablet dosage formulation using LC-MS/MS and stability indicating HPLC methods. Chromatographia 2020; 83: 947-62.
32. Naresh Kumar D. S, Patel D. Stability indicating chromatographic method development and validation for the simultaneous estimation of escitalopram oxalate and flupentixol in its pharmaceutical dosage form by HPLC. WJPR 2017; 6:549-66.
33. Naveen V M K, Veeraswami B, SrinivasaRao G. High response bio analytical validation approach of Nadolol and Bendroflumethiazide by LC-MS/MS on rat plasma. Int J Res Pharm Sci 2021; 12: 1-8.
34. Negut Irina, Grumezescu Valentina, Grumezescu AlexandruMihai. Treatment Strategies for Infected Wounds. Molecules 2018; 23: 2392. doi:10.3390/molecules23092392.
35. Ohar JA, Donohue JF, Spangenthal S.The Role of Guaifenesin in the Management of Chronic Mucus Hypersecretion Associated with Stable Chronic Bronchitis: A Comprehensive Review. Chronic ObstrPulm Dis 2019; 6: 341–49. doi:10.15326/jcopdf.6.4.2019.0139.
36. Potturi Ramadevi, Kantipudi Rambabu. Bio analytical method development and validation for Ezetimibe and Pitavastatin and its application to pharmacokinetic studies in rabbit plasma by using LC-MS/MS. Int J Res Pharm Sci 2021; 11: 7854-62.
37. Rajakumari R, Sreenivasa Rao S et.al., Stress degradation studies and development of a validated RP-HPLC method for determination of Tiagabine in presence of its degradation products. Int J Pharm PharmSci 2016; 8:230-6.
38. Ramachandran D, Anita Kethipalli, Krishnamurthy Mannam. Bio-analytical Method Development and Validation of Daunorubicin and Cytarabine in Rat Plasma by LC-MS/MS and its Application in Pharmacokinetic Studies. J Pharm Sci Res 2020; 12: 381-6.
39. Riley LW. Differentiating Epidemic from Endemic or Sporadic Infectious Disease Occurrence. Microbiology Spectrum 2019; 7. doi:10.1128/microbiolspec.AME-0007-2019.
40. RitivoiuMirela-Elena, Drăgoi Cristina Manuela, MateiDumitru, Stan IustinaVioleta, NicolaeAlinaCrenguţa, CraiuMihai, Dumitrescu Ion-Bogdan, CiolpanAlina Angelica. Current and

Future Therapeutic Approaches of Exocrine Pancreatic Insufficiency in Children with Cystic Fibrosis in the Era of Personalized Medicine. Pharmaceutics 2023; 15: 162. doi:10.3390/pharmaceutics15010162.

41. Sadigh-Eteghad S, Majdi A, Talebi M, Mahmoudi J, Babri S. Regulation of nicotinic acetylcholine receptors in Alzheimer's disease: a possible role of chaperones. Eur J Pharmacol 2015; 755: 34–41. doi:10.1016/j.ejphar.2015.02.047.
42. SaniyeÖzcan, AbeerElriş, SerkanLevent. HPLC method for simultaneous quantification of lumacaftor and ivacaftor bulk and pharmaceutical formulations. European Journal of Life Sciences 2023; 2: 109-17.
43. SenthilRajan D, Muruganathan G, Shivkumar K, Ganesh T. Development and validation of hplc method for simultaneous quantification of vasicine, glycyrrhizin and piperine in poly herbal cough syrup. Int J Curr Pharm Res 2020; 12: 15-19.
44. Sharma S, Hanukoglu A, Hanukoglu I. Localization of epithelial sodium channel (ENaC) and CFTR in the germinal epithelium of the testis, Sertoli cells, and spermatozoa. J MolHistol 2018; 49: 195–208. doi:10.1007/s10735-018-9759-2.
45. Shivani C P, Maheshwari D G. Development and validation of UV spectrometric and HPLC method for estimation of escitalopram oxalate and flupentixol dihydrochloride in combined dosage form. AJPTI 2016; 4: 59-70.
46. Shteinberg M, Haq IJ, Polineni D, Davies JC. Cystic fibrosis. Lancet 2021; 397: 2195–211. doi:10.1016/S0140-6736(20)32542-3.
47. Shteinberg Michal, HaqIram J, PolineniDeepika, Davies Jane C. Cystic fibrosis. The Lancet 2021; 397: 2195–211. doi:10.1016/s0140-6736(20)32542-3.
48. Slocombe L, Al-Khalili JS, Sacchi M. Quantum and classical effects in DNA point mutations: Watson-Crick tautomerism in AT and GC base pairs. Phys. Chem. Chem. Phys.2021; 23: 4141–150. doi:10.1039/D0CP05781A.
49. Sravanthi B, Divya M. Analytical method development and validation of ivacaftor and lumacaftor by RP-HPLC method. IAJPS 2016; 3: 900-04.
50. SubbaRaoYarlagadda, Subba Rao Mannam, Baby PadminiJampani. Stability indicating and cost effective analytical method development and validation of Sotorasib by using RP-HPLC. Int J App Pharm 2021; 13: 154-9.
51. Supriya T, Naresh D, Vijaya Kumar G, Haneer MA. Stability indicating RP-HPLC method development and validation for simultaneous estimation of escitalopram and flupentixol pure and marketed formulation. Asian J Pharm Res 2018; 8:4-10.
52. Swati K, Abhishek P, Sushank S, Bothiraja C, Atmaram P. High-performance liquid chromatography for the simultaneous estimation of cefoperazone and sulbactam in rat plasma and its

importance in therapeutic drug monitoring. Int J Pharm PharmSci 2020; 12: 92-97.

53. Syed Rafi, KantipudiRambabu. Bio analytical method development and validation of Avelumab and Axitinib and its application to pharmacokinetic studies in rabbit plasma by using LC-MS/MS. Int J App Pharm 2021; 13: 198-204.
54. Trehan I, Goldbach HS, LaGrone LN, Meuli GJ, Wang RJ, Maleta KM, Manary MJ. Antibiotics as part of the management of severe acute malnutrition. The New England Journal of Medicine 2013; 368: 425–35. doi:10.1056/NEJMoa1202851.
55. V L N Balaji Gupta T, VenkateswaraRao B, Kishore Babu B. RP-HPLC (stability indicating) based assay method for the simultaneous estimation of Doravirine, TenofovirDisoproxilFumarate and Lamivudine. Int J App Pharm 2021; 13: 153-9.
56. Vijayakumari M, Balasekharreddy Ch. Stability indicating validated hplc method for the determination of zanubrutinib in bulk and pharmaceutical dosage form. Asian J Pharm Clin Res 2020; 13: 159-62.
57. Wang W, Wu Z, Dai Z, Yang Y, Wang J, Wu G. Glycine metabolism in animals and humans: Implications for nutrition and health. Amino Acids 2013; 45: 463–77. doi:10.1007/s00726-013-1493-1.
58. Wang Y, Miller FH, Chen ZE, Merrick L, Mortele KJ, Hoff FL, et al. Diffusion-weighted MR imaging of solid and cystic lesions of the pancreas. Radiographics 2011; 31: E47-64. doi:10.1148/rg.313105174.
59. Warnock L, Gates A. Airway clearance techniques compared to no airway clearance techniques for cystic fibrosis. Cochrane Database Syst Rev 2023; 2023: CD001401. doi:10.1002/14651858.CD001401.pub4.