RESEARCH ARTICLE

Quantitative Determination of Zileuton from Spiked Human Plasma Using Liquid-Liquid Extraction Followed by RP-HPLC and UV Analysis

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ABSTRACT

A rapid, cost-effective and simple "RP-HPLC method with UV detection" was developed for the determination of zileuton from human plasma. The method involved spiking human plasma and validation. Phenacetin (internal standard) and zileuton samples were prepared using LLE in diethyl ether which was used as solvent for extraction. "HiQsil C18 column (250 mm*4.6 mm* 5 m) was used for separation with tetrahydrofuran: water (45:55, v/v) as mobile phase. The flow rate was set at 1-mL/min and UV detection was done at 230 nm. Zileuton showed excellent separation from the internal standard and no interference was observed in plasma samples. A linear calibration curve was obtained in the 500 to 10,000 ng/mL range. The relative error (RE) and relative standard deviation (RSD) were found to be less than 15% for both within the run and between the runs. At lower zileuton concentrations, "weighted least square regression with a weighting factor of 1/X was used to reduce the heteroscedastic effect". Extraction efficiency of LLE method was confirmed by the recovery of zileuton from samples. The stability data showed that "zileuton was stable in human plasma for 6 hours at room temperature for 30 days at -20°C after freeze thaw cycles".

Keywords: Human plasma, Liquid-liquid extraction, Weighted regression, Zileuton.

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INTRODUCTION

Bioanalysis involves the quantitative assessment of biological molecules and xenobiotics.[1] Bioanalysis aims to determine drugs in complex matrices sensitively and selectively with acceptable accuracy and precision.[2] Pharmacologic and pharmacokinetic investigations, which entail figuring out how medications are absorbed, distributed, metabolized, and eliminated in humans and animals, have included estimation of substances from the biological fluids. Bioanalysis also involves the correlation between drug levels in blood tissue and their pharmacologic effects (pharmacodynamics), which is an essential feature during drug development.[3-5] Sample pretreatment is an essential feature to prevent matrix clogging despite the development of highly efficient analytical technologies.[6] Under such circumstances, LLE is a reliable and frequently applied approach for isolating analytes from endogenous interfering molecules in aqueous biological fluids.[7-14] Partitioning between the aqueous and organic phases, determines the separation in LLE.[3,15]

"Zileuton is an antiasthmatic drug which is chemically 1-[1-(1-benzothiophen-2-yl) ethyl]-1-hydroxyurea" (Figure 1). The drug is a racemate mixture of R (+) zileuton and S (-) zileuton enantiomers which have approximately equal 5-lipoxygenase inhibitory activity.[16] 5-lipooxygenase is responsible for catalyzing the production of leukotrienes from arachidonic acid. Leukotrienes trigger various biological reactions that result in symptoms like edema, inflammation, bronchoconstriction in asthmatic patients' airways, and mucus secretion. Zileuton reduces these symptoms through its specific inhibitory activity.[17,18] Patients consuming the recommended dose of 600 mg of zileuton exhibited a peak plasma concentration of 4.41 mg/L.[19] Numerous analytical techniques are reported in the literature for the quantitative estimation of zileuton.[20-29] There aren't many bioanalytical techniques available for determining zileuton in human plasma on its own using LC-MS.[17] RP-HPLC[30] was used for the simultaneous determination of "zileuton and its N-dehydroxylated metabolite from rat urine using micellar liquid chromatography[31] and plasma using HPLC".[16] The HPLC procedures, however,

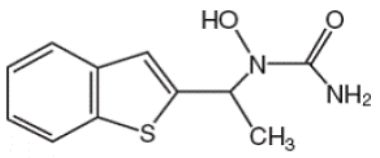

Figure 1: Chemical structure of zileuton.

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demand sophisticated sample preparation technology, such as solid phase extraction. Although the LC-MS approach outlines the use of LLE for sample preparation, the technology is expensive, and the process involves an internal standard that is less frequently used. As a result, the current study proposed a straightforward, simple RP-HPLC-UV method based on LLE for sample preparation and a universally accessible internal standard for measuring zileuton from spiked human plasma.

EXPERIMENTAL

Equipment and Materials

HPLC system with variable wavelength programmable UV detector and manual Rheodyne injector (20 L loop capacity) was used to conduct the analysis. A quaternary pump from Agilent 1200 series was used with HPLC system. The sample was injected using a 50-L Hamilton injection syringe. Chemstation (B.02.01) was used for data analysis. A gift sample of pharmaceutical-grade zileuton was obtained from MSN Labs Ltd. in Hyderabad, Andhra Pradesh, India. An internal standard of phenacetin was acquired from Triveni Chemicals Gujarat, India. Blank human plasma was purchased from the KEM Hospital in Mumbai, India's National Plasma Fractionation Centre. Pooled blank plasma was produced by thoroughly combining plasma from six distinct sources. We acquired all of our chemicals from Molychem in Mumbai, India. Tetrahydrofuran and water used in the analysis were of HPLC grade. Rest all other chemicals used in the investigation were of AR quality. 0.45 µm nylon membrane filters were purchased from "PCI Analytics Pvt. Ltd., Mumbai, India".

Methods

Preparation of Standards for the Calibration Curve (CC)

1000 µg/mL stock solution of zileuton was prepared in methanol. Stock was diluted with methanol to prepare working standards at concentrations of 25, 50, 100, 200, 300, 400, and 500 µg/mL. A total of 20 µL of these standard working solutions was spiked to 1-mL aliquots of blank human plasma to obtain CC standards with concentrations of 500, 1000, 2000, 4000, 6000, 8000 and 10000 ng/mL of zileuton.

Preparation of Quality Control (QC) Sample

The QC samples were prepared similar to CC standards. "Three concentrations of samples were used in study which included 1000 ng/mL for the lower quality control (LQC), 4000 ng/mL for the middle-quality control (MQC), and 8000 ng/mL for the higher quality control (HQC)".

Preparation of Internal Standard Solution

In 1000 µg/mL of phenacetin stock was prepared in methanol. The working standard of IS with a concentration of 250 µg/mL was prepared by diluting the stock solution with methanol.

Selection of LLE Solvent and Preparation of Sample

In 1-mL of spiked human plasma samples and 20 µL of working standard solution of IS (250 µg/mL) were aliquoted in five different glass tubes. The contents were mixed thoroughly for 1 minute using a cyclomixer. In 5 mL of LLE solvents such as dichloromethane, chloroform, diethyl ether, ethyl acetate, and n-hexane were added separately to each tube. The tubes were inclined on a reciprocating shaker which was maintained at 100 strokes per minute for 40 minutes to achieve extraction. The contents of the tube were then centrifuged for 15 minutes at 4°C and 5000 rpm to achieve phase separation. four mL of the organic layer from each tube was transferred to a separate tube and dried under a nitrogen stream. "Each tube's residue was reconstituted in 250 µL of mobile phase and analyzed under optimal chromatographic conditions". The LLE solvent that gave the maximum %extraction recovery of both zileuton and IS was selected. Sample preparation and analysis were performed in six replicates using the selected LLE solvent to evaluate the reproducibility of extraction.

HPLC Analysis

"The analysis was performed on a HiQsil C18 column (250 mm x 4.6 mm, 5 µm, Great Denmow, Essex., UK), which was protected by Hypersil BDS C18 guard column (20 x 4 mm). The mobile phase consisted of a mixture of tetrahydrofuran: water in the ratio of 45:55 (v/v) at a flow rate of 1-mL/min. UV detection was carried out at 230 nm and the run time for analysis was 10 minutes".

Calibration Studies

Six replicates were used to analyze all CC standards. The data on zileuton concentrations and area ratios to IS were subjected to least square linear regression (unweighted and weighted). "Using the regression equations, the interpolated concentrations of the CC standards were calculated, and each CC standard's percentage relative error (%RE) was determined. The calibration model that produced the lowest total %RE for interpolated CC standard concentrations was selected and used in the validation experiments".

Validation Experiments

The developed bioanalytical method was validated in accordance with the "US Food and Drug Administration's Industry Guidance".[32] The lower limit of quantification (LLoQ) of 500 ng/mL was used to assess the procedure's selectivity. "The peak areas provided by blank plasma from six different

Table 1: Area ratio from calibration experiments on zileuton

CC no	*Amount of drug (ng/mL)*	*Area ratio (Mean ± SD, n= 6)*
1	500	0.345 ± 0.009
2	1000	0.574 ± 0.01
3	2000	1.267 ± 0.015
4	4000	2.514 ± 0.038
5	6000	3.770 ± 0.052
6	8000	5.139 ± 0.079
7	10000	6.278 ± 0.114

Table 2: Blank responses and peak areas at LLoQ of zileuton

Sr. no	*Blank response (mAU.sec)*	*Peak areas at LLOQ (mAU.sec)*
1	6.15	42.41
2	5.87	45.69
3	6.43	40.18
4	5.69	44.39
5	5.99	43.41
6	6.32	41.23

Table 3: Results of assessment of accuracy, precision, and % recovery of zileuton

Level	Concentration added (ng/mL)	Intra-day (n=5)			Inter-day (n=5)			% Recovery (n=5)
		Mean concentration found (ng/mL)	RE (%)	RSD (%)	Mean concentration found (ng/mL)	RE (%)	RSD (%)	
LQC	1000	1023.79	2.38	2.77	1059.68	5.97	4.65	78.45
MQC	4000	4217.89	5.45	6.45	3893.69	-2.66	2.78	79.02
HQC	8000	7883.61	-1.45	0.73	8315.61	3.95	3.01	76.98
IS	-	-	-	-	-	-	-	80.21

sources were compared to the peak areas provided by LLoQ samples for comparing the selectivity". Analysis of LQC, MQC, and HQC samples in five replicates over five days was used to determine accuracy (within-run) and precision (between-run). The zileuton concentration in the QC samples was calculated by substituting the zileuton to IS area ratio obtained from the QC sample analysis into the calibration equation generated on the same day.

The stability of zileuton in human plasma was tested at room temperature for 6 hours, at 20°C for a short period of time, and in three freeze-thaw cycles. LQC and HQC samples were used for stability evaluation, and all the analyses were performed in five replicates. The samples were left for 6 hours at room temperature to assess the stability of samples at room temperature. "To assess the short-term stability the samples were stored at -20°C for one month. To assess the freeze-thaw stability, samples were frozen at -20°C for at least 24 hours. The samples were allowed to thaw at room temperature". The concentration of zileuton was determined in stability samples and the % nominal and % RSD values were calculated.

RESULTS AND DISCUSSION

The various chromatographic conditions were standardized by conducting several trials with water containing different mobile phases of acetonitrile/methanol/tetrahydrofuran in different proportions. Sharp peaks with good resolution were obtained with tetrahydrofuran: water (45:55, v/v) for both standard and zileuton. LLE with different immiscible organic solvents showed that diethyl ether was the best followed by ethyl acetate for extraction of zileuton and IS. A summary of the same is presented in Figure 2.

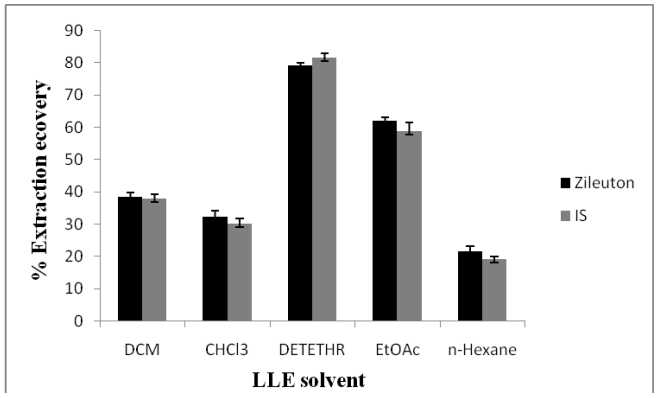

Figure 2: Extraction recovery of zileuton and IS in different LLE solvents.

After conducting several trials with mobile phases consisting of water and different organic solvents (acetonitrile/methanol/tetrahydrofuran) and in different proportions, the chromatographic conditions were fine-tuned. Good peak shape and resolution between zileuton and IS were obtained with the mobile phase when LLE was performed using different immiscible organic solvents. It was discovered that diethyl ether followed by ethyl acetate extracted both zileuton and IS substantially better than n-hexane, as shown in Figure 2. Diethyl ether was selected as LLE solvent since it gave adequate recovery for both zileuton and IS. The mean extraction recovery with diethyl ether for zileuton was 79.13%, while that for IS (phenacetin) was 81.36%.

Table 1 displays the area ratios obtained from calibration experiments. During calibration experiments, there was an increase in the area ratio of the standard deviation with increase in the concentration of standards. "Weighted regression was needed since the unweighted regression calibration model produced heteroscedasticity and a high total% RE".

"The use of a weighting factor of 1/X resulted" in an even distribution of area ratios of CC standards across the calibration zileuton reduces these symptoms through its specific inhibitory activity the equation Y = 0.0003 X + 0.0062 was generated.

While performing validation experiments, the peak area for the LLoQ samples was five times the peak areas afforded by blank plasma obtained from six different sources (Table 2). Thus, the selectivity of the method at the LLoQ of 500 ng/mL was demonstrated. "Chromatogram corresponding to

Table 4: Results of stability studies for zileuton in human plasma (n= 5)

	LQC		HQC	
	%Nominal	RSD (%)	%Nominal	RSD (%)
Stability at room temperature				
2 h	93.2	4.1	106.3	5.2
4 h	105.5	6.9	97.3	8.1
6 h	101.4	5.2	94.2	3.3
Stability at -20^{0}C				
10 days	91.4	8.7	102.8	11.1
20 days	89.3	3.3	105.3	5.2
30 days	93.6	8.9	107.6	6.6
Freeze thaw stability				
Cycle 1	86.2	4.8	88.6	7.2
Cycle 2	94.1	6.6	90.7	6.9
Cycle 3	89.9	9.3	98.2	9.5

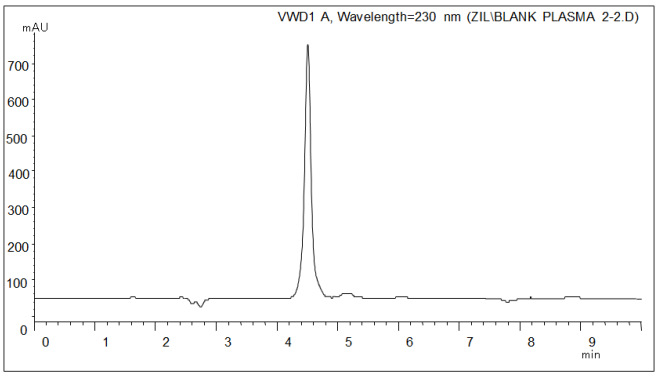

Figure 3: Representative chromatogram of blank plasma extract showing lack of significant interference at the retention times of zileuton and IS, phenacetin.

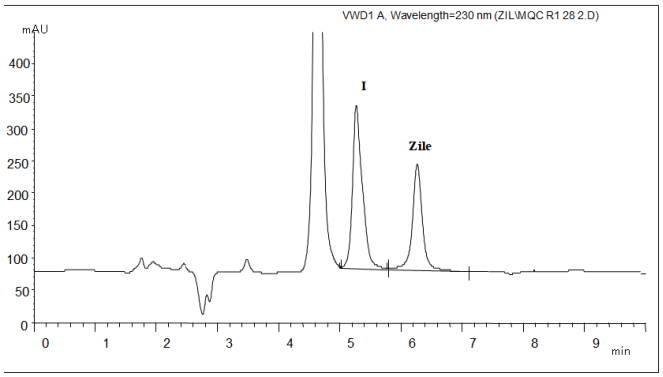

Figure 4: Representative chromatogram of MQC sample showing IS, phenacetin (RT= 5.274 min) and zileuton (RT= 6.276 min)

unspiked plasma (Figure 3) does not show any interference at the retention times" of zileuton and IS, phenacetin. Figure 4 shows a chromatogram of the MQC sample. The results of accuracy, precision, and recovery studies for zileuton at "LQC, MQC, and HQC" and IS are represented in Table 3. The evaluation of accuracy and precision showed that the %RE for accuracy (within-run analysis) was between ±15%, while the % RSD for precision (between-run analysis) was less than 15%, in accordance with the US-FDA Guidance. Likewise, the extraction recovery of zileuton at all three QC levels as well as the recovery of IS was satisfactory and concordant with the selected experimental conditions.

Table 4 shows the results of zileuton stability studies in human plasma of LQC and HQC samples under different stability conditions. The study revealed that the %nominal concentration was between 85 and 115% and the %RSD was less than 15%, which was in the acceptance criteria set by the US-FDA. As a result, zileuton was discovered to be stable in human plasma under the tested conditions. This indicated that during actual bioanalytical study, the drug was unaffected in the time lapse between sample withdrawal and actual analysis, between sample preparation and chromatographic run and in the event of temperature fluctuations during transit from sample collection center and bio-analytical laboratory

CONCLUSION

The proposed HPLC-UV method for determining zileuton in spiked human plasma via liquid-liquid extraction is fast, inexpensive, and easy. The method involved use of easily available chemicals, less expensive instrumentation and a simple sample preparation technique. The method was found to be linear in the concentration range of 500 to 10000 ng/mL. Validation studies demonstrated that the method was selective and showed acceptable precision, accuracy, %recovery and stability. As a result, the method can be used to support bio-availability and bioequivalence studies by routinely analyzing zileuton from human plasma.

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REFERENCES

1. Vuppala PK, Janagam DR, Balabathula P. Importance of ADME and Bioanalysis in the Drug Discovery. Journal of Bioequivalence & Bioavailability. 2013; 5(4):4–5. Available from: https://doi.org/10.4172/jbb.10000e31.
2. Pandey S, Pandey P, Tiwari G, Tiwari R. Bioanalysis in drug discovery and development. Pharmaceutical Methods. 2010; 2(1):14-24. Available from: doi: 10.4103/2229-4708.72223
3. Evans G. A Handbook of Bioanalysis and Drug Metabolism, G. Evans, ed., CRC Press, Boca Raton, 2004.
4. Gonzalez O, Blanco ME, Iriarte G, Bartolome L, Maguregui MI, Alonso RM. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the nonwell defined parameters limit of quantification, robustness and matrix effect. Journal of Chromatography A. 2014; 1353(1):10-27. Available from: http://dx.doi.org/doi:10.1016/j.chroma.2014.03.077
5. Snyder LR, Kirkland JJ, Glajch JL. Practical HPLC Method Development, second ed., John Wiley and Sons, New York, 1997.
6. Weng N, Patel S, Jian W. Bioanalysis of small and large molecule drugs, metabolites, and biomarkers by LC-MS. Identification and Quantification of Drugs, Metabolites, Drug Metabolizing Enzymes, and Transporters. 2020:3–38. Available from: https://doi.org/10.1016/B978-0-12-820018-6.00001-6
7. Abdel-Rehim M, Lee ML, Bojko B. Editorial for the special issue entitled "Extraction and Sample Preparation Techniques in Bioanalysis". Journal of Chromatography B. 2017; 1043:1–2.
8. Chandu BR, Kanala K, Hwisa NT, Katakam P, Khagga M. Bioequivalance and pharmacokinetic study of febuxostat in human plasma by using LC-MS / MS with liquid liquid extraction method, SpringerPlus 2. 2013; (194):1–10. Available from: https://doi.org/10.1186/2193-1801-2-194
9. Iwamoto N, Shimomura A, Tamura K, Hamada A, Shimada T. LC–MS bioanalysis of Trastuzumab and released emtansine using nano-surface and molecular-orientation limited (nSMOL) proteolysis and liquid–liquid partition in plasma of Trastuzumab emtansine-treated breast cancer patients. Journal of Pharmaceutical and Biomedical Analysis. 2017; 145:33–39. Available from: http://dx.doi.org/10.1016/j.jpba.2017.06.032
10. Patel CD, Guttikar S, Patel BH. Development and Validation of Bioanalytical Method for Simultaneous Estimation of Nebivolol

Enantiomers in Human Plasma Using Liquid Chromatography-tandem Mass Spectrometry. Iranian Chemical Society Analytical and Bioanalytical Chemistry Research. 2018; 5(1):131-142. Available from: 10.22036/ABCR.2018.92392.1159

11. Jiang H, Cao H, Zhang Y, Fast DM. Systematic evaluation of supported liquid extraction in reducing matrix effect and improving extraction efficiency in LC-MS/MS based bioanalysis for 10 model pharmaceutical compounds. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences. 2012; 891–892:71–80. Available from: https://doi.org/10.1016/j.jchromb.2012.02.031
12. Elkady EF, Mandour AA, Algethami FK, Aboelwafa AA, Farouk F. Sequential liquid-liquid extraction coupled to LC-MS/MS for simultaneous determination of amlodipine, olmesartan and hydrochlorothiazide in plasma samples: Application to pharmacokinetic studies. Microchemical Journal. 2020; 155:1-26. Available from: https://doi.org/10.1016/j.microc.2020.104757
13. Shahbazi S, Peer CJ, Polizzotto MN, Uldrick TS, Roth J, Wyvill KM, et al. A sensitive and robust HPLC assay with fluorescence detection for the quantification of pomalidomide in human plasma for pharmacokinetic analyses. Journal of Pharmaceutical and Biomedical Analysis. 2014; 92:63–68. Available from: http://dx.doi.org/10.1016/j.jpba.2014.01.001
14. Sun L, Forni S, Schwartz MS, Breidinger S, Woolf EJ. Quantitative determination of odanacatib in human plasma using liquid-liquid extraction followed by liquid chromatography-tandem mass spectrometry analysis. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences. 2012; 885–886:15–23. Available from: https://doi.org/10.1016/j.jchromb.2011.12.004
15. Caballero-Casero N, Lunar L, Rubio S. Analytical methods for the determination of mixtures of bisphenols and derivatives in human and environmental exposure sources and biological fluids. A review. Analytica Chimica Acta. 2016; 908:22–53. Available from: http://dx.doi.org/10.1016/j.aca.2015.12.034
16. Granneman GR, Braeckman RA, Erdman KA. Determination of a New 5-lipoxygenase inhibitor, zileuton, and its inactive N-dehydroxylated metabolite in plasma by high performance liquid chromatography. Clinical Pharmacokinetics. 1995; 29 (2):1–8. Available from: https://doi.org/10.2165/00003088-199500292-00003
17. Prakash K. Development and Validation of a Liquid Chromatography Mass Spectrometry Method for the Determination of Zileuton in Human Plasma. Scientia Pharmaceutica. 2014; 82:571–583. Available from: https://doi.org/10.3797/scipharm.1402-19
18. Bell RL, Young PR, Albert D, et. al. The discovery and development of zileuton: An orally active 5-lipoxygenase inhibitor. International Journal of Immunopharmacology. 1992; 14 (3):505–510. Available from: https://doi.org/10.1016/0192-0561(92)90182-K
19. Awni WM, Braeckman RA, Granmeman GR, et al. Pharmacokinetics and pharmacodynamics of zileuton after oral administration of single and multiple dose regimens of zileuton 600 mg in healthy volunteers. Clinical Pharmacokinetics. 1995; 29 (2):22-33. Available from: https://doi.org/10.2165/00003088-199500292-00005
20. Baezzat MR, Banavand F, Fasihi F. Highly sensitive determination of zileuton using TiO2 nanoparticles and the ionic liquid 1-hexylpyridinium hexafluorophosphate nanocomposite sensor. Ionics. 2019; 25:1835–1844. Available from: https://doi.org/10.1007/s11581-018-2699-8
21. Ganorkar SB, Dhumal DM, Shirkhedkar AA. Development and validation of simple RP-HPLC-PDA analytical protocol for zileuton assisted with Design of Experiments for robustness determination. Arabian Journal of Chemistry. 2017; 10 (2):273-282. Available from: https://doi.org/10.1016/j.arabjc.2014.03.009
22. Rao KVP, Tanuja M, Rao YS, et al. Development and Validation of RP-HPLC method for the estimation of Zileuton in bulk and its dosage form. International Journal of Drug Development & Research. 2015; 7 (1):101-106. Available from: https://www.ijddr.in/abstract/development-and-validation-of-rphplc-method-for-the estimation-ofrnzileuton-in-bulk-and-its-dosage-form-5496.html
23. Anandakumar K, Nareshbabu N. Development and validation of analytical method for estimation of zileuton in bulk and pharmaceutical dosage form by UV spectroscopy. International Research Journal of Pharmacy. 2012;3 (12):154-157. Available from: https://www.researchgate.net/publication/317660752_Development_and_validation_of_analytical_method_for_the_estimation_of_zileuton_in_bulk_and_in_pharmaceutical_dosage_form_by_uv_Spectroscopy
24. Rao KVP, Tanuja M, Rao YS, et al. Validated visible spectrophotometric methods for determination of Zileuton in pharmaceutical formulation. Der Pharma Chemica. 2015; 7(2):25–30. Available from: https://www.derpharmachemica.com/abstract/validated-visible-spectrophotometric-methods-for-determination-of-zileuton-in-pharmaceutical-formulation-3031.html
25. Ganorkar SB, Shirkhedkar AA. Novel HPTLC and UV-AUC analyses: For simple, economical, and rapid determination of Zileuton racemate. Arabian Journal of Chemistry. 2013; 10(3):360-367. Available from: https://doi.org/10.1016/j.arabjc.2013.05.013
26. Hou W, Xia J, Liu C, Li S, Wu T, Huanga Y. Development of a method to screen and isolate bioactive constituents from Stellera chamaejasme by ultrafiltration-liquid chromatography combined with semi-preparative high- performance liquid chromatography and high-speed counter-current chromatography. Journal of Separation Science 2019; 42(22):3421-3431. Available from: https://doi.org/10.1002/jssc.201900772
27. Sreedhar NY, Nayak MS, Prasad KS, et al. Electrochemical Reduction Behaviour of Zileuton at a Dropping Mercury Electrode by Polarography. E-Journal Chem. 2010; 7 (1):166–170. Available from: https://doi.org/10.1155/2010/251415
28. Trivedi JS, Porter WR, Fort JJ. Solubility and stability characterization of zileuton in a ternary solvent system. European Journal of Pharmaceutical Sciences. 1996; 4:109-116. Available from: https://doi.org/10.1016/0928-0987(95)00038-0
29. Ganorkar SB, Dhumal DM, Shirkhedkar AA. Development and validation of simple RP-HPLC-PDA analytical protocol for zileuton assisted with Design of Experiments for robustness determination. Arabian Journal of Chemistry. 2014; 10(2):273-282. Available from: https://doi.org/10.1016/j.arabjc.2014.03.009
30. Awni WM, Granneman GR, Locke CS, et al. Population pharmacokinetics of zileution, a selective 5-lipoxygenase inhibitor, in patients with rheumatoid arthritis. European Journal of Clinical Pharmacology. 1995; 48:155–160. Available from: https://doi.org/10.1007/BF00192742
31. Thomas SB, Albazi SJ. Simultaneous Determination of the 5-Lipoxygenase Inhibitor "Zileuton" and its N-Dehydroxylated Metabolite in Untreated Rat Urine by Micellar Liquid

Chromatography. Journal of Liquid Chromatography & Related Technologies. 1996; 19 (6):977–991. Available from: https://doi.org/10.1080/10826079608001928

32. Armoudjian Y, Mikayelyan A, Zakaryan H, Aleksanyan A, Alaverdyan H. Protein Precipitation Method for Determination of Zileuton in Human Plasma by LC-MS/MS. Journal of Pharmaceutical Innovation. 2020; 15:581–590. Available from: https://doi.org/10.1007/s12247-019-09403-6
33. Bioanalytical Method Validation Guidance for Industry, Available from: http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm (visited on: 15. 09. 2022)