Pharmaceutical Analysis Combined with In-Silico Therapeutic and Toxicological Profiling on Zileuton and its Impurities to Assist in Modern Drug Discovery

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Research Highlights

- Zileuton was subjected to hydrolysis and oxidative stress
- UHPLC-PDA was applied for identification and resolution of degradation products
- Major FDP’s were characterized using LC-MS-QTOF and/or $^1$HNMR
- The drug molecule and FDP’s were comparatively evaluated by in-silico profiling
- Binding affinity, toxicity and possible therapeutic abilities were assessed
- Some FDP’s had higher bio-affinity and indicated as active at other therapeutic targets with lesser toxicity
Abstract
The obligatory testing of drug molecules and their impurities to protect users against toxic compounds seems to provide interesting opportunities for new drug discovery. Impurities, which proved to be non-toxic, may be explored for their own therapeutic potential and thus be a part of future drug discovery. The essential role of pharmaceutical analysis can thus be extended to achieve this purpose. The present study examined these objectives by characterizing the major degradation products of zileuton (ZLT), a 5-lipoxygenase (5-LOX) inhibitor being prevalently used to treat asthma. The drug sample was exposed to forced degradation and found susceptible to hydrolysis and oxidative stress. The obtained Forced Degradation Products (FDP’s) were resolved using an earlier developed and validated Ultra-High-Pressure Liquid Chromatography Photo-Diode-Array (UHPLC-PDA) protocol. ZLT, along with acid- and alkali-stressed samples, were subjected to Liquid-chromatography Mass-spectrometry Quadrupole Time-of-flight (LC/MS-QTOF) studies. Major degradation products were isolated using Preparative TLC and characterized using Q-TOF and/or Proton nuclear magnetic resonance (¹H NMR) studies. The information obtained was assembled for structural conformation. Toxicity Prediction using Komputer Assisted Technology (TOPKAT) toxicity analyses indicated some FDP’s as non-toxic when compared to ZLT. Hence, these non-toxic impurities may have bio-affinity and can be explored to interact with other therapeutic targets, to assist in drug discovery. The drug molecule and the characterized FDP’s were subjected to 3-Dimensional Extra Precision (3D-XP)-molecular docking to explore changes in bio-affinity for the 5-LOX enzyme (PDB Id: 3V99). One FDP was found to have a higher binding affinity than the drug itself, indicating it may be a suitable antiasthmatic. The possibility of being active at other sites cannot be neglected and this is evaluated to a reasonable extent by Prediction of Activity Spectra for Substances (PASS). Besides being antiasthmatic, some FDP’s were predicted antineoplastic, antiallergic and inhibitors of Complement Factor-D.

Keywords: Zileuton; Hydrolysis; Oxidation; Pharmaceutical Analysis; Toxicity Prediction; Bioactivity Prediction
1. Introduction

The ever-increasing pollution and the lack of pure air to breathe increase the number of asthma cases devastatingly throughout the world [1]. However, the generation of new chemical entities and the exploration of their mechanisms is time consuming and costly. The inhibition of 5-LOX, a key enzyme for leukotriene biosynthesis, plays a crucial role in the treatment of asthma [2]. ZLT is a 5-LOX inhibitor which blocks the conversion of arachidonic acid to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) [3]. The literature discusses several analytical determinations of ZLT, such as the preparative separation and analysis of the enantiomers of [14C] zileuton [4], the kinetics and mechanism of chemical degradation in aqueous solutions studied by high-performance liquid-chromatography HPLC [5], the HPLC determination with its N-dehydroxylated metabolite in plasma [6], the simultaneous determination along with N-dehydroxylated metabolite in untreated rat urine using HPLC [7], the solubility and stability characterization of ZLT in a ternary solvent system assisted with HPLC [8], the electrochemical reduction behaviour of ZLT at a dropping mercury electrode by polarography [9], voltammetric methods for determination in serum, urine and pharmaceuticals [10], zero-order UV-spectrophotometric and first-order derivative UV-spectrophotometric determinations [11], and a high-performance thin-layer chromatography (HPTLC) method with UV-spectrophotometry Area-Under-Curve (UV-AUC) determination [12]. Recently, we reported a stability indicating RP-HPLC-PDA protocol, assisted with Design of Experiments (DoE) for robustness evaluation [13]. Further, reports on the electrochemical determination on a nanocomposite sensor with TiO$_2$ nanoparticles and 1-hexylpyridinium hexafluorophosphate (HPFP) as ionic liquid [14], and on a high-performance selective sensor prepared with modified carbon paste electrode [15] have also been reported.

Hence in order to characterize the major forced degradation products (FDP’s) LC/MS-QTOF and $^1$HNMR methods have been applied to assist research objectives. Characterization of the degradation products is thus a usual protocol applied after the forced degradation or the stability assessment of drugs. However, this information allows bridging pharmaceutical analysis and modern drug discovery.

Pharmaceutical research and development is being transformed at a fundamental level in response to the demands of an increasingly difficult drug discovery environment. Much focus is on the application of new technologies to speed up all steps of the drug discovery process [16]. With the
increasing emphasis on identification and the low-level control of potentially genotoxic impurities (GTIs), an increased use of structure-based computational assessments, including the application of computerized models, for the prediction of toxicity and biological activities, is observed [17]. Currently, *in-silico* toxicological methods, such as computational toxicology, predictive quantitative structure–activity relationships (QSAR) modeling of toxicity, *i*-drug discovery, and predictive Absorption, Distribution, Metabolism, Elimination and Toxicity (ADMET)[18], show potential for drug discovery research. They help identifying lead compounds with low toxicological liability. The advanced toxicological and biological activity predictions for FDP’s rely on the use of structure-based *in-silico* methods. The characterization of drug impurities (IMP’s), the establishment of degradation pathways and the prediction/evaluation of the toxicity of IMP’s, including Pharmacopoeia related substances (RS’s) and characterized FDP’s, are goals of the pharmaceutical analyst. The elucidated structures of the IMP’s of all drugs form a large database that could be screened for therapeutic properties. It seems possible when the role of analysis is extended towards *in-silico* toxicological and therapeutic profiling. Such efforts will eventually help in the future drug-discovery process.

The binding affinity of any molecule can be estimated using molecular docking [19]. As the RS’s of a drug molecule (IMP’s and/or FDP’s) are usually structural analogues, they can also be docked on the target receptor to get an idea about their binding affinity. Thus, application of this *in-silico* approach may lead to the extraction of additional information, after characterization of the degradation products, and may be interesting in the drug discovery process. The docking may also provide information about RS’s. The Grid-based Ligand Docking with Energetics (Glide) [20] is prevalently used because of its ability to exhaustively search the positional, orientational and conformational space at good speed. The concise methodology for docking with Schrodinger’s Glide [21] is presented in Section 2.6. After estimating the binding affinity of FDP’s, the approach that can effectively contribute towards the therapeutic repurposing of these RS’s is the prediction of their pharmaceutical/medicinal abilities. This can be achieved, at a preliminary level, without any expenditure, by application of PASS [22].

PASS involves the prediction of biological activity spectra for chemical structures with possible therapeutic use. The prediction is based on the analysis of structure-activity relationships (SAR’s), the average accuracy of prediction is about 95%. The PASS algorithm is found to provide reasonable prediction accuracy. PASS is used by medicinal chemists, pharmacologists and
toxicologists for several years, and its predictions were confirmed by subsequent synthesis and biological testing [23]. Hence the PASS algorithm can be efficiently utilized to predict therapeutic repurposing abilities of the drug impurities.

The best in-silico toxicity prediction tools are Hazard-Expert, TOPKAT, and Deductive Estimation of Risk from Existing Knowledge (DEREK). The effective tool among these is TOPKAT [24] as model extension, alert for structure inclusion and structures out of applicability domain (AD) is possible in extension of training set. Ames mutagenicity can be predicted effectively for closely resembling structures with higher confidence. Carcinogenicity and developmental toxicity require less chemical diversity in the training set. In cases where no structurally similar compounds are available or the model extension does not change the prediction statistics towards more confidence in the prediction, OECD Toolbox is applied [25]. The drug likeliness of the RS’s may be sufficient to foster them as frit (active fragments) or lead (active molecules) for the same or other target diseases.

The present study considers the predictions of toxicities using TOPKAT and of the bioactivity spectrum, using PASS for characterized major FDP’s of zileuton (ZLT), along with known pharmacopoeial impurities, to find positive results for some of the selected chemical structures. After performing forced degradation trials, testing the susceptibility of ZLT towards oxidation and hydrolysis, chromatographic separation, isolation and characterization of the generated FDP’s was done. The primary aim of the present investigation was not just to study the FDP’s generated after hydrolysis and oxidation using LC/MS-QTOF and $^1$H NMR analyses, but also to perform a comparative in-silico toxicological and therapeutic evaluation. The parent drug, ZLT, and its RS’s, i.e. both characterized impurities and the official impurities from USP [26], were first subjected to molecular docking to evaluate the relative affinity for the parent receptor. Considering the RS’s as new frits and leads, they were subsequently subjected to PASS to predict their therapeutic abilities as potential future drug compounds. The relative toxicological abilities of the drug and its impurities were studied by comparative predictive toxicological profiling, using TOPKAT. Though characterization of some FDP’s was successfully carried out, the extension of pharmaceutical analysis to assist in drug discovery was the ultimate target. This has been accomplished with combining forced degradation studies with UHPLC-PDA analysis, LC/MS-QTOF and $^1$H NMR characterization, followed by in-silico profiling.
2. Experimental

2.1 Chemicals

The ZLT drug standard was obtained as a gift from Biophore India (Hyderabad, India). Orthophosphoric acid (OPA) (85 %), hydrogen peroxide (30 %), sodium starch glycolate (SSG) and micro-crystalline cellulose (MCC) were procured from Loba Chemie (Mumbai, India). Methanol (MeOH, HPLC Grade), acetonitrile (ACN, HPLC Grade), sodium hydroxide (NaOH), glacial acetic acid (GAA) and hydrochloric acid (HCl), were purchased from Merck (Mumbai, India). Double-distilled water, prepared in-house using a glass distillation assembly, having a value for total dissolved solutes (TDS) <0.0005 ppm, was used throughout the analysis.

2.2 Instruments and software

The analysis was performed on a UHPLC - LC 20 AD instrument (Shimadzu, Kyoto, Japan) consisting of an LC - 20 AD binary solvent delivery system (pump), an SPD-M20A diode array detector, a CTO 10 AS vp column oven and a Rheodyne injector with a 20 µL loop Hamilton syringe (100 µL). Separations were achieved on a Qualisil® BDS C18 column (4.6 x 250 mm, 5µm), (LC-GC Chromatography Solutions, Mumbai, India). Stress degradation studies were assisted with an i-Therm® AI-7981, thermostatic water bath with digital controller (Disco, Mumbai, India). Data collection and analyses were made with LC-solution(Shimadzu, Japan) and all weighing operations were carried out on Shimadzu AUX-120 analytical balance(Kyoto, Japan). Ultrasonication of samples was performed using an Enertech Ultrasonicator (Mumbai, India).

2.3 UHPLC-PDA Analyses

Preparation of Stock Standard Solution

A 100 µg/mL stock standard solution was prepared in methanol. Further dilutions were made in mobile phase to get appropriate concentrations in the range of 2-12 µg/mL for linearity studies, and 10 µg/mL for analysis of samples generated after forced degradation studies.

Selection of Detection Wavelength

A dilution of 10 µg/mL, prepared from stock standard solution, was scanned in the UV region 200 - 400 nm. The UV spectrum showed 260 nm as wavelength with maximal
absorbance for ZLT and this wavelength was selected for the RP-HPLC-PDA measurements.

**Chromatographic Specifications**

The method was established using reversed-phase mode on a C18 column in isocratic mode. The wavelength selected for the chromatographic measurement was 260 nm ($\lambda_{\text{max}}$ for ZLT). The optimized mobile phase consisted of methanol: water (80:20 v/v). The pH of the mobile phase was adjusted to 3.0 with OPA. The analysis was performed at ambient temperature of about 30°C.

**2.4 Stress Studies**

The optimized LC method was used to study the degradation behavior of the drug under various stress conditions. Stress studies were carried out as per ICH Q2A (R1) [27]. The stressors, choice of concentrations and preparation of samples were based on a pre-developed laboratory protocol. The applied stress conditions are as presented in Table 1. As the drug is insoluble in water; hydrolytic stress was induced by dissolving 10.0 mg drug in 10.0 mL methanolic solution of stressor (0.5 M HCl/0.1 M NaOH). The resulting solution was transferred into a 50.0 mL round bottom flask (RBF); fitted with a reflux condenser and refluxed for a specified period (Table 1). Sample solution (0.1 mL) was withdrawn and neutralized (measured with pH paper) with appropriate counter blank stressor (0.5 M NaOH/0.1 M HCl respectively). The volume of the resulting solution was made up to 10.0 mL with mobile phase. Oxidative stress was induced to the sample by dissolving 1.0 mg/mL drug in hydrogen peroxide (3% v/v). The resulting solution was kept in the dark at room temperature for 6 h to avoid any degradation by combination of exposed light and the oxidative stressor. The sample solutions from all stressed situations were diluted with mobile phase to achieve a final concentration of 10.0μg/mL before injection into the HPLC system. The solutions thus obtained were then subjected to chromatographic determination.

**2.5 TOF-MS, 1HNMR and LC-MS Studies**

The stressed samples, generated after acidic/alkaline hydrolysis and after oxidation, were subjected to UHPLC analysis. The ZLT standard was separately subjected to LC-MS and 1HNMR analysis.
The stressed sample from forced acidic hydrolysis was also subjected to LC-MS analysis to characterize major degradation products.

The isolation of FDP’s with preparative thin-layer chromatography (TLC) was executed for the stressed alkaline and oxidative samples. The separation of the drug and FDP’s was executed on normal-phase aluminium-backed silica gel 60 F\textsubscript{254} TLC plates (10 cm x 10 cm) layer thickness 0.2 mm, (Merck, Darmstadt, Germany). The mobile phase consisted of toluene-MeOH-GAA (7.0:3.0:0.1 v/v), while the detection wavelength was 260 nm. The volume of chloroform used for extraction of scrapped silica was 5.0 mL. After separation, the silica from the appropriate bands was scraped, saturated overnight with chloroform and filtered to collect the extract. The respective extracts were dried by evaporation of solvent at room temperature to get isolated FDP’s. Purity of FDP’s was confirmed by TLC using the same mobile phase to get single spots. The problems faced during the isolation process were the actual quantities of FDP’s obtained (very few), and their consistency (sticky), i.e. not a free-flowing powder. The separated/isolated samples were then subjected to MS and or \textsuperscript{1}H NMR analysis. The mass spectra from the forced alkaline hydrolysis are shown in Figure 1. The NMR spectrum of the major oxidative degradation product is presented in Figure 2a. The NMR spectra of the forced alkaline hydrolytic products are given in Figures 2b and 2c.

LC-MS analysis of the mixture from the acidic hydrolyzed sample was performed on Varian ProStar 410 AutoSampler in combination with Varian 1200 L LC–MS equipment triple quadrupole mass (QqQ) spectrometer with electro-spray-ionization (ESI) source (Varian, Inc., Seattle, WA, USA). The column was 3 µm C18, 100 mm x 2.1mm ID (Phenomex, Torrance, CA, USA). Separation was achieved using water and methanol (20:80 v/v) as mobile phase at a flow rate of 0.2 ml/min for a total run time of 10 min. The ESI source operated in positive ionization mode. The capillary voltage applied was 80.0 V, at normal scanning speed of 3 microscans (3.33/scan). The data rate was 0.30 Hz with max ion time of 250000 µs. The scanning range employed was 1 - 600 Da.

LC-MS analysis for ZLT standard was performed on Waters Micromass Q-Tof Micro LC-MS, with the ESI source operated in positive mode. The column was a 3 µm C18, 100 mm x 2.1mm (Phenomex). Separation was achieved using water and methanol (20:80 v/v) as
mobile phase at a flow rate of 0.1 mL/min. The ESI source operated in positive ionization mode. The total run time was 30 min. The scanning range employed was 1 - 620 Da.

$^1$H NMR spectrum for ZLT and major oxidized degradation product (ODP I) was obtained from Sophisticated Analytical Instrument Facility, (SAIF), Chandigarh, India and chemical shifts are mentioned in ppm relative to tetramethylsilane (TMS) as internal standard. The $^1$H NMR analyses for ZLT and ODP I were performed on multinuclear FT NMR Spectrometer (400 µHz), model Avance-II accompanied by TOPSPIN NMR data system (Bruker, Massachusetts, USA). The instrument is equipped with a cryomagnet of field strength 9.4 T. The solvent used was deuterated chloroform.

The mass spectra for isolated major forced alkaline degradation products were recorded at SAIF, Chandigarh, India on a Waters MICROMASS Q-TOF mass spectrophotometer.

2.6 XP-molecular docking studies

Maestro 9.1 (Schrodinger, LLC, New York, USA) using Glide version 6.8 (Schrodinger) was applied for the molecular docking studies. The optimal lower energy conformers were generated using Ligprep (version 3.5.9) (Schrodinger) from the structures of ZLT and its RS’s. The coordinates for the 5-lipoxygenase enzyme (PDB Id: 3V99) [28] were taken from the RCSB Protein Data Bank and prepared for docking using ‘Protein Preparation Wizard’ in Maestro (version 10.3)(Schrodinger). The bond orders and formal charges were added for heterogroups and hydrogens to all atoms in the structure. Side chains that are not close to the binding cavity and do not participate in salt bridges were neutralized and termini were capped by adding acetyl (ACE) and N-methyl aniline (NMA) residues.

After preparation, the structure was refined using OPLS_2005 force field to optimize the hydrogen bond network. The minimization was terminated when the energy converged or the root mean squared deviation (RMSD) reached a maximum cutoff of 0.30 Å. The Extra Precision (XP) docking mode was applied for all compounds (ZLT, charactized FDP’s and known pharmacopoeial IMP’s-RS’s) on the generated grid of the protein structure. The final evaluation of the ligand-protein binding was done with the Glide score and its comparison for the set of molecules was made. The results of the molecular docking studies for the comparative predictive evaluation of change (increase or decrease) in binding affinity with the target receptor for the drug ZLT and its characterized FDP’s and RS’s are
presented in Table 2. The prototype images for the visualization of ligand binding affinity for ZLT, ARS and IMPD with the selected 5-LOX receptor are shown in Figure 3.

2.7 PASS prediction studies for therapeutic profiling of ZLT and impurities

Being structurally closely related to the drug, the RS’s (FDP’s and IMP’s) have the drug likeliness more than upcoming frits, leads and new chemical entities (NCE’s). The close resemblance (slight variations in structure) may potentiate or reduce bioactivity (predicted with docking) and/or toxicity (from TOPKAT predictions). The chances of being biologically active at other sites and receptors cannot be ignored either and this can be predicted to some extent by evaluating with PASS. This may potentiate further the drug repurposing; and the repurposing of the RS’s, FDP’s and/or IMP’s can be initiated with such predictions. The results for the predictions of bio-activity for ZLT and its related chemical structures are discussed further in Section 3.4.

2.8 TOPKAT toxicity profiling for ZLT and its FDP’s

The toxicity prediction of ZLT, characterized FDP’s and other known pharmacopeial impurities (RS’s) was made by TOPKAT in Discovery Studio 2.5, Accelrys software (Biovia, San Diego, USA). The software first screens the compound structure against the model sub-structural library, determines whether the molecular structure is adequately covered by the model, and automatically chooses the appropriate chemical class-specific QSAR sub-model to generate the toxicity prediction. TOPKAT uses electrotopological descriptors to predict toxicity. The model generated for the prediction of toxicity results in final output values which predict the toxicological properties of the chemical compounds subjected (Table 3). The decision for a positive or negative toxicity prediction is based on the comparison of “Bayesian Score” with the estimated best cut-off value of chemical structures having toxicity. The prediction is considered positive (toxic) if the Bayesian score is above the estimated best cutoff value. Prediction is considered negative (non-toxic) if situation is opposite. The Bayesian score is supported with the “Probability”. The probability indicates whether the estimated sample is in the category of chemical structures selected by the QSPR model.

Genotoxicity of drugs and their impact on the therapeutic ability is a major pharmaceutical concern nowadays. Though the software predicts the genotoxicity for drugs and impurities, it always has been limited by the cost and accessibility to perform such studies. The
prediction of mutagenicity and carcinogenicity adds towards the ability of compounds to be genotoxic. Hence in the present study TOPKAT was used to assess the Ames-mutagenicity and the carcinogenicity using three different models viz. Mouse-Female; Rat-Female and Rat-Male [29]. The prediction of toxicity was also extended to towards the prediction of Developmental Toxicity and Skin Irritancy [30]. The results for these toxicity predictions are given in Table 3.

3. Results and Discussion

3.1 Mobile-Phase Optimization and Evaluation of System Suitability

Optimization of the mobile phase in UHPLC is a crucial phase which affects the method performance during routine estimations. MeOH and ACN were considered as organic modifiers along with water as main solvent of the mobile phase. Initial trials were executed with the selected organic modifiers and the aqueous phase in equal proportions. Appearance of peak splitting led towards adjustment in the mobile-phase pH which was varied from 1.5 to 4.0 considering the pKa of drug (moderately acidic) using additives such as GAA, OPA and FA in concentrations of 0.1-0.5 % in the aqueous phase. Use of GAA and FA led to more peak tailing, while OPA was found to be suitable with a mobile phase pH of 3.0. Optimal retention (~5 min) for rapid analysis within a run time of 10 min was obtained by varying the proportions of MeOH and water. The optimized mobile phase consisted of methanol: water (80:20 v/v). The pH of the aqueous phase was adjusted to 3.0 ± 0.02 with addition of 0.1 % OPA. The chromatogram is shown in Figure 4a). The measurement was performed at about 30 °C (laboratory temperature), while desired peak purity and peak profile were achieved at 260 nm, as shown in Figures 4b) and 4c), respectively. The mobile phase flow rate was adjusted to 1.0 mL/min. The retention time (Rt) for ZLT was found to be 4.21 ± 0.05 min (n=6) with detection performed at 260 nm. According to USP, system suitability tests are an integral part of liquid chromatographic methods. Retention time, retention factor, number of theoretical plates, asymmetry factor were calculated for standard solutions. The measurements have shown a retention time of 4.21 (± 0.44%) min, peak area 628114 (± 1.05%) theoretical plates (N) > 7431 (±1.07%), tailing factor (A,.) ≤ 1.36 and retention factor (k') ≤ 1.33.

3.2 Forced degradation of ZLT
Forced degradation studies (FDS) by acidic hydrolysis, resulted in three degradation products indicated as ADP I, ADP II and ADP III (Figure 4d); two degradation products, BDP I and BDP II, were produced during alkaline hydrolysis (Figure 4e). Oxidative stress in 3% hydrogen peroxide led to two degradation products ODP I and ODP II (Figure 4f).

3.3 LC-MS-QTOF and $^1$HNMR characterization of ZLT and FDP’s

The resolution of ZLT from the FDP’s was successfully achieved. LC-MS and $^1$HNMR analyses were utilized effectively to characterize the major DP’s. Characterization of the major FDP’s of ZLT was carried out, hence efforts were made to isolate the major alkaline FDP (BDP II) using preparative TLC, while the mixture of degradation products from acidic hydrolysis was subjected directly to LC-MS. The isolated major ODP and BDP’s were subjected to MS and NMR analysis separately. The drug ZLT was also subjected to LC-MS and $^1$HNMR studies to establish the LC-MS compatibility of the method and to compare the drug characterization of FDP’s with ZLT.

The LC-DAD and LC-QTOF chromatograms of ZLT response are shown in Figure 5a), while the TOF mass spectrum for ZLT standard is shown in Figure 5b). The $^1$HNMR spectrum for the ZLT standard is as depicted in Figure 5c). The LC-MS chromatogram for the sample containing acidic hydrolysis degradation products resulted in the resolution of two peaks (expected to be one as a preparative isolation of was performed prior to LC-MS). The corresponding mass spectrum for the major peak led to the identification of the compound as ADP I, (E)-1-(benzo[b]thiophen-2-yl)ethanone oxime with molecular ions peaks at 192 as M+1, and at 193 as M+2. It is the non-pharmacopoeial impurity IMPD, which is represented in Figure 1. The second peak resulted into resolution of two compounds and led to the characterization of an ADPII substance N-(1-(benzo[b]thiophen-2yl)ethyl)hydroxylamine, found to be a non-pharmacopoeial impurity (IMPE) with a molecular ion peak at 192 as M-H as presented in Figure 1. The ZLT standard, (1-(1-(benzo[b]thiophen-2-yl)ethyl)-1-hydroxyurea), was also subjected to LC-MS, studies which resulted into a molecular ion peak at 236.1 and a base peak at 261.0.

$^1$H NMR analyses pertaining to ZLT and FDP’s from alkaline hydrolysis and oxidative stress led to δ ppm values of 9.1 (s, 1H, OH), 7.23-7.77(m, 5H, Ar), 6.0(s, 2H, NH$_2$), 5.71-5.69(m, 1H, CH), and 1.60-1.62(d, 3H, CH$_3$) for ZLT. The δ ppm values of the major FDP from the oxidized sample (ODP I) were 7.26-7.79 (m, 5H, Ar), 6.47(s, H, NH), 6.0(s, 2H,
NH\(_2\)), 5.71-4.47(m, 1H, CH), and 1.27 (d, 3H, CH\(_3\)) which led to the characterization of 1-(1-(benzo[b]thiophen-2-yl)ethyl)urea. This was found to be related substance A (ARS) as designated in USP. One of the major FDP, isolated using preparative TLC from alkaline hydrolysis named as BDP I. This degradant was characterized using \(^1\)HNMR analysis as bis(benzo[b]thiophen-2-yl)methanone (BRS) with \(\delta\) ppm values 7.79 (s, 2H, thiophene), 7.50 (d, 2H, Ar), 7.36 (d, 2H, Ar), 7.26 (s, 4H, Ar). The QTOF-ESI-MS analysis resulted in a mass spectrum depicting a peak at 293.2 corresponding to the molecular weight of BRS as M-1. However, the fragments at 134 and 166 also correspond to the predicted structure. The additional peaks are more distant probably due to the formation of Na/K adducts or impurities remained unresolved and thus are uncorrelated, the information along with NMR corresponds to compound’s probable identity as shown in Figure 6a). Another alkaline hydrolysis FDP, BDP II when subjected to \(^1\)HNMR analysis was identified as 1-benzo[b]thiophen-2-yl-ethanone (CRS) with \(\delta\) ppm values 7.79 (s, 2H, thiophene), 7.50 (d, 2H, Ar), 7.36 (d, 2H, Ar), 7.26 (s, 4H, Ar), and 2.47 (s, 3H, CH\(_3\)). Further structural confirmation was done with the QTOF-ESI-MS analysis depicting a peak at 177 corresponding to the molecular weight of CRS as presented in Figure 6b).

### 3.4 Assessment of comparative bio-affinity with XP-Molecular Docking

The RS’s and FDP’s (Table 2) (characterized and pharmacopoeial) were subjected to molecular docking studies on 5-LOX enzyme to evaluate a possible increase or decrease in binding affinities. Relative to the ZLT standard the changes witnessed for some of the compounds were considerable. For instance, ARS (benzo-thiophene-urea) was found to have higher docking score, while IMPD had a comparable docking score to the ZLT standard, which indicates some changes in the binding affinity. Thus ARS and IMPD may be more or similarly bioactive than the ZLT and are worthwhile to be subjected to biological evaluations. The ARS (Table 2) according to USP is 1-(1-(benzo[b]thiophen-2-yl)ethyl)urea which was a characterized major FDP, while IMPD a characterized non-pharmacopoeial impurity is\((E)-1-(benzo[b]thiophen-2-yl)ethanone oxime.

The binding poses for the RS’s and DP’s of ZLT were duly studied. ARS having an interesting docking score was visualized in the 5-LOX enzyme. ARS and ZLT were found to have similar binding poses and were oriented towards the same hydrophobic region, constituted with amino acid residues Phe555, Gln557, Tyr558, Leu607, and Ser608 in the
active site of the 5-LOX enzyme. ZLT belongs to the group of chelating inhibitors of the 5-LOX, chelating the active iron (Fe) site of the enzyme. The hydroxyl and carbonyl groups of the hydroxy urea part of the ZLT form metal interactions with iron (Fe) in the active site of the 5-LOX enzyme. Similarly, the carbonyl group from the urea part of ARS is found to stabilize the ligand-enzyme complex by forming also metal interaction with Fe. Apart from metal interactions, hydroxyurea of ZLT and urea of ARS also formed a hydrogen bond with Val671. In addition, the amino moiety of urea in ARS and IMPD shows similar interactions and hydrogen bondings with Ala672. BRS, CRS and IMPE found to have docking scores and glide energies lesser than the ZLT thus indicted their chances of being less active as antiasthmatic.

3.5 Prediction of bioactivities for the drug standard and its RS’s and FDP’s

After the in-silico molecular docking evaluation, the ZLT standard, its RS's and FDP’s were screened via PASS for their bioactivity potential. Results indicated that ZLT (Pa: 0.986) and ARS (Pa: 0.985) had the highest probabilities for being active (Pa) as lipoygenase inhibitors and thus good anti-asthmatic agents. BRS (Pa: 0.949) and CRS (Pa: 0.947) were found to have a high probability for being an inhibitor of the Complement Factor D, thereby potentially acting as antitumor agents. These compounds were also predicted to be good as aspulvinone dimethylallyl-transferase inhibitors, and glutamyl endopeptidase II inhibitors. BRS (Pa: 0.773), CRS (Pa: 00.773), IMPE (Pa: 0.710) and IMPG (Pa: 0.794) showed good probabilities for being bioactive as glycosyl phosphatidyl inositol phospholipase D inhibitors. Unique predictions for being antineoplastic (Pa: 0.819) and antiseborrheic (Pa: 0.757) were found for BRS. BRS (Pa: 0.751) and CRS (Pa: 0.751) were also predicted to be good thiorredoxin inhibitors.

3.6 Comparative toxicity prediction studies

The prediction of bioactivities using PASS and the corresponding results motivated us to predict the toxicity of the ZLT impurities as a next step in the evaluation of their potential as new leads. TOPKAT, being best in such predictions, was applied to predict the toxicity. The ZLT standard and related compounds were subjected to evaluations for AMES-mutagenicity, carcinogenicity using three models Mouse-female; Rat-female and Rat-male. Results (Table 3) indicated that ARS may be non-mutagenic with negative Baysian
Scores of -1.514 and -0.770, respectively compared to the best lowest cut off values for being either mutagenic (0.433) or non-mutagenic (-0.795). The carcinogenic potential for ARS and IMPD was small and they were indicated as non-carcinogenic. In contrast, ZLT has an intermediate Baysian score and is evaluated to be slightly mutagenic and carcinogenic. ZLT can be considered as needing a structural fine tuning to make the molecule still less toxic.

The compounds BRS, CRS and IMPE were found to have higher Baysian scores and thus higher chances of being carcinogenic. The same compounds, except IMPD were also predicted to be carcinogenic. All the compounds were predicted to have slight abilities to cause developmental toxicities and all the FDP’s were predicted to be skin non-irritants.

Conclusions
Forced degradation studies were applied and the drug was exposed to hydrolysis (acidic, alkaline and neutral); oxidative and thermal stress conditions. The drug was found to be susceptible to hydrolysis and oxidation. The generated FDP’s were resolved from the drug standard by the developed LC method. The stability results were extended towards LC-MS and 1H NMR analyses for the FDP’s. This led to the characterization of major degradation products. XP-Molecular docking, to assess the binding affinities (Glide score and Glide Energy), led to identification of ARS as an impurity with a binding affinity higher than ZLT. Another molecule IMPD was also found to have a Glide score comparable to that of ZLT. The binding affinities for the other impurities were found to be less. Thus one related substance and one impurity were found to have an interesting binding affinity for 5-LOX enzyme, indicating their possibility of being bioactive in\textit{in-vivo} studies. ZLT and ARS were found to have the highest probability for being active as 5-LOX inhibitors. Consequently, ARS has a good chance of being a good antiasthmatic agent.

Structural resemblance between ZLT and its RS’s reflects their ability of being bio-active. Impurities BRS, CRS and IMPG have the highest probability for being bio-active as Complement factor D inhibitors. The prediction results for BRS showed also high probability to be antiseborrheic. Thus the possibility that related compounds are bio-active on the same or on other target receptors than the initial drug molecule cannot be denied and hence these predictive evaluations could be beneficial to some extent.
TOPKAT predictions of toxicity to assess mutagenicity and carcinogenicity of the drug and its impurities were carried out and the results allowed interesting conclusions. The drug ZLT was predicted to have slight abilities to be mutagenic or carcinogenic while ARS was predicted to be non-mutagenic and less carcinogenic than ZLT. For the other compounds varying toxicological properties were found. All compounds were indicated to be safe for developmental toxicity and skin irritancy. These investigations thus led to some interesting findings, which, may prove beneficial to abridge the pharmaceutical drug analysis with drug discovery.

Acknowledgements
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References


Table 1: Stress conditions for zileuton, generated degradation products with corresponding retention times

<table>
<thead>
<tr>
<th>Stress conditions</th>
<th>Stressor &amp; Concentration</th>
<th>Exposure condition</th>
<th>Duration of Exposure</th>
<th>Degradants and respective retention times ($R_t$) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis Acidic</td>
<td>0.5 M HCl</td>
<td>Reflux 60°C</td>
<td>12 h</td>
<td>ADP I (3.39) and ADP II (4.04) ADP III (5.86)</td>
</tr>
<tr>
<td>Hydrolysis Basic</td>
<td>0.1 M NaOH</td>
<td>Reflux 60°C</td>
<td>4 h</td>
<td>BDP I (3.42) BDP II (4.04)</td>
</tr>
<tr>
<td>Oxidation</td>
<td>3 % H$_2$O$_2$</td>
<td>RT</td>
<td>6 h</td>
<td>ODP I (4.63)</td>
</tr>
</tbody>
</table>

‘RT’- room temperature, ADP I and ADP II - FDP’s from acidic hydrolysis, BDP I, and BDP II- FDP’s from alkaline hydrolysis, and ODP I - FDP from oxidative stress
Table 2: XP-molecular docking results for ZLT and its major FDP’s

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Docking Score</th>
<th>Glide Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZLT</td>
<td><img src="image" alt="Structure" /></td>
<td>-6.11</td>
<td>-31.82</td>
</tr>
<tr>
<td>ARS</td>
<td><img src="image" alt="Structure" /></td>
<td>-6.85</td>
<td>-27.37</td>
</tr>
<tr>
<td>IMPD</td>
<td><img src="image" alt="Structure" /></td>
<td>-6.25</td>
<td>-20.87</td>
</tr>
<tr>
<td>BRS</td>
<td><img src="image" alt="Structure" /></td>
<td>-5.72</td>
<td>-34.77</td>
</tr>
<tr>
<td>IMPE</td>
<td><img src="image" alt="Structure" /></td>
<td>-5.39</td>
<td>-23.32</td>
</tr>
<tr>
<td>CRS</td>
<td><img src="image" alt="Structure" /></td>
<td>-5.02</td>
<td>-21.93</td>
</tr>
<tr>
<td>IMPF</td>
<td><img src="image" alt="Structure" /></td>
<td>-5.28</td>
<td>-22.43</td>
</tr>
<tr>
<td>IMPG</td>
<td><img src="image" alt="Structure" /></td>
<td>-4.89</td>
<td>-20.15</td>
</tr>
</tbody>
</table>

‘ZLT’-Zileuton (111406-87-2#) 1-(1-(benzo[b]thiophen-2-yl)ethyl)-1-hydroxyurea
‘ARS’(USP)(171370-49-3#) 1-(1-(1H-inden-2-yl)ethyl)urea
‘IMPD’ (ADP I)N-(1-Benzo[b]thiophen-2-yl-ethyl)-hydroxylamine
‘BRS’ (USP) (97978-07-9#) 2-(Benzo[b]thien-2-yl)benzo[b]thiophene, Benzo[b]thien-2-yl ketone, Bis(benzo[b]thien-2-yl)methanone
‘IMPE’ (ADP II)(E)-1-(benzo[b]thiophen-2-yl)ethanone oxime

‘CRS’ (USP)1-(benzo[b]thiophen-2-yl)ethanone

‘IMPF’ (118564-89-9*) N-(1-Benzo[b]thiophen-2-yl-ethyl)-hydroxylamine

‘IMPG’2-Ethyl-benzo[b]thiophene
Table 3: TOPKAT toxicity analyses for Zileuton (ZLT) and its impurities

<table>
<thead>
<tr>
<th>Models</th>
<th>Drug Characterized Impurities</th>
<th>Known Impurities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZLT</td>
<td>ARS</td>
</tr>
<tr>
<td>Ames-mutagenicity</td>
<td>0.756</td>
<td>0.707</td>
</tr>
<tr>
<td></td>
<td>0.849</td>
<td>-1.514</td>
</tr>
<tr>
<td></td>
<td>-0.795</td>
<td>-0.690</td>
</tr>
<tr>
<td>Carcinogenicity (Mouse-Female)</td>
<td>0.580</td>
<td>0.605</td>
</tr>
<tr>
<td></td>
<td>0.230</td>
<td>1.537</td>
</tr>
<tr>
<td></td>
<td>-0.526</td>
<td>-0.555</td>
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<tr>
<td>Carcinogenicity (Mouse-Male)</td>
<td>0.089</td>
<td>0.465</td>
</tr>
<tr>
<td></td>
<td>-1.468</td>
<td>-1.468</td>
</tr>
<tr>
<td>Carcinogenicity (Rat-Female)</td>
<td>0.548</td>
<td>0.537</td>
</tr>
<tr>
<td></td>
<td>2.043</td>
<td>1.289</td>
</tr>
<tr>
<td></td>
<td>-0.575</td>
<td>-0.575</td>
</tr>
<tr>
<td>Carcinogenicity (Rat-Male)</td>
<td>0.616</td>
<td>0.627</td>
</tr>
<tr>
<td></td>
<td>0.671</td>
<td>1.178</td>
</tr>
<tr>
<td></td>
<td>-0.497</td>
<td>-0.497</td>
</tr>
<tr>
<td>Developmental Toxicity</td>
<td>0.496</td>
<td>0.526</td>
</tr>
<tr>
<td></td>
<td>0.453</td>
<td>0.197</td>
</tr>
<tr>
<td></td>
<td>-0.422</td>
<td>-0.512</td>
</tr>
<tr>
<td>Skin Irritancy Mild vs Mod/Sev</td>
<td>0.268</td>
<td>0.245</td>
</tr>
<tr>
<td></td>
<td>-1.482</td>
<td>-0.758</td>
</tr>
<tr>
<td></td>
<td>-0.650</td>
<td>-0.210</td>
</tr>
</tbody>
</table>

*Values in bold highlight significant non-toxic responses for each model; Top - Probability; Middle - Bayesian score, Bottom - Estimated best cutoff*
Figure Captions

**Fig. 1:** LC-MS studies for the acid stressed sample showing (a) the TIC chromatogram, (b) Q-TOF-mass spectrum for ADP I (IMPD) and (c) ADP II (IMPE)

**Fig. 2:** 1H NMR spectra for the oxidative and basic hydrolytic FDP’s of ZLT, a) ODP-I - Oxidized degradation product I b) BDP-I - Basic degradation product I, and c) BDP-II Basic degradation product II

**Fig. 3:** 3D - XP-docking images showing receptor interactions for a) ZLT, b) ARS Characterized (ODP I), and c) IMPD (ADP I)

**Fig. 4:** a) UHPLC-PDA chromatogram of ZLT (λ<sub>max</sub> = 260 nm), b) Peak purity spectrum, c) Peak profile at different wavelengths (210, 220...300 nm), d) acidic hydrolysis products (ADP I - 3.392 min, ADP II - 4.041 min and ADP III - 5.862 min) e) alkaline degradation (BDP I - 3.415 min and BDP II - 4.036 min), and f) Chromatogram for oxidative (ODP - 4.629 min)

**Fig. 5:** ZLT standard, a) LC-DAD/TOF-MS Chromatogram, b) TOF-MS spectrum, and c) 1H NMR spectrum

**Fig. 6:** QTOF ESI-MS spectrum for a) BDP I (BRS) and b) BDP II (CRS)
Fig. 1: LC-MS studies for the acid stressed sample showing (a) the TIC chromatogram, (b) Q-TOF-mass spectrum for ADP I (IMPD) and (c) ADP II (IMPE)
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