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Liquid chromatography electrospray ionization tandem mass spectrometry study of nilutamide and its stress degradation products: *in silico* toxicity prediction of degradation products[†]

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ABSTRACT: Nilutamide, a nonsteroidal anti-androgen drug, widely used in the treatment of prostate cancer, was subjected to hydrolytic, photolytic, thermal and oxidative stress conditions as per International Conference on Harmonization guidelines Q1A (R2). Nilutamide showed significant degradation under basic hydrolysis and photolytic stress conditions, while it was stable to neutral, acidic and thermal stress conditions. Five degradation products were formed and the chromatographic separation of nilutamide and its degradation products was achieved on a Waters C₁₈ column (4.6 × 250 mm, 5 μm) using a mobile phase consisting of acetonitrile and 0.1% of formic acid in an isocratic elution method. All these degradation products were characterized by LC/MS/MS in negative ion mode, combined with accurate mass measurements. To assign likely structures for the observed degradation products, the fragmentation patterns of the deprotonated drug and its degradation products were compared. The *in silico* toxicity of the drug and its degradation products was also assessed using TOPKAT software. The carcinogenicity probability of the degradation products, DP-I–IV, was greater than that of nilutamide. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: nilutamide; degradation products; LC/ESI-MS/MS; accurate mass measurements

Introduction

Nilutamide (NLM) is a nonsteroidal anti-androgen drug primarily used in the treatment of prostate cancer. NLM {5,5-dimethyl-3-[4-nitro-3-(trifluoromethyl) phenyl] imidazolidine-2, 4-dione; Scheme 1} belongs to the class of nitroaromatic compounds. It blocks the action of androgens of adrenal and testicular origin, which stimulate the growth of normal and cancerous prostatic tissue (Harris *et al.*, 1993). Reports on the lung toxicity of drugs containing nitro aromatic groups explain their toxicity as resulting from ring oxidation and nitro reductive biotransformation pathway (Kedderis and Miwa, 1988; Foth, 1995; Gram, 1997). Acute liver failure, interstitial pneumonitis and mild elevations in serum enzymes (ALT and alkaline phosphatase) have been reported during NLM therapy (<http://livertox.nih.gov/Nilutamide.htm>). These led us to investigate the chemical stability of drug. The drug substance monograph on NLM in the British Pharmacopoeia listed four impurities (WHO, 2009).

Stability studies are a critical part of the drug development process and it is highly desirable to study the degradation behavior of the drug under various stress conditions and also to characterize the degradation products. The stress conditions for systematic forced degradation studies are given in International Conference on Harmonization (ICH) and World Health Organization (WHO) guidelines. The stability testing guidelines Q1A (R2) developed by ICH experts require validated stability-indicating analytical procedures (ICH, 2003). These analytical procedures involve the identification of degradation products and degradation pathways, and the investigation of the intrinsic stability of drugs. Characterization of degradation products and

understanding of the degradation pathways are crucial for the assessment of the safety and potency of the drugs in pre-clinical studies. LC/MS/MS in combination with accurate mass measurements is increasingly being used for the structural characterization of degradation products of drugs formed under various stress conditions (Novak *et al.*, 2009; Murakami *et al.*, 2008; Shah *et al.*, 2008; Feng *et al.*, 2001; Raju *et al.*, 2011; Borkar *et al.*, 2012, 2013; Purna Chander *et al.*, 2013). The information from the structural characterization of degradation products is expected to be useful for the development of new drugs with improved stability.

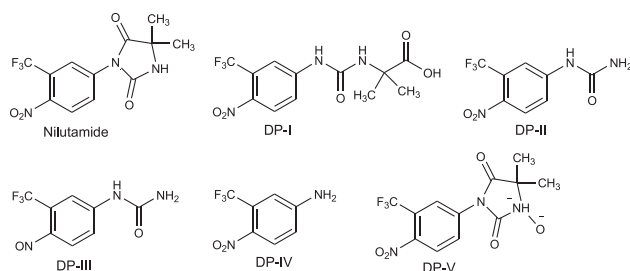
As there is no published LC/MS method for the investigation of the degradation behavior and stability of NLM, we have studied the identification and structural characterization of the degradation products of NLM by negative ion LC/ESI-MS/MS and accurate mass measurements. A further objective was to assess the *in silico* toxicity of all the degradation products using TOPKAT software (Enslein *et al.*, 1994) followed by comparison

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Abbreviations used: ESI, electrospray ionization; ICH, International Conference on Harmonization; NLM, nilutamide; WHO, World Health Organization.



Scheme 1. Structure of nilutamide and proposed structures of degradation products (DP-I to DP-V).

of the results with those for NLM. The results of the present study may also be helpful for generic pharmaceutical industries in ensuring the quality of production and supporting site or other changes to the product. The *in silico* toxicity studies may also be useful in establishing the toxicity profile of NLM.

Experimental

Chemicals and reagents

Pure nilutamide was purchased from Sigma Aldrich (St Louis, MO, USA). Acetonitrile, methanol, formic acid, sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). All reagents used were of analytical grade and the acetonitrile and methanol were HPLC grade. Water was purified by a Milli-Q® system (Millipore, Milford, MA, USA).

Instrumentation

High-performance liquid chromatography and mass spectrometry.

The high-performance liquid chromatography (HPLC) analysis was performed on Waters 2695 HPLC system consisting of an auto sampler, a P4000 pump, photo diode array detector and column compartment (Waters Corp., Milford, MA, USA). For liquid chromatography (LC/MS) analysis, a Waters HPLC system was coupled to a Quattro Micro LC-triple-quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) source. The data acquisition was under the control of Masslynx software (version 4.1; Micromass). The ESI mass spectra were recorded by scanning MS1 in negative ion mode. The capillary voltage was maintained between 3.5 and 4.0 kV, and nitrogen was used as the desolvation and nebulization gas at flow rates of 500 and 60 L/h, respectively. The source and desolvation temperatures used were 130 and 300 °C, respectively. The scan time and the interscan delay time were fixed at 1.0 and 0.1 s, respectively. All the compounds studied yielded abundant $[M - H]^-$ ions. For MS/MS experiments, keeping MS1 static, the precursor $[M - H]^-$ ion of interest was selected with an isolation width of 1 m/z unit, and the product ion spectra were obtained by scanning MS2. Argon was used as the collision gas for MS/MS experiments. The collision energy used was 20–30 eV.

Accurate mass measurements were performed using a quadrupole time-of-flight mass spectrometer (QSTAR XL; Applied Biosystems/MDS Sciex, Foster City, CA, USA), equipped with an ESI source. Data acquisition was under the control of Analyst QS software (Applied Biosystems/MDS Sciex). The source conditions were: capillary voltage, 5 kV; declustering potential, 70 V; focusing potential, 280 V; declustering potential 2, 10 V; collision energy 15–25 eV; mass resolution 8000 (full-width half-maximum). The samples were infused into the ESI (negative ion mode) source at a flow rate of 10 μ L/min using an in-built syringe pump. Mass spectra were acquired from m/z 50 to 1000. Ultra high pure nitrogen was used as the curtain gas and collision gas, whereas zero air was used as the nebulizer gas. The $[M - H]^-$ ions were selected as precursors by the quadrupole

and allowed to collide with nitrogen gas in the collision cell. The product ion spectra were then acquired by a time-of-flight mass analyzer.

A water bath equipped with a temperature controller was used to perform degradation studies. A controlled temperature dry air oven was used for the solid-state thermal stress studies. A photostability chamber (Sanyo, Loughborough, UK), with both UV and fluorescent lamps, was used for the photo degradation study. A calibrated lux meter and a UV meter were used for the energy measurements. All pH measurements were carried out on a Metrohm 780 pH meter (Metrohm AG, Herisau, Switzerland) and weighing was performed on a Sartorius CD 225 D balance (Sartorius, Göttingen, Germany).

Stress degradation studies

Stress degradation studies of NLM were carried out under the ICH prescribed conditions of hydrolysis (acidic, alkaline, and neutral), photolysis, oxidation and thermal stress. Acidic, basic and neutral hydrolysis was carried out by refluxing NLM with 0.5 M HCl for 48 h, 0.01 M NaOH for 24 h and water for 48 h, respectively, at room temperature. An oxidative study was carried out with 6% H_2O_2 at room temperature for 72 h. The photostability study was carried out by exposing NLM to 1.2×10^6 lx h of fluorescent light and 200 W h/m² of UV light in a photostability chamber (ICH, 1996). For the thermal degradation study, NLM was spread over a petri dish and kept at 60 °C for 10 days.

Sample preparation

All stress samples were diluted 10 times with the mobile phase. All degradation studies were carried out with a drug concentration of 1 mg/mL. All solutions were filtered through 0.22 μ m pore size nylon 66 membrane filter before HPLC and LC/MS analysis.

Results and discussion

The separation of NLM and its degradation products was achieved on a Waters C₁₈ column (4.6 \times 250 mm, 5 μ m) using a mobile phase consisting of acetonitrile (A) and 0.1% of formic acid in water (B) in the ratio of 70:30 at a flow rate of 0.6 mL/min.

Figure 1(a–c) shows the LC/ESI-MS total ion chromatograms of the unreacted drug, and the degradation products formed under

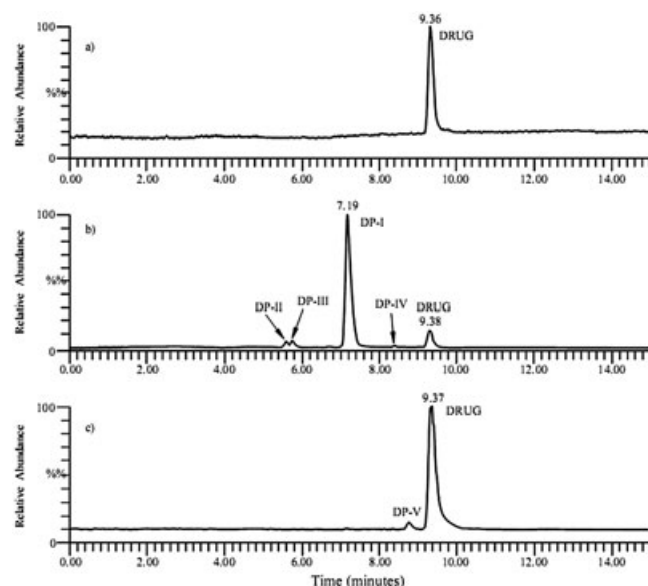


Figure 1. LC/ESI-MS total ion chromatograms of the drug (a), its degradation products under base hydrolysis (b) and under photolysis (c).

basic and photolytic conditions, respectively. The drug forms four degradation products, DP-I (retention time, $R_t = 7.9$ min), DP-II ($R_t = 5.6$ min), DP-III ($R_t = 5.8$ min) and DP-IV ($R_t = 8.4$ min) under

alkaline hydrolysis, and fifth, DP-V ($R_t = 8.8$ min), under photolytic conditions, while it was stable to neutral, acidic and thermal stress conditions (Scheme 1).

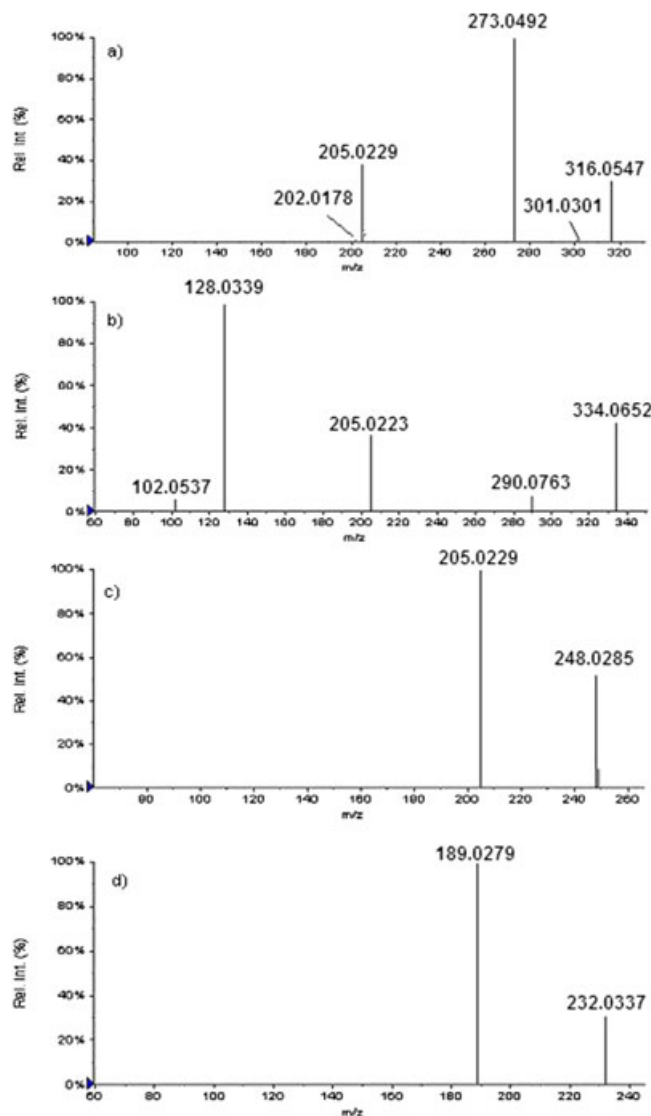


Figure 2. LC/MS/MS quantitative time-of-flight-MS/MS spectra of deprotonated (a) nilutamide, (b) DP-I, (c) DP-II and (d) DP-III.

ESI-MS/MS of the $[M - H]^-$ ion of NLM

The negative ion ESI-MS of NLM shows an abundant $[M - H]^-$ ion at m/z 316 whose MS/MS spectrum displays product ions at m/z 301 (loss of CH_3), m/z 273 (loss of $HNCO$), m/z 205 and m/z 84 (Fig. 2a and Scheme 2).

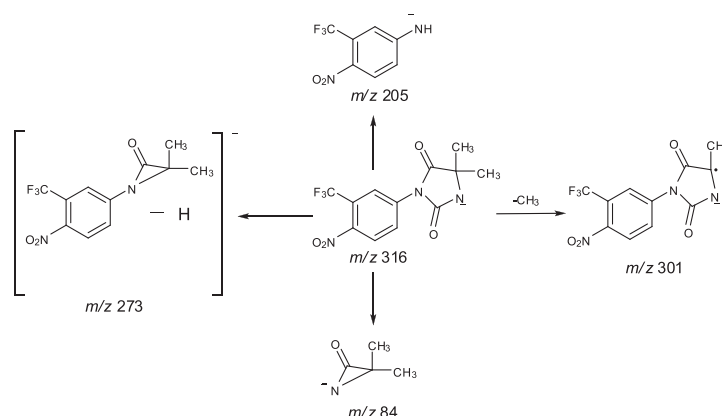
Characterization of degradation products using LC/MS/MS

DP-I. The LC/ESI-MS spectrum of DP-I shows an $[M - H]^-$ ion at m/z 334 with an elemental composition of $C_{12}H_{11}N_3O_5F_3^-$. Its MS/MS spectrum exhibits product ions at m/z 290 (loss of CO_2), m/z 205 [the 4-nitro-3-(trifluoromethyl)benzenamine anion], m/z 128 and m/z 102 (Fig. 2b and Scheme 3). The ion at m/z 290 formed by the loss of CO_2 suggests the presence of a carboxylic acid group in DP-I. The formation of DP-I may be explained by nucleophilic (OH^-) attack on the C-4 carbonyl group resulting in the ring opening reaction (Scheme 3; Blagoeva *et al.*, 1978; Bergon and Calmon, 1978). Based on the structure of NLM and the observed fragmentation of the deprotonated molecule, with accurate mass measurement data (Table 1), the 2-methyl-2-[3-[4-nitro-3-(trifluoromethyl)phenyl]ureido] propanoic acid structure is proposed for DP-I.

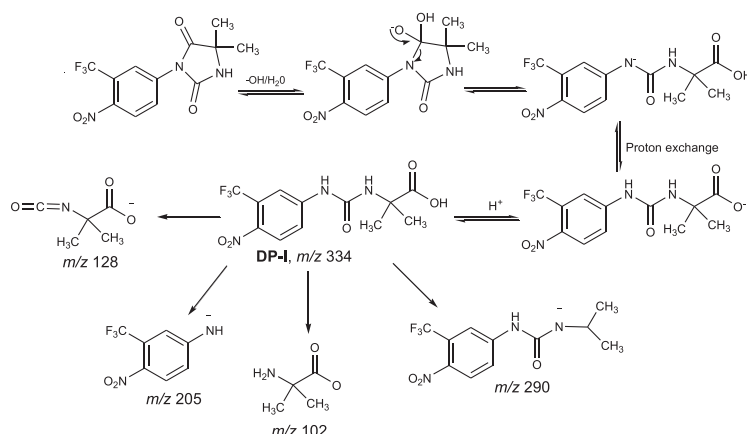
DP-II. The LC/ESI-MS spectrum of DP-II shows an $[M - H]^-$ ion at m/z 248 whose MS/MS spectrum gives a product ion at m/z 205 corresponding to the 4-nitro-3-(trifluoromethyl) benzenamine anion (Scheme 4). The elemental composition of deprotonated DP-II (m/z 248) was found to be $C_8H_5F_3N_3O_3^-$. Based on the MS/MS spectrum of m/z 248 and accurate mass measurement data, the structure of DP-II is proposed as 1-[4-nitro-3-(trifluoromethyl) phenyl] urea.

DP-III. The LC/ESI-MS spectrum of DP-III shows an $[M - H]^-$ ion at m/z 232 whose MS/MS spectrum exhibits a product ion at m/z 189 (loss of $HNCO$; Scheme 4). The elemental composition of deprotonated DP-III was found to be $C_8H_5F_3N_3O_2^-$. Based on these data, the structure of DP-III was assigned as 1-[4-nitroso-3-(trifluoromethyl) phenyl] urea.

DP-IV and DP-V. The LC/ESI-MS spectra of DP-IV and DP-V show $[M - H]^-$ ions at m/z 205 and m/z 332 with elemental composition $C_7H_4N_2O_2F_3^-$ and $C_{12}H_9N_3O_5F_3^-$, respectively. As can be



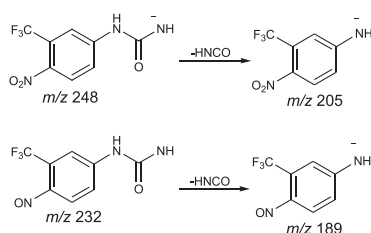
Scheme 2. Proposed fragmentation pathway of deprotonated nilutamide (m/z 316).



Scheme 3. Proposed fragmentation pathway of deprotonated DP-I (m/z 334).

Table 1. Elemental compositions of ions formed for nilutamide and its degradation products

[M – H] [–] of the drug/ degradation product	Calculated mass (m/z)	Observed mass (m/z)	Proposed formula	Error (ppm)
Nilutamide	316.0545	316.0547	C ₁₂ H ₉ N ₃ O ₄ F ₃ [–]	–0.99
	301.0310	301.0301	C ₁₁ H ₆ N ₃ O ₄ F ₃ [–]	6.84
	273.0487	273.0492	C ₁₁ H ₈ N ₂ O ₃ F ₃ [–]	–0.73
	205.0224	205.0229	C ₇ H ₄ N ₂ O ₂ F ₃ [–]	1.03
DP-I	334.0650	334.0652	C ₁₂ H ₁₁ N ₃ O ₅ F ₃ [–]	–0.24
	290.0752	290.0763	C ₁₁ H ₁₁ N ₃ O ₃ F ₃ [–]	3.96
	205.0224	205.0223	C ₇ H ₄ N ₂ O ₂ F ₃ [–]	–2.86
	128.0347	128.0339	C ₅ H ₆ NO ₃ [–]	–7.56
DP-II	248.0283	248.0285	C ₈ H ₅ F ₃ N ₃ O ₃ [–]	0.80
	205.0224	205.0229	C ₇ H ₄ N ₂ O ₂ F ₃ [–]	2.01
DP-III	232.0334	232.0337	C ₈ H ₅ F ₃ N ₃ O ₂ [–]	1.35
	189.0276	189.0279	C ₇ H ₄ N ₂ OF ₃ [–]	1.73
DP-IV	205.0224	205.0227	C ₇ H ₄ N ₂ O ₂ F ₃ [–]	1.03
DP-V	332.0494	332.0498	C ₁₂ H ₉ N ₃ O ₅ F ₃ [–]	1.11



Scheme 4. Proposed fragmentation pathway of deprotonated DP-II (m/z 248) and deprotonated DP-III (m/z 232).

seen from Fig. 1, these DPs are formed in very low concentrations; hence, their [M – H][–] ions could not be subjected to MS/MS experiments. However, based on the fragmentation of deprotonated NLM, which forms a product ion at m/z 205 with an identical elemental composition, the 3-trifluoromethyl-4-nitro aniline structure can be proposed for DP-IV. A mass difference of 16u, from an additional oxygen atom, in the elemental composition of deprotonated DP-V, from deprotonated NLM suggests that DP-V corresponds to *N*-oxide nilutamide (Scheme 1). From the present study, it is difficult to make further comment on this.

In silico toxicity prediction

The *in silico* toxicities of NLM and its degradation products were predicted by TOPKAT software. The TOPKAT calculations were based on a quantitative structure–toxicity relationship model. Probability values from 0.0 to 0.30 are considered low probabilities, and are expected to produce a negative response in an experimental assay, while probability values >0.70 are considered high, and are expected to produce a positive response in an experimental assay. Probabilities of 0.30–0.70 are considered as indeterminate. Table 2 shows the results of the predicted toxicity and carcinogenicity for NLM and its degradation products. The toxicities of NLM and its degradation products were assessed and compared in different animal models, and both NLM and its degradation products showed toxicity and carcinogenicity. The probabilities of degradation products DP-I to -4 being toxic were higher than that of NLM. Developmental toxicity potential and skin irritation were observed only in NLM whereas Ames mutagenicity was observed in DP-III and DP-IV. The toxicity of the degradation products was assessed as being similar to that of the drug. For example, the Skin Sensitization MLD/MOD v SEV (v6.1) model indicated high probabilities for toxicity for NLM and for all the degradation products except DP-I. On the other hand the Ocular Irritancy MLD vs NON (v5.1)

Table 2. Probability values of different toxicity models of nilutamide and its degradation products by TOPKAT^a analyses

Models	Nilutamide	DP-I	DP-II	DP-III	DP-IV
NTP Carcinogenicity Call (Male Rat) (v3.2)	0.003	0.004	0.002	0.007	0.334
NTP Carcinogenicity Call (Female Rat) (v3.2)	0.000	0.196	0.000	0.752	0.008
NTP Carcinogenicity Call (Male Mouse) (v3.2)	0.000	0.002	0.000	1.000	0.000
NTP Carcinogenicity Call (Female Mouse) (v3.2)	0.000	0.000	0.000	0.000	0.000
FDA Carcinogenicity Male Rat Non vs Carc (v3.1)	0.031	0.992	1.000	0.000	0.842
FDA Carcinogenicity Male Rat Single vs Mult (v3.1)	1.000	1.000	1.000	1.000	1.000
FDA Carcinogenicity Female Rat Non vs Carc (v3.1)	0.000	0.000	0.000	0.007	0.000
FDA Carcinogenicity Female Rat Single vs Mult (v3.1)	0.000	0.000	0.000	1.000	0.000
FDA Carcinogenicity Male Mouse Non vs Carc (v3.1)	1.000	0.106	0.993	0.991	0.951
FDA Carcinogenicity Male Mouse Single vs Mult (v3.1)	0.000	0.000	0.000	0.000	0.000
FDA Carcinogenicity Female Mouse Non vs Carc (v3.1)	0.001	0.000	0.000	0.000	0.000
FDA Carcinogenicity Female Mouse Single vs Mult (v3.1)	0.000	0.000	0.000	0.000	0.000
Weight of Evidence Carcinogenicity Call (v5.1)	0.000	0.665	0.000	0.055	0.000
Ames Mutagenicity (v3.1)	0.000	0.000	0.977	1.000	0.004
Developmental Toxicity Potential (DTP) (v3.1)	1.000	0.001	0.630	0.005	0.516
Rat Oral LD ₅₀ (v3.1) (g/kg)	5.800	4.000	964.7	2.00	1.3
Rat Maximum Tolerated Dose – Feed/Water (v6.1) (mg/kg)	79.8	681.2	86.2	922.7	32.2
Rat Inhalational LC 50 (v6.1) (g/m ³ /h)	10.00	10.00	6.3	4.1	10
Chronic LOAEL (v3.1) (mg/kg)	6.3	1.9	113.3	565.9	110.3
Skin Irritation (v6.1)	1.000	0.000	0.000	0.000	0.158
Skin Sensitization NEG v SENS (v6.1)	0.016	0.105	0.620	0.910	0.173
Skin Sensitization MLD/MOD v SEV (v6.1)	1.000	0.672	1.000	1.000	1.000
Ocular Irritancy SEV/MOD vs MLD/NON (v5.1)	1.000	0.889	0.860	0.093	0.831
Ocular Irritancy SEV vs MOD (v5.1)	1.000	0.078	0.003	0.000	0.000
Ocular Irritancy MLD vs NON (v5.1)	0.000	1.000	0.999	0.989	0.736
Aerobic biodegradability (v6.1)	0.000	0.000	0.000	0.000	0.000
Daphnia EC ₅₀ (v3.1) (μg/L)	850.1	836.4	3.8 ^b	4.5 ^b	4.7 ^b

^aEnslein et al. (1994).^bmg/L.

model revealed higher toxicity for the degradation products than for NLM.

Conclusions

The LC/MS method described in the present study can resolve all the degradation products from NLM as well as from each other under various stress conditions. The drug formed four degradation products in alkaline condition and one degradation product in photolytic condition, while it was stable to other degradation conditions. These degradation products were characterized using LC/ESI-MS/MS experiments combined with accurate mass measurements. *In silico* toxicity studies were used to predict the relative toxicities of the degradation products. In considering the probabilities of carcinogenicity and the ocular irritancy MLD vs NON (v5.1) model, the degradation products DP-I–IV showed higher potential values than NLM.

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