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Forced degradation of fentanyl: Identification and analysis of impurities and degradants☆

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ABSTRACT

Fentanyl, N-(1-phenethylpiperidin-4-yl)-N-phenylpropionamide is a rapid-acting, powerful opioid analgesic used extensively for anesthesia and chronic pain management. A forced degradation study of fentanyl active pharmaceutical ingredient (API) was performed using light, acid, base, heat and oxidation. Under acidic conditions, fentanyl was shown to degrade to N-phenyl-1-(2-phenylethyl)-piperidin-4-amine (PPA[1]). Fentanyl was stable to light exposure and base treatment with no degradation observed. Oxidation with hydrogen peroxide produced fentanyl N-oxide by rapidly oxidizing the nitrogen on the piperidine ring. Five degradants were formed during thermal degradation of fentanyl. The two known degradants included propionanilide (PRP[2]) and norfentanyl (NRF[3]). The three unknown degradants were first identified by mass using LC/MS, and postulated compounds were synthesized and confirmed by LC/MS and ^{1}H NMR. These degradants were identified as 1-phenethylpyridinium salt (1-PEP[4]), 1-phenethyl-1H-pyridin-2-one (1-PPO[5]), and 1-styryl-1H-pyridin-2-one (1-SPO[6]). In addition to the seven degradants, three known process impurities, acetyl fentanyl, pyruvyl fentanyl and butyryl fentanyl were also detected by reverse-phase high performance liquid chromatography (HPLC) with UV detection. All degradants and impurities were identified and confirmed using authentic materials. Method validation was performed for the assay of fentanyl and its related compounds in accordance to ICH guideline Q2(R1), and the method was demonstrated to be specific, linear ($r > 0.999$ for fentanyl assay and $r > 0.996$ for related compounds), accurate (recovery > 99.6% for fentanyl assay and recovery > 91.0 for related compounds), precise (%RSD < 0.8% for fentanyl assay and <4.8% for related compounds), sensitive (limit of detection = 0.08 μg/mL or 0.016% of nominal concentration), robust and suitable for its intended use. The chemical structures for the degradants and impurities were submitted to three *in silico* toxicity programs to identify any structural alerts.

1. Introduction

Fentanyl is a controlled substance and has been categorized as a Schedule II drug under the "Controlled Substance Act". Forced degradation samples produced seven degradants, phenethylpyridinium salt (1-PEP), norfentanyl (NRF), propionanilide (PRP), N-phenyl-1-(2-phenylethyl)-piperidin-4-amine (PPA), 1-phenethyl-1H-pyridin-2-one (1-PPO), fentanyl N-oxide, and 1-styryl-1H-pyridin-2-one (1-SPO). In addition, three known process impurities, acetyl, pyruvyl and butyryl fentanyl were also monitored. Structures for fentanyl degradants and process impurities are depicted in Table 1.

Previously, Rabinowitz et al. [1] compared the purity of fentanyl starting material (>99%) to fentanyl powder heated on a hot plate at 300 °C, which showed approximately 30% degradation. Two major and three minor peaks were observed in the chromatogram for the fentanyl material heated on a hot plate, but no identification was provided.

A forced degradation study on fentanyl was reported by Lambropoulos et al. [2] using light, acid, base, heat and oxidation. PPA formation was reported under acidic conditions, but unknown peaks generated by light, base, heat and oxidation were not identified. Chen et al. [3] developed an analytical method for the quantitation of PPA, which was suggested as a potential genotoxic compound due to the aniline moiety in the structure. The European Pharmacopoeia (EP) has designated fentanyl impurities by

[1] N-Phenyl-1-(2-phenylethyl)-piperidin-4-amine.
[2] Propionanilide.
[3] Norfentanyl.
[4] 1-Phenethylpyridinium salt.
[5] 1-Phenethyl-1H-pyridin-2-one.
[6] 1-Styryl-1H-pyridin-2-one.

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Table 1
Impurities/degradants trends in fentanyl API by changing heating time.

Peak	MW	RRT	RRF	Area % of impurity/degradant					
				Process impurity	Light	Acid	Base	Heat	Oxidation
1-PEP[a]	184	0.36	0.54	–	–	–	–	0.27	–
NRF[b]	232	0.55	0.86	–	–	–	–	1.38	–
PRP[c]	149	0.81	0.74	–	–	–	–	3.77	–
PPA[d]	280	0.79	0.50	–	–	34.64	–	–	–
Unknown	ND	0.84	–	–	–	–	–	0.19	–
Unknown	ND	0.86	–	–	–	–	–	0.11	–
Acetyl fentanyl	322	0.89	0.87	Present in fentanyl base at 0.04%	0.04	0.04	0.05	0.05	0.03
Unknown	ND	0.93	–	–	–	–	–	1.47	–
Fentanyl base	336	1.00	1.00	–	99.96	64.78	98.8	87.5	92.3
1-PPO[e]	199	1.08	1.06	–	–	–	–	0.12	–
Fentanyl N-oxide, RRT 1.11	352	1.11	0.83	–	–	–	–	–	6.51
Fentanyl N-oxide, RRT 1.18	352	1.18	–	–	–	–	–	–	0.56
Unknown	ND	1.19	–	–	–	–	–	0.26	–
1-SPO[f]	197	1.25	1.52	–	–	–	–	0.06	–
Unknown	ND	1.45	–	–	–	–	–	0.37	–
Unknown	ND	1.78	–	–	–	–	–	0.95	-

MW = molecular weight; RRT = relative retention time; RRF = relative response factor; ND = not determined.
[a] 1-Phenethylpyridinium salt.
[b] Norfentanyl.
[c] Propionanilide.
[d] N-Phenyl-1-(2-phenylethyl)-piperidin-4-amine.
[e] 1-Phenethyl-1H-pyridin-2-one.
[f] 1-Styryl-1H-pyridin-2-one.

letters [4], where A: N-oxide, B: NRF, C: acetyl, D: PPA, E: benzaldehyde, F: aniline, and G: PRP. EP also describes a fentanyl HPLC method where acetonitrile and ammonium carbonate (pH 9.0) is used with tetrahydrofuran as an additive. The EP method has low sensitivity since fentanyl is practically insoluble at pH 9.0. No other information on forced degradation of fentanyl was found in the literature.

There is an increased interest in the identification and control of potentially genotoxic impurities. In 2007, the European Medicines Agency (EMEA) issued guidelines on the limits of genotoxic impurities [5], and the Food and Drug Administration (FDA) issued draft guidance on the same subject in December of 2008 [6]. Because of the structure of fentanyl, and the possibility of generating aromatic amines or amine derivatives via degradation, we identified the impurities resulting from common degradation pathways, and validated a stability-indicating method to separate fentanyl from its process impurities and degradants.

2. Experimental

2.1. Materials

Fentanyl base was purchased from Mallinckrodt (Hazelwood, MO, USA). USP standard fentanyl citrate was used for HPLC quantitation. All impurities and degradants including 1-PPO, 1-SPO, 1-PEP, PRP, NRF, acetyl, pyruvyl and butyryl fentanyl were synthesized and qualified as standards at Alexza Pharmaceuticals Inc. PRP and NRF were also available from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile from J.T. Baker (Phillipsburg, NJ, USA) was used in the mobile phase. HPLC grade methanol from Honeywell Burdick & Jackson (Muskegon, MI, USA) was used as the sample extraction solvent. Sodium phosphate monobasic (Ultrapure LC grade), trifluoroacetic acid (TFA), 5N hydrochloric acid and 5N sodium hydroxide (both Baker analyzed reagents) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Hydrogen peroxide and HPLC grade phosphoric acid were obtained from Sigma Aldrich (St. Louis, MO, USA) and EMD (Gibbstown, NJ, USA), respectively. Milli-Q water was produced at Alexza Pharmaceuticals Inc. using a water purification system from Millipore (Billerica, MA, USA).

2.2. High performance liquid chromatography

The HPLC system consisted of a Waters (Milford, MA) Alliance HPLC 2695 equipped with a 2996 photo diode array (PDA) detector and an Empower 2 data acquisition system. The analyses were carried out on a Phenomenex (Torrance, CA) Gemini C18, 150 mm × 3.0 mm, 5 μm particle diameter column. Mobile Phase A was phosphate buffer (pH 2.0), prepared by dissolving 8 g of sodium phosphate monobasic in 1 L of deionized water, and the pH adjusted to 2.0 with phosphoric acid. Mobile phase B was a 1:1 mixture of mobile phase A and acetonitrile. UV detection was carried out at 215 nm and the flow rate was kept at 0.5 mL/min. The data acquisition time was 35 min. The pump gradient program was as follows: time (min)/A (v/v):B (v/v); $T_{0.0}$/82:18, $T_{2.0}$/82:18, $T_{31.0}$/0:100, $T_{32.0}$/82:18, $T_{35.0}$/82:18. The fentanyl target assay concentration was 500 μg/mL.

2.3. LC/MS analysis

LC/MS analysis was conducted using an Applied Biosystems (Foster City, CA, USA) 3200 Q-Trap coupled to an Agilent (Santa Clara, CA, USA) 1100 HPLC equipped with a diode array detector. Electrospray ionization mass spectrometry (ESI-MS) and atmospheric pressure chemical ionization mass spectrometry (APCI-MS) were used to produce ions. Analyst® Software was used for data acquisition and data processing. The LC/MS spectra were acquired from m/z 80–800 amu. The analysis was carried out using Waters SymmetryShield RP18, 100 mm × 3.0 mm column with 3.5 μm particle size. Mobile phase A consisted of 0.1% TFA in Milli-Q water and mobile phase B was a mixture of 40% of deionized water and 60% of acetonitrile mixed with 0.1% TFA. UV detection was carried out at 215 nm and flow rate was kept at 0.8 mL/min. The data acquisition time was 35 min. The gradient program was as follows: time (min)/A (v/v):B (v/v); $T_{0.0}$/95:5, $T_{25.0}$/35:65, $T_{29.0}$/0:100, $T_{30.0}$/95:5, $T_{35.0}$/95:5.

2.4. NMR spectroscopy

The ^{1}H NMR samples were analyzed by Acorn NMR Inc. (Livermore, CA, USA) using deuterated methanol (CD_3OD) as the solvent and tetramethylsilane (TMS) as an internal standard.

2.5. Forced degradation

2.5.1. API control

A fentanyl base sample was prepared in methanol at the target method concentration and analyzed with forced degradation samples.

2.5.2. Thermal degradation

Fentanyl base was weighed and placed in a sealed glass tube. The glass tube was placed into a heated tube furnace (Lindberg/Blue) at 350 °C for 5 min to mimic the conditions used by Rabinowitz et al. [1]. The glass tube was then allowed to cool to room temperature. Fentanyl was dissolved in methanol for HPLC analysis.

2.5.3. Oxidation degradation

Hydrogen peroxide (0.3%, 1 mL) was added to 10 mL of fentanyl API solution in acetonitrile to give a fentanyl concentration of 500 μg/mL. This sample was then analyzed at 0, 2, 4, 6 and 24 h time points until about 10% degradation of fentanyl was achieved. An oxidation control was prepared by adding 1 mL of 0.3% hydrogen peroxide to 10 mL of acetonitrile.

2.5.4. Acid degradation

HCl (5N, 3 mL) was added to about 6 mg of fentanyl and heated at 70 °C for 24 h. After 24 h, the solution was brought to ambient temperature and neutralized with 3 mL of 5N NaOH. To this solution, 6 mL of acetonitrile was added. An acid control was prepared by heating 2 mL of 5N HCl at 70 °C for 24 h. After 24 h this solution was brought to ambient temperature and neutralized with 2 mL of 5N NaOH. To this solution, about 4 mL of acetonitrile was added.

2.5.5. Base degradation

NaOH (5N, 3 mL) was added to about 6 mg of fentanyl and heated at 70 °C for 24 h. After 24 h this solution was brought to ambient temperature and neutralized with 3 mL of 5N HCl. To this solution, 6 mL of acetonitrile was added. A base control was prepared by heating 2 mL of 5N NaOH at 70 °C for 24 h. After 24 h this solution was brought to ambient temperature and neutralized with 2 mL of 5N HCl. To this solution, about 4 mL of acetonitrile was added.

2.5.6. Photo-degradation

Fentanyl API (about 1.5 mg) was weighed and transferred into a clear scintillation vial. A dark control sample was prepared by weighing fentanyl API into clear scintillation vial, and covering with aluminum foil. The sample and dark control vials were both exposed to UV light at 365 nm by using the Spectroline UV lamp (output: 300 mW/cm^2 at 6") and white fluorescent light under ambient conditions. After one week, the light source was removed and each sample was extracted with about 3 mL of methanol.

2.6. Method validation

The method was validated according to the ICH guidelines Q2(R1) with respect to specificity, accuracy, precision, linearity range, limit of detection (LOD), limit of quantitation (LOQ) and robustness. The specificity of the HPLC method for fentanyl was evaluated in the presence of potential process impurities and forced degradation products. The peak purity of fentanyl was assessed with PDA and mass spectrometer detectors. Linearity test solutions for fentanyl assay were prepared at five concentrations ranging from approximately 50 to 150% of the 500 μg/mL target analyte concentration (283, 452, 565, 709, and 904 μg/mL). Linearity test solutions for impurity testing were prepared from limit of quantitation 0.05% (LOQ) to approximately 2% (0.25, 1, 5, 8, and 10 μg/mL). The peak area was plotted against the concentration at each level and a calibration curve was generated by least-squares linear regression analysis. The accuracy of the assay method was assessed in triplicate at concentration levels of 283, 565 and 904 μg/mL. The accuracy for the impurity testing was assessed in triplicate at 0.25, 5, and 10 μg/mL. Precision was assessed from nine analyses at assay and impurity concentrations. The %RSD of the %recovery was calculated across three levels for $n = 9$ samples. The LOD and LOQ were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively. Verification of the LOD and LOQ was performed with six replicate injections of 0.08 and 0.25 μg/mL, respectively for 1-PEP, NRF, PRP, PPA, acetyl fentanyl, pyruvyl fentanyl, 1-PPO, N-oxide, butyryl fentanyl, 1-SPO and fentanyl standard solutions. Critical parameters of flow rate, column temperature and mobile phase concentrations in the method were altered ±10% for the robustness evaluation. The normal flow rate of 0.50 mL/min was adjusted to 0.45 and 0.55 mL/min. The normal column temperature of 30 °C was adjusted to 27 and 33 °C. The initial mobile phase with 18% solvent B was adjusted to 16.2 and 19.8% solvent B.

3. Results and discussion

3.1. Forced degradation studies

The forced degradation results for fentanyl API are summarized in Table 1. The structures of fentanyl impurities and degradants are shown in Table 2. For each set of samples, a methanol blank, resolution solution (see Fig. 1) and fentanyl API controls were injected. The peak purity angles of the fentanyl in the fentanyl API controls were much less than the thresholds and the mass spectra across the peaks were homogenous. This demonstrated that the fentanyl peak was pure and free of co-eluting impurities/degradants. The relative response factors (RRFs) were calculated for each impurity as the ratio between the response factor of each impurity to the response factor of fentanyl base; RRF values for PRP and PPA were determined as 0.74 and 0.50, respectively (Table 1). It should be noted that these RRFs are outside the Pharmacopeial Forum [7] acceptable range of 0.8–1.2, and these two degradants would be underestimated if quantitation was determined by peak area percents.

3.1.1. Thermal degradation

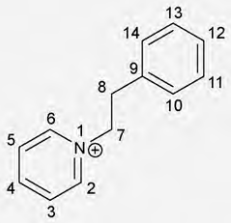
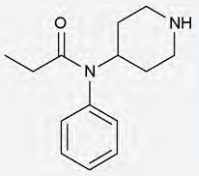
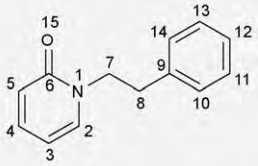
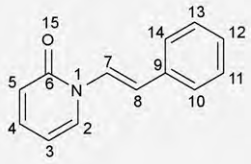
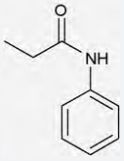
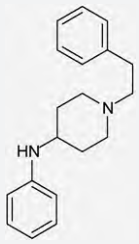
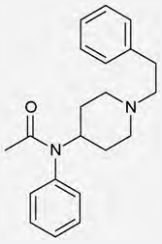
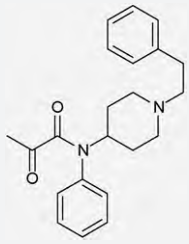
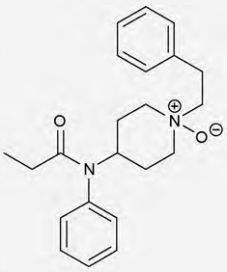
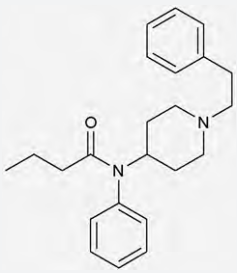
Five main fentanyl degradants were observed by heating fentanyl API for 5 min at 350 °C, which was consistent with the information reported by Rabinowitz et al. [1]. NRF and PRP were known degradants and authentic materials were commercially available. Unknown degradants at RRTs 0.36, 1.08 and 1.25 were observed, which were identified and confirmed as 1-PEP, 1-PPO and 1-SPO, respectively (see Section 3.2).

3.1.2. Oxidation

Oxidation of fentanyl with 0.3% hydrogen peroxide selectively produced diastereomers of fentanyl N-oxide at RRTs 1.11 and 1.18, Table 1. From time 0 to 24 h, the levels of fentanyl N-oxide diastereomers (Fig. 2) at RRTs 1.11 and 1.18 increased from 0.44 to 6.51% and 0.04 to 0.56%, respectively. LC/MS analysis of the diastereomers of fentanyl N-oxide gave the same m/z (353 amu).

^{1}H NMR of the fentanyl N-oxide standard supported the presence of two diastereomers of fentanyl N-oxide with the minor diastereomer present at slightly above a level of 10% This was also supported by the HPLC analysis of the standard, where the ratio of minor to major diastereomer was about 12%. Nuclear overhauser effect spectroscopy (NOESY) was also performed, but did not provide enough information to determine whether the major isomer

Table 2
Structures of fentanyl process impurities and degradants.

Compound	Structure	Compound	Structure
1-PEP[a] Degradant MW 184		NRF[b] Degradant MW 232	
1-PPO[c] Degradant MW 199		1-SPO[d] Degradant MW 197	
PRP[e] Degradant MW 149		PPA[f] Degradant MW 280	
Acetyl fentanyl Process impurity MW 322		Pyruvyl fentanyl Process impurity MW 350	
Fentanyl N-oxide Degradant MW 352		Butyryl fentanyl Process impurity MW 351	

[a] 1-Phenethylpyridinium salt.
[b] Norfentanyl.
[c] 1-Phenethyl-1H-pyridin-2-one.
[d] 1-Styryl-1H-pyridin-2-one.
[e] Propionanilide.
[f] N-Phenyl-1-(2-phenylethyl)-piperidin-4-amine.

was the α- or β- diastereomer. Based on structural stability, the β-form should predominate.

3.1.3. Acid degradation

Acid degradation selectively produced PPA at a level of 34.64%. UV scans were compared for the PPA peak in both the samples and in the commercially available reference material, which were identical. PPA formation is expected by the acid catalyzed hydrolysis of fentanyl, Table 1.

3.1.4. Base degradation

Fentanyl was very stable to base degradation, and remained mostly intact after 24 h of heating with 5N NaOH, Table 1. No known or unknown degradants were detected.

3.1.5. Photo-degradation

Fentanyl was very stable to light and did not degrade after 7 days of light exposure to UV light at 365 nm and white fluorescent light under ambient conditions, Table 1. No known or unknown degradants were detected.

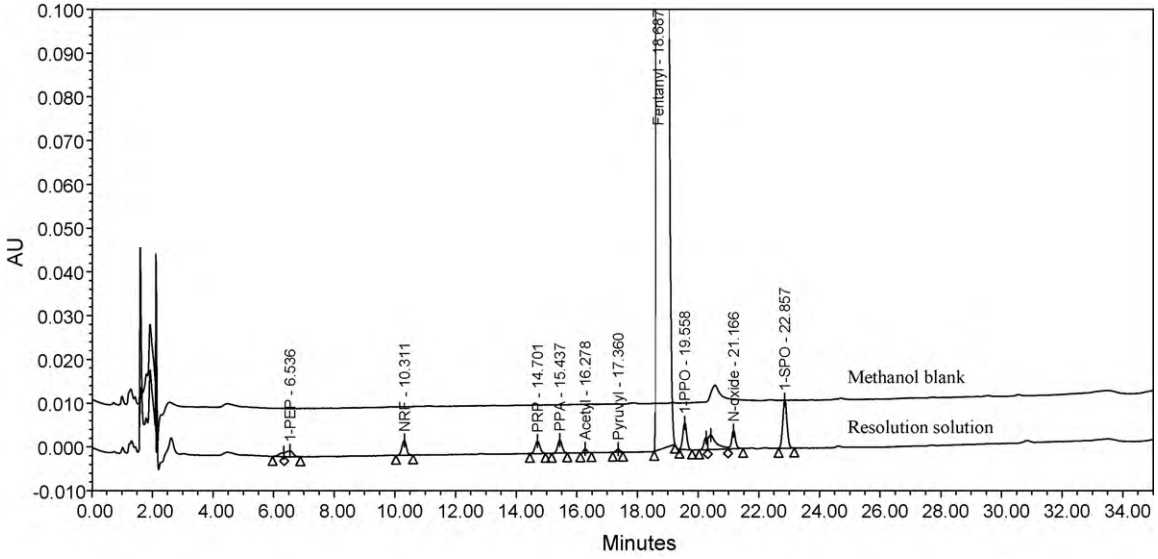


Fig. 1. Chromatogram of fentanyl standard spiked with impurities.

3.2. Identification of degradants at RRTs 0.36, 1.08 and 1.25

3.2.1. Thermal degradant peak at RRT 0.36

ESI-MS of the peak at RRT 0.36 showed a molecular ion peak at *m*/*z* 184 [M]+ in the positive ionization mode (Fig. 3). The molecular weight of this impurity was 152 amu less than that of fentanyl. The molecular mass indicated that a large piece of fentanyl was lost in formation of this degradant; the lost piece was identified as the amide side of fentanyl molecule. The other prominent molecular ion peak at *m*/*z* 105 [MH]+ was proposed as styrene [8]. Based on the molecular weight, 1-phenethylpyridinium (1-PEP) salt was postulated and synthesized. The RRT, UV and LC/MS spectra of synthesized 1-PEP compound matched with the degradant at RRT 0.36. The identity of this degradant was further confirmed by the ^{1}H NMR spectra (Table 3). From the spectral data and the synthesized compound, the structure was characterized as 1-PEP.

3.2.2. Thermal degradant peak at RRT 1.08

This compound was difficult to ionize using ESI-MS. APCI-MS of the peak at RRT 1.08 showed a molecular ion peak at *m*/*z* 200 [MH]+

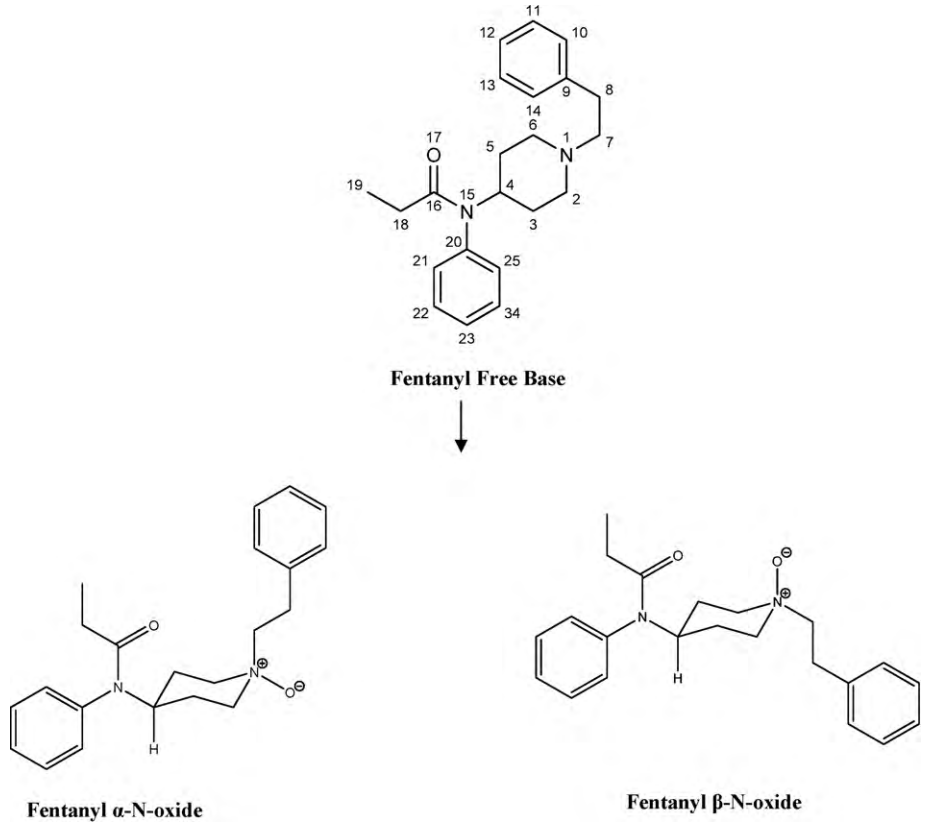


Fig. 2. Structures for forced degradation of fentanyl by oxidation; diastereomer of N-oxide (α- and β-predictions).

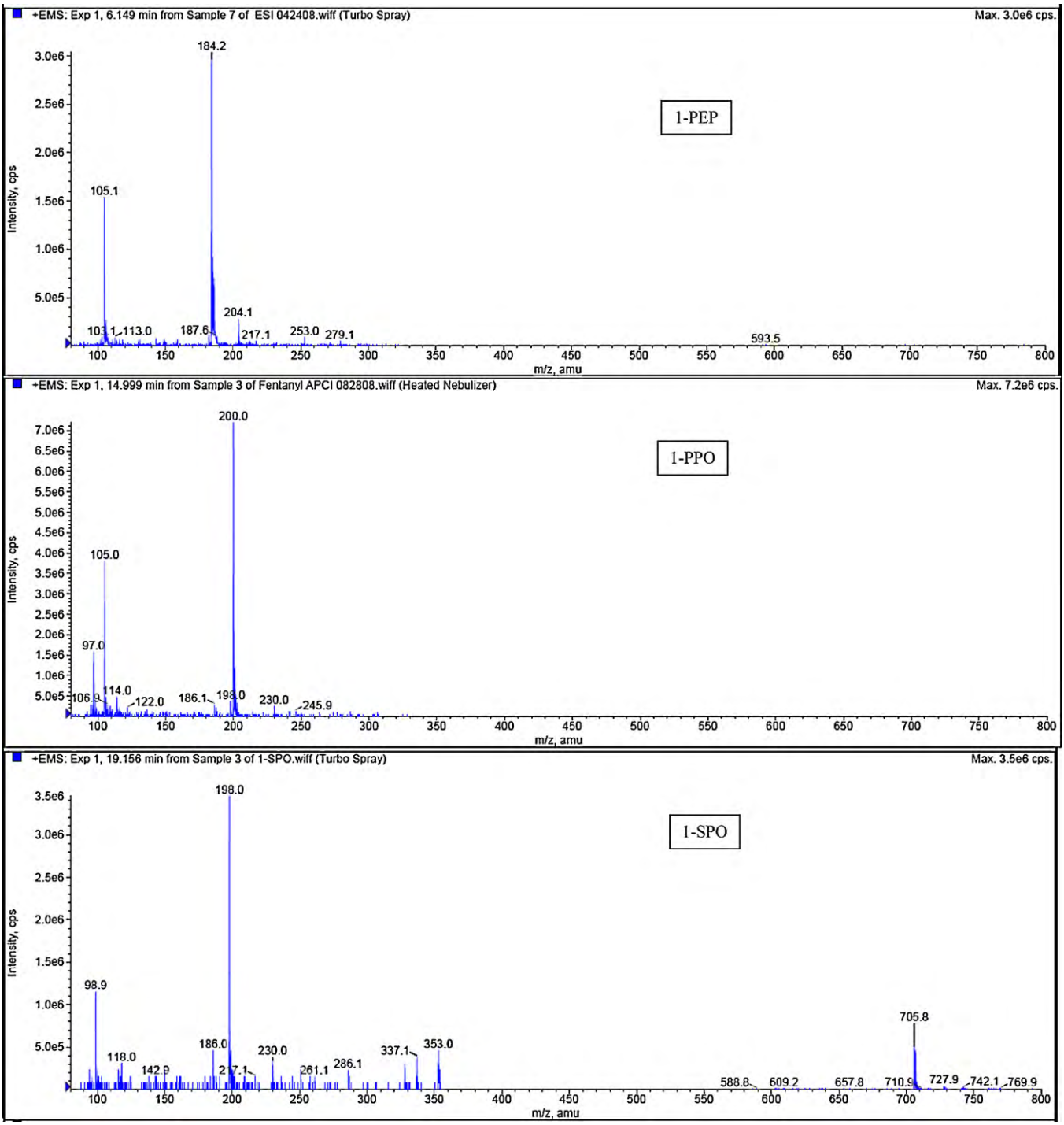


Fig. 3. LCMS of 1-PEP, 1-PPO, and 1-SPO.

in the positive ionization mode (Fig. 3). The molecular weight of this impurity was 137 amu less than that of fentanyl. The other prominent molecular ion peak at *m*/*z* 105 [MH]+ was proposed as styrene. Based on the molecular weight, 1-phenethyl-1H-pyridin-2-one (1-PPO) was postulated and synthesized. The RRT, UV and LC/MS spectra of the synthesized 1-PPO compound matched with the degradant at RRT 1.08. The identity of this degradant was further confirmed by the ^{1}H NMR spectra (Table 3). From the spectral data and the synthesized compound, the structure was characterized as 1-PPO.

3.2.3. Thermal degradant peak at RRT 1.25

ESI-MS of the peak at RRT 1.25 showed a molecular ion peak at *m*/*z* 198 [MH]+ in the positive ionization mode (Fig. 3). The molecular weight of this impurity was 139 amu less than that of fentanyl. Based on the molecular weight, 1-styryl-1H-pyridin-2-one (1-SPO) was postulated and synthesized. The RRT, UV and LC/MS spectra of synthesized 1-SPO compound matched with the degradation peak at RRT 1.25. The identity of this degradant was further confirmed by the ^{1}H NMR spectra (Table 3). From the spectral data and the synthesized compound the structure was characterized as 1-SPO.

Table 3
Comparative ^{1}H NMR assignments for 1-PEP, 1-PPO and 1-SPO.

Position[a]	1-PEP δ (ppm relative to TMS)	1-PPO δ (ppm relative to TMS)	1-SPO δ (ppm relative to TMS)
1	–	–	–
2	8.83	7.30	7.98
3	8.03	6.21	7.32
4	8.55	7.46	7.95
5	8.03	6.55	7.40
6	8.83	–	–
7	4.91	4.20	7.58
8	3.35	3.03	6.99
9	–	–	–
10	7.27	7.18	7.55
11	7.13	7.25	7.37
12	7.27	7.18	7.36
13	7.13	7.25	7.37
14	7.27	7.18	7.55

[a] Refer structures for numbering (Table 2).

3.3. Formation of degradants

3.3.1. 1-PEP

Fentanyl can be converted to phenethylpiperidiene via solvent mediated thermal β-elimination [9], followed by N-oxidation, protonation and rapid dehydration. Phenethylpiperidiene goes through N-oxidation followed by protonation and rapid dehydration to form 1-PEP (Fig. 4).

3.3.2. 1-PPO

1-PEP can undergo further oxidation in an oxygen-rich environment in the presence of heat and water and causes the pyridinium ring to oxidize. A water molecule was lost to form 1-PPO (Fig. 5).

3.3.3. 1-SPO

1-SPO originates from phenethylpiperidiene, which can undergo N-oxidation followed by protonation and rapid dehydration. The resulting compound undergoes N-oxidation followed by protonation and rapid dehydration. Further oxidation results in the insertion of a hydroxyl group followed by dehydration to form 1-SPO (Fig. 6).

3.4. Method validation

3.4.1. Specificity

All compounds were well separated. The closest eluting impurity/degradants to fentanyl were pyruvyl fentanyl and 1-PPO, and USP resolution values from fentanyl were 4.2 and 3.2, respectively. The fentanyl peak was considered to be pure (i.e. free of co-elution) if the purity angle calculated by the Empower software was less than the purity threshold, and also if the threshold was less than 1.0. The peak purity criteria were met, with a purity angle of 0.082 and purity threshold of 0.271. Uniformity of the mass spectra throughout the fentanyl peak also showed the absence of co-elution.

3.4.2. Linearity and range for assay

Peak area was plotted against concentration for five standard levels ranging from 283–904 μg/mL. A non-weighted linear curve fitting was applied to generate the calibration curve, and the method was demonstrated to be linear with a correlation coefficient of 0.9995 (Table 4).

3.4.3. Accuracy and precision for assay

Calculations for % recovery were based on fentanyl concentrations derived from the calibration curve and the theoretical values. For the three levels tested for accuracy, the % recovery ranged from 99.6 to 101%. The accuracy results are summarized in Table 5. The precision of the assay was reflected by the low %RSD (0.8%) of the % recoveries of the triplicate samples at low, mid and high assay levels.

Table 4
Linear regression data for fentanyl assay and impurity testing.

Parameter	Assay	Impurity testing
Concentration range (μg/mL)	283–904	0.23–11.30
Slope	0.060558	0.007430
Intercept	1,260,000	1640
Correlation coefficient (r)	0.9995	0.9996
LOD/LOQ (μg/mL)	–	0.08/0.25

3.4.4. Low-level linearity, accuracy, and precision for impurity/degradant testing

Low-level linearity, accuracy and precision for impurity/degradant testing were assessed for 1-PEP, NRF, PRP, PPA, acetyl fentanyl, pyruvyl fentanyl, 1-PPO, N-oxide, butyryl fentanyl, and 1-SPO and fentanyl (to represent an unknown degradant). The linearity range tested was from 0.05% (LOQ) to 2.0% of the nominal fentanyl concentration where the method was demonstrated to be linear with a correlation coefficient of 0.999 for fentanyl (Table 4) and 0.996–0.999 for impurities/degradants. The average % recoveries from triplicate sample analyses at levels of 0.25, 5 and 10 μg/mL ranged from 91.0 to 100.3%. The %RSD for low-level fentanyl and impurities/degradants at the low, mid and high levels (n = 9) was less than 4.8%, which demonstrated that precision (<10%) was achieved for impurity/degradant testing.

3.4.5. Limit of detection and limit of quantitation (LOQ)

The fentanyl limit of detection (LOD) was determined to be 0.08 μg/mL with a signal-to-noise ratio of 3:1. The limit of quantitation (LOQ) was determined to be 0.25 μg/mL with a signal-to-noise ratio of 10:1. This LOQ was verified by the %RSD of six replicate analyses of fentanyl solutions at this level. A peak area %RSD of 3.2 and 2.3–3.4% was obtained for fentanyl and impurities/degradants, respectively which demonstrated that adequate precision (<10%) was achieved at this LOQ level.

3.4.6. Robustness

When eluent flow, column temperature and initial mobile phase concentrations were altered by ±10%, the peak tailing factors and resolutions between fentanyl and the impurities/degradants did not show any significant change; demonstrating that the method was robust with respect to changes in critical parameters of the method.

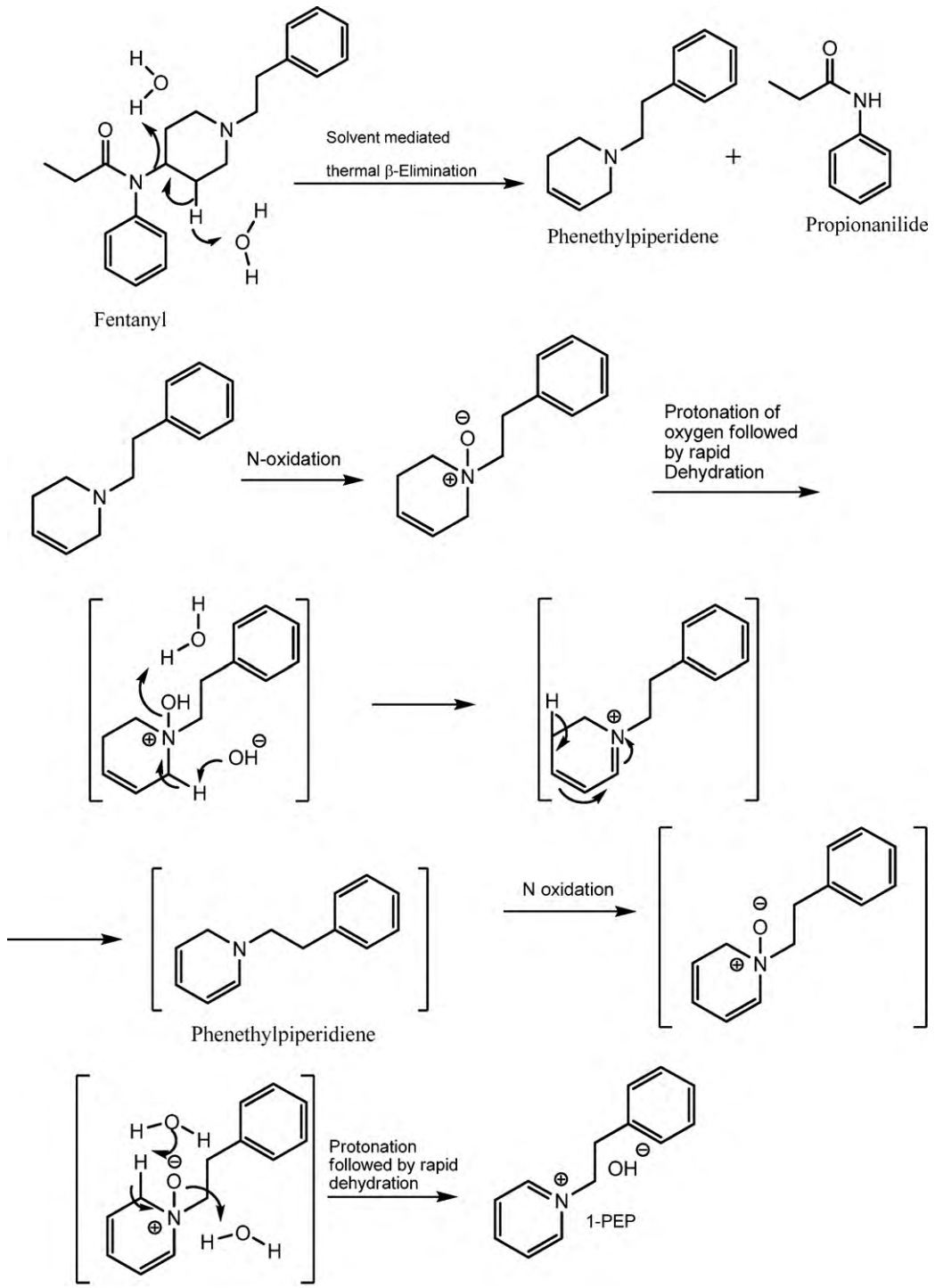


Fig. 4. Reaction mechanism for the formation of 1-phenethylpyridinium salt (1-PEP).

Table 5
% accuracy and precision data for fentanyl assay and impurity testing.

Levels	Assay concentration (μg/mL)	Recovered (%)	Impurity testing concentration (μg/mL)	Recovered (%)
Low ($n = 3$)	282.5	99.6	0.23	91.4
Mid ($n = 3$)	565.0	101.0	5.65	99.9
High ($n = 3$)	904.1	99.6	11.30	99.5
Average	–	100.1	–	96.9
%RSD ($n = 9$) i.e. precision	–	0.8	–	4.6

n = number of replicate samples.

3.4.7. System suitability

A methanol blank injected prior to the start of the system suitability run showed a clean baseline. Six consecutive injections of the resolution solution (500 μg/mL fentanyl with 1% of each 1-PEP, NRF, PRP, PPA, acetyl fentanyl, pyruvyl fentanyl, 1-PPO, N-oxide, butyryl fentanyl, and 1-SPO) were performed. A methanol blank was injected after the last injection of the resolution solution, and no carryover was observed. The %RSD for six consecutive injections of the resolution solution for average peak area and average retention time was 0.19–1.67% and 0.05–0.12%, respectively. The tailing factor for the fentanyl peak was less than 2.0. The capacity factor for all the peaks was greater than 3.5.

3.5. Structural alerts for fentanyl and related substances

Derek for Windows, Ver. 11.0.0, Leadscope Model Applier Ver. 1.0, and ACD/Tox Suite Ver. 2.95 were used to determine the genetox structural alerts for fentanyl and all identified impurities and degradants listed in Table 1. No genetox alerts were found in *Derek*. *Leadscope* generated positive predictions for PRP and PPA in 1 of the 20 models in their genetox suite. The DNA damage in unscheduled DNA synthesis (UDS) *in vitro* rat hepatocytes for PRP was positive; however, the UDS *in vitro* (which contains a larger training set than UDS *in vitro* rat hepatocytes) predicted negative for PRP. The gene mutation *in vivo* drosophila bacteria for PPA was at the probability cut-off for positives/negatives. In the *ACD/Tox Suite*, 1-PPO and 1-SPO were identified as Ames hazards. These compounds were also predicted with higher probabilities of being Ames positive, although predictions performed by the probabilistic model were considered inconclusive due to low reliability indices.

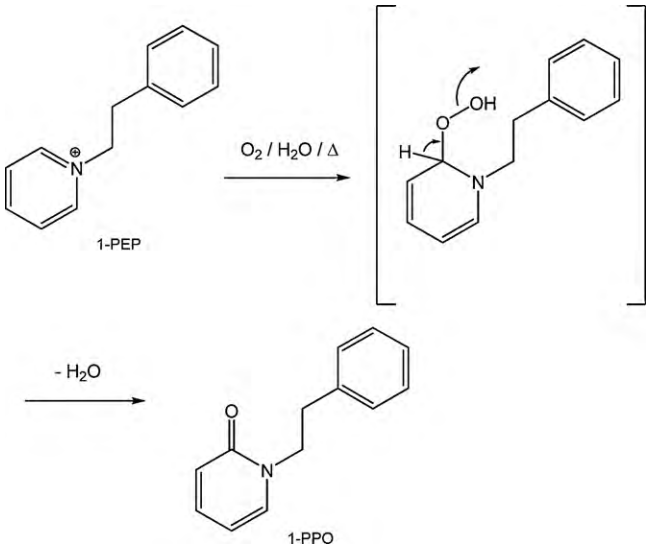


Fig. 5. Reaction mechanism for the formation of 1-phenethyl-1H-pyridin-2-one (1-PPO).

Upon further examination of the result for PPA, which was also discussed as a possible genetox alert by Chen et al. [3], this was most likely an alert in the Leadscope software due to the presence

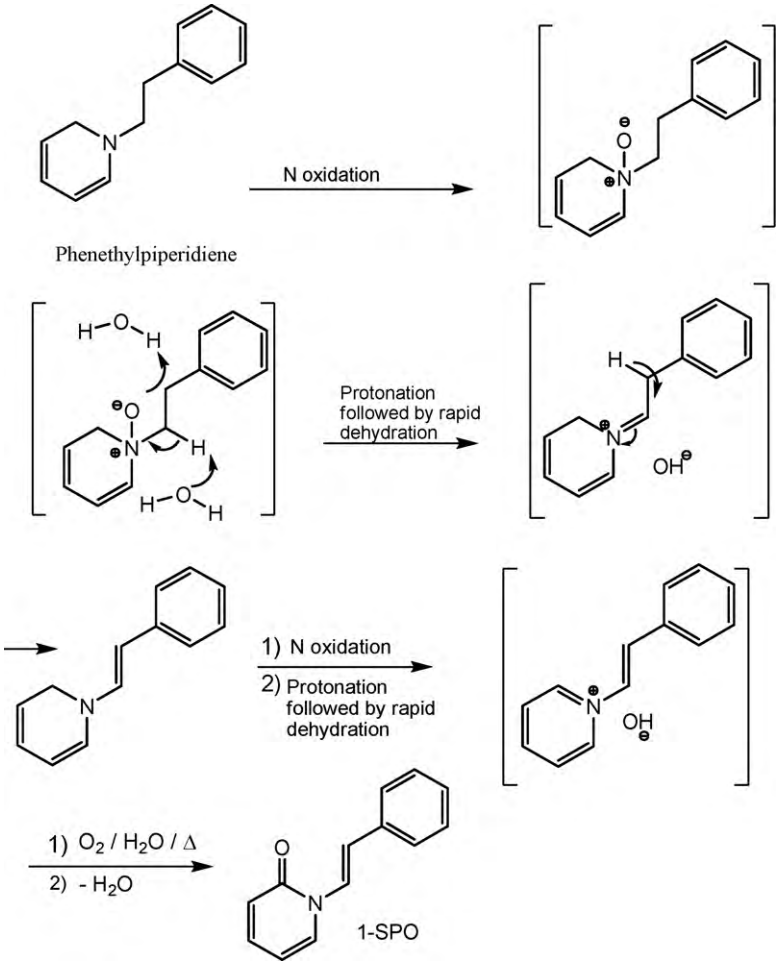


Fig. 6. Reaction mechanism for the formation of 1-styryl-1H-pyridin-2-one (1-SPO).

of the aromatic amino group, which is a well-recognized genotoxicophore. Compounds possessing this structural feature can induce DNA damage and metabolic transformation to hydroxyl amines. While this may be true in a general case, Bailey et al. [10] and Benigni and Bossa [11] identified secondary or tertiary amines as hazards only if the substituents at the amino group were not larger than an ethyl group. PPA is a secondary amine with a large aromatic substituent, and therefore, would not be expected to be a hazard. In the case for the alert for PRP, the same authors suggest including only formamide and acetamides as hazards. Each of the three software programs predicted different outcomes, and the results are consistent with that described recently by Matthews et al. [12] where it was advised that in order to predict a better overall performance; multiple *in silico* programs should be used in combination with each other. In addition, we would add that any output from *in silico* toxicity programs should undergo further review.

4. Conclusions

A forced degradation study for fentanyl was performed using light, acid, base, and oxidation degradation on fentanyl API. Fentanyl was very stable to light and base treatment, no degradation was seen. Oxidation selectively produced diastereomers of fentanyl N-oxide. Acid degradation exclusively generated PPA. Thermal degradation produced the degradants 1-PEP, NRF, PRP, 1-PPO, and 1-SPO. Unknown degradants 1-PEP, 1-PPO, and 1-SPO were synthesized and identified using LC/MS and ^{1}H NMR spectroscopy. A stability-indicating HPLC method for the assay of fentanyl and its related compounds was validated and demonstrated to be specific, precise, linear, accurate, sensitive, robust and suitable for the intended use.

No toxicological alerts for genotoxicity were conclusively found for any of the fentanyl impurities and degradants using three separate *in silico* toxicity programs.

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Supporting Information Available

Synthesis information for fentanyl related substances is available from the corresponding author.

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