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HPLC METHOD DEVELOPMENT, VALIDATION AND IMPURITY CHARACTERIZATION FOR AN ANTITUMOR HSP90 INHIBITOR -- PU-H71 (NSC 750424)

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Abstract

An HPLC method for the assay of the heat shock protein 90 inhibitor, PU-H71 (NSC 750424), has been developed and validated. The stress testing of PU-H71 was carried out in accordance with ICH guidelines Q1A (R2) under aqueous, acidic, alkaline, oxidative, thermolytic and photolytic conditions. The separation of PU-H71 from its impurities and degradation products was achieved within 50 min on a Mac-Mod ACE 3 C18 column (150 mm × 4.6 mm i.d., 3 μm) with a gradient mobile phase comprising 20–95% acetonitrile in water, with 0.1% trifluoroacetic acid in both phases. LC - Quadrupole TOF/MS was used to obtain accurate mass data on various components as well as on their fragments for characterization of impurities and degradation products. The proposed HPLC assay method was validated for specificity, linearity (concentration range 0.1–0.3 mg/mL, $r = 0.9998$), accuracy (recovery 99.7–101.1%), precision (intra-lab RSD 1.39%, inter-lab RSD 0.91%), sensitivity (LOD 0.08 μg/mL), and ruggedness. The developed method was suitable for the assay and stability monitoring of PU-H71 drug substance.

Keywords

PU-H71; NSC 750424; forced degradation; HPLC validation; stability study; impurity and degradation product characterization

1. Introduction¹

PU-H71, 8-(6-Iodo-benzo[1,3]dioxol-5-ylsulfanyl)-9-(3-isopropylaminopropyl)adenine dihydrochloride, is a potential anticancer agent that tightly binds and deactivates heat shock protein 90 (Hsp90) [1]. Hsp90 is a molecular chaperone that facilitates protein folding, intracellular transport, and maintenance and degradation of proteins as well as transmission

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¹Abbreviation list: Hsp, heat shock protein; ATP, adenosine triphosphate; ADP, adenosine diphosphate; TFA, trifluoroacetic acid; ACN, acetonitrile; PDA, photodiode array; RRT, relative retention time; S/N, signal to noise ratio; Q-TOF, quadrupole time-of-flight.

of cell signaling. It has more than 200 client proteins, covering almost all the cellular processes. Hsp90 accounts for 1–2% of the total protein in unstressed cells and is up-regulated to 4–6% under stress [2]. Hsp90 has become an attractive target for anti-cancer therapeutics in recent years. The usefulness of Hsp90 inhibitors in cancer therapy lies in the fact that many of its client proteins are oncogenic and hence their degradation simultaneously blocks a variety of cancer-causing signaling pathways. The diverse array of signaling pathways associated with its client proteins may be correlated with its wide-range anticancer effects and a lesser propensity for developing resistance.

Hsp90 exists as a homodimer where each monomer is consisted of three highly conserved domains: an N-terminal ATP-binding domain (25kDa), a middle domain (40kDa) and a C-terminal dimerization domain (12kDa) [3, 4]. The N-terminus of Hsp90 contains a specific ATP binding pocket [4, 5]. The major role of the middle domain is to discriminate various types of client proteins and adjust the molecular chaperone for proper substrate activation. [4, 6]. The C-terminal dimerization domain strengthens the weak association between the two N-terminal domains of the Hsp dimer [7]. The C-terminal domain has a conserved pentapeptide (MEEVD) implicated in binding to the tetratricopeptide repeat domain of co-chaperones.

Current Hsp90 inhibitors are categorized into several classes based on distinct modes of inhibition, including i) blockade of ATP binding, ii) disruption of co-chaperone/Hsp90 interactions, iii) antagonism of client/Hsp90 associations, and iv) interference with post-translational modification of Hsp90. The most explored class of inhibitors act through blockade of the N-terminal ATP binding. The N-terminal domain contains a unique nucleotide-binding pocket that binds both ATP and ADP. Conformational changes that occur upon binding and hydrolysis of ATP regulate the ability of the chaperone to bind its client proteins [8]. The ATPase activity of Hsp90 drives the chaperone cycle and directs binding, induction of the active conformation, and release of its client proteins. Because this pocket is distinct from that of other ATPase it has become an attractive target for inhibition [9]. Benzoquinone ansamycins, represented by geldanamycin, 17-AAG, 17-DMAG and IPI-504, are among the first class of natural compounds and derivatives that bind to the N-terminal ATP pocket. However, the binding of these ansamycin drugs to Hsp90 may induce potential drug resistance due to the inhibition of apoptosis signaling resulting from Hsp70 up-regulation through the heat shock factor-1 (HSF-1) stress response [4]. Purine scaffold and pyrazole scaffold are the two important classes of synthetic small molecules that were developed to bind to the N-terminal ATP pocket of Hsp90. PU-H71, an inhibitor with purine scaffold, was synthesized and shown to be a potent inhibitor of Hsp90 [1]. PU-H71 has shown anticancer activity in a wide variety of animal tumor models including small-cell lung cancer [10], triple-negative breast cancer [11], B-cell lymphomas [12], pancreatic cancer [13], and myeloma [14, 15]. PU-H71 has also been used as a research tool to reveal the set of abnormal proteins coordinated by Hsp90 in tumor cells [16]. PU-H71 is currently in Phase I clinical trial in patients with metastatic solid tumor and lymphoma.

In spite of the vast number of publications on the synthesis, efficacy and mechanism studies on PU-H71, no published report is available regarding the HPLC analysis and validated analytical method for PU-H71.

The goal of this work is to develop and validate a stability-indicating HPLC method for PU-H71 in accordance with ICH guideline Q2 (R1), and to identify the impurities present in the drug substance, as well as degradation products observed in stress testing. The study was carried out with HPLC, UV-visible spectroscopy and high-resolution accurate mass spectrometry.

2. Material and methods

2.1. Chemicals and reagents

PU-H71 (NSC 750424) was provided by the National Cancer Institute (Bethesda, MD, USA). Anhydrous trifluoroacetic acid (TFA) was purchased from Thermo Scientific (Rockford, IL, USA). HPLC grade acetonitrile (ACN) and hydrogen peroxide (H₂O₂) 30% solution were purchased from Mallinckrodt (Paris, KY, USA). Water was purified through a Millipore Super-Q Pure Water System (Waltham, MA, USA). Solutions of hydrochloric acid (HCl) and sodium hydroxide (NaOH) were prepared from Dilute-it Analytical Concentrate (J.T. Baker, Phillipsburg, NJ, USA).

2.2. HPLC

An Agilent 1100 HPLC system (Wilmington, DE, USA) equipped with a solvent degasser, pump, autosampler, and PDA detector was used in the study. Agilent ChemStation for LC (A 10.02) software was used for instrument operation control and data collection. The HPLC was performed on a Mac-Mod ACE 3 C18 column (3 μ m, 150 $\times$ 4.6 mm I.D.; Chadds Ford, PA, USA). The column was held at 25°C. The mobile phase was a combination of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in ACN). The gradient program was: 0–15 min, 20% solvent B and 80% solvent A; 15–30 min, linear gradient to 95% solvent B and 5% solvent A; 30–40 min, hold at 95% solvent B and 5% solvent A; 40–50 min, re-equilibrate at 20% solvent B and 80% solvent A before the next injection. The injection volume was 10 μ L. The elution flow rate was 1.0 mL/min, and the detection wavelength was set at 285 nm. The second validation in the other laboratory employed a Shimadzu LC-2010CHT system with a Waters 2487 dual absorbance detector and a PE-Nelson TotalChrom (v.6.3.0) data system.

LC-MS was performed on an Agilent LC/MS system consisting of an Agilent 1200 binary LC pump, a temperature-controlled autosampler, a PDA UV detector, and a 6530 Accurate Mass Q-TOF mass spectrometer (Wilmington, DE, USA). The mass spectrometer was equipped with a JetStream[®] ESI probe operating at atmospheric pressure. The ESI source parameter settings were: mass range m/z 100–1000, gas temperature 350°C, gas flow 10 L/min, nebulizer 50 psi, sheath gas temperature 400°C, sheath gas flow 12 L/min, capillary voltage (Vcap) 3500 V, nozzle voltage 500 V, fragmentor 200 V, skimmer 65 V, octopole RF (OCT 1 RF Vpp) 750 V. Tandem mass spectrometry was performed using ramped collision energy at slope 3 and offset 10. The LC conditions used for identification of impurities and decomposition products of PU-H71 were the same as those described above. Matching of the peak identity between the LC/UV system (Agilent 1100) and LC/MS system (Agilent 1200) was achieved by relative retention time and relative UV peak intensity. Each forced degradation solution was divided into two portions and analyzed by both systems on the same day to ensure comparable peak intensities.

2.3. Environmental chamber for photo stressed decomposition

The forced degradation study under UV and visible light was carried out in the ES 2000 Environmental Chamber (Environmental Specialties, Inc., Raleigh, NC, USA), equipped with a cool white lamp (8.0 kilolux) and a UV-A lamp (14.00 W/m²), in conformance with the ICH Q1B option 2 for photostability testing. Temperature and humidity conditions were set at 25°C/60% RH.

2.4. Sample preparation

An acetonitrile and water mixture (20:80, v/v) with 0.1% TFA was used as the sample diluent in the following preparations.

For forced decomposition in solutions, approximately 2 mg of PU-H71 was dissolved in 1 mL of the respective decomposition reagents: H₂O, 1N HCl, 1N NaOH, or 3% H₂O₂. The solutions were then heated in a heating block at 80°C for 18 hours for the H₂O, HCl and NaOH solution, and 2 hours for the H₂O₂ solution. The acid and base samples were neutralized with an equivalent of base and acid after heating. Then all the solutions were quantitatively transferred to a 10 mL volumetric flask and diluted to the mark with the diluent to obtain solutions of 0.2 mg/mL nominal concentration.

For solid dry heat decomposition, approximately 2 mg of PU-H71 was accurately weighed and heated in a heating block at 80°C for 14 days. The sample was then quantitatively transferred to a 10 mL volumetric flask and diluted to the mark with the diluent.

For solid photo decomposition and the control sample, approximately 2 mg each of two portions of PU-H71 were accurately weighed into two 1-dram vials. One of the vials was wrapped with aluminum foil to be used as the control. These two vials were placed in an environmental chamber at 25°C/60%RH with both UV-A light and cool white light on for 72 hours, followed by only the cool white light for an additional 240 hours. After the light exposure, the samples were quantitatively transferred into two separate 10 mL volumetric flasks and diluted to the mark with the diluents, respectively.

For validation of linearity, accuracy and precision, accurately weighed 1–3 mg portions of PU-H71 were dissolved in 10.0 mL of the diluent to yield solutions at 0.1, 0.15, 0.2, 0.25, and 0.3 mg/mL.

3. Results and Discussion

3.1. HPLC method development

The chemical structure of PU-H71 is presented in Figure 1. The compound is a di-basic molecule with a calculated pK_a approximately of 8–11. Its structure predicts that the compound will be suitable for analysis by reversed-phase mode of liquid chromatography. A low pH (pH < 3) mobile phase would fully protonate the compound, resulting in a substantial retention time that would not be affected by small variations in pH. Low pH mobile phase also suppresses the silanols in the stationary phase and minimizes the tailing of basic compounds.

A pilot gradient elution with 5–95% ACN in water containing 0.1% TFA (pH 2) exhibited an 11 min retention time for PU-H71. Resolution of the closest impurity at relative retention time (RRT) 0.80 (compound **13** in Section 3.3) to PU-H71 is 3.3. A small degradation product (compound **16** in Section 3.3) in H₂O₂ stressed solution was not resolved under this condition. After several trials, 20% ACN appeared to provide the optimal retention and overall resolution of all impurities and degradation products including compound **16**. Trifluoroacetic acid, in addition to providing sufficient acidity, also acts as an ion-pairing agent to increase the compound's retention on the hydrophobic C18 bonded phase. Replacing TFA with formic acid resulted in a significant reduction of the target compound's retention time (from 11 min to 7 min), as formic acid, with its smaller molecular size, is less likely to form ion pairs.

Methanol was inferior to ACN as the organic modifier in terms of peak shape and separation. Isocratic elution at 35% of methanol gave similar retention of the compound (16 min). However, all peaks were broader, resulting in lower resolution and sensitivity. Resolution of PU-H71 and the RRT 0.80 peak (compound **13**) decreased from 6.6 to 6.2, while the peaks of PU-H71 and compound **16** were not resolved. The signal to noise ratios (S/N) also decreased by 40%.

The presence of TFA in the sample diluent also proved to be critical to HPLC performance. As PU-H71 is highly soluble in methanol, it was chosen as the initial diluent. However, when the methanolic solution of the compound was injected into the HPLC system using a mobile phase starting at 20% ACN containing 0.1% TFA, a server broad peak (bulge) between 2 to 10 min leading the PU-H71 peak was observed (Figure 2a). As discussed, the additional peak was probably due to a certain amount of the non-ion-paired species of PU-H71. Adding 0.1% TFA to the sample diluent significantly reduced the leading peak. For the optimal performance and solubility, a solvent mixture that matched the initial mobile phase composition, which is ACN/H₂O (20:80) containing 0.1% TFA, was chosen as the sample diluent. In this acidic mixture, PU-H71 was allowed to form ion pairs with TFA before injection, and appears as a single peak (Figure 2b). The total area of the bulge (2–10 min) plus the area of the main peak (12.7 min) in Figure 2a was equivalent to the area of the main peak in Figure 2b, consistent with the assumption that PU-H71 was partitioned into the non-ion-paired and ion-paired fractions.

In order to obtain a decomposition profile that simulates actual stability, forced degradation was targeted at a level which is equal to 5–20% decrease of the active drug [17]. The conditions that led to the targeted decrease, found after several trial runs, are presented in Section 2.4, Sample preparation. The optimized LC condition described in Section 2.2 separates PU-H71 from all impurities and degradation products. Additionally, major degradation products are adequately resolved from one another. In all forced degradation sample chromatograms the peak of PU-H71 passed the peak purity check performed by the Agilent ChemStation® software. When examined by LC/MS, no impurity was embedded within the peak of PU-H71 in any forced degradation sample chromatogram. These findings indicate that the method is specific for analysis of PU-H71. Mass balance was assessed by comparing the decrease in PU-H71 and the increase in all detectable degradants. The total peak area in the degraded sample was not less than 95% of that before degradation, indicating that no major degradation product was overlooked by UV detection.

3.2. HPLC method validation

The proposed method was validated in accordance with the current ICH guideline Q2 (R1). Figure 2c shows a typical chromatogram of the HPLC assay and system suitability criteria. The peak purity results from the forced degradation study (Section 3.1) have demonstrated the specificity of the method. The method was validated in two independent laboratories, SRI International (Menlo Park, CA, USA) and Midwest Research Institute (Kansas City, MO, USA), for intermediate precision and reproducibility.

Linearity of the method was demonstrated by standard curves in the range of 0.1–0.3 mg/mL (50–150 % of the target concentration). The sample peak area (A) versus drug concentration (C) was analyzed by linear least square regression to obtain the equation of $A = \text{slope} \times \text{concentration} + \text{intercept}$. Comparable slopes (18,681 and 18,723) and intercepts (–34.734 and –18.839) from the two laboratories were obtained. Linearity was shown by the excellent correlation between peak area A and the drug concentration C in both laboratories ($r = 0.9998$ and 1.0000 , Supplementary Table 1).

Accuracy and precision were established by evaluating recoveries and RSD values obtained with three test solutions each at concentration of 0.1, 0.2, and 0.3 mg/mL corresponding to 50%, 100% and 150% of the target concentration. Recovery was calculated by comparing the theoretical concentration calculated from the calibration curve and the nominal concentration. The accuracy results showed recoveries between 99.7% and 101.0% (Supplementary Table 2). The intra-lab and intermediate (inter-labs) precision values are less than 1.39% and 0.91% RSD, respectively (Supplementary Table 3).

The stability of PU-H71 in the sample diluent, ACN/H₂O (20:80, v/v) with 0.1% TFA, was monitored at 5°C and at room temperature for eight days. Two 0.2 mg/mL sample solutions at each temperature were tested on 0, 1, 3 and 8 days. On each day of analysis, the sample solutions were assayed against a freshly prepared standard solution, and compared to the initial assayed value at day 0. All test solutions remained stable in the sample diluent for 8 days at both temperatures. The 0, 1, 3 and 8 days average recoveries are $100.6 \pm 1.1\%$ for room temperature solutions, and $101.1 \pm 1.4\%$ for 5°C solutions.

Robustness of the method was evaluated by small but deliberate variation of the following three method parameters: percentage of acetonitrile ($\pm 0.5\%$ absolute, $\pm 2.5\%$ relative), column temperature ($\pm 2^\circ\text{C}$), and percentage of TFA ($\pm 0.01\%$ absolute, $\pm 10\%$ relative). Column-to-column variation was also tested. The robustness was tested with a 0.2 mg/mL sample solution, and described by the effect of parameter variations on retention time, capacity factor, USP tailing factor and resolutions. The robustness results indicate that the retention time was significantly affected by minor changes of the ACN percentage. Variation in column temperature, TFA percentage, and changing to a different column did not have significant impact on the retention time. The USP tailing factor was moderately different between the two columns tested. Resolution between the active drug and the RRT 0.80 peak (compound **13**) was similar for the tested variations (Supplementary Table 4).

The proposed assay method is of high sensitivity. The LOD and LOQ were with 0.08 $\mu\text{g/mL}$ and 0.2 $\mu\text{g/mL}$ PU-H71 solutions, respectively. The 0.08 $\mu\text{g/mL}$ solution gave an S/N of 5.4, higher than the commonly accepted S/N = 3 criterion for LOD. The 0.2 $\mu\text{g/mL}$ solution gave an S/N of 24.7, also higher than the commonly accepted S/N = 10 criterion for LOQ. The peak area RSD of six injections of the 0.2 $\mu\text{g/mL}$ solution was 1.6%. Based on the S/N = 3 and the S/N = 10 criteria, the LOD and LOQ are at least 0.08 $\mu\text{g/mL}$ and 0.2 $\mu\text{g/mL}$, respectively.

3.3. Impurity and degradation products characterization

The expanded scale chromatograms of a fresh sample and the representative stressed samples are shown in Figure 3. Impurities and degradation products are labeled with numbers on the chromatograms. There are a total of 10 impurity peaks in the untreated sample (Figure 3a) and an additional 12 degradation product peaks in various stressed samples (Figure 3b–f). Mass and UV spectra of impurities and degradation products are obtained through LC with MS and PDA detections, and a summary of the available UV λ_{max} and protonated molecular ions (m/z) is presented in Table 1. Peaks **1**, **15**, **16** and **17** are too small to obtain good quality UV spectra, and no molecular ions for peak **1** and **3** could be detected. MS/MS experiments were performed on all peaks for which a molecular ion was available. Product ions are also provided in Table 1. The UV, MS and MS/MS spectra of most impurities and degradation products can be found in Supplementary Figures 1–3.

Based on physical data and synthetic scheme, the proposed chemical structures of the majority of impurities and degradation products are provided in Figure 4. The calculated accurate mass value of the proposed formulae and measured accurate mass value of the corresponding peak were all within ± 3 ppm (Table 1).

The impurities and degradation products can be categorized to nine classes: (A) impurities and degradation products through alkylation on the purine N-7; (B) impurities with altered alkyl chain; (C) impurities and degradation products through substitution, loss or addition of iodine; (D) degradation products through oxidation of sulfur; (E) impurities and degradation products through hydrolysis of the methylene dioxy group; (F) degradation products of the adenylyl NH₂ group; (G) degradation products through nucleophilic reaction on the purine

C-8; (H) degradation products through the loss of the isopropyl group from the amino-containing sidechain; and (I) impurity through dimerization.

Spectral data of PU-H71 are an important reference for interpretation of the impurity and degradation product spectral data. The UV, MS and MS/MS spectra of PU-H71 are shown in Figure 5. The protonated molecular ion of PU-H71 is m/z 513.0560, and the UV λ_{max} are 220 nm and 290 nm. The predominant fragment from MS/MS is 453.9825, resulting from the loss of the isopropyl amine.

Supplementary Table 5 provides the characterization basis for all the proposed compounds. The MS/MS data and interpretation plays an important role in supporting the proposed structure. The proposed fragmentation mechanism for PU-H71 and all impurities and degradation products are presented in Supplementary Figures 4–6.

One universal fragmentation pathway was the cleavage of the C-N bond between the isopropyl amine and the *n*-propyl carbon, followed by the dehydrogenation. The resulting neutral leaving group is the isopropyl amine [(CH₃)₂CHNH₂]. Elimination of the isopropyl amine or other alkyl amine from the side chain was observed in the ESI MS/MS spectra of PU-H71 and all 20 impurities and degradation products. Two common fragmentation pathways were observed in the oxidized products **7**, **16** and **17**. One is the cleavage of the C-S bond between the sulfoxide and the purine position 8 carbon. The oxidized sulfur makes the C-S bond more polar and more susceptible to cleavage. The other pathway is the cyclization between the isopropyl amine and purine position 8 carbon (observed in products **7** and **16**).

The cleavages of the C-S bond of non-oxidized sulfur are observed in products **12** and **18** accompanied by McLafferty rearrangement that yield the m/z 174 and 175 fragments in **12** and **18**, respectively.

4. Conclusion

An HPLC assay method has been developed and validated for PU-H71. The HPLC method separates PU-H71 from its impurities and forced decomposition products. Identities of the impurities and decomposition products have been elucidated by MS spectral data. PU-H71 is generally stable to hydrolysis and thermolysis. The most susceptible site in the molecule is the sulfur and the C-S bond under oxidative condition, which generated the sulfoxide product **7** and the 7*H*-purine-8-one product **2**. The other susceptible group is the methylene dioxy, which led to the substituted catechol degradant **5**. The assay method has been validated to be specific, linear ($r = 0.9998$), accurate (recovery 99.7–101.0%), precise (intra-lab RSD = 1.39%, intermediate RSD = 0.91%) and sensitive (LOD 0.08 $\mu\text{g/mL}$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- An HPLC method for a potent antitumor Hsp90 inhibitor, PU-H71, was developed
- Presence of TFA in both diluent and eluent is critical to HPLC method's performance
- The HPLC method was validated to be stability-indicating per ICH guidelines
- Total of 20 related compounds were identified through HRMS and MS/MS fragments
- Sulfur oxidation and hydrolysis of methylene dioxy group are observed degradations

PU-H71.2HCl: $C_{18}H_{21}IN_6O_2S \cdot 2HCl$

Molecular Weight: 585.29

Free Base Exact Mass: 512.0491

Figure 1.

The chemical structure of PU-H71. 2HCl, 8-(6-Iodo-benzo[1,3]dioxol-5-ylsulfanyl)-9-(3-isopropylaminopropyl)adenine dihydrochloride

Figure 2.

- (a) Expanded scale chromatograms of PU-H71, 0.2 mg/mL in methanol;
 (b) Expanded scale chromatograms of PU-H71, 0.2 mg/mL in diluent, ACN/H₂O (20:80, v/v) with 0.1 % TFA;
 (c) Full scale chromatogram of PU-H71, 0.2 mg/mL in diluent, ACN/H₂O (20:80, v/v) with 0.1 % TFA.

Figure 3. PU-H71 forced degradation sample expanded scale chromatograms

(a) 0.2 mg/mL in diluents, un-stressed reference.

(b) 2 mg/mL in H₂O, heated at 80°C for 18 hours, then diluted to 0.2 mg/mL with diluent.

(c) 2 mg/mL in 1N HCl, heated at 80°C for 18 hours, then neutralized with 1N NaOH, and diluted to 0.2 mg/mL with diluent.

(d) 2 mg/mL in 1N NaOH, heated at 80°C for 18 hours, then neutralized with 1N HCl, and diluted to 0.2 mg/mL with diluent.

(e) 2 mg/mL in 3% H₂O₂, heated at 80°C for 2 hours, then diluted to 0.2 mg/mL with diluent.

(f) exposed to UV for 72 hours and cool white light for 312 hours in solid state at 25°C/60% RH, then dissolved and diluted to 0.2 mg/mL with diluent.

Figure 4. The structures of impurities and degradation products of PU-H71, Impurities-enclosed with solid lines; Degradation products-enclosed with dotted lines

- A. impurities and degradation products through alkylation on the purine N-7;
- B. impurities with altered alkyl chain;
- C. impurities and degradation products through substitution, loss or addition of iodine;
- D. degradation products through oxidation of sulfur;
- E. impurities and degradation products through hydrolysis of the methylene dioxy group;
- F. degradation products of the adenyl NH_2 group;
- G. degradation products through nucleophilic reaction on the purine C-8;
- H. degradation products through the loss of the isopropyl group;
- I. impurity through dimerization

Figure 5.
UV, MS and MS/MS spectra and fragment interpretation of PU-H71

Table 1
Summary of Chromatographic and Spectral Data for Impurities and Degradation Products

Degradation Product	RT (min)	λ_{max} , nm	Measured m/z	Calculated m/z	Error (ppm)	MS/MS Major Product Ions m/z
1	1.7	N/A	N/A	N/A	N/A	N/A
2	1.7	220, 280	251.1609	251.1615	2.39	192.0876, 164.0561
3	2.7	215, 255, 295	N/A	N/A	N/A	N/A
4	2.8	220, 280	233.1503	233.1509	2.57	191.1034
5	4.1	220, 280	501.0566	501.0564	-0.40	441.9820
6	5.0	210, 285	387.1593	387.1598	1.29	328.0855
7	6.1	210, 285	529.051	529.0513	0.57	469.9768, 294.8911, 233.1501, 191.1025
8	6.5	220, 285	471.0094	471.0095	0.21	453.9823, 413.9505, 287.0465
9	7.2	215, 255, 295	485.0247	N/A	N/A	453.9816, 413.9470, 287.0479, 174.0745
10	7.8	210, 285	499.04	499.0408	1.60	453.9827
11*	8.2	215, 285	444.2178	444.2176	-0.45	385.1437, 265.1222, 208.0645, 179.0933
11'*	8.2	215, 285	421.1212	421.1208	-0.95	362.0465
12	9.0	285	499.0406	499.0408	0.40	453.9821, 287.0459, 174.0763
13	9.4	215, 285	465.0696	465.0703	1.51	405.9966
14	9.8	285	513.0565	513.0564	-0.19	453.9823
15	10.6	N/A	444.2176	444.2176	0.00	385.1432, 343.0962
16	11.4	N/A	529.0508	529.0513	0.95	469.9767, 294.8911, 233.1501, 191.1025
PU-H71	11.8	215, 285	513.056	513.0564	0.78	453.9825
17	18.0	N/A	503.9827	503.9833	1.19	485.9703, 314.0689, 194.0482
18	19.1	210, 270	514.0418	514.0404	-2.72	454.9748, 175.0593
19	19.1	205, 270	503.072	503.0721	0.20	486.0436, 426.9698, 403.9641, 386.9397, 100.1107
20	19.7	220, 285	541.0872	541.0877	0.92	482.0126
21	20.7	230, 285	638.952	638.9531	1.72	579.8775
22	21.1	205, 290	966.0324	966.0321	-0.31	553.0908, 453.9861

* **11** and **11'** co-eluted