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# Identification and Structure Elucidation of Novel Forced Degradation Products of Gimeracil

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## Abstract

**Rationale:** The study aims to explore the degradation products of gimeracil via forced degradation analysis, necessitating the development of an analytical method for liquid chromatography-mass spectrometry (LC-MS). **Methods:** Gimeracil was subjected to various stress conditions in accordance with International Conference on Harmonization guidelines, including acidic, alkaline, oxidative, photolytic, and thermal conditions. The analytical method was optimized to effectively separate gimeracil from its potential degradation products. **Results:** Under acidic, alkaline, photolytic, and thermal conditions, minimal degradation of gimeracil was observed. However, oxidative stress led to significant degradation, resulting in the identification of fourteen previously unreported degradation products. Structural elucidation of these products was achieved through orbitrap HRMS (high-resolution mass spectrometry) and HRMS/MS spectra analysis, with pathways for the formation of various daughter ions provided for some degradation products. **Conclusion:** Forced degradation analysis revealed the susceptibility of gimeracil to oxidative stress, leading to the generation of numerous degradation products. The developed LC-MS method proved effective in separating gimeracil from its degradation products, enabling comprehensive structural elucidation and identification.

## Keywords

Gimeracil, Forced Degradation, Degradation Products, Nuclear Magnetic Resonance Spectroscopy, HRMS, Structure Elucidation

## 1. Introduction

Cancer stands as the second leading cause of mortality globally, as documented by the World Health Organization's 2018 data [1] [2]. Among the spectrum of malignancies, gastric cancer emerges as the second most prevalent type, contributing significantly to patient mortality [3]. Decades ago, combination therapies demonstrated efficacy in combating various cancers [4]. In 1957, Heidelberger *et al.* proposed the anticancer potential of fluorine-substituted purines and pyrimidines [5]. While diverse first- and second-line treatment modalities have since emerged, recent advancements include oral fluoropyrimidines, serving as inactive prodrugs of 5-fluorouracil. These compounds undergo absorption intact through the gastrointestinal mucosa, converting to 5-fluorouracil via enzymatic systems [3] [6]. Notably, the combination of tegafur, gimeracil, and oteracil gained approval from the European Medicines Agency in March 2011 and is marketed as "Tysuno" [7]. Tegafur, the main active compound, acts as a prodrug for 5-fluorouracil (5-FU), halting cancer cell growth by incorporating into DNA and RNA strands and preventing their replication. In the liver, the cytochrome P-450 enzyme gradually converts tegafur into 5-FU. Additionally, uracil competitively inhibits the enzyme dihydropyrimidine dehydrogenase (DPYD), which breaks down 5-FU, leading to higher levels of the drug in the bloodstream [7] [8]. Gimeracil (5-chloro-2,4-dihydroxypyridine) is a crucial inhibitor of dihydropyrimidine dehydrogenase (DPYD), the enzyme responsible for breaking down 5-fluorouracil (5-FU) and other pyrimidines. By inhibiting DPYD, gimeracil prevents the degradation of 5-FU in the blood, ensuring sustained levels of the drug. This mechanism is particularly effective when gimeracil is combined with the oral fluoropyrimidine derivative S-1, as it helps maintain the active presence of 5-FU in the body, especially in serum and tumor tissues. Consequently, gimeracil plays a vital role in prolonging the therapeutic effect of 5-FU [9]-[12]. Similarly, Oteracil potassium is a chemoprotective agent that primarily functions within the gastrointestinal (GI) tract. It inhibits the enzyme orotate phosphoribosyl-transferase (OPRT), thereby slowing the conversion of 5-fluorouracil (5-FU) into its active form, 5-fluorouridine-5'-monophosphate. This enzymatic inhibition significantly reduces the GI toxicity commonly associated with 5-FU. Furthermore, oteracil potassium prevents the conversion of the prodrug tegafur into 5-FU within the GI tract, thereby minimizing gastrointestinal side effects [10] [13]. Oteracil potassium has consistently been used alongside tegafur and gimeracil in cancer treatment. Tegafur, gimeracil, and oteracil potassium are commonly used medications for treating cancers of the gastrointestinal tract, including those affecting the oral cavity, esophagus, colon, rectum, and pancreas, as well as non-small cell lung cancers. In 2001, these drugs were also approved for the treatment of progressive or recurrent head and neck tumors [14].

The combination of tegafur, gimeracil, and oteracil potassium was evaluated using liquid chromatography-mass spectrometry (LC-MS) [15]-[20]. A literature review identified an LC-MS/MS method for the determination of tegafur, 5-

fluorouracil, gimeracil, and oteracil in human plasma [21]. However, this method involves laborious derivatization steps and internal standard usage. Additionally, a patented HPLC method exists for estimating related substances in combination drug capsules [22]. Another publication describes the simultaneous estimation of tegafur and gimeracil using an LC-MS/MS method [23]. Furthermore, a patented HPLC method exists for the estimation of tegafur, 5-FU, and oteracil [24]. Matsushima *et al.* reported an HPLC and GC ESI negative method for determining a combination of tegafur, 5-fluorouracil, and 5-chloro-2,4-dihydroxy pyridine [25], noting a slight structural difference between gimeracil and 5-chlor-2,4-dihydroxy pyridine due to a ketone group at the 2nd position [25]. Sun *et al.* described an HPLC method for determining tegafur, gimestat, and potassium oxonate in compound tegafur capsules, similar to those used in our studies [26]. Gimeracil has also been estimated by HPLC [27] and LC-MS [28] [29] in various studies, mostly in human plasma and blood samples withdrawn from subjects.

Forced degradation studies conducted on both active pharmaceutical ingredients (APIs) and formulations aim to identify potential degradation products that may arise during storage until use [30]. While various stress conditions are instrumental in impurity identification, oxidative degradation is a primary route for generating degradation impurities [31]-[33]. Oyler *et al.* identified and characterized novel oxidative degradation products of rapamycin [34]. Notably, oxidative degradation poses unique challenges due to the complexity of degradation pathways [35]-[38]. Despite its importance, regulatory guidance on conducting forced degradation studies remains limited due to the diversity and complexity of such studies [39]-[41]. Liquid chromatography-mass spectrometry (LC-MS) plays a crucial role in identifying and elucidating the structures of degradation products, particularly in cases where multiple isomeric degradation products are observed [38] [42].

This study aims to develop an HPLC method for gimeracil estimation in the presence of its degradation products. Additionally, an LC-MS compatible method was developed to characterize major forced degradation products based on their mass values. Forced degradation studies followed the International Conference on Harmonization guidelines to estimate the threshold of degradation products for further identification and characterization [43]-[46]. Both methods underwent validation according to ICH guideline Q2 (R1), encompassing accuracy, method precision, linearity, limit of detection, limit of quantitation, robustness, and ruggedness [47]. These methods are anticipated to be valuable for pharmaceutical industries in quantitatively estimating gimeracil content and its degradation products.

## 2. Materials and Methods

### 2.1. Chemicals, Reagents and Materials

The active pharmaceutical ingredient gimeracil, sourced from Sun Pharmaceuticals Industries Limited, Vadodara, India, high-performance liquid chromatography

(HPLC) grade acetonitrile from Merck, Mumbai, India, and Sigma Aldrich's formic acid (LCMS Grade) were employed for the preparation of the mobile phase. Milli-Q water was obtained through the Milli-Q® Integral water purification system. For stress degradation studies, hydrogen peroxide (30%, Perhydrol®) for analysis from Merck's EMSURE® ISO line, and hydrochloric acid (34% - 37%, Trace Metal grade) from Fischer Scientific, UK, were employed. Additionally, sodium hydroxide pellets of AR grade from Rankem, Mumbai, India, were utilized to simulate alkali stress conditions.

## 2.2. Instrumentation and Analytical Condition

**High Resolution-Mass Spectrometry**

The HPLC system (Waters Alliance 2695) equipped with a PDA detector and with the Empower 3.0 software was used for chromatographic studies. The pH of the buffer solution was adjusted using Eutech (Model: PH-510) pH meter. Ultrasonic cleaner (Leelasonic-500) was used for degassing the mobile phase and other solutions. Analysis was conducted using a Waters X-Bridge C18 column (250 × 4.6 mm, i.d., 3.5 µm) maintained at room temperature. Mobile phase-A comprised a solution of 10 mM potassium dihydrogen orthophosphate, 10 mL triethylamine, and 1.00 g 1-octane sulfonic acid salt per liter, with pH adjusted to 2.50 using orthophosphoric acid. Mobile phase-B consisted solely of acetonitrile. Diluent optimization involved a 50:50 mixture of mobile phase-A and acetonitrile. Gimeracil estimation occurred at a wavelength of 248 nm, with a flow rate of 0.5 mL/min and an injection volume of 10 µL. The gradient transitioned linearly from 0% to 30% mobile phase-B over 30 minutes, followed by a five-minute equilibration period with initial mobile phase compositions.

**Modifications in MS-Compatible Method**

The high-resolution mass spectrometry (HRMS) Orbitrap Q-Exactive plus of Thermo system was used for this study. For MS compatibility, mobile phase-A was prepared with a 0.05% formic acid solution in water, while mobile phase-B remained acetonitrile. Diluent comprised of water. Utilizing a YMC Pack pro C18 column (250 × 4.0 mm, 3 µm) enhanced peak shape for HRMS analysis. Wavelength, gradient, flow rate, injection volume, and other parameters were retained from the original LC method, ensuring methodological consistency across analyses.

The instrument and method parameters are same as mentioned in HPLC method. Instrument method parameters for HRMS instrument were set as per below listed in Table 1.

## 2.3. Forced Degradation Experimental

The forced degradation experiments were performed according to ICH Q1A (R2) and Q1B guidelines to test the stability of gimeracil under the following conditions: acidic and alkaline hydrolysis, oxidation, heat and light. Sample solutions utilized for these experiments maintained a concentration of 0.25 mg/mL in a diluent comprising a 50:50 mixture of mobile phase-A and acetonitrile.

Table 1. HRMS instrument method parameters.

| **Full MS/dd-MS² (TopN)** |
|---|
| **General** |
| Runtime: 0 to 35 min |
| Polarity: Positive and negative |
| In-source CID: 0.0 eV |
| Default charge state: 1 |
| Inclusion: NA |
| Exclusion: NA |
| Tags: NA |
| **Full MS** |
| Microscans: 1 |
| Resolution: 70,000 |
| AGC target: 1e6 |
| Maximum IT: 100 ms |
| Number of scan ranges: 1 |
| Scan range: 100 to 1000 m/z |
| Spectrum data type: Profile |
| **dd-MS²/dd-SIM** |
| Microscans: 1 |
| Resolution: 17,500 |
| AGC target: 5e5 |
| Maximum IT: 100 ms |
| Loop count: 5 |
| MSX count: 1 |
| TopN: 5 |
| Isolation window: 2.0 m/z |
| Isolation offset: 0.0 m/z |
| Scan range: 200 to 2000 m/z |
| Fixed first mass |
| Instrument method: sema_alc |
| Saturday, June 12, 2021 12:07:45 page 5 of 6 |
| (N)CE/stepped (N)CE nce: 30 |
| Spectrum data type: Profile |
| **dd Settings** |
| Minimum AGC target: 5.0e3 |
| Intensity threshold: 5.0e4 |
| Apex trigger |
| Charge exclusion |
| Multiple charge states: all |
| Peptide match preferred |
| Exclude isotopes on |
| Dynamic exclusion: 30.0 s |
| If idle… do not pick others |

**Continued**

| Tune page parameters |
|---|
| Scan type: Full MS |
| Source: HESI source |
| Sheath gas flow rate: 60 |
| Aux gas flow rate: 20 |
| Sweep gas flow rate: 0 |
| Spray voltage (kV): 3.5 |
| Spray current (μA): 0 |
| Capillary temperature (˚C): 320 |
| S-lens RF level: 55 |
| Aux gas heater temp (˚C): 450 |

### Acidic Condition

In acidic conditions, approximately 1 mg of gimeracil was introduced into a 10 mL volumetric flask, followed by the addition of 1 mL of 1M hydrochloric acid solution. The solution underwent heating at 60˚C for 15 minutes and was subsequently neutralized with 1M sodium hydroxide solution before dilution to volume with diluent. Corresponding blank solutions were prepared under identical conditions, omitting the test sample.

### Alkali Condition

For alkali degradation, approximately 1 mg of gimeracil was transferred into a 10 mL volumetric flask and subjected to heating at 60˚C for 15 minutes. Neutralization was achieved with 1M hydrochloric acid solution prior to dilution to volume with diluent. Blank solutions were prepared similarly without the inclusion of gimeracil.

### Oxidation Condition

Under oxidation conditions, 1 mg of gimeracil was added to a 10 mL volumetric flask followed by the addition of 1 mL of 30% hydrogen peroxide solution. The solution underwent heating at 60˚C for 15 minutes and was then diluted to volume with diluent. Blank solutions were prepared analogously, devoid of gimeracil.

### Photolytic Condition

Gimeracil samples (1 mg) were introduced into 10 mL volumetric flasks and diluted with diluent. Additionally, 10 mg of gimeracil was directly exposed to UV-visible light in a photo-stability cabinet for 24 hours. Blank solutions were prepared in parallel without gimeracil.

### Thermal Condition

For thermal degradation, 1 mg of gimeracil was transferred into a 10 mL volumetric flask, diluted with diluent, and subjected to heating at 60˚C for 60 minutes. Blank solutions were prepared similarly without gimeracil.

Subsequently, all degradation samples underwent HPLC analysis employing the analytical conditions detailed in the results and discussion section. Chromatograms were recorded to detect any generated degradation products. Following confirmation of degradation under oxidative conditions, the oxidative degradation sample

was subjected to LC-MS and HRMS analysis to ascertain the exact mass and fragmentation patterns of degradation products.

## 3. Results and Discussion

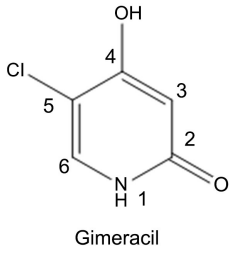
### 3.1. Forced Degradation Results

In our study, gimeracil exhibited remarkable stability under various stress conditions, with the exception of oxidative degradation. Specifically, no significant degradation products were observed in acidic, thermal, and photolytic conditions, see the Declaration for details [48].

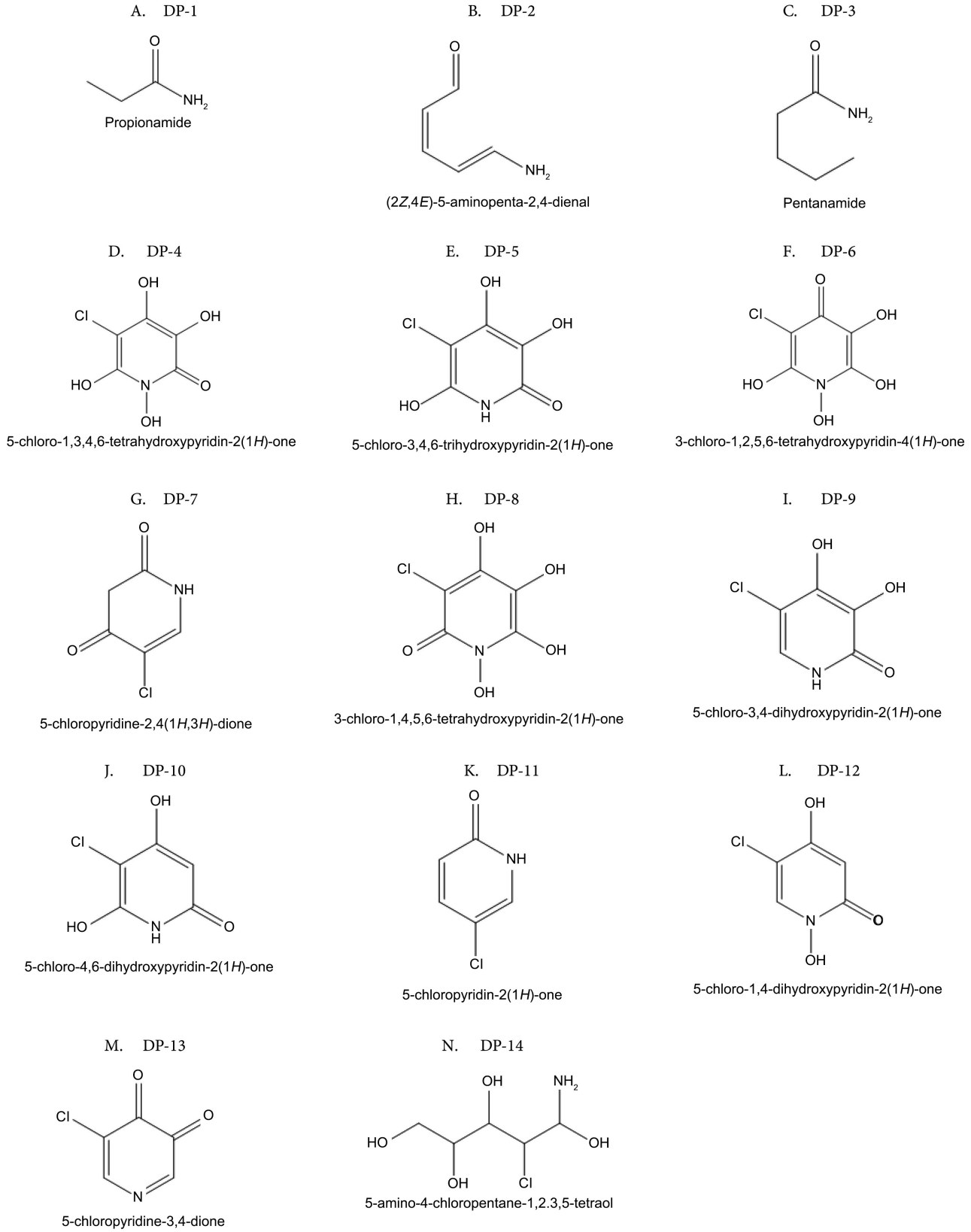
However, under alkaline stress, notably severe conditions of 1 M NaOH and heating at 80˚C for 1 hour, two impurity peaks emerged at RRT 0.88 and 1.11. Despite this, when subjected to milder conditions of 1 M NaOH and heating at 70˚C for 30 minutes, a concentration of 250 µg/mL of gimeracil exhibited no major degradation products.

In the oxidative degradation scenario, while the main peak of gimeracil experienced approximately a 50% reduction, traditional UV/PDA detection failed to identify degradation products even after extending the runtime to 90 minutes. Recognizing this limitation, we pursued the development of an LC method coupled with a MS detector. Consequently, further investigation was carried out via HRMS analysis.

Remarkably, HRMS analysis of the degraded sample under oxidative conditions revealed the presence of 14 degradation product peaks in the TIC chromatogram, each accompanied by exact MS and MS/MS spectra (**Figure 1**, **Figure 2** and see the Declaration). Seven of these degradation products (DP-4, DP-5, DP-6, DP-8, DP-9, DP-10, and DP-12) were identified as oxidative products of gimeracil, with incorporation of one, two, or three oxygen atoms into the molecular structure. Out of these seven DPs, three isomeric products were noted with addition of three oxygen molecules mass in gimeracil and three isomeric products were noted with addition of one oxygen molecules mass Notably, isomeric variations were observed, attributed to the multiple potential attachment sites for oxygen molecules within the gimeracil structure. Other than these degradation products, remaining seven degradation products can also be clearly identified based on accurate HRMS and MS-MS data which have been discussed in individual section, as outlined in **Figure 2** and **Figure 3**, and summarized in **Table 2**.

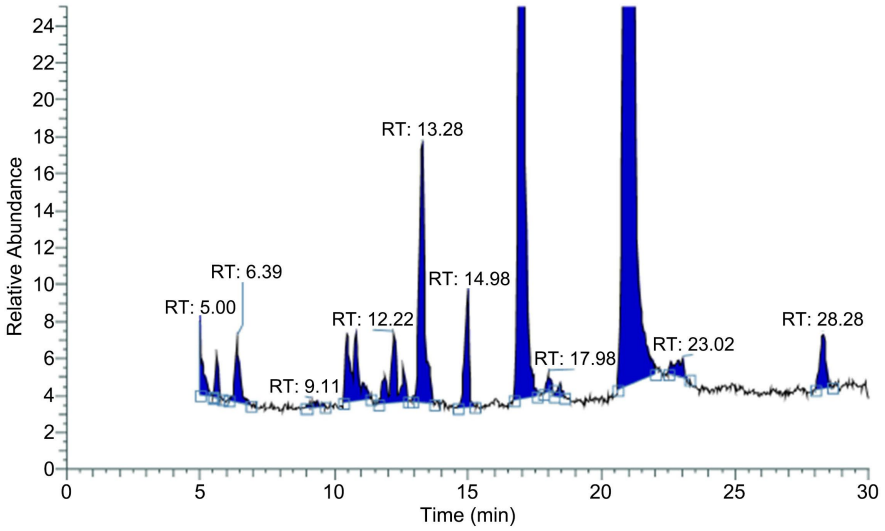



Molecular structure of gimeracil with identification number




Molecular structure of degradation products of gimeracil

**Figure 1.** Molecular structure of gimeracil and its degradation products.




| Name | RT (Min) | Peak Area | % Area |
|---|---|---|---|
| | 5.00 | 1820480023.52 | 0.76 |
| | 5.64 | 1363373724.92 | 0.57 |
| | 6.39 | 2964009581.80 | 1.24 |
| | 9.11 | 123418280.62 | 0.05 |
| | 9.22 | 100279002.29 | 0.04 |
| | 9.34 | 118231282.50 | 0.05 |
| | 10.44 | 3324780947.63 | 1.39 |
| | 10.78 | 2920983579.82 | 1.22 |
| | 11.05 | 1108850172.55 | 0.46 |
| | 11.88 | 1390362132.75 | 0.58 |
| | 12.22 | 2628697985.90 | 1.1 |
| | 12.56 | 995866463.31 | 0.42 |
| | 13.28 | 15425365599.70 | 6.44 |
| | 14.98 | 5163000275.08 | 2.16 |
| | 16.99 | 48533938053.88 | 20.27 |
| | 17.79 | 245962792.80 | 0.10 |
| | 17.98 | 1664004586.19 | 0.70 |
| | 18.43 | 579288890.58 | 0.24 |
| | 21.07 | 145507785570.59 | 60.77 |
| | 28.28 | 3443197369.83 | 1.44 |

**Figure 2.** TIC chromatogram of gimeracil in oxidative degradation condition.

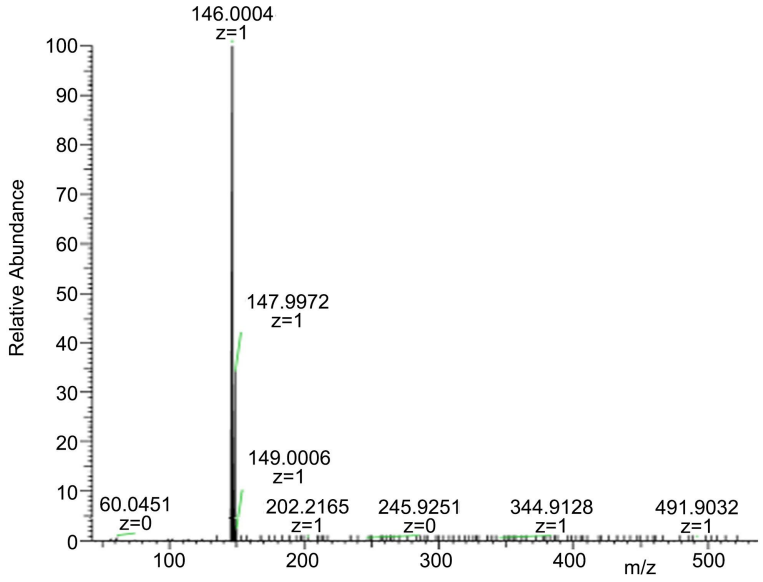



**Figure 3.** HRMS spectrum of gimeracil peak.

**Table 2.** Degradation products summary of gimeracil under oxidative degradation condition in HRMS study.

| Degradation products | RT of impurity | RRT | Observed molecular ion peak (m/z) | Further MS-MS fragmentation molecular ion peaks (m/z) | | | | | | Degradation products |
|---|---|---|---|---|---|---|---|---|---|---|
| DP-1 | 5.64 | 0.27 | **74.097** | 57.0706 | | | | | | Propionamide or (E)-3-aminoprop-1-en-1-ol |
| DP-2 | 6.39 | 0.30 | **98.9845** | 80.9607 | 78.9565 | | | | | (*Z*)-penta-2,4-dienoic acid |
| DP-3 | 10.4 | 0.50 | **102.128** | 74.097 | 58.658 | | | | | Pentanamide |
| DP-4 | 10.8 | 0.51 | **194.1386** | 176.1282 | 162.1124 | 133.1096 | 116.1072 | 100.1124 | 88.0762, 74.097, 58.0658 | Gimeracil + 3O |
| DP-5 | 11.1 | 0.52 | **178.1438** | 102.128 | 74.097 | | | | | Gimeracil + 2O |
| DP-6 | 11.9 | 0.56 | **194.1387** | 176.1281 | 162.1124 | **148.0968** | 135.0442 | 118.854 | 100.0761, 74.097, 58.0658 | Gimeracil + 3O |
| DP-7 | 12.2 | 0.58 | **146.1539** | **128.1436** | 86.097 | | | | | Converstion of hydroxy of gimeracil to ketone |
| DP-8 | 12.6 | 0.60 | **194.1388** | **86.097** | 58.0058 | 211.9407 | | | | Gimeracil + 3O |
| DP-9 | 13.3 | 0.63 | **162.1488** | 144.1383 | **130.1227** | 101.1203. 102.0917 | 86.0969 | 72.0814 | 58.0658 | Gimeracil + O |
| DP-10 | 15 | 0.71 | **162.1488** | **116.1071** | 139.9821 | 144 | | | | Gimeracil + O |
| DP-11 | 17 | 0.81 | **130.159** | 74.097 | 57.0706 | | | | | 5-chloropyridine-2(1H)-one or 3-chloropyridin-4-ol |
| DP-12 | 18 | 0.85 | **161.9953** | **124.0009** | | | | | | Gimeracil + O |
| DP-13 | 18.4 | 0.87 | **144.1747** | 121.9664 | 88.1125 | 57.0706 | | | | Double bond creation at N-H position of gimeracil |
| Gimeracil | 21.1 | 1.00 | **146.0004** | 160.016 | 127.9894 | 103.9899 | 90.0113 | 78.0111 | 68.9977, 54.9529 | Gimeracil main peak |
| DP-14 | 28.3 | 1.34 | **186.2218** | 130.1592 | 100.0758 | 80.0978 | 74.0971 | 57.0707 | | 1-amino-4-chloropentane-1,2,3,5-tetraol |

Our findings underscore the importance of employing advanced analytical techniques such as LC-MS and HRMS to comprehensively characterize degradation pathways and products, thereby facilitating the development of robust pharmaceutical formulations.

## 3.2. Structure Elucidation of Degradation Products

### 3.2.1. Structure Elucidation of DP-1

DP-1 exhibited an elution time of 5.64 minutes, with a molecular ion peak observed at 74.0970 m/z (see the Declaration). Considering the structural components of gimeracil, the breakdown may yield propionamide, featuring a molecular mass of 73.0950. Notably, the MS/MS fragmentation spectra revealed a prominent peak at 57.0706 m/z, consistent with the exact mass of ionized propionaldehyde (58.0800), accounting for one proton less (see the Declaration [49]). This fragmentation pattern suggests the plausible formation of propionamide through further degradation of gimeracil under elevated collision energy conditions. Thus, DP-1 is confidently identified as propionamide, as illustrated in **Figure 1**.

### 3.2.2. Structure Elucidation of DP-2

The molecular ion peak observed at 98.9845 [23] from the gimeracil structure prompted consideration of three potential structural modifications, see the Declaration for details. In each scenario, the common alteration involved the removal of the chloride functional group and the cleavage of the bond between the -NH and -C=O positions (positions 1 and 2, respectively).

Initially, the first possibility explored entailed solely the removal of the chloride

group and bond cleavage, resulting in a structure with a molecular mass of 97.1170. However, this structure yielded a molecular mass one proton less than expected for the observed molecular ion peak of 98.9845, rendering it implausible.

Subsequently, attention turned to the second possibility, which involved an increment of one proton mass by converting -C=O to -C-OH. However, this modification would introduce two protons into the structure, resulting in a molecular mass one proton higher than anticipated for DP-2, thus negating this likelihood.

Finally, the third probability involved the formation of a carboxylic acid derivative from the gimeracil structure, alongside the removal of the chloride group and bond cleavage between positions 1 and 2. This proposed structure exhibited an exact mass of 98.1010, precisely aligning with the observed molecular ion peak at 98.9845 m/z. Moreover, the MS/MS spectra further supported this hypothesis, with the molecular ion peak at 80.9607 m/z indicative of the removal of a terminal -OH group and the formation of a double bond between positions 5 and 6 of gimeracil (see the Declaration). Additionally, the major MS/MS fragment at 78.0700 m/z corresponded to the formation of another triple bond between positions 1 and 2 within the same structure (see the Declaration).

### 3.2.3. Structure Elucidation of DP-3

DP-3 exhibited an elution at RRT 0.50 (RT 10.4 min), accompanied by a molecular ion peak at 102.128 m/z. Notably, two characteristic MS/MS fragmentation peaks at 74.097 m/z (propionamide) and 58.658 m/z (propionaldehyde) were observed (see the Declaration), as discussed in the DP-1 section, indicating the presence of the -$CONH_2$ group in the DP-3 structure.

Given this evidence, the most probable structure for DP-3 is that of pentanamide (see the Declaration), with an exact molecular mass of 101.1490.

Additionally, another potential structure with a mass near 101.128 was considered, involving the retention of the chloride group in the structure with an aliphatic carbon chain only. However, the mass of this structure was obtained as 102.5610 which is having one proton mass higher. As there was no scope in the structure to reduce the mass by a single proton, this probability was ruled out (see the Declaration).

### 3.2.4. Structure Elucidation of DP-4, DP-6 and DP-8

DP-4, DP-6, and DP-8 displayed elution at RRT 0.51, 0.56, and 0.60 (RT 10.8 min, 11.9 min, and 12.6 min, respectively), each with a molecular ion peak of approximately 194.1386 m/z, 194.1387 m/z, and 194.1388 m/z, respectively (see the Declaration). Considering that the molecular ion peak of gimeracil is 146.0004 m/z, the observed molecular ions of these DPs at ~194 strongly suggest the addition of three oxygen molecules (146.0004 + 48 = 194.0004).

Besides the two oxygen molecules present in gimeracil's structure (a hydroxy group at the 4th position and a ketone group at the 2nd position), three potential sites for oxygen molecule incorporation exist: the 1st, 3rd, and 6th positions. The first probable structure involves the addition of hydroxy groups (-OH) at these

positions, resulting in an exact mass of 193.5390, which correlates precisely with the observed molecular ion peak at 194.1386 m/z (**Figure 1**).

Given the structure of DP-4, it is highly likely that multiple isomers could form if the position of the ketone functional group is altered to either the 4th or 6th position, yielding the same molecular mass. Accordingly, DP-6 and DP-8 represent these isomeric structures. Thus, the structures and chemical names of DP-4, DP-6, and DP-8 can be inferred from **Figure 1**.

### 3.2.5. Structure Elucidation of DP-5

DP-5 eluted at RRT 0.52 (RT 11.1 min) with a molecular ion peak at 178.1438 m/z (**Figure 2** and see the Declaration). This molecular ion peak indicates the addition of two oxygen molecules to the structure of gimeracil (146.0004 + 32 = 178.0004). The most probable sites for incorporating oxygen molecules in the gimeracil structure are the 3rd and 6th positions.

The addition of a hydroxy group, rather than a ketone group, is logically favored. Adding a hydroxy group would only increase the molecular mass by 16 for each position, as the hydrogen of the -CH group at the 3rd or 6th position would be replaced, but an additional hydrogen would accompany the -OH hydroxy functional group. Conversely, if a ketone were to form at the 3rd or 6th position, it would add 15 + 15 molecular mass for each position, as the hydrogen of the -CH group would be removed. Thus, this would not align with the observed molecular ion peak, where a 16 + 16 molecular mass increase is evident.

The MS/MS spectrum of DP-5 exhibits a prominent peak at 102.1281 m/z, suggesting the formation of pentanamide due to the breakdown of DP-5 at high collision energy in HRMS (see the Declaration). Taking these observations into account, the structure and chemical name of DP-5 can be depicted as shown in **Figure 1**.

### 3.2.6. Structure Elucidation of DP-7

DP-7 eluted at RRT 0.58 (RT 12.2 min) in the HRMS chromatogram with a molecular ion peak at 146.1539 m/z, which is almost identical to the gimeracil molecular ion peak of 146.0004 m/z (see the Declaration).

The isomeric degradation product of gimeracil that can be formed under oxidative degradation conditions would only be possible if the hydroxy group of gimeracil is converted to a ketone group. This conversion is highly likely to occur with slight modifications in the oxidative degradation conditions. While other isomeric forms could be considered, they would require more intense modifications to the gimeracil structure, which are unlikely to occur during normal drug storage.

Therefore, based on the observed molecular ion peak and the plausible mechanism of oxidative degradation, the structure and chemical name of DP-7 can be depicted as shown in **Figure 1**.

### 3.2.7. Structure Elucidation of DP-9, DP-10 and DP-12

DP-9, DP-10, and DP-12 eluted at RRT 0.63, 0.71, and 0.85 (RT 13.3 min, 15.00 min, and 18.00 min respectively) with molecular ion peaks at 162.1488 m/z, 162.1488

m/z, and 161.9953 m/z respectively (see the Declaration). The molecular ion peaks suggest the addition of one oxygen molecular mass to the structure of gimeracil (146.0004 + 16 = 162.0004).

As discussed in the sections on DP-4, DP-6, and DP-8, there are three positions in the gimeracil structure where an oxygen molecule can be incorporated. These positions are the 1st, 3rd, and 6th positions of the gimeracil structure. If all these positions were occupied and ketone functional groups were changed, three isomers of DP-4, DP-6, and DP-8 would be created. If two of these positions were filled with hydroxy groups, it would create DP-5. However, if only one of these three positions were occupied, isomeric degradation products, namely DP-9, DP-10, and DP-12, would be generated with a molecular mass of 161.5410.

In conclusion, DP-9, DP-10, and DP-12 are isomeric degradation products with the addition of a hydroxy group at the 1st, 3rd, or 6th positions of the gimeracil structure. Another noteworthy point about the difference in these isomeric degradation products is their polarity. The oxidative degradation products with 2 or 3 hydroxy groups incorporated in the structure appear to be more polar than those with only one hydroxy group. Hence, DP-4, DP-5, DP-6, and DP-8 eluted earlier in the chromatogram (*i.e.* at RRT 0.51, 0.52, 0.56, and 0.60 respectively), whereas DP-9, DP-10, and DP-12 eluted later in the chromatogram (*i.e.* at RRT 0.63, 0.71, and 0.85 respectively). The MS/MS spectra of these isomeric degradation products and gimeracil exhibit characteristic peaks such as 74.097 m/z and 58.0658 m/z.

Considering these facts, the molecular structures and chemical names of DP-9, DP-10, and DP-12 can be depicted as shown in **Figure 1**.

### 3.2.8. Structure Elucidation of DP-11

DP-11 was detected at RRT 0.81 (RT 17.00 min) with a molecular ion peak at 130.159 m/z (see the Declaration). This suggests the removal of one oxygen molecule from the gimeracil structure (146.0004 − 16 = 130.0004). Gimeracil contains two oxygen molecules, one in the keto functional group and the other in the hydroxy functional group. The removal of either of these oxygen molecules leads to two possibilities with the same molecular mass (see the Declaration).

An intriguing observation is the fragmentation pattern, which also yields almost identical molecular masses. The MS/MS fragmentation peaks of 73.095 m/z and 58.0800 m/z are evident in both possibilities (see the Declaration). Consequently, the molecular structure and chemical name of DP-11 can be inferred as depicted in **Figure 1**.

### 3.2.9. Structure Elucidation of DP-13

DP-13 was identified at RRT 0.87 with a molecular ion peak at 144.1747 m/z (see the Declaration). The discrepancy between the molecular ion peaks of DP-13 and gimeracil is approximately two protons (146.0004 − 144.1747 = 1.8257). This suggests the potential removal of two protons from the gimeracil structure by the generation of a double bond at certain positions.

Initially, one possibility considered was the conversion of the hydroxy group at

the 4th position to a ketone functional group. However, this alteration would remove one hydrogen atom from the hydroxy group while adding two hydrogen atoms to the adjacent carbon at the 3rd position due to double bond formation between the 3rd and 4th positions. Consequently, this possibility was ruled out.

Another scenario examined involved creating a double bond between the 1st and 2nd positions, accompanied by converting the ketone functional group into a hydroxy functional group due to double bond formation. However, this modification would not result in a reduction of hydrogen molecular mass, as the hydrogen molecule removed from the -NH group at the 1st position would be compensated by the addition of one hydrogen molecule in the form of a hydroxy group at the 2nd position. Thus, the overall molecular mass would remain the same as gimeracil.

The third possibility involves creating a double bond between the 1st and 2nd positions while shifting the ketone functional group from the 2nd to the 3rd position carbon. Since the ketone functional group is present at the 3rd position, no double bond would exist between the 3rd and 4th positions, thereby converting the hydroxy functional group at the 4th position to a ketone functional group.

Based on this analysis, the molecular structure and chemical name of DP-13 can be depicted as illustrated in **Figure 1**.

#### 3.2.10. Structure Elucidation of DP-14

DP-14 was detected at RRT 1.34 (28.3 min) with a molecular ion peak at 186.2218 (see the Declaration). The disparity between the molecular ion peak of DP-14 and gimeracil is 40.2214 (186.2218 − 146.0004 = 40.2214), indicating the incorporation of either three oxygen molecules (48) into the gimeracil structure and the subsequent removal of 8 hydrogen atoms by the inclusion of four double bonds in the same structure, or the formation of a ketone group from a hydroxy group.

However, given that the gimeracil structure already contains two double bonds, there is limited potential for the creation of additional double bonds within the structure. Therefore, an alternative method to increase the molecular mass by 40 is to incorporate two oxygen molecules (32) and subsequently add 8 hydrogen atoms to the structure by breaking existing double bonds and disrupting the ring system at the 1st and 2nd positions.

This process yields an aliphatic structure, as depicted in **Figure 1**, with an exact mass of 185.6040, which aligns closely with the molecular ion peak obtained at 186.2218 m/z. No other feasible pathways have been identified for the generation of this molecular mass degradation product.

Hence, the structure and chemical name of DP-14 can be delineated as illustrated in **Figure 1**.

## 4. Conclusions

In this study, we conducted a comprehensive investigation into the novel degradation products of gimeracil through forced degradation analysis. Our innovative analytical approach, optimized for liquid chromatography-mass spectrometry, enabled

effective separation and identification of each potential degradation product peak from the gimeracil peak.

Gimeracil demonstrated remarkable stability under various stress conditions, except for oxidative stress, which led to significant degradation, resulting in the formation of fourteen previously unreported degradation products. These degradation products were meticulously analyzed using orbitrap HRMS, with detailed structural elucidation achieved through HRMS/MS spectra. Furthermore, pathways for the formation of various daughter ions (MS/MS) of these degradation products were provided.

The structural elucidation of these degradation products sheds light on the intricate mechanisms underlying gimeracil degradation under oxidative conditions. By employing advanced analytical techniques such as LC-MS and HRMS, we were able to comprehensively characterize the degradation pathways and products, facilitating the development of robust pharmaceutical formulations.

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## Declaration

Due to the sensitive/confidential nature of the experimental data, these results (Supporting Information S1-A-S11-B) cannot be included in the published manuscript. However, interested researchers may contact the corresponding author directly to request access to the dataset.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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## Abbreviations List

| | |
|---|---|
| DPs | Degradation products |
| ICH | International conference on harmonization |
| PDA | Photodiode array |