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Original Research Article

Stability study on an anti-cancer drug 4-(3,5-bis(2-chlorobenzylidene)-4-oxo-piperidine-1-yl)-4-oxo-2-butenoic acid (CLEFMA) using a stability-indicating HPLC method

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CLEFMA, 4-(3,5-bis(2-chlorobenzylidene)-4-oxo-piperidine-1-yl)-4-oxo-2-butenoic acid, is a new chemical entity with anti-cancer and anti-inflammatory activities. Here, we report its stability in solution against stress conditions of exposure to acid/base, light, oxidant, high temperature, and plasma. The identity of the degradation products was ascertained by mass and proton nuclear magnetic resonance spectroscopy. To facilitate this study, we developed and validated a reverse phase high performance liquid chromatography method for detection of CLEFMA and its degradation. The method was linear over a range of 1–100 µg/mL; the accuracy and precision were within acceptable limits; it was stability-indicating as it successfully separated *cis-/trans*-isomers of CLEFMA as well as its degradation product. The major degradation product was produced from amide hydrolysis at maleic acid functionality caused by an acidic buffer, oxidant (3% hydrogen peroxide), or temperature stress (40–60 °C). The log *k*-pH profile showed that CLEFMA was most stable at neutral pH. In accelerated stability study we found that the shelf-life ($T_{90\%}$) of CLEFMA at 25 °C and 4 °C was 45 days and 220 days, respectively. Upon exposure to UV-light (365 nm), the normally prevalent *trans*-CLEFMA attained *cis*-configuration. This isomerization also involved the maleic acid moiety. CLEFMA was stable in plasma from which it could be efficiently extracted by an acetonitrile precipitation method. These results indicate that CLEFMA is sensitive to hydrolytic cleavage at its maleic acid moiety, and it is recommended that its samples should be stored under refrigerated and light-free conditions, and under inert environment.

1. Introduction

CLEFMA, 4-(3,5-bis(2-chlorobenzylidene)-4-oxo-piperidine-1-yl)-4-oxo-2-butenoic acid, is an anti-proliferative compound that is being tested at pre-clinical stage as an anti-cancer agent. In our previous work we reported that CLEFMA potently inhibited the viability of H441 lung adenocarcinoma cells [1,2]. CLEFMA resembles a broader group of electrophilic chemicals carrying a diphenyldihaloketone core (Fig. 1). It is also classified as a curcuminoid because of the presence of a chalcone backbone which is also present in the curcumin structure [1,3,4]. Like the other molecules of chalcone class, CLEFMA possesses structural features that have also been reported to inhibit the NF-κB pathway [5,6]. Such molecules exhibit a wide spectrum of pharmacological activity such as anti-microbial, anti-inflammatory, anti-oxidant, immunomodulating, and anti-neoplastic actions [7,8].

As a drug in its initial stage of developmental process, it is important to understand CLEFMA's physiochemical properties, including the factors and mechanisms which influence its stability. The inherent stability of a drug substance is a critical component of the pre-formulation studies which provides important insights into the choices of materials and processes during the development of a drug-product. Earlier, we reported a liposome formulation of CLEFMA [9]. To develop an effective liposomal anti-cancer drug product of CLEFMA, the formulation must be designed to maintain stability of the encapsulated drug, ensure its quality until it is administered to patients, and exert maximum potency [10]. However, the stability of CLEFMA has not been investigated so far.

According to the guidelines issued by the International Conference on Harmonization (ICH), stability studies can be classified as the long-term, accelerated, and stress-stability ones [11–13]. The long-term studies are carried out to determine the shelf-life, which is nominally defined as the time taken for the amount of active ingredient to fall by 10% of the original amount. The accelerated stability studies are carried out under elevated environmental conditions. As compared to

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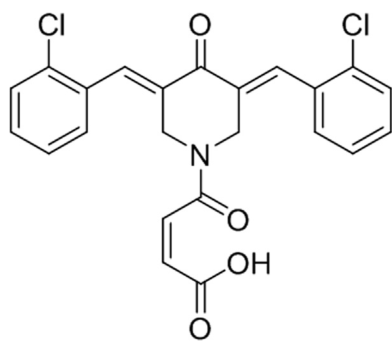


Fig. 1. Chemical structure of CLEFMA.

the long-term studies, the accelerated studies reduce the time required to accomplish the goals of stability studies and predict shelf-life of a drug-product. Lastly, the stress-stability studies are carried out under more stressful conditions to determine the intrinsic property of the drug and its expected degradation pathways. The stress-stability testing also helps with the validation of analytical method for its ability to detect and separate active pharmaceutical ingredient from its degradation product.

The primary objective of this study was to examine the solution-state stability of CLEFMA. The solution-stability of chalcones as a class has also not been reported in the literature. An earlier work showed that curcumin exhibits some instability in the presence of light and in neutral-to-alkaline pH; it is stable in acidic conditions [14]. We hypothesized that curcuminoid CLEFMA will show a stability profile similar to the one reported for curcumin. Since the primary requirement for stability studies is the availability of a robust stability-indicating analytical method, the secondary objective of this study was to develop and validate a high performance liquid chromatography (HPLC) method for assay of CLEFMA to monitor its degradation. No analytical method is currently available for detection and quantification of CLEFMA.

2. Experimental

2.1. Chemicals

The synthesis and characterization of CLEFMA and internal standard EFNAC (N-Acetyl-3,5-bis(2-fluorobenzylidene)–4-piperidone) have been reported in a previous article [1]. All chemicals and solvents used in this study were of analytical reagent grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA) or various manufacturers represented by VWR Scientific (West Chester, PA, USA). For the stability studies in solution phase, we solubilized CLEFMA in acetonitrile (1 mg/mL solution).

2.2. Analytical method and method validation

We employed System Gold (Beckman Coulter, Indianapolis IN, USA) for peak separation and analyses. The isocratic chromatography of CLEFMA was performed on a C_{18} Sonoma analytical column (250 mm×4.6 mm, 10 µm, 100 Å) from ES industries (West Berlin, NJ, USA). The mobile phase, consisting of acetonitrile and water (45:55, v/v) and containing 0.1% trifluoroacetic acid (TFA), was pumped at 1.5 mL/min. The peak detection was carried out at 318 nm using a Dynamax UV-1 detector (Rainin, Woburn, MA, USA). The HPLC profile data were analyzed using PeakSimple software (SRI Instruments, Earl St. Torrance, CA, USA). The quantification was done by measuring the peak-area count. The CLEFMA standards were prepared in acetonitrile in the range of 1–100 µg/mL; a constant volume of 20 µL was injected each time. The lower limit of quantification (LLOQ) and the limit of detection (LOD) were determined by the signal-to-noise ratio (s/n) from the calibration curve.

Method validation was performed in accordance with the ICH guidelines [15]. System suitability was determined by injecting 100 µg/mL of CLEFMA in triplicate to determine the resolution factor, tailing factor, theoretical plate number, and peak capacity factor. The precision of the method was determined in terms of inter-day and intra-day variability. Intra-day variability (repeatability) was determined by injecting CLEFMA standards of 100, 20 and 5 µg/mL in triplicate on the same day. The relative standard deviation (RSD) was determined as a measure of precision. Inter-day variability was determined by injecting the same CLEFMA standard solutions on 3 different days. The mean values and the RSD were determined for each concentration from the peak area. The precision was calculated as % RSD =(SD/Mean) ×100. The percentage recovery, a measure of accuracy, was determined as how close the calculated values of CLEFMA concentration were from the actual values. It was determined by spiking a known amount of standard in triplicate.

2.3. Acid-base catalyzed degradation of CLEFMA (pH-dependent stability)

CLEFMA solution was buffered in a pH range of 2–10 by using buffers consisting of HCl/KCl (pH 2.2), acetic acid/sodium acetate (pH 3.5 and 5.0), Na_2HPO_4/KH_2PO_4 (pH 7.4), and $Na_2CO_3/NaHCO_3$ (pH 8.2 and 9.6). The strength of all the buffers was 0.2 M. The degradation was studied over 2 weeks. To characterize the degradation product from the acid-catalyzed reaction, we separated it on a preparative thin layer chromatography (TLC). Briefly, a solution of CLEFMA in acetonitrile (100 mg/mL) was acidified with HCl (to approximately 0.15 M) and stirred for 6 h. The mixture was diluted with 5 mL water and the acid-degraded product was extracted with ethyl acetate. After drying the extract with sodium sulfate, it was evaporated to dryness. The crude product was spotted on glass-backed silica gel (Cat # 021013 Analtech, Newark, DE, USA) and separated using ethyl acetate/hexane system (90:10, v/v). After detection by iodine-staining, the degraded product was extracted from silica gel spot in ethyl acetate. The dried product was subjected to ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) in $CDCl_3$ (Cambridge Isotope Lab, Tewksbury, MA, USA). The NMR spectra were recorded at 25 °C with a Varian Mercury 300 MHz spectrometer. Mass spectroscopy of the degradation product was performed in the core facility of the University of Oklahoma Health Sciences Center (Oklahoma City, OK,USA).

2.4. Temperature-dependent stability of CLEFMA (accelerated stability)

CLEFMA solution was stored at 4 °C, 25 °C, 40 °C, and 60 °C. At various times, the samples were collected and subjected to HPLC analyses. The shelf-life of CLEFMA at different temperatures was calculated as $T_{90\%} = 0.105/k_{obs}$. According to the United States Food and Drug Administration (US FDA) drug stability guidelines, a product must recover 90% of its original value throughout its shelf-life [16]. $T_{90\%}$ also remains a typical manufacturer's quality assurance criteria for drug products [13,17]. The rate constant (k_{obs}) for the degradation reaction was related to the absolute temperature (T) by the Arrhenius equation: $\log k = \log A - E_a/2.303RT$, where A, E, and R are prefactor constant, activation energy, and gas constant, respectively [18].

2.5. Photo-stability and oxidative degradation of CLEFMA

According to the ICH guidelines, a drug should be exposed to a light source with an overall illumination not less than 1.2 million lx h and an integrated near ultraviolet energy of not less than 200 W h/m^2. The UV fluorescent lamp should have a spectral distribution between 320 and

400 nm with maximum spectra between 350 and 370 nm [19]. The sensitivity of CLEFMA to UV (wavelength 365 nm) for 2 h or ambient light for 6 weeks was studied in a quartz vial as per the ICH guidelines for the photo-stability studies [11,19]. The control sample of CLEFMA was wrapped in an aluminum foil and stored in darkness. To investigate the identity of the photo-degraded product, a solution of CLEFMA in acetonitrile was irradiated by a 365 nm lamp for 2 h. The solvent was evaporated to dryness and the ^{1}H NMR spectrum was acquired, without further purification, in $CDCl_3$. The degradation product was also submitted to the mass spectroscopy.

It is reported that subjecting the solutions to 0.1%–3% H_2O_2 at neutral pH and room temperature for 7 days or to H_2O_2 concentration required for 20% degradation could potentially generate relevant degradation products [13,20]. The stability of CLEFMA against oxidation was achieved by exposing CLEFMA solution to 3% H_2O_2 (1 part CLEFMA solution +4 parts H_2O_2). The samples were collected at 2, 4, 8, and 24 h of exposure to the oxidative conditions. In order to ascertain the identity of the degradation product after oxidative stress, we extracted the 24 h sample with ethyl acetate. After drying the extract under nitrogen stream, the dry product was subjected to mass spectroscopy and HPLC.

2.6. CLEFMA stability in biological matrix (bio-stability)

To study the stability of CLEFMA in biological matrix, we first established a method of recovering CLEFMA from plasma samples (described below). The bench-top stability studies were performed on rat plasma containing 50 µg/mL CLEFMA. The mixture was left at room temperature for 4 h before extraction and subsequent HPLC analysis. The plasma solution of CLEFMA was also tested for freeze-thaw stability. Briefly, 50 µg/mL and 20 µg/mL plasma solutions of CLEFMA were subjected to three freeze-thaw cycles at 12 h intervals by freezing at −80 °C and thawing to room temperature. The HPLC conditions for determining the bio-stability of CLEFMA were identical to those described in Section 2.2.

For extraction of CLEFMA from plasma samples, a protein precipitation method was employed, which has been previously described for curcumin and other drugs [21,22]. The plasma samples were doped with 2 µg of internal standard (EFNAC) per 100 µL of plasma. After thorough mixing, 500 µL of acetonitrile was added to the plasma and the mixture was vigorously vortexed for 1 min, followed by centrifugation (12,000 rpm for 10 min, at 4 °C) to separate the supernatant. The pellets were re-extracted two more times to ensure maximum extraction. The collected supernatants were evaporated to dryness and reconstituted in 100 µL acetonitrile for the HPLC analysis. The standard curve of CLEFMA in plasma was obtained by extracting CLEFMA from its solutions in rat plasma (0.5, 2, 5, 10, 20 and 50 µg/mL). The extraction efficiency of CLEFMA in plasma samples was determined at both high (50 µg/mL) and low (5 µg/mL) concentrations. As an alternative, we also tested liquid-liquid extraction with ethyl acetate (1:1, v/v) as the organic solvent.

2.7. Cell viability assay

The effect of CLEFMA on cell viability of human non-small cell lung cancer cell line H441 was determined by 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assay. The H441 cells were obtained from American Type Culture Collection (Manassas, VA, USA), and maintained at 37 °C with 5% CO_2 in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 50 µg/mL gentamicin. The cells were seeded in 96-well flat-bottom tissue culture plate at a density of 2,500 cells per well. The cells were allowed to adhere and grow overnight, followed by treatment with CLEFMA for 48 h (10 µM). At the end of incubation, 20 µL of MTT solution (5 mg/mL) was added into each well. After 2 h, the formazan crystals in each well were dissolved in 100 µL of dimethylsulfoxide and quantified at 562 nm by using a Synergy 2 plate reader (BioTek, Winoosky, VT, USA).

2.8. Data analysis

All reported conditions were assessed in three separate experiments. Wherever applicable, the data are presented as the mean ± standard error of mean (SEM) or ± standard deviation (SD) calculated using Prism 6 software (GraphPad, San Diego, CA, USA). The viability of cells was calculated as percentage of control. For statistical comparisons, we employed a two-tailed *t*-test or analysis of variance (for multiple comparison, Tukey post-hoc test); $P < 0.05$ was taken as the cut-off for significance.

3. Results

3.1. Analytical method and method validation

An HPLC method was developed, which allowed the separation and estimation of CLEFMA (Fig. 2A). The detection wavelength was decided on the basis of its UV–visible spectrum, which showed a peak at 318 nm. The mobile phase was optimized after several trials with acetonitrile and water in various proportions to obtain a sharp peak. The best results (peak width and retention time, ~14.5 min) were obtained when the mobile phase consisted of acetonitrile and water in the volume ratio of 45:55 (v/v) containing 0.1% TFA (v/v). The concentration of TFA significantly affected the peak shape of CLEFMA in C_{18} column. On reducing the percentage of TFA to 0.05%, a significant drift in CLEFMA-peak was observed. Further

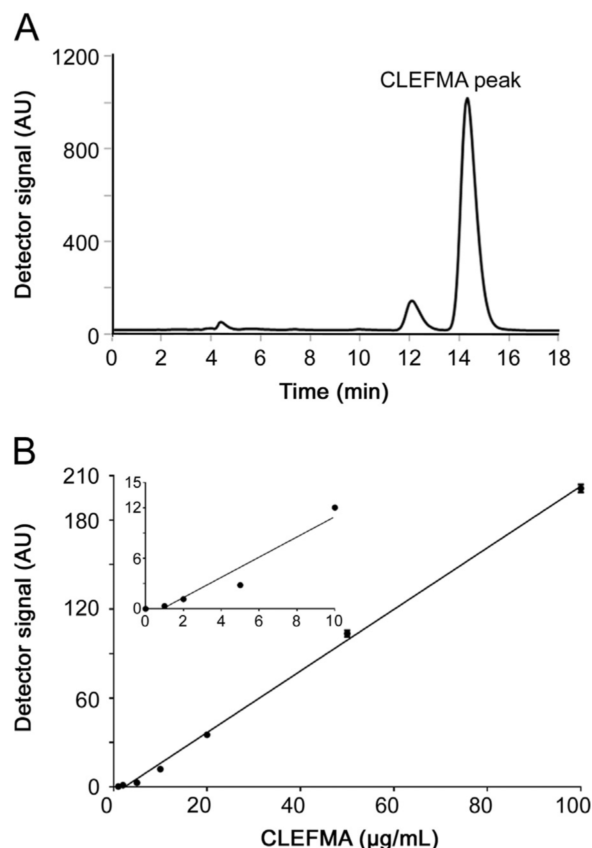


Fig. 2. Reversed phase HPLC of CLEFMA. (A) A representative chromatogram of CLEFMA with a prominent peak at 14.5 min, and a minor peak at 12.5 min. (B) Calibration curve of CLEFMA (1–100 µg/mL). The Inset shows relationship between concentration and absorbance in a lower range of 0–10 µg/mL. The RSD was less than 2% for all concentrations, and R^2 =0.998. The bars show the SEM values for 3 repeats for each concentration.

Table 1
Precision data for the HPLC method.

Concentration (μg/mL)	Intra-day variability (RSD, %)	Inter-day variability (RSD, %)
5	2.0	1.9
20	1.5	1.5
100	1.0	0.7

reduction in TFA concentration resulted in poor resolution of the CLEFMA-peak, suggesting that TFA served as an important ion-pairing agent in mobile phase for CLEFMA. A relatively small peak at ~12 min was also observed in CLEFMA solutions that were not freshly made. A standard curve showing a correlation between the CLEFMA concentration and the absorbance is provided in Fig. 2B.

The suitability parameters of the HPLC method for CLEFMA, namely resolution (1.2), theoretical plate number (3,132), tailing factor (0.75), and capacity factor (5.76), indicated that the standardized chromatographic conditions are adequate for the quantification of CLEFMA. The linearity of the method was determined by preparing standard solutions of CLEFMA at various concentrations, and analyzing the calibration curve by least square regression analysis. The response was a linear function of analyte concentration (R^2 =0.998) in the range of 1–100 μg/mL and the regression equation for the calibration plot was y=2.078x – 4.885 (Fig. 2B). The LOD was calculated to be 500 ng/mL (s/n > 3) whereas LLOQ was estimated to be 2 μg/mL (s/n > 10). The inter-day and intra-day precision of the method is shown in Table 1. The RSD values for inter-day variability and intra-day variability were within the limits (≤2.0%) specified in the ICH guidelines, indicating that the standardized method showed acceptable precision. Moreover, the method also demonstrated an excellent recovery of CLEFMA (1.001 ± 0.017, [Actual]/[Estimated] ± SD).

3.2. Acid-base catalyzed degradation of CLEFMA (pH-dependent stability)

The most important variable affecting drug degradation in solution phase is the pH. We investigated the stability of CLEFMA in the buffered pH range of 2–10 at 37 °C for 2 weeks. Fig. 3A shows the degradation profile of CLEFMA in different pH conditions and a representative chromatogram of CLEFMA buffered at pH 5.0 is shown in Fig. 3B. A sharp peak appeared at 4.5 min in the CLEFMA samples stored in acidic conditions. No significant degradation was observed at pH 7.4 and 8.2; however, mild degradation was detected at pH 9.6, especially by the end of the second week (Fig. 3A). The accumulation of the degradation product with respect to time is shown in Fig. 3C, and a plot of log k versus pH is provided in Fig. 3D.

The ^{1}H NMR of the acid-degraded product (4.5 min-peak) showed the disappearance of the olefinic proton signals of CLEFMA at 6.14 and 5.9 ppm (Fig. 3E; Figs. S1 and S2). These protons are present in the maleic moeity of CLEFMA structure (Fig. 1), indicating the possibility of an acid-catalyzed hydrolysis of the amide bond. We analyzed the 4.5 min-peak by mass spectroscopy (Fig. S3). The [M+H]$^+$ peak of 344 Da in the mass spectroscopy data confirmed that the structure of the degraded product was 3,5-*bis*[(2-chlorophenyl)methylene]–4-piperidone (Fig. S4). The ^{1}H and ^{13}C NMR data of acid degradation product are provided in Figs. S5 and S6, respectively.

3.3. Temperature-dependent stability of CLEFMA (accelerated stability)

The effect of temperature was investigated by incubating CLEFMA solution in acetonitrile at various temperatures. As shown in Fig. 4A, the rate of degradation followed the first order kinetics and was dependent on temperature. A representative chromatogram of CLEFMA solution stressed at 60 °C for 4 days is shown in Fig. 4B. A new peak at ~4.5 min appeared in addition to the characteristic CLEFMA- peaks at 14.5 min and 12.5 min. Just like in the pH stability study, this new 4.5 min-peak was identified as the large fragment of CLEFMA cleaved at the amide bond (discussed above). This stability study provided the information about the potential shelf-life of CLEFMA ($T_{90\%}$ or time required for the drug to degrade more than 10% of its original amount). The values of k_{obs} and $T_{90\%}$ calculated at different temperatures are summarized in Table 2. The dependence of k_{obs} on temperature followed a linear relationship as observed from the Arrhenius plot (Fig. 4C).

3.4. Photo-stability and oxidative degradation of CLEFMA

Upon exposure of CLEFMA to 365 nm UV-light and subsequent HPLC, we found a remarkable rise in the peak at 12.5 min within 2 h of UV-exposure (Fig. 5A). The elevation of 12.5 min-peak was also significant when CLEFMA was exposed to ambient light for 6 weeks, although several other unidentified peaks also appeared in this long-term exposure experiment (Fig. 5B). In contrast, CLEFMA kept in darkness was stable and showed no additional peaks (data not shown). The molecular mass of the compound collected as 12.5 min-peak was found to be 442 Da [M+H]$^+$(Fig. S7). Given that the mass of 12.5 min-peak and that of CLEFMA are identical, we hypothesized that in the presence of light, CLEFMA undergoes an isomerization.

The ^{1}H NMR of CLEFMA (Fig. 5C) indicated two doublets centered at 6.14 ppm (with coupling constant 12 Hz) and 5.90 ppm (with coupling constant 11.8 Hz), which are typical of *trans*-coupling constants for the olefinic protons. When we compared this NMR spectrum with that of the irradiated-CLEFMA (Fig. 5C), we found a significant reduction in the intensity of these *trans*-proton signals at 6.05 ppm (with coupling constant 13.2 Hz) and 5.97 (with coupling constant 12.7 Hz). At the same time, we observed an appearance of a multi-plet at 6.30 ppm with a coupling constant of ~ 5.1 Hz, which is a characteristic coupling constant for *cis*-olefinic protons. The rest of the other signals remained unchanged, although the signals at 7.9 and 7.8 ppm were split probably due to a long-range coupling. The ^{1}H and ^{13}C NMR of cis-iosmer of CLEFMA obtained after irradiation are provided in Figs. S8 and S9, respectively. These NMR spectroscopy results suggest that CLEFMA undergoes *cis-trans* conversion upon UV exposure, and the peak which consistently appear at 12.5 min is that of *cis*-CLEFMA.

In order to assess the effect of *cis-trans* isomerization on biological activity of CLEFMA, we separated *cis*-CLEFMA on a preparative TLC after UV-exposure of CLEFMA. The effect of original CLEFMA (*trans*-CLEFMA) and *cis*-CLEFMA preparations on the viability of H441 cells was studied. As shown in Fig. 5D, approximately 60% of H441 cells were non-viable after *trans*-CLEFMA treatment. The anti-proliferative activity of *cis*-CLEFMA was slightly reduced (P < 0.05, *trans*-CLEFMA versus *cis*-CLEFMA). We also tested the activity of *cis*-CLEFMA when it was allowed to 'recuperate' for 2 h after UV-exposure (*r*CLEFMA). There was no difference in the viability of cells when they were exposed to *cis*-CLEFMA and *r*CLEFMA (Fig. 5D).

The effect of oxidizing conditions (3% H_2O_2) on the CLEFMA stability is shown in Figs. 5E and F. Approximately 85% of CLEFMA was found to be degraded within 24 h of peroxide-exposure. The profile of degradation was biphasic, with the initial degradation faster (slope =0.09) than the degradation in the later phase (slope =0.02). The major degradation product appeared as a 4.5 min-peak in the HPLC profile and its mass spectroscopy indicated a peak [M+H]$^+$ at 344 Da (Fig. S10).

3.5. CLEFMA stability in biological matrix (bio-stability)

As CLEFMA is being developed as a therapeutic drug, the effect of biologic material on its stability is important to know. We performed

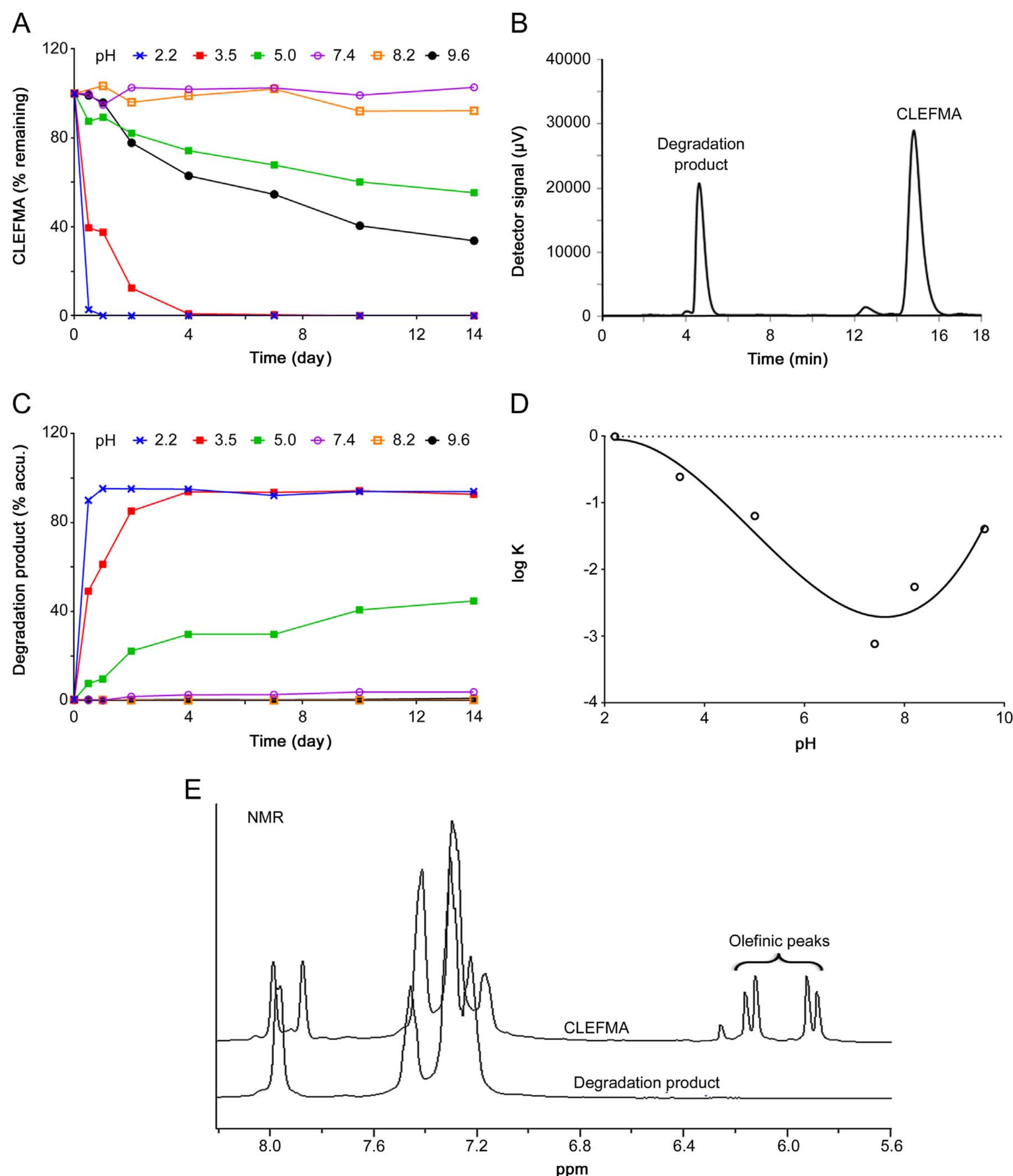


Fig. 3. The pH-dependent degradation profile of CLEFMA. (A) Degradation of CLEFMA solution stored in buffers of various pH values over 14 days. Percentage of CLEFMA remaining as 14.5 min-peak is plotted with respect to time. (B) A chromatogram of CLEFMA solution buffered at pH 5.0, showing the appearance of a 4.5 min degradation peak. (C) Accumulation of degradation product (4.5 min-peak) with respect to time in CLEFMA solutions buffered at various pH values. (D) A plot of log *k* versus pH units shows the sensitivity of CLEFMA in a pH range of 2-10. (E) An overlapping representation of NMR spectrum of CLEFMA and its acid-catalyzed degradation product. ^{1}H NMR of acid-degraded product (300 MHz, $CDCl_3$): δ 7.92 (s, 2 H, C=CH), 7.50-7.10 (m, 8 H, Ar-H) ppm. ^{1}H NMR of CLEFMA (300 MHz, $CDCl_3$): δ 7.99 and 7.87 (2 s, 2 H, C=CH), 7.50-7.01 (m, 8 H, Ar-H), 6.14 (d, 1 H, HC=CH, *J*=11.8 Hz), 5.90 (d, 1 H, HC=HC, *J*=11.9 Hz) ppm.

bench-top and freeze-thaw stability tests on CLEFMA solutions in presence of rat plasma. Our sole objective in these experiments was to understand CLEFMA's stability in plasma samples that would be subjected to room temperature and multiple freeze-thaw conditions during pharmacokinetic and pharmacodynamic studies.

In order to account for the efficiency of CLEFMA extraction from plasma, we used EFNAC as an internal standard. EFNAC is a close congener of CLEFMA and it has similar physiochemical characteristics (inset Fig. 6A). The flow rate of 1.5 mL/min provided satisfactory separation of EFNAC (retention time ~17 min) from CLEFMA (retention time ~14.5 min). The HPLC profile of EFNAC is shown in Fig. 6A.

The linearity of the HPLC method for CLEFMA extracted from plasma is shown in Fig. 6B. We found no interference from the endogenous materials present in plasma. The linear relationship between the concentration and the peak-ratio (CLEFMA-to-EFNAC) was maintained in the extracted samples, with inter-day variations less than 15%. A representative chromatogram of CLEFMA and EFNAC, extracted from plasma, is shown in Fig. 6C. The extraction efficiency of CLEFMA from plasma by acetonitrile-precipitation and ethyl acetate-based liquid-liquid extraction was 76.4% ± 2.1% and 57.4% ± 0.7%, respectively (Fig. 6D, $P < 0.05$). As such, we employed the acetonitrile-precipitation method for testing CLEFMA stability in plasma.

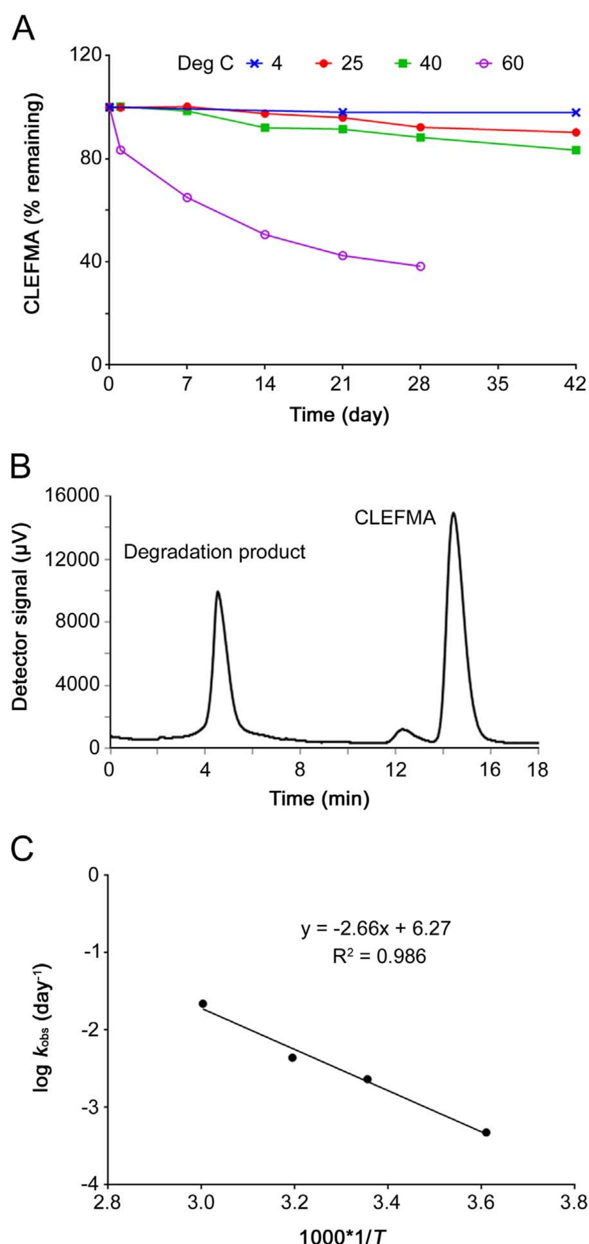


Fig. 4. The effect of temperature on degradation of CLEFMA. (A) CLEFMA was stored at different temperatures as solution in acetonitrile, and the percent of CLEFMA remaining was plotted with respect to time. (B) A representative chromatogram of CLEFMA exposed to 60 °C for 4 days, showing the appearance of a 4.5 min-peak of degradation product. (C) Arrhenius plot of CLEFMA degradation. The activation energy calculated from the curve was 53 kJ/mole.

Table 2
Rate of degradation, rate constants and $T_{90\%}$ values of CLEFMA, as calculated from the data presented in Fig. 3.

Temp (°C)	Rate of degradation (% degradation/day)	k_{obs}(day^{-1})	$T_{90\%}$ (day)
4	0.048	0.0005	220
25	0.232	0.0023	45
40	0.396	0.0040	27
60	2.203	0.0220	5

The stability of CLEFMA in plasma is shown in Table 3. CLEFMA was found to be stable in rat plasma; the HPLC-estimated concentration (14.5 min-peak) after 4 h of incubation at room temperature was 97% of the actual concentration (50 µg/mL). Similarly, when the CLEFMA-plasma mixture was subjected to three deep freeze-thaw cycles, the HPLC-estimated concentration was 94% and 97% of the actual concentrations (50 and 20 µg/mL, respectively). These results suggest that CLEFMA is stable in plasma and the samples can tolerate multiple freeze-thaw cycles.

4. Discussion

Since the introduction of the first stability guidelines in 1984 by the US FDA and harmonization of the ICH principles in the early 1990 s, stability studies have now become integral to the new drug development processes [14,16]. The main purpose of the drug stability studies is to test for the propensity of a drug to degrade under the influence of various environmental conditions including temperature, pH, light, etc., and predict the mechanisms responsible for the degradation. These studies also empower researchers with possible solutions to overcome drug instability during sample preparation. Moreover, the information available from stability studies is useful in determining the shelf-life of a drug and the storage conditions under which a drug can maintain its stability for a prolonged duration. As such, it is now common to conduct stability studies at each stage of drug development, from the discovery of the drug to its launch in the market, and even during its stay in the market. A significant reduction in bench-to-bedside failures could be achieved by incorporating the inferences drawn from such studies.

In order to conduct a stability study, the ICH recommends that the analytical methods applied to new substances should be validated and should be able to detect and quantify the degradation products [11]. HPLC is one of the most commonly used analytical techniques for the study of drug stability. It can quantify diverse compounds and distinguish between the active pharmaceutical ingredient and its degradation products [23]. Here, we standardized a reverse phase HPLC method to study the stability of CLEFMA as a potential active pharmaceutical ingredient in an anticancer drug-product. As defined in Table 1, the method was characterized by acceptable suitability parameters, linearity, precision, and resolution. The method was also capable of separating a hitherto unknown *cis*-isomer of CLEFMA indicated by a 12.5 min-peak. However, the primary objective of setting up this HPLC method for CLEFMA analysis was to obtain knowledge about CLEFMA stability so that it could be handled in a proper manner during its initial phase of pre-clinical testing.

It has been noted that the molecular structure of a drug determines its degradation pathways and that substituent around the reaction center can strongly influence its reactivity towards environmental stress stimuli [24]. The results described in this study showed that CLEFMA stability was influenced by multiple factors such as temperature, pH, and light. Temperature is one of the most important factors that influence the stability of drugs in storage and usage. The Arrhenius plot of CLEFMA indicated that the degradation mechanism did not change over the temperature range studied in these experiments, and the activation energy required to initiate the degradation of CLEFMA was approximately 53 kJ/mol (slope of the Arrhenius plot). CLEFMA appeared to be stable at 4 °C ($T_{90\%}$ =220 days), but its stability was drastically lower at elevated temperatures. From the data in Table 2, we conclude that the shelf-life of CLEFMA at room temperature is approximately 45 days. It must be noted that these values are for CLEFMA in solution form, and the stability of CLEFMA as pure solid may be significantly different.

As with many other drugs, the degradation rate of CLEFMA is influenced by pH. These degradation pathways are catalyzed by hydronium or hydroxide ions, and water itself serves as a critical reactant [24]. Analyses of the rate of degradation at different pH values revealed that with every 1.5 unit fall of pH, the rate of degradation quadrupled. A minimum in the Fig. 3D shows that CLEFMA is most stable in neutral conditions. When we compared the V-shape of this log k_{obs} versus pH plot with the classic pseudo-first order kinetics (k_{obs} = k_{H^+} + k_{H_2O} + k_{OH^-}), the degradation appears to be contributed by

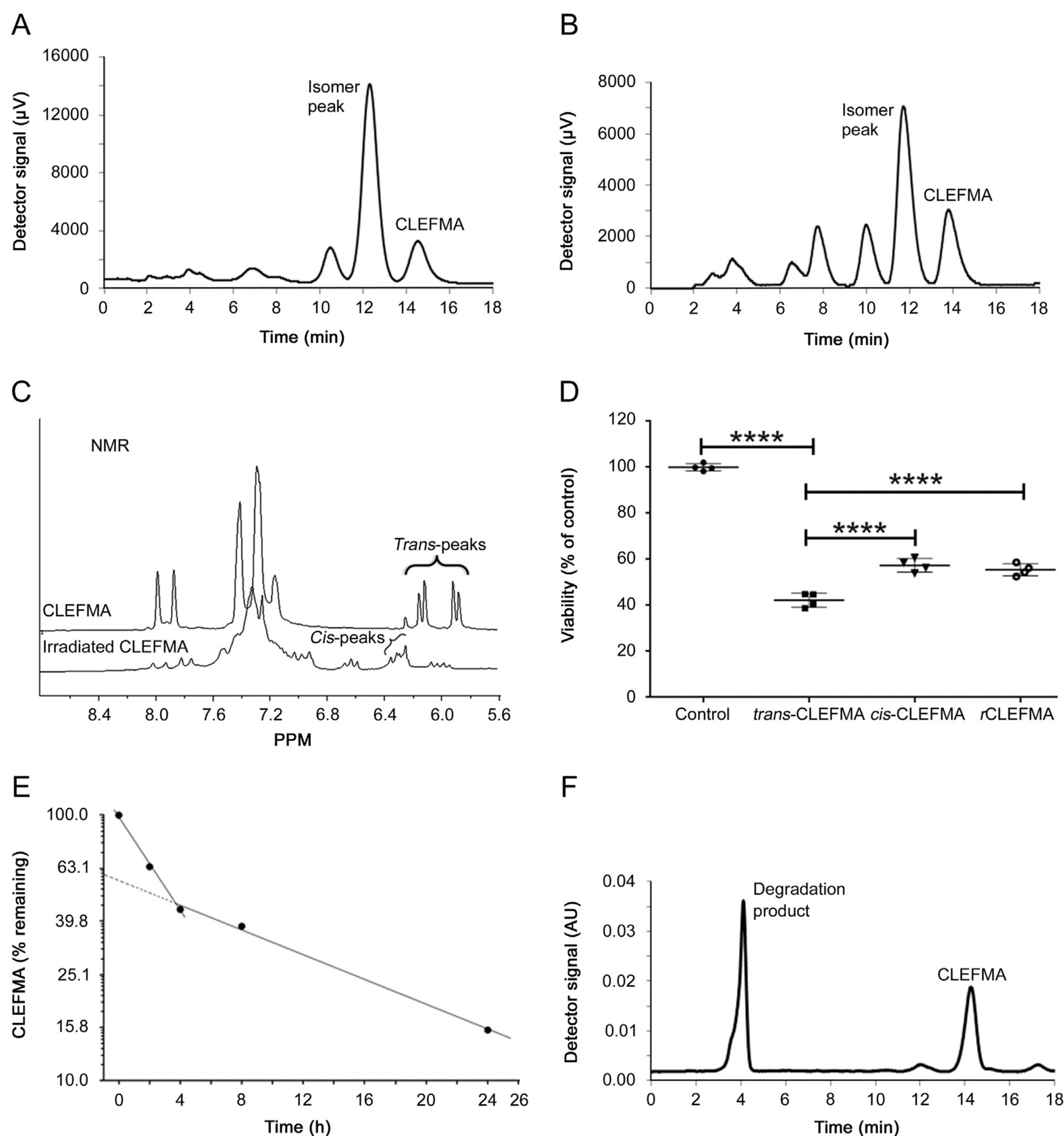


Fig. 5. Photo-stability and oxidative degradation of CLEFMA. Chromatogram of CLEFMA exposed to (A) UV light 365 nm for 2 h and (B) ambient light for 6 weeks. (C) An overlapping representation of NMR spectrum corresponding to CLEFMA and its UV-exposed isomer. (D) Viability of H441 cells exposed to 10 µM of *trans*-CLEFMA, *cis*-CLEFMA and *r*CLEFMA for 48 h (mean ± SD,**** $p < 0.0001$). (E) Oxidation of CLEFMA by 3% H_2O_2 showing a biphasic degradation. (F) A representative chromatogram of CLEFMA that was exposed to 3% H_2O_2 for 24 h.

both hydronium and hydroxide ions [24]. Moreover, since CLEFMA is a weakly acidic drug with significant capacity of undergoing ionization in alkaline conditions, both ionic and nonionic forms of CLEFMA would be subject to hydroxide ion attack. For the degradation product of mass 344 Da, we hypothesized a degradation scheme shown in Scheme 1A. The acid-catalyzed production of the degradation product 2 was confirmed by separating it on a preparative TLC and analyzing its NMR spectrum.

Another drug degradation mechanism is catalyzed by light, which also plays an important role in thermal oxidation [25]. However, the composite effect of light on drug stability is difficult to measure because of the dependence of degradation on the wavelength. These photolytic reactions can result in either oxidative or non-oxidative degradation. Isomerization, rearrangements, alkylation, de-alkylation, decarboxylation, and cleavage of X-C hetero bonds are some examples of the non-oxidative photo-degradation. Our study suggests that CLEFMA is sensitive to light and its major photolytic degradation product appears as a 12.5 min-peak in the HPLC. Because the molecular mass of the 12.5 min-peak was the same as that of CLEFMA ($[M+H]^+$=442 Da), we investigated the UV-exposed CLEFMA samples by NMR and found that it isomerized via a *cis*-*trans* non-oxidative isomerism involving the double bond in the maleic acid tail. The scheme to this effect, indicated by H_a and H_b atoms around olefinic bond, is shown in Scheme 1B. We also found that this isomerization is not reversible as withdrawal of the UV-exposure did not convert *cis*-isomer back to its baseline equilibrium dominated by *trans*-CLEFMA (data not shown). Moreover, we compared the anti-proliferative activity of the *cis*-CLEFMA with that of control *trans*-CLEFMA, and found that there was no significant difference between the biological activities of *cis*-CLEFMA and *trans*-CLEFMA (Fig. 5D).

In contrast to the non-oxidative photolysis, reactions involving free radicals are of oxidative type. For example, singlet oxygen can react

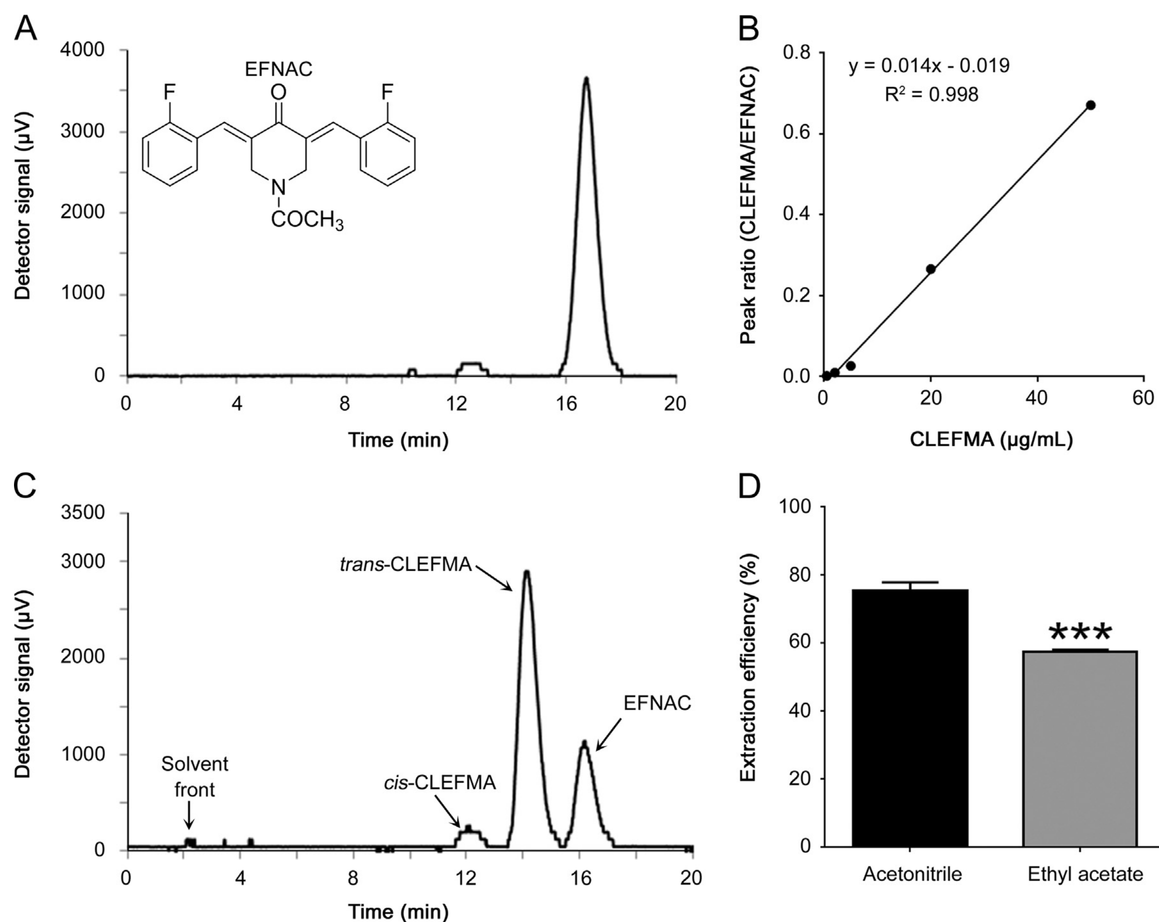


Fig. 6. Extraction of CLEFMA from plasma samples. (A) A representative chromatogram of internal standard EFNAC showing a peak at 17 min. The structure of EFNAC is shown as an inset. (B) A calibration curve showing linear relationship between CLEFMA concentration and the ratio of peak areas corresponding to CLEFMA and internal standard EFNAC (R^2 > 0.99). The LLOQ was estimated to be 2 μg/mL. (C) An HPLC profile of CLEFMA and internal standard EFNAC extracted from plasma samples. (D) A comparison of extraction efficiency (mean ± SEM) of CLEFMA from plasma samples by acetonitrile-precipitation method and ethyl acetate-based liquid-liquid extraction method (***P < 0.0001, t-test).

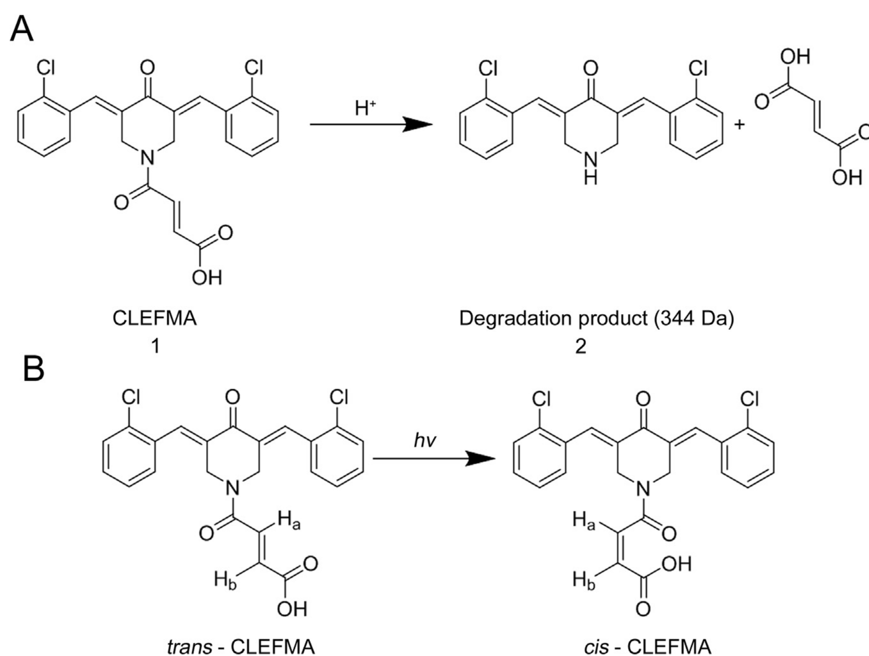
Table 3
Stability study of CLEFMA in rat plasma.

Stability	Actual concentration (μg/mL)	Recovery (%)
Bench-top stability (4 h)	50	97.1 ± 4.2
Freeze-thaw stability	50	94.2 ± 3.8
(3 cycles)	20	97.2 ± 6.0

with an unsaturated bond of drugs containing polynuclear aromatic ring or alkenes. Also, a triplet oxygen may react with free radicals of drug to form peroxides [26]. Metal ion impurities potentiate these reactions by reacting with oxygen and forming free radicals [18]. Although the ICH guidelines mention the necessity of carrying out oxidation stability studies as part of stress stability evaluation, the conditions in which these studies should be conducted are not clearly stated. Hence, most groups set up their own procedures for conducting oxidative stability studies with some free radical initiators such as H_2O_2 in the concentrations of 3%–30% for a few hours [26]. We exposed CLEFMA to 3% H_2O_2 and found that CLEFMA was highly sensitive to oxidation (Fig. 5F). The appearance of a 4.5 min-peak (and not a 12.5 min-peak) suggested that in contrast to the photo-catalyzed isomerization of CLEFMA, H_2O_2-induced oxidative stress degraded CLEFMA by a different mechanism. We hypothesized that the degradation product of CLEFMA after oxidation is the same as the degradation product 2 obtained after acid-hydrolysis. This hypothesis was proved true because the mass spectroscopy provided its mass as 344 Da and the ^{1}H NMR spectrum showed the disappearance of characteristic olefinic double bond present in the maleic acid tail.

5. Conclusions

CLEFMA is a new drug which is under investigation as an anticancer agent. The stability studies of CLEFMA provide valuable information about the expected degradation mechanisms and degradation products of CLEFMA. This knowledge empowers us to choose appropriate conditions of handling and storage of CLEFMA-containing samples during the ongoing pre-formulation and pre-clinical studies. Later in the development phase, these issues will also determine the choice of container and packaging as well as formulation ingredients. Specifically, we recommend that neutral solutions of CLEFMA should be kept at low temperatures, away from direct light exposure, and in inert environment. However, it should be noted that the stability of CLEFMA in a dosage form might show a different profile. In addition, how the concurrent presence of two or more destabilizing factors would impact CLEFMA stability remains to be a matter of future investigations. Furthermore, to satisfy the pharmaceutical, regulatory, and clinical-safety requirements of a drug, accurate quantitation of degradants is needed based on relative response factors of drug and degradants at a given wavelength [27]. Although the present study does not provide this information for CLEFMA, this could be accomplished by purifying or synthesizing the CLEFMA degradants and determining their response factors at 318 nm. Another interesting approach is the use of a chemiluminescent nitrogen-specific detectorin conjunction with a UV detector [28]. In this approach, the



Scheme 1. Degradation pathways suggested for CLEFMA. (A) Prediction of the acid-catalyzed degradation of CLEFMA into a degradation product 2 of mass 344 Da. The same degradation pathway was ascertained when CLEFMA was exposed to high temperature or hydrogen peroxide. (B) Prediction of the *cis-trans* isomerization of CLEFMA upon stimulation with UV exposure (365 nm).

chemiluminiscence is directly proportional to the number of moles of nitrogen in each eluting peak which can be used to determine the relative amounts of each nitrogen-containing impurity present in the sample [28].

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2016.09.004.

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