Stability of β-Lapachone upon Exposure to Various Stress Conditions: Resultant Efficacy and Cytotoxicity

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β-Lapachone, a natural naphtoquinone originally isolated from the bark of the lapacho tree (Tabebuia avellanedae), can be a novel drug with promising biological and pharmacological activity against many diseases. 1,3 Recently, it has been investigated for antineoplastic, 3 anti-inflammatory, 4 antibacterial, antifungal, 5 and antiviral 5 activity. It may also promote wound healing activity. 7 Effects of β-lapachone on collagen biosynthesis in human dermal fibroblasts (HDFs) recently reported provide a basis for its potential applicability as an active ingredient for cosmeceuticals. It enhances type I collagen synthesis through the activation of Smad signaling. The underlying mechanism of β-lapachone-mediated cell response is the activation of a specific transforming growth factor-β receptor type I (TGF/βRI) kinase-dependent Smad signaling cascade to stimulate collagen synthesis.

One of the issues in the development of β-lapachone products is its instability during not only formulation but also manufacturing and storage. Especially, during the development of topical and cosmetic preparations, such as emulsion, cream, or ointment, the active ingredient might be exposed to various aqueous and non-aqueous environments, which might accelerate its degradation and complicate stabilization strategies. Since the formation of degradation products or impurities may incur a loss of efficacy and cause side effects, a systematic investigation of the chemical stability under various conditions is indispensable to assure quality and safety of the active ingredient. 8–10 There are a couple of reports on β-lapachone stability under light condition. 10 However, the chemical stability and degradation mechanisms of β-lapachone under various stress conditions including oxidation and heat have not been investigated yet. One of the common assumptions in stability evaluation is that efficacy is dependent on the stability of the active ingredient. Thus, stability proportionate to efficacy would provide robust backgrounds for formulation development. In accordance to International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines, 9 different conditions were applied to simulate the degradation of an ingredient, where degradation can occur along many pathways such as acidic and basic hydrolysis, photo-degradation, thermal-degradation, or oxidation.

The most common analytical technique to determine degradation products in the recent past, was high-performance liquid chromatography (HPLC). 11 Liquid chromatography-mass spectrometry (LC-MS) techniques in combination with accurate mass measurements have evolved as a tool for the structural characterization of drug impurities and degradation products. 8,12,13 Besides its sensitivity and ease of use, LC-MS has potential to provide unequivocal characterization of the structure of major and minor components, except enantiomers and epimers. 14 Previous reports on β-lapachone 5 and its metabolites 6 also used HPLC and LC-MS for identification and quantitation. However, for a systematic understanding of degradation mechanisms and a correlation between the stability and efficacy, there is a need for a reliable and validated

Key words chemical stability; kinetics; HPLC; calorimetry; degradation product; oxidation

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stability indicating method.\textsuperscript{10,17}

In the present study, HPLC with photo diode array detector (DAD) and LC-MS were used to evaluate the degradation mechanisms of \( \beta \)-lapachone and identify degradation products under stress conditions like oxidation, light, heat, acidic, and basic condition. Major degradation products were isolated and its structure was determined. Moreover, a validated stability-indicating method, according to the ICH guidelines, was presented for quantitative determination of \( \beta \)-lapachone in the presence of its degradation products, which have provided degradation kinetic data with regard to specific stress conditions. The results of the stability test may be helpful and have important implications for formulation development, quality control, and clinical uses.\textsuperscript{18,19} Correlation between stability and cellular efficacy of \( \beta \)-lapachone was investigated subsequent to the stability tests. Collagen synthesis assay in HDF cells and in vitro cytotoxicity study were performed to evaluate the biological safety and efficacy of an active ingredient, with individual impurity or degradation products.\textsuperscript{20}

Experimental

Materials \( \beta \)-Lapachone, Dulbecco’s modified Eagle’s medium (DMEM), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). HDF cells were obtained from Life Technologies (Carlsbad, CA, U.S.A.). Fetal bovine serum (FBS) and antibiotic/antimycotic (AA) solution were purchased from Life Technologies. Hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Acetonitrile (ACN) was purchased from Avantor Performance Materials (Center Valley, PA, U.S.A.). All other reagents were of analytical or HPLC grade and were used as received.

HPLC Analytical Method and Method Validation An HPLC system (1100 Series, Agilent Technologies, Santa Clara, CA, U.S.A.) equipped with a photo diode array detector was used as a stability indicating method to analyze the degradation products of \( \beta \)-lapachone, and determine its degradation kinetics. The wavelength of the UV detector was set at 258 nm. An Eclipse Plus C18 5 \( \mu \)m (4.6\texttimes{}150 mm) column (Agilent Technologies) was used and maintained at 30°C. The mobile phase was a mixture of acetonitrile and water at a volume ratio of 65:35. The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 10 \( \mu \)L.

The method was validated according to the ICH guidelines Q2 (RI) with respect to specificity, linearity, range, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), and robustness. The specificity of the HPLC method for \( \beta \)-lapachone was determined in the presence of its degradation products. A resolution between peaks was analyzed by Chemstation software (Agilent Technologies) and the peak purity of \( \beta \)-lapachone was measured by a diode array detector. A stock solution of 1000 \( \mu \)g/mL \( \beta \)-lapachone was prepared by dissolving 100 mg of \( \beta \)-lapachone in 100 mL ACN. A serial dilution at 100, 50, 25, 10, and 5 \( \mu \)g/mL with 50% ACN was used for linearity test solutions. The peak area was plotted against the concentration of \( \beta \)-lapachone and a calibration curve was obtained by the least square linear regression method. The accuracy and precision of the method was assessed in triplicate at 3 concentration levels of 5, 25, and 100 \( \mu \)g/mL. The accuracy was expressed as % recovery, and calculated from the slope and \( y \)-intercept of the calibration curve. The precision was expressed as relative standard deviation (R.S.D.) of the concentration of each sample. The LOD and LOQ for \( \beta \)-lapachone were calculated based on the standard deviation of response (\( y \)-intercept) (S.D.) and the mean slope of the calibration curve (S) according to the following equation:

\[
\text{LOD} = \frac{\text{S.D.}}{3.3} \times 3.3 \quad \text{LOQ} = \frac{\text{S.D.}}{5} \times 10
\]

The effects of variations in mobile phase composition, column temperature, and flow rate by \( \pm 10\% \) was measured for robustness evaluation. The mobile phase with 35% water was adjusted to 31.5 and 38.5%. The normal column temperature of 30°C was adjusted to 27 and 33°C. The flow rate was adjusted to 0.9 and 1.1 mL/min. After adjustment, changes in peak elution orders, peak tailing factors, and the resolution between \( \beta \)-lapachone and degradation products were evaluated.

LC-MS Analysis To evaluate the molecular weight of degradation products with their molecular structures, LC-MS analysis was conducted using a LCMS 2020 system (Shimadzu Corporation, Kyoto, Japan) with 2 LC-20AD pumps, an SIL-20A autosampler, a CTO-20A column oven, a SPD-20A diode array detector, CBM-20A controller, and an LCMS 2020 single quadruple mass spectrometer. Detection wavelength, column, column oven temperature, mobile phase composition, and flow rate of the mobile phase were the same as the HPLC method.

Mass analysis was performed using an electrospray ionization (ESI) source, which produces ions, in both the positive and negative ionization mode. The LC-MS spectra were acquired from \( m/z \) 50 to 500. The following parameters were used: ESI interface temperature (350°C), desolvation line (DL) temperature (250°C), heat block temperature (200°C), nebulizing gas flow (1.5 L/min), drying gas flow (15 L/min), ESI interface voltage (4.5 kV), and detector voltage (1.2 kV). Tuning of the mass spectrometer was conducted using an auto-tuning function of LabSolutions LCMS software (Shimadzu Corporation).

Forced Degradation Studies To evaluate degradation products and kinetics of \( \beta \)-lapachone, forced degradation studies were conducted under various stress conditions, according to previously reported procedures.\textsuperscript{10,21,22}

Photo Degradation

Photo degradation studies were carried out in solution and solid states. \( \beta \)-Lapachone solutions (100 \( \mu \)g/mL in 50% ACN) were conditioned in a clear glass vial. Dark control samples were prepared by covering the vial with aluminum foil and stored in a dark place at 45°C. The sample was exposed to a xenon light lamp (Suntest CPS; Atlas Material Testing Tech., Chicago, IL, U.S.A.) at a constant temperature of 45°C. The xenon light source is a full spectrum light source with both, UV and visible outputs (320–800 nm). Illumination condition was 110 klux and the UV irradiation level was 46.25 W/m\(^2\). The samples were analyzed by HPLC and LC-MS at predetermined time intervals.

For stability testing of \( \beta \)-lapachone in the solid state, approximately 25 mg of \( \beta \)-lapachone powder was weighed and transferred to glass vials as a thin layer (2 mm thickness). Experiments were performed at 2 different levels (0 or 75%) of relative humidity (RH) by storing the samples in a silica gel or in an environment created by a saturated solution of NaCl, to evaluate the effects of humidity on photo-stability.
of β-lapachone powder. Other experimental conditions were similar to those of the solution test. Content change of β-lapachone powder was also analyzed by differential scanning calorimetry (DSC) (Q-2000, TA Instruments, New Castle, DE, U.S.A.) in addition to HPLC and LC-MS analysis. Approximately 3 mg of β-lapachone standard powder and sample, which was exposed to light, were sealed in an aluminum pan. A blank pan was used as a reference. The pans were kept at 0°C for 5 min before initiating the analysis to confirm isothermal starting conditions. DSC measurements were performed at a scan rate of 10°C/min from 0 to 260°C under a nitrogen flow of 50 mL/min.

Acidic and Basic Degradation

Acidic and basic degradation studies were conducted on 5 mL of β-lapachone solutions (200 µg/mL in ACN) mixed with 5 mL of 0.1 M HCl and 0.1 M NaOH. Control solution (pH 7.0 phosphate buffer) was also prepared. All samples were covered with aluminum foil and stored at constant temperature (25°C). One milliliter of sample solutions was withdrawn at predetermined time intervals (0, 2, 4, 6, 10 h). The solutions were neutralized and diluted with 0.5 mL of 0.1 M NaOH or 0.1 M HCl and ACN as well. The samples were analyzed by HPLC and LC-MS.

Oxidative Degradation

For oxidative degradation study, 1 mL of H2O2 solutions (0.3, 3, 30%) were added to 10 mL of β-lapachone solution in 50% ACN to prepare a β-lapachone concentration of 100 µg/mL. All samples were covered with aluminum foil and stored at a constant temperature of 25°C. One milliliter of sample solutions were withdrawn at predetermined time intervals (0, 2, 4, 6, 10 h) and analyzed by HPLC and LC-MS.

Thermal Degradation

β-Lapachone solutions (100 µg/mL in 50% ACN) were stored at 4, 25, 40, and 60°C for the thermal degradation study. All samples were covered with aluminum foil. At predetermined time intervals (0, 2, 6, 24, 48, 72 h), 1 mL of sample solutions were withdrawn and transferred into vials placed in a refrigerator in order to stop the reaction. The samples were immediately analyzed with HPLC and LC-MS.

Cell Activity and Toxicity Test

Collagen assay was performed to evaluate the correlation between stability and cellular activity of β-lapachone. Moreover, cell viability assay was conducted to determine if β-lapachone was nontoxic to the cell at an effective concentration. HDFs were cultured in DMEM supplemented with 10% FBS and 1% AA solution in humified atmosphere containing 5% CO2 in air at 37°C. Cells at between 10 and 13 passage number were used for the study, and the culture medium was changed every 2 d. HDFs were seeded in 12-well plates (1×105 cells/well) and cultured in a serum-free medium for 24 h, followed by replacement with fresh medium. The cells were then cultured with or without 0.1 µg/mL β-lapachone standard and reduced content β-lapachone powder (80, 2: #2: 62, #3: 46, #4: 7%). The aliquots were assayed for procollagen type I levels with a Procollagen Type I C-peptide EIA Kit (TaKaRa, Shiga, Japan). The assay was performed according to the manufacturer’s protocol. For comparing the negative control, β-lapachone standard, and photo-degraded samples, statistical analysis was performed using the student’s t-test with a p-value <0.05 or <0.01 considered to be significant or very significant.

The MTT assay was performed to determine cell viability. HDFs were seeded in 96-well plates at a concentration of 5×103 cells/well and cultured for 24 h. The cells were incubated with and without β-lapachone standard and reduced content β-lapachone powder (80, 2: #2: 62, #3: 46, #4: 7%) for 48 h, after which the culture medium was removed and replaced with 1 mg/mL MTT solution dissolved in DMEM, and incubated for an additional 4 h. The MTT solution was then removed and dimethyl sulfoxide was added. The absorbance of the dissolved formazan crystals was measured at 570 nm wavelength using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Results and Discussion

HPLC Method Validation

A symmetrical standard peak was obtained at a retention time of 3.42 min. LC-MS spectrum of β-lapachone gave the base peak at m/z 243.0 [M+H]+ in the positive ionization mode and hence m/z 242 was considered as its molecular weight in the mass spectrum. In a specificity evaluation, the resolution value between β-lapachone and its nearest degradation product peak was 8.28 indicating satisfactory separation of the peaks. U.S. Food and Drug Administration (FDA) guidelines recommend well separated peaks, with resolution >2 between the peak of interest and the closest potential interfering peak for quantification. The purity factor was within the calculated threshold limit, indicating that analyte peak was pure and not attributable to more than one component. The linearity of the method was established at the tested concentration range of 5–100 µg/mL. The calibration curve of the peak area versus β-lapachone concentration was y = 34.556x + 2.420, with a correlation coefficient of 0.99999. Accuracy and precision results at low, mid, and high concentration are summarized in Table 1. The % recovery ranged from 99.50 to 100.35% for the 3 levels tested. The precision of the method was demonstrated by low % R.S.D. (0.21–0.32%). Therefore, based on the accuracy and precision results, the analytical method of β-lapachone has been established to be accurate for the intended purpose, with good repeatability.

The LOD and LOQ of β-lapachone were found to be 0.078 and 0.235 µg/mL, respectively. Robustness is the capacity of an analytical method to remain unaffected by small variations in parameters. When mobile phase composition, column temperature, and flow rate was altered by ±10%, peak elution orders did not change significantly. Additionally, peak tailing factors and resolution between β-lapachone and degradation products did not show any significant change, suggesting that the method was robust over an acceptable working range of its HPLC operational conditions.

Forced Degradation Studies

Forced degradation studies were carried out to assess the stability indicating applicability of the analytical method to investigate the stability and quality of the compound under the influence of different conditions. HPLC analysis method with photo diode array and MS detector was used to obtain the degradant profiles of the stressed

| Level and assay conc. (µg/mL) | Found conc. (µg/mL) | Accuracy (%) | Precision (%) |
|------------------------------|--------------------|--------------|---------------|
| Low, 5 (n=3)                 | 4.98±0.02          | 99.50        | 0.30          |
| Mid, 25 (n=3)                | 25.09±0.09         | 100.35       | 0.32          |
| High, 100 (n=3)              | 100.03±0.24        | 100.03       | 0.21          |
samples. The proposed molecular structures of β-lapachone degradation products on exposure to various conditions are summarized in Fig. 1.

Photo Degradation

As shown in Fig. 2, HPLC chromatogram of β-lapachone after exposure to light revealed that 3 degradation products

Fig. 1. Proposed Degradation Pathways of β-Lapachone on Exposure to Various Stress Conditions

Fig. 2. HPLC Chromatogram of β-Lapachone after Exposure to Light Condition, Revealed 3 Degradation Products: Benzomacrolactone (m/z 274), Methylated β-Lapachone (m/z 256), and Demethylated β-Lapachone (m/z 228)
presenting a higher polarity were eluted before β-lapachone. Degradation product at retention time of 1.23 min showed a molecular ion peak at \( m/z \) 275 [M+H]\(^+\) in positive ionization mode and its molecular weight was found to be 274, 32 atomic mass unit (amu) higher than that of β-lapachone. Based on the MS result and previous report, \(^9\) the degradation product was assumed to be benzomacrolactone, which was produced by the addition of two oxygen atoms to β-lapachone. Two unknown degradation products at retention times of 1.79 min and 2.09 min showed molecular ion peaks at \( m/z \) 257 [M+H]\(^+\) and 229 [M+H]\(^+\), respectively, in the positive ionization mode. The molecular weight of the degradation products were 14 amu higher or less than that of β-lapachone. The structure of degradation products were assumed as methylated β-lapachone and demethylated β-lapachone, respectively (Fig. 1). In addition, UV absorbance spectrum of degradation products was different compared to β-lapachone suggesting that the molecular structure of β-lapachone was changed after exposure to light conditions.

Figure 3a shows the change of β-lapachone concentration in solution depending on exposure time to light conditions. The correlation coefficients (\( r^2 \)) of the plots of the remaining β-lapachone concentration versus time were above 0.99, suggesting the reaction of β-lapachone degradation might be following zero-order kinetic. The kinetic model can be expressed as the following equation:

\[
C_t = C_0 - kt
\]

where, \( C_0 \) is the concentration of β-lapachone at time zero, \( C_t \) is the concentration of β-lapachone after reaction time (\( t \)), and \( k \) is the reaction rate constant. It degraded much faster under light conditions than dark conditions (Fig. 3a). Half-life values at 45°С under light and dark conditions were found to be 4.0 and 449.5 h, respectively. The rate constant under light conditions was 112 times higher than that of dark conditions. Moreover, no change in color was observed in the dark control sample (Fig. 3b). However, after exposure to light, the dark
yellow color of β-lapachone solution faded to light yellow. The results suggested that light significantly accelerated the degradation of β-lapachone, which recommended that certain actions such as an addition of sun-screening excipients to the formulation or using a light resistant container might be necessary.

In case of solid samples, experiments were performed at 2 different levels of RH (0 and 75%). Figure 4a shows the change of β-lapachone concentration in solid state after exposure to light. It degraded faster under light conditions and high humidity accelerated the photo degradation. Thus, light and RH have a synergistic effect on the degradation of β-lapachone in the solid state as with other pharmaceutical raw materials.27,28 Moreover, the orange color powder became darker after exposure to light, as shown in Fig. 4b.

Figure 4c shows DSC thermograms of the standard and sample after exposure to light. A single endothermic peak at 155.84°C due to melting was observed for the standard. On the contrary, the melting temperature of the irradiated sample decreased slightly to 155.34°C and the melting peak became broader. Moreover, heat of fusion decreased from 100.60 J/g to 93.68 J/g. The results suggested that β-lapachone lost its crystallinity to some degree and degraded after exposure to light,29–31 which supported the above HPLC results.

Acidic and Basic Degradation

Figure 5a shows the change of β-lapachone concentration after exposure to acidic, neutral, and basic conditions. The correlation coefficients ($r^2$) of the semi-logarithmic plots of the remaining β-lapachone concentration versus time were above 0.99, suggesting that the reaction of β-lapachone degradation might be first-order kinetic. The kinetic model can be expressed as the following equation:

$$\ln C_t = \ln C_0 - kt$$

Especially, under basic condition, the chemical reaction formula and reaction rate could be expressed as the following equation:

$$\beta$$-Lapachone + NaOH \rightarrow Degradation products

Reaction rate = $k$[β-Lapachone][NaOH]

The reaction kinetic would be second-order. However, NaOH existed in excess and its concentration had changed very little during the reaction. Therefore, the reaction rate might be expressed as the following equation:

Reaction rate = $k'$[β-Lapachone][NaOH] = $k'\beta$-Lapachone

where $k' = k$[NaOH].

Therefore, the reaction kinetic could be pseudo first-order. Another assumption was that β-lapachone degradation mechanism in the basic conditions could be a catalytic reaction by hydroxyl groups. In other words, NaOH might be a catalyst and reaction kinetics could be first-order reaction. As shown in Fig. 5a, β-lapachone degraded much faster in basic conditions than in acidic and neutral conditions. Half-life values of β-lapachone at 25°C in acidic, neutral, and basic conditions were found to be 866.3, 2178.4, and 2.5h, respectively. Additionally, the yellow color of β-lapachone solution changed to red under basic conditions suggesting the degradation of β-lapachone in solution (Fig. 5b). On the contrary, no change in color was observed for the acidic sample. It might be important to know the effects of pH on the stability because it is essential for the development of liquid and solid dosage forms for drug delivery.

Figure 6 shows the change of β-lapachone chromatogram after exposure to basic conditions. Two degradation products were eluted before β-lapachone. Degradation product at retention time of 2.05min showed a molecular ion peak at $m/z$ 243 [M+H]⁺ in the positive ionization mode and the molecular weight was found to be 242, which was identical to β-lapachone. Moreover, UV spectrum of the degradation product showed maximum absorbance at 252, 282, and 334nm, which was the special absorbance of α-lapachone,32 suggesting the isomerization of β-lapachone. Degradation product at retention time 2.84min showed a molecular ion peak at $m/z$ 282 [M+H]⁺ and at $m/z$ 306 [M+Na]⁺ in positive ionization mode. Molecular weight of the degradation product was found to be 283, 41 amu higher than that of β-lapachone. The degradation mechanism was assumed to be base-mediated cyanomethylation, which was addition reaction of acetonitrile to an aldehyde or ketone in basic condition.33,34

Oxidative Degradation

Figure 7a shows the HPLC chromatogram of β-lapachone after exposure to oxidative conditions. One degradation peak at retention time of 1.22min was observed before the solvent peak. The degradation product showed a molecular ion peak
at $m/z$ 165 [M−H]$^-$ in negative ionization mode and molecular weight of the degradation product was 166, 76 amu less than that of β-lapachone. Based on the MS result and DAD absorbance data, the degradation product was assumed to be phthalic acid.\textsuperscript{35)} Figure 7b shows the change of β-lapachone concentration after exposure to $\text{H}_2\text{O}_2$. It degraded faster as the concentration of $\text{H}_2\text{O}_2$ increased and its degradation kinetic was dependent on the concentration of $\text{H}_2\text{O}_2$. Moreover, the dark yellow color of β-lapachone solution faded to light yellow after exposure to $\text{H}_2\text{O}_2$, suggesting that oxidative condition accelerated the degradation of β-lapachone. It might be in need of careful attention when to store the oxidation sensitive materials. The reaction of β-lapachone degradation was assumed to follow first-order kinetic in the experiment using the 30% $\text{H}_2\text{O}_2$. However, when using 0.3 and 3% $\text{H}_2\text{O}_2$, the degradation rate was greatly reduced after 2h, which might be attributed to insufficient amount of $\text{H}_2\text{O}_2$ for the reaction.
Thermal Degradation

Figure 8 shows the change of β-lapachone concentration after exposure to thermal conditions. It degraded faster as the temperature increased. However, it was relatively stable in thermal conditions with slight degradation compared to other stress conditions. The dark yellow color of β-lapachone solution was not changed even at 60°C indicating stability to thermal conditions. The degradation reaction was too slow to permit investigation of degradation products and reaction kinetics.

Cell Activity and Toxicity Test

Effects of β-Lapachone on Collagen Synthesis

β-Lapachone solutions (100 µg/mL in 50% ACN) were exposed to xenon light lamp as in the photo degradation method. Sample solutions were withdrawn at a predetermined time intervals (#1: 1.5, #2: 3, #3: 4.5, #4: 7 h), and solvents were evaporated using a rotary evaporator (EYELA N-1100, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to collect the remaining β-lapachone powder. Aliquots of powder were dissolved in 50% ACN and analyzed by HPLC to determine the contents of β-lapachone in the powder. It was observed that the contents of β-lapachone were 80 (#1), 62 (#2), 46 (#3), and 7% (#4) compared to the standard. Figure 9 shows the collagen secretion with the β-lapachone standard and photo-degraded samples from #1 to #4. The sample concentration of 0.1 µg/mL was selected based on the preliminary study. Increased collagen synthesis (about 160% compared to control) (p<0.01) was observed with the standard. However, as the contents decreased, collagen synthesis decreased significantly (p<0.05 or p<0.01) in comparison to the reference standard and control. In other words, the cellular efficacy of β-lapachone was dependent on its contents remained. This result might suggest that maintaining chemical stability would be one of the key factors to achieve efficacy of the active ingredient. Therefore, certain actions would be necessary to stabilize the products with identifying instability mechanisms.

Cytotoxicity of β-Lapachone

The MTT cytotoxicity assay in HDFs was performed to evaluate the probable cytotoxic potential of β-lapachone and degraded samples to the cell. Figure 10 shows the cell viability after exposure to different concentrations of standard and photo-degraded samples from #1 to #4. The results obtained indicated that β-lapachone and degraded samples did not show significant cytotoxic effects to the cell after 48h of exposure. The results were not different from low (0.001 µg/mL) to effective concentration (0.1 µg/mL), suggesting that the β-lapachone standard and photo-degraded samples might be non-toxic to HDFs at the concentration range tested. The results might suggest the importance of biological assays in stability studies for the investigation of cytotoxic potential of active ingredients and degradation products.

Conclusion

In this study, the stability-indicating analytical method for β-lapachone was validated according to ICH guidelines.

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Fig. 9. Effects of β-Lapachone Standard and Photo-degraded β-Lapachone Samples (from #1 to #4) on Collagen Synthesis
Total collagen contents were measured in cultured HDFs using a Procollagen Type I C-Peptide (PIP) EIA kit. Values represent the mean±S.D. of triplicate determinations. For comparing the negative control, β-lapachone standard and photo-degraded samples, statistical analysis was performed with a p-value <0.05 (*) or <0.01 (**).

Fig. 10. Cytotoxic Effects of the β-Lapachone Standard and the Photo-degraded Samples (from #1 to #4)
HDFs were cultured in a 96-well plate in the presence of the samples for 48h. Viability was subsequently assessed by MTT assay. Values represent the mean±S.D. of triplicate determinations.

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Chemical stability of β-lapachone was investigated under various stress conditions such as light, oxidation, thermal, acidic, and basic conditions. Degradation products were confirmed by HPLC-DAD and LC-MS. β-Lapachone was unstable under light and basic conditions with its degradation kinetics following zero-order and first-order reaction, respectively. β-Lapachone was relatively stable in thermal and acidic conditions with slight degradation. In the cellular activity test, the β-lapachone standard increased the synthesis of collagen compared to control. However, degraded samples showed decreased efficacy suggesting that chemical stability might have an influence on the efficacy of β-lapachone. Therefore, in order to achieve efficacy and safety, the chemical stability of β-lapachone needs to be carefully considered and the control of stability may be essential during formulation development, manufacturing, and storage.

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Conflict of Interest The authors declare no conflict of interest.

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