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# An HPLC method to evaluate purity of a steroidal drug, loteprednol etabonate

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**Abstract**

Validation of an analytical method for impurities and degradation products in an active pharmaceutical ingredient is important to assessment of quality and safety in a new pharmaceutical product. In the present study, a high-performance liquid chromatographic method was validated to evaluate purity of loteprednol etabonate (LE). LE and its four related substances, major process impurities and degradation products (PJ-90, PJ-91, LE-11-keto and LE-methyl ester) were well resolved using a phenyl-stationary phase under isocratic conditions. Two photo-degradation products were identified as chloromethyl 17α-ethoxycarbonyloxy-11β-hydroxy-5α-methyl-2-oxo-19-norandrosta-1(10),3-diene-17β-carboxylate and chloromethyl 17α-ethoxycarbonyloxy-11β-hydroxy-1-methyl-3-oxo-6(5→10α)-*abeo*-19-norandrosta-1,4-diene-17β-carboxylate. A photo-degradation product, chloromethyl 1β,11β-epoxy-17α-ethoxycarbonyloxy-2-oxo-10α-androsta-4-ene-17β-carboxylate, was not abundant by ultraviolet detector. The risk depending on only ultraviolet detection should be noted. Calibration curves for PJ-90, PJ-91, LE-11-keto and LE-methyl ester showed linearity over the range of 0.05–2.0% levels in LE with correlation coefficient of 0.999. Accuracy ($n = 3$) at the concentration of 0.5% level in LE for PJ-90, PJ-91, LE-11-keto and LE-methyl ester were 2.0, 2.0, 2.3 and 2.0%, respectively. Intra-day repeatability ($n = 6$) at the concentration of 0.5% level in LE for PJ-90, PJ-91, LE-11-keto and LE-methyl ester were 1.4, 1.4, 1.8 and 1.4%, respectively. The lower limits of detection for PJ-90, PJ-91, LE-11-keto and LE-methyl ester were 0.002, 0.001, 0.004 and 0.003% levels in LE, respectively.


*Keywords:* Validation; High-performance liquid chromatography; Impurity; Loteprednol etabonate

## 1. Introduction

An ophthalmic preparation containing a steroidal anti-inflammatory component such as betamethasone sodium phosphate is often used for treatment of inflammation, but its instillation may cause ocular hypertension. Loteprednol etabonate (LE, chloromethyl 17α-[(ethoxycarbonyl)oxy]-11β-hydroxy-3-oxoandrosta-1,4-diene-17β-carboxylate) is a new glucocorticoid drug that was developed for topical use (Fig. 1) [1–3]. LE is derived from the inactive metabolite of prednisolone (PJ-90, 11β,17α-dihydroxy-3-oxoandrosta-1,4-diene-17β-carboxylic acid) [3] by introduction of a chloromethyl ester and ethylcarbonate ester to the 17β- and 17α-positions of PJ-90, respectively [1,4]. The 17β-chloromethyl ester is easily hydrolyzed to form an inactive compound, 17α-ethoxycarbonyloxy-11β-hydroxy-3-oxoandrosta-1,4-diene-17β-carboxylic acid (PJ-91) and then to PJ-90 with nonspecific esterases [5,6]. Thus accidental systemic side effects are avoided. Additionally, LE demonstrates a low tendency to raise the intraocular pressure due to its rapid metabolism to inactive metabolites in the eye [7].

From the quality and safety, analysis of impurities and degradation products in an active pharmaceutical ingredient is important for development of a new pharmaceutical product [8]. Therefore the analytical method should be validated if it is suitable for the evaluation of the impurity profile [9].

Several high-performance liquid chromatographic (HPLC) methods have been reported to assay LE and its metabolites in biological fluids for pharmacokinetic and tissue permeability evaluation studies [5,10–12]. In this study, we developed an HPLC method to evaluate purity of LE.

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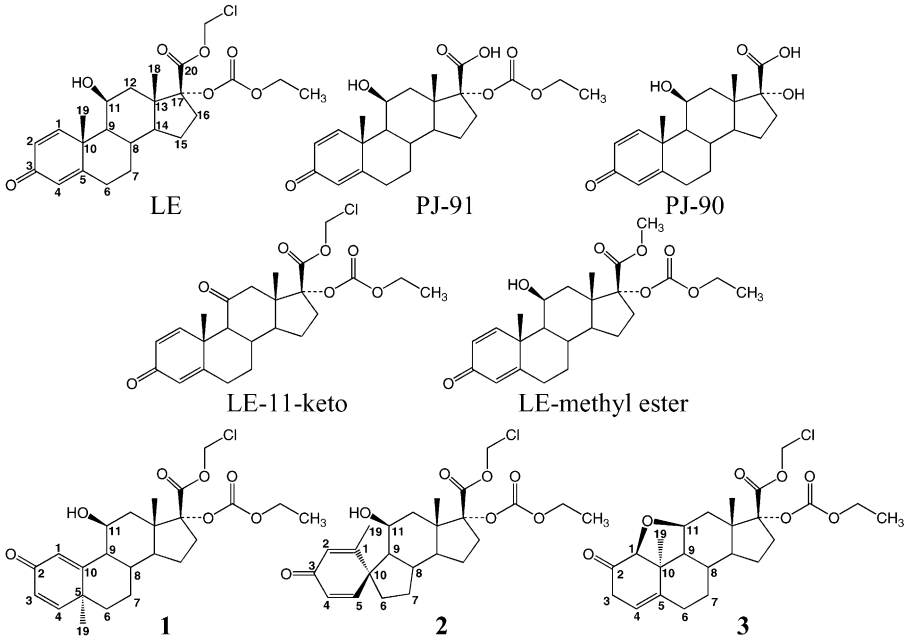


Fig. 1. Loteprednol etabonate and its related substances. Loteprednol etabonate (LE): chloromethyl 17α-[(ethoxycarbonyl)oxy]-11β-hydroxy-3-oxoandrosta-1,4-diene-17β-carboxylate, PJ-91: 17α-ethoxycarbonyloxy-11β-hydroxy-3-oxoandrosta-1,4-diene-17β-carboxylic acid, PJ-90: 11β,17α-dihydroxy-3-oxoandrosta-1,4-diene-17β-carboxylic acid, LE-11-keto: chloromethyl 3,11-dioxo-17α-ethoxycarbonyloxy-androsta-1,4-diene-17β-carboxylate, LE-methyl ester: methyl 17α-ethoxycarbonyloxy-11β-hydroxy-3-oxoandrosta-1,4-diene-17β-carboxylate, **1**: chloromethyl 17α-ethoxycarbonyloxy-11β-hydroxy-5α-methyl-2-oxo-19-norandrosta-1(10),3-diene-17β-carboxylate, **2**: chloromethyl 17α-ethoxycarbonyloxy-11β-hydroxy-1-methyl-3-oxo-6(5→10α)-*abeo*-19-norandrosta-1,4-diene-17β-carboxylate and **3**: chloromethyl 1β,11β-epoxy-17α-ethoxycarbonyloxy-2-oxo-10α-androsta-4-ene-17β-carboxylate.

The method allowed the quantitation of LE-related substances, PJ-91, PJ-90, and process impurities, chloromethyl 3,11-dioxo-17α-ethoxycarbonyloxy-androsta-1,4-diene-17β-carboxylate (LE-11-keto) and methyl 17α-ethoxycarbonyloxy-11β-hydroxy-3-oxoandrosta- 1, 4-diene- 17β-carboxylate (LE-methyl ester). Although a photo-degradation product of chloromethyl 1β,11β-epoxy-17α-ethoxycarbonyloxy-2-oxo-10α-androsta-4-ene-17β-carboxylate (**3)** was not abundant by ultraviolet detector, photo-degradation products of chloromethyl 17α-ethoxycarbonyloxy-11β-hydroxy-5α-methyl-2-oxo-19-norandrosta-1(10),3-diene-17β-carboxylate (**1**) and chloromethyl 17α-ethoxycarbonyloxy-11β-hydroxy-1-methyl-3-oxo-6(5→10α)-*abeo*-19-norandrosta-1,4-diene-17β-carboxylate (**2**) were detected [13].

## 2. Experimental

### 2.1. Materials

LE was purchased from PPG-Sipsy (Cedex, France). PJ-90, PJ-91, LE-11-keto and LE-methyl ester whose purity was over 98% were obtained from Alchem Laboratories (Alachus Florida, USA). Acetonitrile (HPLC grade) was obtained from Wako Pure Chemicals (Osaka, Japan). Water was purified with a Milli-Q purification system (Millipore, Tokyo, Japan). Other reagents and solvents were HPLC grade or the highest grade commercially available, and used without further purification.

### 2.2. Chromatographic equipment

An HPLC system (LC-10A, Shimadzu, Kyoto, Japan) was composed of an autosampler (SIL-10ADvp), a pump (LC-10AD or LC-10ADvp), a column oven (CTO-10Acvp or CTO-10ASvp), a UV detector (SPD-10AV or SPD-10AVvp) and a data processor (CLASS-LC10 or CLASS-VP). Detection was performed at 244 nm, and the injection volume was 20 μl throughout the work.

An HPLC system (L-7000, Hitachi Instruments Service, Tokyo, Japan) was also used for the analysis of decomposed products of LE. The equipment was composed of an autosampler (L-7200), a pump (L-7100), a column oven (L-5030), and a data processor (D-2500) with a UV detector (L-7400) at 244 nm or a photodiode array detector (SPD-M10Avp, Shimadzu). The injection volume was 100 μl.

### 2.3. Chromatographic conditions

Separations were carried out on a phenylsilica column (Alltima Phenyl, 250 mm, 4.6 mm i.d., Alltech, Deerfield Illinois, USA) using a mixture of water, acetonitrile and acetic acid (57.0:42.5:0.5 v/v%) as mobile phase at a flow-rate of 1.8 ml/min at 25 °C.

An octadecylsilica column (YMC-Pack ODS-A, 250 mm, 4.6 mm i.d., YMC, Kyoto, Japan) was also used to select an appropriate stationary phase for the separation of LE and its related substances. The analysis was performed using a mixture of water, acetonitrile and acetic acid (55.0:44.5:0.5 v/v%) as mobile phase at a flow-rate of 1.0 ml/min at 25 °C.

### 2.4. Optimization studies for the separation of LE and its four related substances

Stock solutions of LE, PJ-90, PJ-91, LE-11-keto and LE-methyl ester were separately prepared by accurately weighing 10 mg of each compound in a 100 ml volumetric flask followed by dissolution in methanol. The stock solutions were prepared just before use, although they were stable for at least 24 h at 25 °C. Standard solution (50 μg/ml each) of a mixture of LE, PJ-90, PJ-91, LE-11-keto and LE-methyl ester was prepared by diluting the stock solutions with methanol. A portion (20 μl) of the solution was injected into the HPLC column.

### 2.5. Degradation of LE in suspension

LE (1 g) was suspended in water (200 ml) or 0.1 M hydrochloric acid (200 ml), and the suspension was kept at 100 °C for 24 h, and allowed to cool to room temperature. The mixture after keeping in hydrochloric acid was neutralized with 5 M sodium hydroxide solution. LE (1 g) was suspended in 0.1 M sodium hydroxide solution (200 ml), and the suspension was kept at 100 °C for 1 h, and allowed to cool to room temperature. The mixture was neutralized with 5 M hydrochloric acid solution. LE (1 g) was suspended in water (200 ml) in a clear glass bottle, and the suspension was exposed to light providing an overall illumination of 1.2 million lx h and an integrated UV energy of 200 W h/m$^2$ at 25 °C. Samples of decomposed LE (0.5 mg/ml) were prepared by pipeting the uniform suspensions (1 ml each) into 10 ml volumetric flasks followed by dilution with the mobile phase. A portion (100 μl) of the solution was injected into the HPLC column.

### 2.6. Stress testing of LE in solid state

LE was kept at 60 °C and 75% relative humidity in a sealed glass bottle or in an open container for 4 weeks. LE was also exposed to light providing an overall illumination of 1.2 million lx h and an integrated near-ultraviolet energy of 200 W h/m$^2$ in a sealed clear glass bottle at 25 °C. Sample solution (1.0 mg/ml) of LE was prepared by accurately weighing 25 mg of the compound into 25 ml volumetric flasks followed by dissolution in methanol. A portion (20 μl) of the solution was injected into the HPLC column. The sample solution was prepared just before use, although it was stable for at least 24 h at 25 °C. The reporting thresholds of PJ-90, PJ-91, LE-11-keto and LE-methyl ester were set to their quantitation limits. The reporting threshold of unknown impurities was also set to 0.007% in LE. It was established from the quantitation limit of LE, because content of unknown impurities was calculated from a standard of LE (data not shown).

### 2.7. Linearity

Linearity of the calibration curves was observed for PJ-90, PJ-91, LE-11-keto and LE-methyl ester. Standard solutions of these compounds were prepared by diluting the stock solutions with methanol. The slope and the other statistical parameters of the calibration curves were calculated by linear regression analysis.

### 2.8. Detection limit and quantitation limit

Detection limit and quantitation limit for PJ-90, PJ-91, LE-11-keto and LE-methyl ester were determined using Eqs. (1) and (2) [14]:

$$\text{Detection limit} = 3.3\sigma/S \quad (1)$$

$$\text{Quantitation limit} = 10\sigma/S \quad (2)$$

where $\sigma$ is the standard deviation of the $y$-intercept and $S$ is the slope of the calibration curve.

### 2.9. Accuracy

Three preparations were used for accuracy analysis. The sample solutions spiked with 0.5, 5.0 and 20 μg/ml (0.05, 0.5 and 2.0% level in LE, respectively) for PJ-90, PJ-91, LE-11-keto and LE-methyl ester were prepared.

### 2.10. Repeatability

Six preparations for PJ-90, PJ-91, LE-11-keto and LE-methyl ester were used for repeatability analysis. Samples (0.5, 5.0 and 20 μg/ml (0.05, 0.5 and 2.0% level in LE, respectively)) were prepared from the stock solutions of PJ-90, PJ-91, LE-11-keto and LE-methyl ester.

### 2.11. Intermediate precision

Preparations were made changing combinations of parameters for two analysts, two systems and two columns over a six-day period (Table 1). Sample (5.0 μg/ml (0.5% level in LE, respectively)) was prepared from the stock solutions of PJ-90, PJ-91, LE-11-keto and LE-methyl ester.

Table 1
Combinations of analytical parameters for intermediate precision

| | | | | | | |
|---|---|---|---|---|---|---|
| Day (6 different days): | 1 | 2 | 3 | 4 | 5 | 6 |
| Chemist (A or B): | A | A | B | B | A | B |
| Equipment (A or B): | A | B | A | B | A | B |
| Column (A or B): | A | B | A | B | B | A |

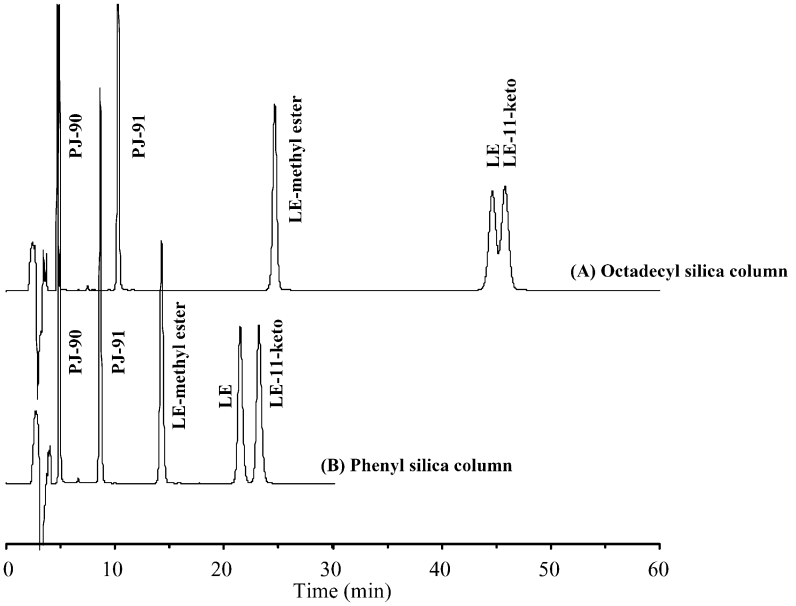


Fig. 2. Analysis for the mixed standard solution of LE, PJ-90, PJ-91, LE-11-keto and LE-methyl ester using an octadecyl silica column (A), and a phenyl silica column (B).

## 3. Results and discussion

### 3.1. Selection of stationary phase

We examined two stationary phases (octadecylsilica and phenylsilica) for the separation of LE and its four related substances. Fig. 2(A) shows the separation of major process impurities and the degradation products (PJ-90, PJ-91, LE-11-keto and LE-methyl ester) using an octadecylsilica column. Fifty minutes period was required for elution of all components, but good resolution between LE and LE-11-keto was not achieved. We also examined the use of a phenylsilica column. LE and its four related substances were well resolved under isocratic condition (Fig. 2(B)). The separation between LE and LE-11-keto was improved probably due to π–π interaction between LE-11-keto and phenols on the phenylsilica. Furthermore, the separation was performed within 25 min, and we chose a phenylsilica column in the present study.

### 3.2. Optimization studies for the separation of LE and its four related substances

Fig. 3 shows the relationship between composition of mobile phase and elution times. With increase of water, LE and LE-11-keto was obviously eluted later. As shown in Fig. 2, resolution between LE and LE-11-keto was the most important in this analysis, and a mobile phase (water–acetonitrile–acetic acid: 57.0–42.5–0.5 v/v%) was chosen.

### 3.3. Degradation of LE in suspension

Fig. 4(A) shows a chromatogram obtained after keeping preparations under heat conditions in neutral suspension (see Section 2). Two peaks at 3.0 and 5.5 min were identified as PJ-90 and PJ-91, respectively, by comparing their retention times and photodiode array spectra, also identified by LC-MS spectrum (data not shown).

Fig. 4(B) shows a chromatogram obtained after keeping preparations under heat conditions in acidic suspension. The peaks at 3.0 and 5.5 min were also confirmed as PJ-90 and PJ-91, respectively. Fig. 4(C) shows a chromatogram obtained after keeping preparations under heat conditions in basic suspension. Although PJ-90 and PJ-91 were also observed at 3.0 and 5.5 min, the required time for degradation

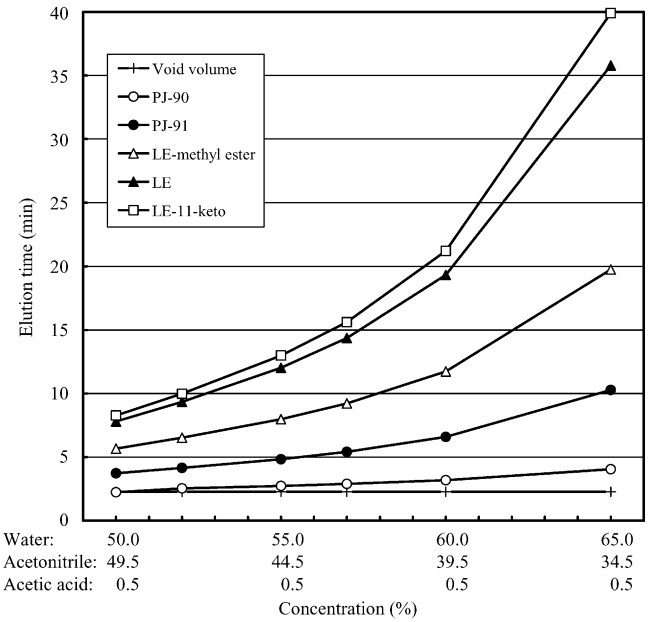


Fig. 3. Effect of water concentration on elution time of LE, PJ-90, PJ-91, LE-11-keto and LE-methyl ester.

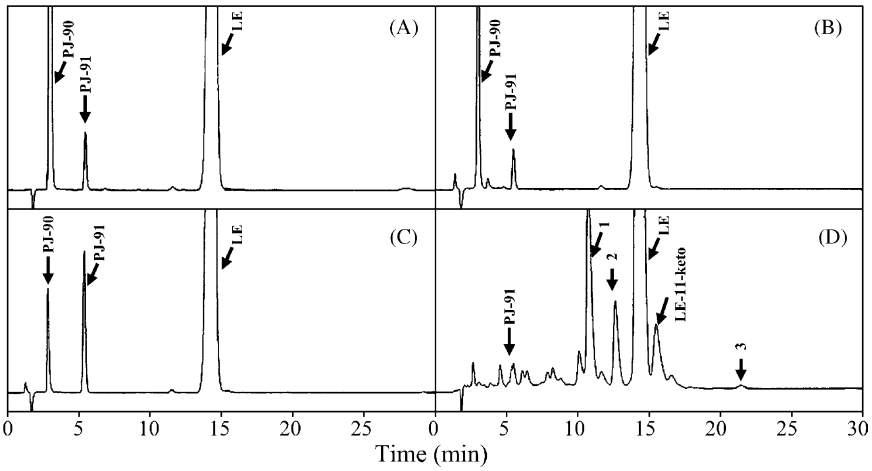


Fig. 4. Analysis of the degradation samples in suspension: heat degradation sample in neutral suspension (A), in acidic suspension (B), and in basic suspension (C). Photodegradation sample in neutral suspension (D).

was 1 h in basic suspension. Relative ratios were different from those in Fig. 4(A) and (B). These results suggested that PJ-91 was first generated by hydrolysis of a chloromethyl ester at 17β-position, and then PJ-90 was generated by hydrolysis of an ethylcarbonate ester at 17α-positions. Hydrolysis of LE easily occurred in wide pH range, and the hydrolysis rate in basic suspension was faster than in acidic suspension as expected. Fig. 4(D) shows a chromatogram obtained after keeping the preparation under irradiation with light in neutral suspension. Three major peaks at 11.0, 12.5 and 15.5 min were observed. The peak at 15.5 min was confirmed as LE-11-keto by comparing the retention time and photodiode array spectra. Identification of the peaks at 11.0 and 12.5 min was previously reported by Shirasaki et al. [13]. The peaks at 11.0 and 12.5 min were chloromethyl 17α-ethoxycarbonyloxy-11β-hydroxy-5α-methyl-2-oxo-19-norandrosta-1(10),3-diene-17β-carboxylate (**1**) and chloromethyl 17α-ethoxycarbonyloxy-11β-hydroxy-1-methyl-3-oxo-6(5→10α)-*abeo*-19-norandrosta-1,4-diene-17β-carboxylate (**2**), respectively (Fig. 1). The peak at 22 min was also identified as chloromethyl 1β,11β-epoxy-17α-ethoxycarbonyloxy-2-oxo-10α-androsta-4-ene-17β-carboxylate (**3**) (Fig. 1) [13]. Photo-degradation products of **1**, **2** and **3** were identified using UV, IR, NMR and MS spectrum after isolation by HPLC (data not shown) [13]. Photolysis of predonisolone and its 21-acetate in solution afforded to the corresponding 1,11-epoxy steroids as the major products [15,16]. Although the peak (**3**) at 22 min was not abundant by ultraviolet detection, the relative abundance was quite high in the total ion chromatogram obtained from LC-MS (Fig. 5). This was due to loss of quinoid structure by formation of epoxide between C1 and C11.

### 3.4. Stress testing in solid state

Fig. 6(A) shows a chromatogram of intact LE. We could find the peak of LE-methyl ester as well as those of PJ-91 and LE-11-keto. LE-methyl ester was due to manufacturing process. We could not find LE-methyl ester from the chromatograms of neutral suspension (Fig. 4(A)), because LE-methyl ester was probably hydrolyzed to PJ-91 in the suspension.

Fig. 6(B) shows a chromatogram of a stress sample after keeping under 60 °C/75%RH for 4 weeks in a sealed glass bottle. The peaks of PJ-91, LE-methyl ester and LE-11-keto were observed. Fig. 6(C) shows a chromatogram of a stress sample after keeping under 60 °C/75%RH for 4 weeks in an open container. PJ-90, PJ-91, LE-methyl ester and LE-11-keto were observed. No other degradation products were found. Fig. 6(D) shows a chromatogram of a stress sample after keeping under irradiation with light in solid state. PJ-91, LE-methyl ester and LE-11-keto were observed. Degradation products **1** and **2** were also observed. It is supported that degradation products **1** and **2** were generated by light exposure in solid state as well as in suspension. These results are summarized in Table 2.

After keeping under 60 °C/75%RH for 4 weeks in a sealed glass bottle, amounts of PJ-91, LE-methyl ester and

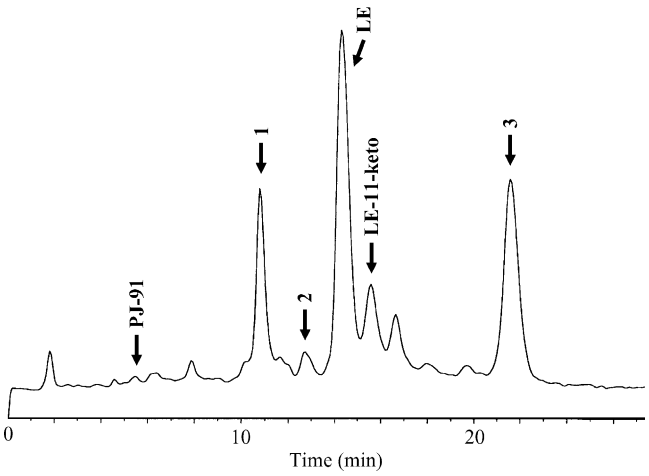


Fig. 5. Total ion chromatogram obtained from LC-MS of the light irradiated sample in neutral suspension.

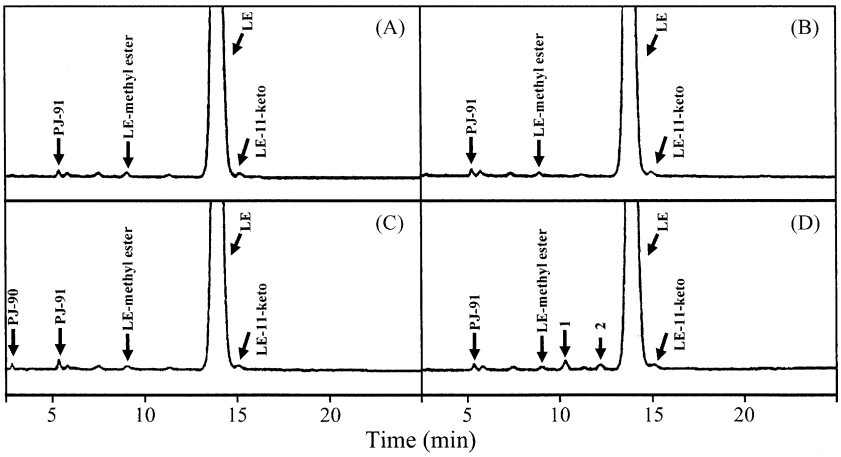


Fig. 6. Analysis of the stress samples in solid state: intact LE (A), heat degradation sample with sealed container (B), heat degradation sample with open container (C), and photo-degradation sample (D).

LE-11-keto were almost the same as that of intact LE. PJ-90 and PJ-91 were slightly increased after keeping under 60 °C/75%RH for 4 weeks in an open container. LE was probably hydrolyzed to PJ-90 and PJ-91 under the high moisture condition. Amounts of PJ-91 and LE-methyl ester were almost the same as that of intact LE after keeping under irradiation with light in solid state. However, LE-11-keto was slightly increased, and other degradation products including **1** and **2** were remarkably increased. It seems that photo-degradation pathway of LE was different from heat-degradation pathway.

### 3.5. Linearity

Linearity of the calibration curves was determined for PJ-90, PJ-91, LE-11-keto and LE-methyl ester by a linear regression analysis. The results are shown in Table 3.

Calibration curves for PJ-90, PJ-91, LE-11-keto and LE-methyl ester showed good linearity over the range of 0.05–2.0% levels in LE with correlation coefficients of 0.999. The values of sum of squares for residuals were $1.83 \times 10^7$, $2.56 \times 10^7$, $2.12 \times 10^6$ and $1.94 \times 10^7$ for PJ-90, PJ-91and LE-methyl ester and LE-11-keto, respectively. The random distribution of residuals was confirmed using residual plots (plots not shown). Ninety-five per cent confidence interval of the intercepts for PJ-90, PJ-91and LE-methyl ester and 98% confidence interval for LE-11-keto included the theoretical value of zero ($P = 0.20$, 0.91, 0.51, 0.02 for PJ-90, PJ-91, LE-methyl and LE-11-keto, respectively). We calculated relative response factors of PJ-90, PJ-91, LE-11-keto and LE-methyl ester against LE from the slopes, and found that these values were almost the same as that for LE (Table 3).

### 3.6. Detection limit and quantitation limit

Detection limits and quantitation limits for each compound were shown in Table 3. The detection limits of PJ-90, PJ-91, LE-11-keto and LE-methyl ester were 0.002, 0.001, 0.004 and 0.003% levels in LE, respectively. The quantitation limits of PJ-90, PJ-91, LE-11-keto and LE-methyl ester were 0.005, 0.004, 0.013 and 0.009% levels in LE, respectively. Recoveries for each compound at the quantitation limit were 123.7 ± 4.0%, 154.7 ± 5.5%, 119.6 ± 6.7% and 139.0 ± 4.5% ($n = 6$) for PJ-90, PJ-91, LE-11-keto and LE-methyl ester, respectively. These results indicated that the HPLC method had sufficient sensitivity to analyze the impurity in LE.

Table 2
Quantitation of impurities and degradation products of LE after keeping preparation under stress conditions of heat and light in solid state

| | Initial (time = 0) | 60 °C/75%RH for 4 weeks | | Light exposure |
|---|---|---|---|---|
| | | Sealed glass bottle | Open container | |
| PJ-90 | UQL | UQL | 0.06% | UQL |
| PJ-91 | 0.11% | 0.13% | 0.17% | 0.11% |
| LE-11-keto | 0.23% | 0.24% | 0.25% | 0.30% |
| LE-methyl ester | 0.09% | 0.12% | 0.12% | 0.12% |
| Other | 0.32% | 0.33% | 0.31% | 0.79% |
| Total | 0.75% | 0.82% | 0.91% | 1.32% |

UQL: Under quantitation limit.

Table 3
Summary of linearity, detection limit and quantitation limit

| | Range (% level in LE) | Regression line[a] | Correlation coefficient | Relative response factor | Detection limit (% level in LE) | Quantitation limit (% level in LE) |
|---|---|---|---|---|---|---|
| PJ-90 | 0.05–2.0% | $y = 2.70 \times 10^4 x + 2.57 \times 10^3$ | 0.999 | 1.21 | 0.002 | 0.005 |
| PJ-91 | 0.05–2.0% | $y = 2.34 \times 10^4 x + 1.34 \times 10^3$ | 0.999 | 1.05 | 0.001 | 0.004 |
| LE-11-keto | 0.05–2.0% | $y = 2.16 \times 10^4 x - 2.29 \times 10^3$ | 0.999 | 0.97 | 0.004 | 0.013 |
| LE-methyl ester | 0.05–2.0% | $y = 2.36 \times 10^4 x - 2.53 \times 10^2$ | 0.999 | 1.06 | 0.003 | 0.009 |

[a] Where $y$ is the response and $x$ is the concentration (μg/ml).

Table 4
Accuracy, repeatability and intermediate precision

| | Concentration (% level in LE) | Accuracy[a] | | Repeatability[b] | | Intermediate precision[c] | |
|---|---|---|---|---|---|---|---|
| | | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
| PJ-90 | 0.05 | 108.5 | 1.3 | 109.3 | 1.2 | 100.8 | 0.7 |
| | 0.5 | 101.4 | 2.0 | 101.4 | 1.4 | | |
| | 2.0 | 99.1 | 0.6 | 99.0 | 0.9 | | |
| PJ-91 | 0.05 | 102.6 | 5.7 | 101.6 | 3.7 | 100.0 | 0.7 |
| | 0.5 | 101.1 | 2.0 | 101.1 | 1.4 | | |
| | 2.0 | 100.1 | 0.8 | 99.9 | 1.1 | | |
| LE-11-keto | 0.05 | 91.2 | 2.4 | 92.4 | 2.2 | 99.5 | 0.9 |
| | 0.5 | 100.3 | 2.3 | 100.5 | 1.8 | | |
| | 2.0 | 99.7 | 0.8 | 99.7 | 1.0 | | |
| LE-methyl ester | 0.05 | 100.8 | 2.0 | 99.5 | 2.5 | 99.9 | 0.7 |
| | 0.5 | 100.8 | 2.0 | 100.9 | 1.4 | | |
| | 2.0 | 99.5 | 0.7 | 99.5 | 0.9 | | |

[a] $n = 3$ within 1 day.
[b] $n = 6$ within 1 day.
[c] $n = 12$ over a 6-day period.

### 3.7. Accuracy and precision

Accuracy was determined by making injections of spiked samples for PJ-90, PJ-91, LE-11-keto and LE-methyl ester during a day. Relative standard deviations were 1.3, 5.7, 2.4 and 2.0% at 0.05% level in LE, 2.0, 2.0, 2.3 and 2.0% at 0.5% level in LE, and 0.6, 0.8, 0.8 and 0.7% at 2.0% level in LE for PJ-90, PJ-91, LE-11-keto and LE-methyl ester, respectively (Table 4).

Repeatability was determined by making injections of six preparations for PJ-90, PJ-91, LE-11-keto and LE-methyl ester during a day. Relative standard deviations were 1.2, 3.7, 2.2 and 2.5% at 0.05% level in LE, 1.4, 1.4, 1.8 and 1.4% at 0.5% level in LE and 0.9, 1.1, 1.0 and 0.9% at 2.0% level in LE for PJ-90, PJ-91, LE-11-keto and LE-methyl ester, respectively (Table 4).

Influence of within-laboratories variations such as two different analysts, two HPLC systems and two columns over a 6-day period was investigated (Table 1) [9]. We chose one concentration of 0.5% level of LE for PJ-90, PJ-91, LE-11-keto and LE-methyl ester as representative concentration, because we believed that it was sufficient to investigate the influence of typical variations such as days, people, equipment and columns. Relative standard deviations were 0.7, 0.7, 0.9 and 0.7% for PJ-90, PJ-91, LE-11-keto and LE-methyl ester, respectively (Table 4).

In the present study, we reported an HPLC method to evaluate purity in LE. We found that LE was hydrolyzed to PJ-91 and PJ-90 in the presence of water such as high humidity condition and in aqueous suspension. Moreover LE generated photo-degradation products different from heat-degradation products. Photo-degradation product **3** was not abundant by ultraviolet detector. We have to note the risk depending on only ultraviolet detection. We demonstrated that the method has appropriate precision for routine work of quality control, and also could be used for a stability indicating method. An HPLC method using a phenylsilica column in an isocratic condition was effective for separation of LE and its related substances.

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