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Conversion Investigation for Lovastatin and Its Derivatives By HPLC

Zhibing Huang, Yang Xu*, Yanping Li, and **Yanhua Wang**

Sino-Germany Joint Research Institute, State Key Laboratory of Food Science , Nanchang University, Nanchang 330047, China

Abstract

A high-performance liquid chromatography (HPLC) method was developed for the separation of lovastatin (LT) and its derivatives. The conversion of LT and its derivatives in alkaline or acidic solution in different storage times was also investigated by HPLC. The results showed that LT was present in different forms: as a lactone, as lovastatin acid (LA), and as its methyl ester (LM) under acidic condition. Well-resolved peaks of three forms compounds of lovastatin were separated on a Symmetry C_{18} column (4.6 × 250 mm i.d., particle size 5 µm) using acetonitrile–water (77:23, v/v) as the mobile phase at pH 3.0. The retention time of LA, LT, LM was 6.41 ± 0.25 min, 8.89 ± 0.25 min, 9.73 ± 0.25 min, respectively. LT only converted to LA when LT was treated with 0.1 M NaOH. Under the acidic condition, with the increase of storage time, LT converted to LA, following LA would be transformed to LT and LM. Apparently, containing high concentrations of methanol in acidic methanol solutions might facilitate the conversion of LA to LM, and conversion of LT, LA, and LM would almost reach equilibrium after 60 h. The concentration of methanol and the storage time would also change the form of the LT when LA, LT, and LM were extracted by methanol–water in acidic condition. So the determination and separation of LA and LT should possibly exclude methanol in acidic condition.

Introduction

Lovastatin (LT) is an important fungal secondary metabolite inhibiting 3-hydroxy-methyl-3-glutaryl-coenzyme A (HMG-CoA), which catalyzes a rate-limiting step in the biosynthesis of cholesterol. LT was first reported from *Monascus* rubber (1) and by Alerts et al. (2) from *Aspergillus terreus*. Endo et al. (3) and Alberts (4) also indicated that many strains of *Monascus* as well as a variety of other filamentous fungi, such as *Gymnoascus*, *Trichoderma*, *Hypomyces*, *Doratomyces*, *Penicillium*, *Phoma*, and *Eupenicillium*, were found to produce LT. It is present in the fermentation broth largely in hydroxycarboxylare [lovastatin acid (LA)] form, but the lactone form (LT) also can be found (5–7). Li et al. (8) have also identified the monacolins in *M. purpureus*-fermented rice existing in both lacton form and hydroxy acids form by liquid chromatography–mass spectrometry (LC–MS).

At present, numerous reports had carried on determination the LT by gas chromatography–mass spectrometry (GC–MS) (9), high-performance liquid chromatography (HPLC), and HPLC–MS (10–13). In contrast, the LA was seldom provided with determination method as the isolation and purification of free LA was seriously hampered by the fact that it was structurally unstable. Rajh et al. (14) made a comparison of capillary electrophoresis (CE) and HPLC methods for determining LT and its oxidation products after exposure to an oxidative atmosphere. Recently, Lee et al. (15) also reported a synchronous analysis method for detection of citrinin and the lactone and acid forms of LT in red mold rice. Alvarez-Lueje et al. (16) studied the assessment of the hydrolytic degradation of LT by HPLC.

There were a few reports on the conversion of LT from lactone forms to its corresponding hydroxyl acid forms. Ye et al. (17) have investigated the conversions of LT to LA in plasma at different temperature, such as 37°C, room temperature, –20°C, and –70°C. Their results showed that the complete transformation of LT to LA would be for 21.5 and 121.5 h at 37°C and room temperature, respectively; moreover, LT was not altered significantly at –20 and –70°C for four months.

Yang and Hwang (18) measured the preparation of LA with 0.1 M NaOH or 0.05 M KOH (prepared with 25, 50, 75, and 90% methanol in water or 100% water) and modified to prepare the alkaline solutions with 25, 50, 75, 90% acetonitrile in water. Their results showed that LT could be converted to LA entirely in 0.1 M NaOH and 0.05 M KOH (prepared with 25–90% methanol in water); nevertheless, they would be further transformed to the methyl ester of the hydroxy acid form, and the transformation increased as methanol raised. Also, they found that the conversions would be better if lactone form were placed in 0.1 M NaOH or 0.05 M KOH solutions prepared with 25 or 50% acetonitrile in water.

Jozica et al. (7) have investigated LT in fermentation broth after the addition of acid and extraction with methanol using a mobile phase at pH 3.0. They found that after extraction with methanol, LT was present in the broth samples in three forms: as LA, LT, and LM. And the same forms were found in aged solution of the standard, indicating that under acidic conditions the LT slowly transformed to acid, which further reacted with methanol to from an ester (Figure 1).

These reports have only investigated the conversion of LT and LA in different conditions, but there were few detailed reports on conversion of LA to LM in alkaline and acidic methanol solution over different times by HPLC. Apparently, under alkaline or acidic conditions, the LM was also one of important compound and was structurally stable. So LM

* Author to whom correspondence should be addressed: e-mail xuyang1951@yahoo.com.cn.

should be considered, when the content of LA or LT was determined by methanol extracted from fermentation samples. It is necessary to investigate the conversion of LT, LA, and LM in various solution over different storage times, which could be important to determine the content of LT or LA in the fermentation sample by *Monascus* rubber and *Aspergillus terreus* or clarify the pharmacokinetics and pharmacodynamics so as to further increase of potency of these compounds.

In the present study, a HPLC method was developed for the simultaneous separation of LT, LA, and LM. Meanwhile, the conversion from LT to LA and LA to LM over different storage time in alkaline and acidic solution was studied.

Experimental

Apparatus

The HPLC system consisted of a Waters 510 solvent delivery pump (Milford, MA), a manual injector system (7725) equipped with a 20-µL loop, and a model UV200 detector equipped with WDL-95 chromatography manager for integration (Dalian Elite Analytical Instruments, Dalian, China). Chromatographic separation was achieved at room temperature using a Waters Symmetry C_{18} column (4.6 × 250 mm i.d., particle size 5 µm). The LC–MS analyses were performed using Waters LC/ZQ 2695/4000 LC–MS system.

Reagents

LT standard was purchased from Sigma (St. Louis, MO). LA was prepared in our laboratory from LT standard. Their chemical structures are shown in Figure 1. Methanol and acetonitrile (ACN) were HPLC-grade from Shanghai General Chemical Reagent Factory (Shanghai, China). Sodium hydroxide (NaOH), hydrochloric acid (HCl), and phosphoric acid were analytical-grade from ChromTech (Shanghai, China). All other chemicals were analytical-grade from ChromTech (Shanghai, China) and were not further purified. De-ionized water was obtained with an in-house Milli-Q Plus System (Millipore, Billerica, MA) at 18.2 MΩ, this de-ionized water was referred to as "water" hereafter. It was degassed under vacuum and filtered through a 0.2-µm membrane filter (nylon) before use in the HPLC analysis.

Preparation of the stock solution and standards

LT stock solution (100 µg/mL) was prepared in the following manner. LT (2.5 mg) was dissolved in 100% methanol in a 25-mL low actinic volumetric flask and stored at 4ºC before further dilutions.

Working solutions were prepared daily at concentrations 0.5, 5, 10, 30, 50, and 75 µg/mL by serial dilutions of stock solution with methanol and were stored at 4°C before use. A 10-µL aliquot was injected into HPLC. The amount of LT was calculated using the calibration curve of LT.

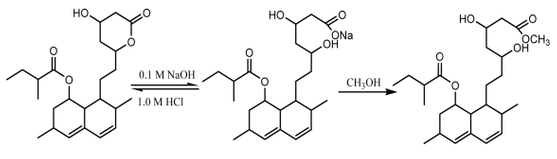

Figure 1. Chemical structures of LT and its derivatives.

The conversion of LT to LA

To obtain LA, LT was converted to acid through sodium salt according to Brown et al. (19). Twenty milliliters of LT standard preparation was accurately transferred into another 25-mL low actinic volumetric flask and 5 mL of 0.1 M NaOH was added and the solution was allowed to be kept at 25°C for 60 min. Subsequently, to neutralize the NaOH, the solution was adjusted to pH 7.0 with 1.0 M HCl, filtered through a 0.22-µm membrane filter (Millipore), diluted to the concentration of 10 µg/mL, and used as standard preparation of LA.

The conversion of LA, LT, and LM under alkaline condition

0.01 mL NaOH (0.1 M) was added to 1.0 mL MK (10 ug/mL), in which the concentration of methanol was 99%, over different storage time at 25°C, and then analyzed with HPLC.

0.01 mL NaOH (0.1 M) was added to 1.0 mL MK (10 ug/mL), followed 2.0 mL H_2O to reduce the concentration of methanol, in which the concentration of methanol was 33%, over different storage times at 25°C, and then analyzed with HPLC.

The conversion of LA, LT, and LM under acidic condition

0.05 mL HCl (1.0 M) was added to 1.0 mL MK (100 µg/mL), in which the concentration of methanol was 95%, over different storage times at 25°C, and then analyzed with HPLC.

0.05 mL HCl (1.0 M) was added to 1.0 mL MK (100 µg/mL), followed by 2.0 mL H_2O added to reduce the concentration of methanol, in which the concentration of methanol was 33%, over different storage time at 25°C, and then analyzed with HPLC.

The conversions of LT to LA and LM after treatment with NaOH and HCl

0.1 mL NaOH (0.1 M) was added to 1.0 mL LT (100 µg/mL), kept at 25°C for 30 min, followed by neutralization with 0.05 mL HCl (1.0 M) to make the solution in acidic conditions, in which the concentration of methanol was 87% over different storage time (from 10 to 60 min) at 25°C, and then analyzed with HPLC.

0.05 mL NaOH (0.1 M) and 1.0 mL H_2O were added to1.0 mL LT (100 µg/mL) and kept at 25°C for 30 min, followed by neutralization with 0.05 mL HCl (1.0 M) to make the solution in acidic conditions, in which the concentration of methanol was 48% over different storage time at 25°C, and then analyzed with HPLC.

0.05 mL NaOH (0.1 M) and 1.0 mL H_2O were added to1.0 mL LT (100 µg/mL), then 0.05 mL HCl (1.0 M) was immediately added to neutralize the NaOH and to make the solution in acidic conditions, in which the concentration of methanol was 48% over different storage time at 25°C, and then analyzed with HPLC.

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Results and Discussion

Optimization of chromatography conditions

LT, LA, and LM contain the same hydride naphthalene ring system with highly polarities. Therefore, method development for the analysis of LT, LA, and LM using HPLC can be time-consuming and complicated. Normally, there are some factors that have some obvious effect on the separation, peak shape, and retention time of analyte, sensitivity, noise, and resolution as well as column efficiency of HPLC. The factors concern pH value, concentration of buffer, and amount of organic modifier, etc. The following are the results of optimization and investigations.

Influence of buffer pH

The first step in the method development process was the selection of optimum pH value. LT can be dissolved in the alkaline solution, and the LT can be converted to its acidic form. However, the velocity of conversion was slow, and the conversion was not completely at low pH. In this study, low pH was selected in order to make LT can be slowly converted to LA, and then LA would be further transformed to LM; so buffer solutions ranging from pH 2.5 to pH 8.0 were investigated in this study. Experimental results revealed that the separation of LA was very poor because the LA could be an converted its salt in the studied higher pH range, and the retention time of the salts was about 2.0 min. Considering low pH could be influence on the HPLC column, we chose pH 3.0 as the optimized pH value at which LT could be converted to its acidic form, LA could be converted to LM, and the peak shape of LA, LT, and LM were good.

Influence of buffer concentration

The effect of phosphate buffer concentration on the separation was studied by varying it from 0.01 to 0.1 M. The results indicated that buffer concentration had no influence on retention time, resolution, and peaks shape of targeted analytes. However, the high concentration of buffer led to higher pressure on the HPLC column. So 0.01 M phosphate buffer or phosphoric acid was selected for the mobile phase of the pH adjusted to 3.0 and for the further experiments as a compromise between resolution, peak shape, and analysis time.

Influence of organic modifier

During the method development, the percentage of the mobile phase organic solvents was varied using different combinations of acetonitrile–phosphate buffer (0.01 M, pH 3.0) (85:15, 77:23, 70:30, and 60:40, v/v). The results indicated that the analysis time was prolonged with the decrease of acetonitrile concentration. Finally, acetonitrile–phosphate buffer (77:23, v/v) was selected as a compromise between resolution and analysis time.

Optimized conditions

Finally, the following optimized conditions was performed: The mobile phase was a mixture of acetonitrile–water (77:23, v/v) with the pH adjusted to 3.0 with 0.01 M phosphate buffer or phosphoric acid. All separations were carried out at room temperature and a flow rate of 0.8 mL/min. The UV detector was set at 237 nm. Under this condition, a good peak shape, satisfactory resolution, and relatively short analysis time of LA, LT, and LM can be achieved by HPLC analysis. The retention times of LA, LT, and LM were 6.41 ± 0.25 min, 8.89 ± 0.25 min, and 9.73 ± 0.25 min, respectively. The single run time was about 12 min for the baseline separation of LA, LT, and LM (Figure 2).

Identification of LA, LT and LM by LC–electrospray ionization-MS

LT was administrated as its lactone forms while LA was administered as sodium salts of the active hydroxy acids. Therefore, LA was monitored as free acidic forms, and negative-ion mode (Figure 3B) was expected to be more appropriate for the analyte. As shown in Figure 3, the molecular ion of the predominant peak at t_R = 6.56 min was 421.3 (M-1) (Figure 3C), identified as LA. The responses of LT and LM were measured in the positive mode (Figure 3D), the molecular ion of the predominant peak at t_R = 8.99 min was 405.3 (M+1) (Figure 3E), identified as LT. And the molecular ion of the predominant peak at t_R = 9.86 min was 437.3 (M+1) (Figure 3F), identified as LM. Experimental results reveal that LT was present in three different forms: as a lactone, as the lovastatin acid (LA), and as its methyl ester (LM), when LT was treated with 0.1 M NaOH and 1 M HCl.

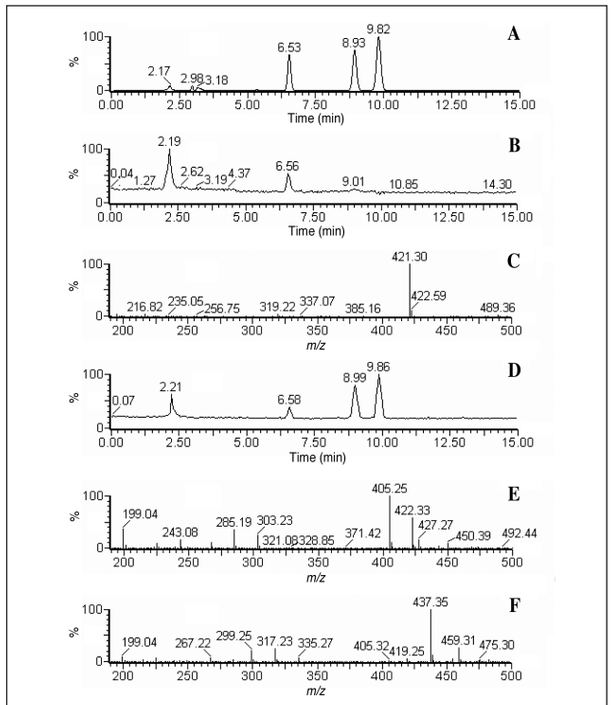

Figure 2. Chromatograms of LT treated with 1.0 M HCl in (A) 95% and (B) 33% methanol solution and kept at 25°C for 30 min. The peaks from left to right are ordered LA (6.62 min), LT (9.11 min), and LM (10.00 min).

Method validation

Validity of a newly developed analytical technique needs to be strictly demonstrated before its application to actual determination of LA, LT, and LM. In this work, various validation criteria of the developed method of HPLC, such as specificity, linearity of response, detection limit (LOD), quantification limit (LOQ), etc. were assessed as described below.

Specificity of method

Specificity of a newly developed analytic method should be firstly demonstrated due to its key importance and priority. In the demonstration of this paper, numerous samples of LT treated with 0.1 M NaOH or 1.0 M HCl were analyzed repeatedly under different conditions. The purpose of the analysis was to make sure whether or not the peaks of LA, LT, and LM in the chromatograms was exclusive under the given experimental conditions optimized previously. Furthermore, we checked the peak purity by UV diode array detection and identified LA, LT, and LM. The results indicated that no interference was noted for LT and its derivatives. All of the experiments discussed previously evidently indicate the specificity of the developed HPLC method for the separation of LA, LT, and LM.

Linearity

The linearity of detector response versus concentration of analyte was determined by constructing a calibration curve from a set of standard solutions of LT with six different concentrations on the optimized conditions. The linear range was from 0.5 to 75 µg/mL. The regression equations of LT was expressed for $y = 11023.1x + 3037$ with $r = 0.9998$ ($n = 6$),

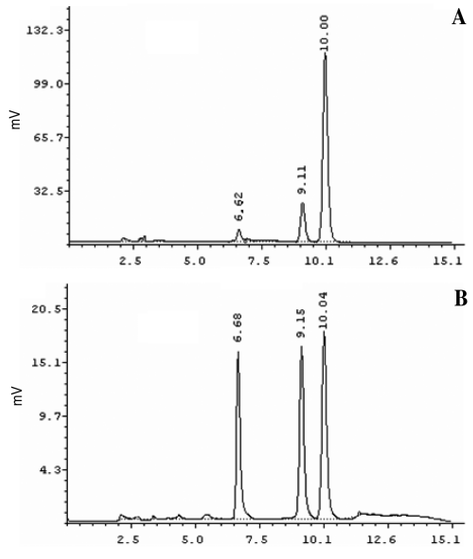

Figure 3. LC–MS for LT treated with 0.1 M NaOH and 1 M HCl in a mobile phase of 77% acetonitrile and 23% aqueous 3.0-mM formic acid. (A) Diode array detection of LA, LT, and LM. The retention times of LA, LT, and LM were 6.53 min, 8.93 min, 9.82 min, respectively; (B) ESI negative-ion mode of LA, LT, and LM; (C) mass spectrum of LT in ESI negative-ion mode; (D) ESI positive-ion mode of LA, LT, and LM; (E) mass spectrum of LT (tR = 8.99 min) in positive-ion model; and (F) mass spectrum of LM (tR = 9.86) in positive-ion mode.

where x, y, r is the concentration of LT in µg/mL, the peak area, and the regression coefficient, respectively.

Limits of detection and quantification

The LOD is a standard which reflects the sensitivity of the method and equipment. The LOQ reflects the reliability of the method when used for the determination of low concentration LT. The LOD and LOQ were calculated by setting the signal-to-noise ratio of 3:1 and 10:1, respectively. The LOD and LOQ of the developed method were 0.15 and 0.5 µg/mL, respectively.

Conversions of LT to LA and LM under alkaline condition

Shen et al. (6) prepared LA from their LT by adding 0.1 M NaOH water solution or 0.05 M KOH in aqueous methanol. In addition, they indicated that methanolysis would take place if LT was stored in neat methanol at 25°C for two days, and LM would be formed. Jozica et al. (7) also found that LT, LA, and LM was observed in the samples as well as in the standard solution after some days of storage in the refrigerator.

In order to confirm the better storage time under alkaline condition, the change of the peak areas of LA, LT, and LM in 33% and 99% methanol was investigated by HPLC. The results showed that LT could be only converted to LA in 0.1 M NaOH methanol solution. With the increasing of storage time, the peak areas of LA scarcely changed in 33% and 99% methanol water solution. The results were different from those reported by Yang and Hwang (18); they indicated that LA would be further transformed to the methyl ester of the hydroxy acid form, and the transformation increased as methanol raised in alkaline methanol solution. In fact, the generation of LM might be due to the mobile phase in acidic pH.

Conversions of LT to LA and LM under acidic condition

In this section, the conversions of LA, LT, and LM were investigated over different storage times under acidic condition. The results showed that LT slowly transformed to LA, and then LA would convert to LM in 33% and 95% methanol. It also showed that the containing high concentrations of methanol in acidic solutions might facilitate the conversion of LA to LM (Figure 2). The complete transformation would almost be for 60 h. This result is shown in Figure 4A.

Conversions of LT to LA and LM under NaOH and HCl

As shown in Figure 4B, LT can mostly convert LA in alkaline solution; however, HCl was added to neutralize the reaction and make the methanol water in acidic condition with the storage time increasing (from 10 to 60 min). The peak areas of LM and LT slowly increased, and the peak areas of LA gradually reduced. Apparently, LA can convert to LT and LM in acidic solution.

From Figure 5, it is showed that LT could be mostly converted to LA when LT is treated with NaOH kept at 25°C in 48% methanol–water solution for 30 min, then HCl added to neutralize the reaction, and immediately injected to the chromatography. Thus, there was no LM generated (Figure 5A). However, when LT was treated with NaOH in 48% methanol–water solution, HCl was added immediately and immediately injected to the chromatography, thus LM was

formed (Figure 5B). It appeared that the time of HCl added to alkaline methanol water solution would affect the generation of LM.

With the increase of storage time, the peak area values of LA decreased rapidly, the peak area values of LT increased firstly then reduced, and then peak area values of LM increased from 0 h to 60 h. But the peak area values of LA, LT and LM slightly changed after 60 h. This was because equilibrium existed between LT and LA; thus, when LT was treated with 0.1 M NaOH, base hydrolysis of the lactone ring took place, and total conversion to the hydroxy acid occurs. This result is shown in Figure 6A.

As can also be seen in Figure 6B, when LT is treated with NaOH and HCl is added immediately to neutralize the reaction and make the methanol water in acidic condition, LA, LT, and LM were found in these conditions. With the increase of storage time, most of LT transformed to LA. Moreover, LA mostly converted to LM from 0 h to 60 h, and LT increased firstly then reduced, but the peak area values of LA, LT, and LM slightly changed after 60 h.

Compared with Figure 6A, the peak area values of LM were also more than the values of LA and LT after 60 h, and the peak area values of LA, LT, and LM were similar after 60 h. It also appeared that the LM in acidic solutions was more stable than LA and LT.

From Figure 7A, it is shown that LT could be converted to LM and LA when LT is treated with NaOH kept at 25°C in 91% methanol–water solution, then HCl was immediately added. Compared with Figure 5B, the peak area values of LM were apparently increased in high concentration of methanol.

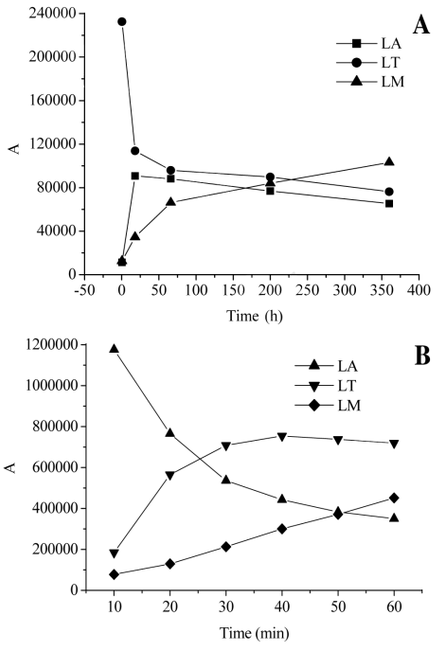

Figure 4. The change of peak areas as storage time for LT treated with HCl kept at 25°C in 33% methanol (A). The change of peak areas as storage time (from 10 to 60 min) for LT treated with NaOH and HCl in 87% methanol (B). All peak area values are means obtained by triplicate analyses, and the relative standard deviation (RSD) range was from 1.5 to 2.3%.

As shown in Figure 7B, with the increase of storage time, the peak area values of LM increased from 0 to 60 h, when LT was treated with NaOH kept at 25°C in 91% methanol–water solution, then HCl was immediately added; however, the peak area values of LA, LT, and LM slightly changed after 60 h. Compared with Figure 6B, it was concluded that the concentration of transformation of LM from LA increased as methanol raised.

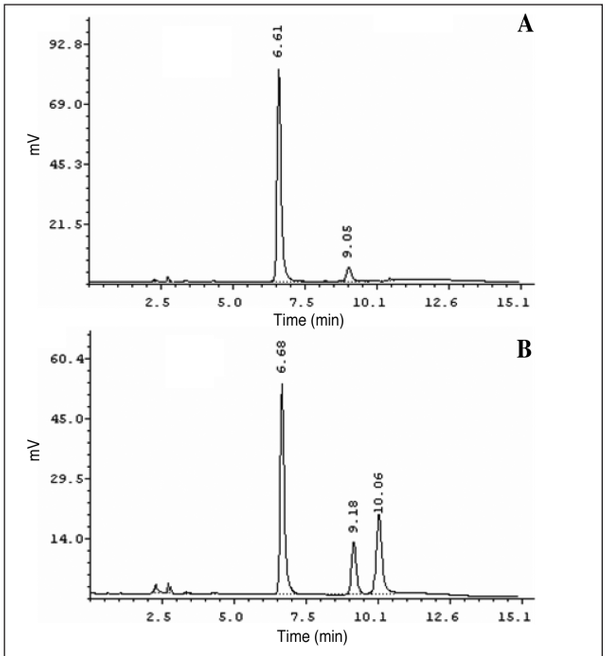

Figure 5. Chromatograms of (A) LT treated with NaOH at 25°C in 48% methanol for 30 min, then HCl was added; (B) LT treated with NaOH, then HCl added immediately. The peaks from left to right area ordered LA, LT, and LM.

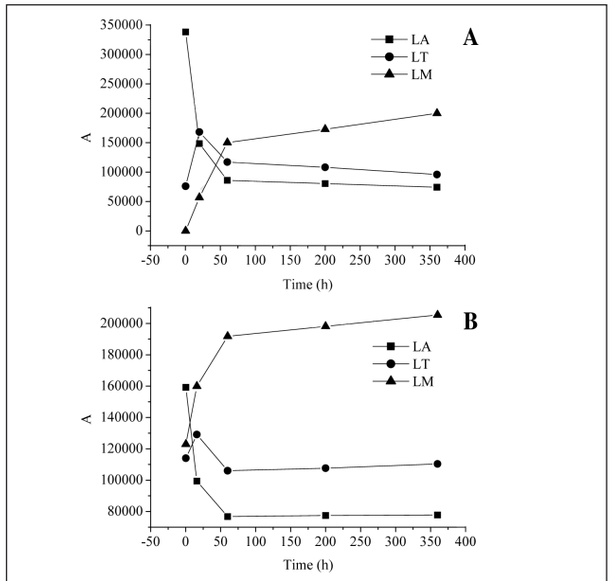

Figure 6. The change of peak areas as storage time for (A) LT treated with NaOH at 25°C in 48% methanol for 30 min, and then HCl was added; (B) LT treated with NaOH, then HCl was added immediately. All peak area values were obtained by triplicate analyses, and the RSD range was from 1.0 to 2.3%.

Conclusion

This work results also showed that LT only converted to LA when LT was treated with 0.1 M NaOH. With the increase of the storage time, the peak areas of LA scarcely changed in 33% and 99% methanol–water solution. Under acidic condition, with the increase of storage time, LT converted to LA, resulting in LA being transformed to LM. Compared with 95% and 33% methanol–water solution, apparently, the containing high concentrations of methanol in acidic solutions might facilitate the conversion of LA to LM. The LT could also convert to LA, then LA would further convert to LT and LM when LT is treated with 0.1 M NaOH, and then 1.0 M HCl added. It appeared that the time of HCl added to alkaline methanol water solution would affect the generation of LM. The peak area of LM was apparently increased when the HCl was added immediately. The peak area of LM increased as methanol increased. In addition, the conversion of LT, LA, and LM would almost reach to equilibrium after 60 h. The HPLC method would be suitable for simultaneous separation and determination the LA, LT, and LM in the fermentation sample by *Monascus* rubber and *Aspergillus terreus*. It was also to show a basic mobile phase of methanol–water in acidic condition, if used for HPLC, would change the form of the LT. The concentration of methanol and the storage time would also change the form of the LT when LA, LT, and LM were extracted by methanol–water in acidic condition. So the determination and separation of LA and LT should possibly avoid using methanol in acidic condition.

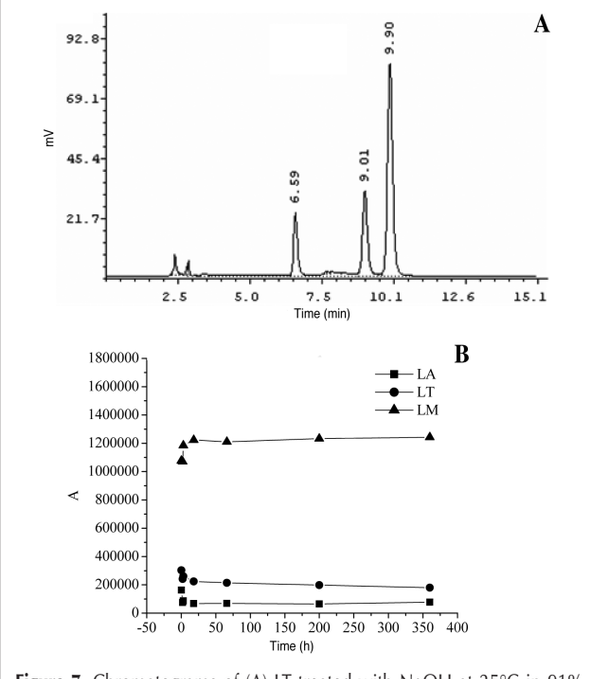

Figure 7. Chromatograms of (A) LT treated with NaOH at 25°C in 91% methanol, then HCl was added immediately and (B) LT treated with NaOH at 25ºC in 91% methanol, then Hcl was added immediately. All peak area values are means obtained by triplicate analyses, and the RSD range was from 1.2 to 2.8%. The peaks from left to right are ordered LA (6.59 min), LT (9.01 min), and LM (9.90 min).

Acknowledgements

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