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# Kinetics and mechanism of degradation of lithospermic acid B in aqueous solution

Yong-Xue Guo [a], Zhi-Long Xiu [a,*], Dai-Jia Zhang [a], Hui Wang [a], Long-Xing Wang [b], Hong-Bin Xiao [b]

[a] *Department of Bioscience & Biotechnology, Dalian University of Technology, Linggong Road 2, Dalian 116024, PR China*
[b] *Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, PR China*

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**Abstract**

The degradation of lithospermic acid B (LAB) was investigated as a function of buffer concentration, pH and temperature. Stability tests were performed using a stability-indicating high-performance liquid chromatography (HPLC) with UV–vis detection. The degradation followed pseudo-first-order kinetics under all experimental conditions. The maximum stability of LAB was observed at pH 2.0. The $\log k_{pH}$–pH profile described by specific acid–base catalysis and water molecules agreed with the experimental results. The overall degradation rate constant as a function of the temperature under the given conditions obeyed the Arrhenius equation. The chemical fate of LAB in mild acidic solution was investigated, and nine degradation products were detected and tentatively identified by LC–MS analysis. The primary degradation pathway involving the cleavage of ester bond and ring-opened of benzofuran in the LAB are proposed.


*Keywords:* Lithospermic acid B; Kinetics; Reaction mechanism; Hydrolysis; LC–MS

## 1. Introduction

Lithospermic acid B (LAB) is the most common and the most abundant compound in danshen, which is a Chinese traditional medicine. Recent pharmacological studies indicated that LAB has strong antioxidant and free radical scavenging activity [1–3]. In addition, LAB is indicated to protect against renal dysfunction, liver damage and lung fibrosis [4–6].

The structure of LAB is shown in Fig. 1. It is a conjugate of two 3-(3, 4-dihydroxyphenyl) lactic acid (also known as danshensu, DSU) and a dimer of caffeic acid. The ester linkage is liable to hydrolysis. A recent study showed that LAB is unstable in aqueous solution, and the degradation of LAB during decoction will be accelerated in heating extraction process [7,8]. However, there was no systematic report about chemical stability and degradation mechanism of the LAB in aqueous solution. In our recent research, the hydrolysis kinetics of LAB extract in relation to pH and temperature has been studied [9]. But it is difficult to propose a degradation mechanism of LAB, because of the interference of impurities in extract.

In this study, LAB was purified and the influences of buffer concentration, pH and temperature on the hydrolysis kinetics of LAB in aqueous solution were investigated. The degradation products were identified by HPLC and LC–MS, and a new degradation mechanism was proposed. This information will be useful for understanding the chemical stability of LAB and the development of suitable formulations and proper storage conditions.

## 2. Materials and methods

### 2.1. Materials

LAB was isolated from the roots of danshen in Dalian Institute of Chemical Physics. DSU and protocatechuic aldehyde were purchased from the National Institute for the Control of Biological and Pharmaceutical Drugs (Beijing, PR China), the purity of these three compounds was above 98.5%. All other chemicals used in this experiment were analytical or HPLC grade. The double-distilled water was used for mobile phase.

* Corresponding author. Tel.: +86 411 84706369; fax: +86 411 84706369.
*E-mail address:* zhlxiu@dlut.edu.cn (Z.-L. Xiu).

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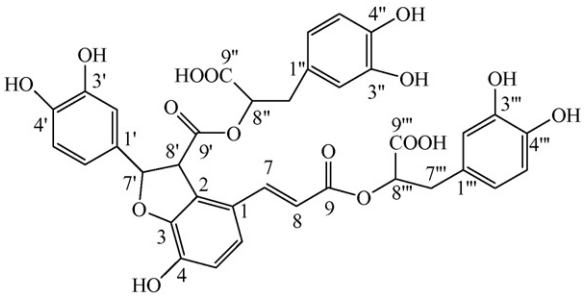



Fig. 1. Structure of LAB (M.F. $C_{36}H_{30}O_{16}$, M.W. 718).

### 2.2. HPLC analyses

HPLC-UV analysis was carried out on Agilent 1100 Series with G1314A UV–vis detector using an Agilent RP-8 column (200 mm × 4.6 mm, 5 μm). The column was maintained at 30 °C. Detection wavelength was 288 nm. The flow rate was 1.0 ml/min. A gradient elution comprising Solvent A (1%, v/v acetic acid in water) and Solvent B (acetonitrile:methanol, 3:2, v/v) was used. The mobile phase composition was changed using a linear gradient from 80:20 (A:B, v/v) to 70:30 (A:B, v/v) over 0–15 min, then remained constant at 70:30 (A:B, v/v) over 15–25 min. The system was recovered by returning to a composition of 80:20 (A:B, v/v) over 2 min and equilibrating the column for 8 min.

### 2.3. HPLC-ESI/MS analyses

The HPLC conditions for LC–MS analysis were as described above. A Finnigan (San Jose, CA, USA) TSQ (Triple-Stage Quadrupole) mass spectrometer equipped with an APCI interface was employed with nitrogen as the nebulising gas and the auxiliary gas. The parameters of APCI were as follows: auxiliary gas pressure 20 arbitrary units; sheath gas pressure 40 psi; spray voltage 4.5 kV; capillary temperature 325 °C. Initially, the mass spectrometer was programmed to perform full scans between 120 and 1200 *m/z* in order to obtain molecular ion signals as well as fragments or adducts of the possible degradation products in negative ion mode.

### 2.4. Stability studies of LAB

In the stability studies, LAB was dissolved in double-distilled water to prepare a stock solution with a concentration of 10 mg/ml. The final concentration for all stability samples was about 0.4 mg/ml, which was obtained by mixing a 4:96 ratio of the stock solution with the appropriate buffer solution. Stability samples were sealed in screw-topped test tubes and then laid in a thermostat bath at predefined temperature. Aliquots of the solutions periodically withdrew during a kinetic run were rapidly cooled in ice to quench the reaction, or diluted with acetate at pH 2–3 when it was necessary. Solutions were stored in an ice bath for analysis within 6 h. Ten microlitres of sample was injected into a reversed-phase HPLC column for analysis.

### 2.5. Effect of buffer ions

The influence of buffer ions on $k_{obs}$ was determined for acetate and phosphate by performing experiments at buffer concentration between 20 and 200 mM at constant pH. The ionic strength was adjusted to 0.5 by using sodium chloride.

### 2.6. Effect of pH

The influence of pH on $k_{obs}$ was determined between pH 0.35 and 7.05. Buffers used were HCl below pH 1.20, acetate in the pH range 3.5–5.5, phosphate in the pH range 2.0–3.0, and 6.0–7.05, respectively. All the pH measurements were performed on a pH meter (Sartorius, Germany) equipped with a combination electrode, which was calibrated with primary buffer solution (Tianjin Kermel Chemical Reagent Co., Ltd.) of pH $4.01 \pm 0.01$, $6.86 \pm 0.01$ and $9.18 \pm 0.01$. The ionic strength of buffers was adjusted to 0.5 by using sodium chloride. Data acquisition was analysed using origin V 7.0 software (OriginLab company, USA).

### 2.7. Effect of temperature

The hydrolysis kinetics and its observed rate constant ($k_{obs}$) were determined at 50, 60, 70, 80 and 90 °C. Arrhenius plots were used to determine the activation energy ($E_a$) and the half-life of LAB. The influence of temperature on $k_{obs}$ was determined at pH 0.93 and 2.0 using 0.2 M hydrochloric acid and 48 mM phosphate buffer solution, respectively.

## 3. Results and discussion

### 3.1. Hydrolysis of LAB

Eleven peaks were selected to monitor during LAB degradation studies as labelled in Fig. 2. Peaks 10 and 11 were consistent with 9″-methyl lithospermate B and 9‴-methyl lithospermate B reported in previous work [10] and were observed in small

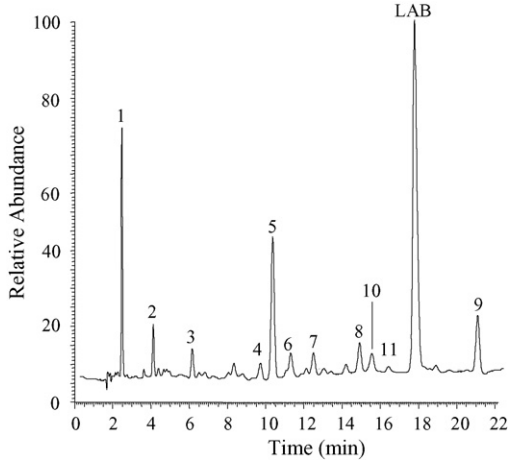



Fig. 2. HPLC chromatogram of LAB solution at pH 0.93 after heating 7 h at 90 °C (hydrolysis not completed).

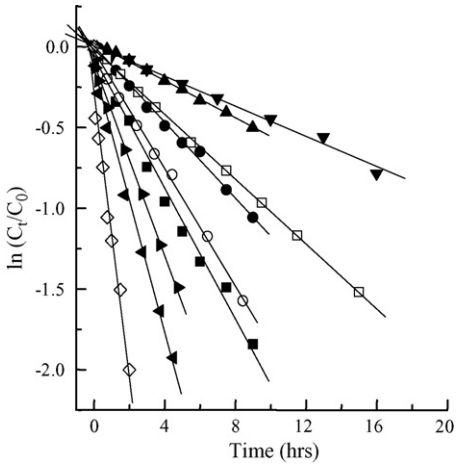



Fig. 3. Pseudo-first-order plots for the hydrolysis of LAB in HCl and 0.05 M buffer solutions at 90 °C. (■) pH 0.35, (●) pH 0.66, (▲) pH 1.20, (▼) pH 2.0, (□) pH 3.0, (○) pH 4.0, (►) pH 5.0, (◄) pH 6.0, and (◊) pH 7.0.

amounts in the purified LAB sample. The other peaks (1–9) were further analysed by LC–MS.

### 3.2. Determination of first-order rate constants

For a given initial concentration of LAB at constant temperature and pH, the change of LAB concentration versus time could be depicted as shown in Fig. 3. The linear relationship between $\ln(C_t/C_0)$ and time shows that the degradation of LAB followed a first-order reaction kinetics. The pseudo-first-order rate constants ($k_{obs}$) were calculated from first-order plots based on the following Eq. (1):

$$\ln\frac{[C_t]}{[C_0]} = -k_{obs}t \quad (1)$$

where the initial concentration of LAB is $[C_0]$, the time-dependent concentration is $[C_t]$, and $t$ is time. Each study was comprised of eight or more assays spaced to provide changes of ~0.1 $[C_0]$ per sampling interval. The observed first-order rate constant ($k_{obs}$) was obtained by linear regression for data in the range from $[C_0]$ to ~0.25 $[C_0]$. The first-order plots under all experimental conditions yielded correlation coefficient over 0.99 ($p < 0.0001$).

### 3.3. Influence of buffer catalysis

In the kinetic studies, the catalytic effect of phosphate and acetate buffer on the observed degradation rate constant ($k_{obs}$) was performed at the concentration of 0.4 mg/ml LAB, with the changing of the total buffer concentration. Fig. 4 illustrates the dependency of the observed first-order rate constants for LAB degradation on the buffer concentration according to Eq. (2):

$$k_{obs} = k_{pH} + k_{buffer}[B] \quad (2)$$

where $k_{pH}$ is the value in the absence of buffer catalysis and $k_{buffer}$ is the catalytic constant for buffer at 90 °C. The results indicate that the observed rate constants increased linearly with

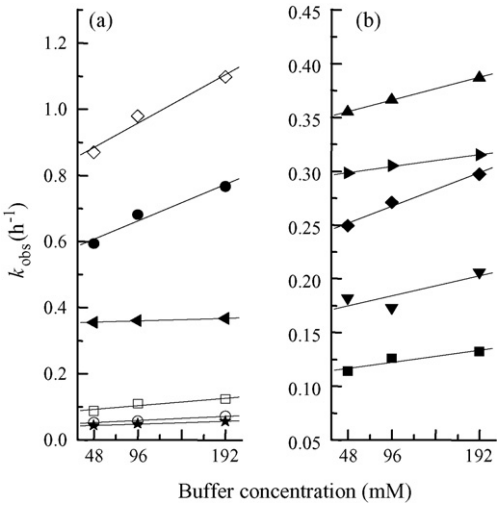



Fig. 4. Plots of $k_{obs}$ vs. the total buffer concentration at 90 °C and ionic strength of 0.5 for the degradation of LAB. Buffer solution: (a) phosphate and (b) acetate. pH values: (◊) pH 7.05, (●) pH 6.5, (◄) pH 6.0, (▲) pH 5.5, (►) pH 5.0, (♦) pH 4.5, (▼) pH 4.0, (■) pH 3.5, (□) pH 3.0, (○) pH 2.5, and (⋆) pH 2.0.

the increase of buffer concentration. As intercepts, $k_{pH}$, was obtained by extrapolation of the curves for each pH values.

### 3.4. pH-rate profile of LAB

The stability of LAB over the pH range 0.35–7.05 was investigated at 90 °C using different buffers. The pH-rate profile of LAB as shown in Fig. 5 demonstrated that the maximum stability was obtained at about pH 2.0. The hydrolysis was catalyzed by hydrogen ion, hydroxyl group and water molecules. At pH < 2, the rate of degradation is rapid, indicating specific hydrogen-ion catalysis. At pH 2.0–7.0, the rate of degradation increases with pH value. The curve exhibited an inflection in the region of pH 4.0–6.5. The inflections are frequently observed near p$K_a$ of substrate, when the reactivity of protonated and unpronated forms are sufficiently different. The similar profiles were obtained for ceftazidime [11] and temocillin [12]. Thus, the pH-rate profile

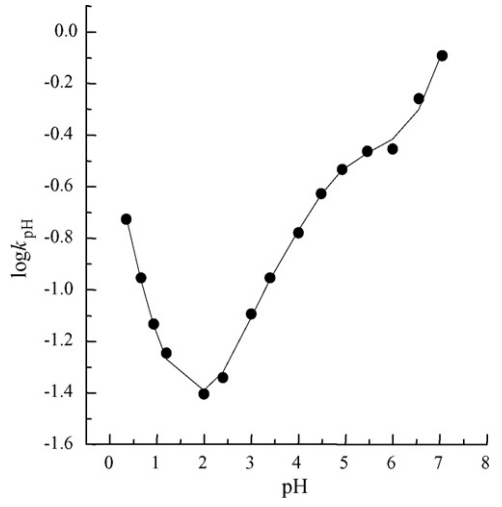



Fig. 5. Effect of pH on degradation rate constant ($k_{pH}$) of LAB at 90 °C and a constant ionic strength of 0.5.

Table 1
Rate constants from the reaction in solution at various temperatures

| pH | $k_{obs}$ ($h^{-1}$)[a] | | | | |
|---|---|---|---|---|---|
| | 50 °C | 60 °C | 70 °C | 80 °C | 90 °C |
| 0.93 | 0.00414 | 0.00874 | 0.02360 | 0.04357 | 0.06932 |
| 2.0 | 0.00391 | 0.00765 | 0.01864 | 0.02759 | 0.04326 |

[a] Results are presented as means for replicate analyses.

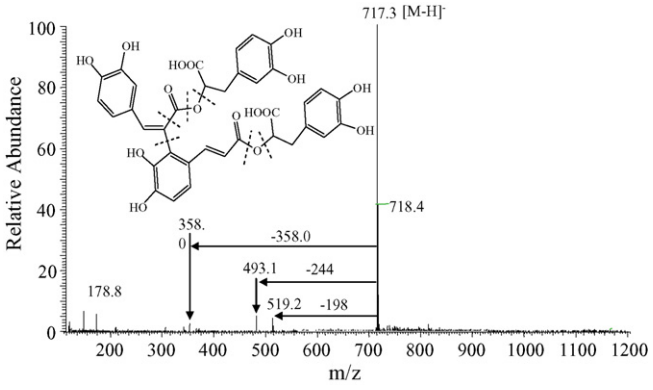



Fig. 6. Proposed structure and tandem mass spectra of Degradate **9**.

is described by the Eq. (3):

$$k_{pH} = k_{H1}(a_{H^+})f_1 + k_{H2}(a_{H^+})f_2 + k_{H3}(a_{H^+})f_3 + k_{H_2O}f_3 + k_{OH^-}(a_{OH^-})f_3 \quad (3)$$

where $k_{H1}$, $k_{H2}$ and $k_{H3}$ are catalytic second-order rate constants ($M^{-1}\,min^{-1}$) for hydrogen-ion activity ($a_{H^+}$), $k_{OH}$ the catalytic second-order rate constant ($M^{-1}\,min^{-1}$) for hydroxyl ion activity ($a_{OH^-} = K_w/[a_{H^+}]$) and $k_{H_2O}$ is the first-order constant ($h^{-1}$) for spontaneous hydrolysis. The values for $K_w$ as a function of temperature were obtained from Harned and Owen [13]. LAB has two carboxylic acid groups (Fig. 1), the fractions of LAB as the dicarboxylic acid ($f_1$), the monocarboxylate ($f_2$) and the dicarboxylate ($f_3$) were calculated from Eqs. (4)–(6) with the dissociation constants ($K_{a1}$ and $K_{a2}$):

$$f_1 = \frac{a_{H^+}^2}{a_{H^+}^2 + a_{H^+}K_{a1} + K_{a1}K_{a2}} \quad (4)$$

$$f_2 = \frac{a_{H^+}K_{a1}}{a_{H^+}^2 + a_{H^+}K_{a1} + K_{a1}K_{a2}} \quad (5)$$

$$f_3 = \frac{K_{a1}K_{a2}}{a_{H^+}^2 + a_{H^+}K_{a1} + K_{a1}K_{a2}} \quad (6)$$

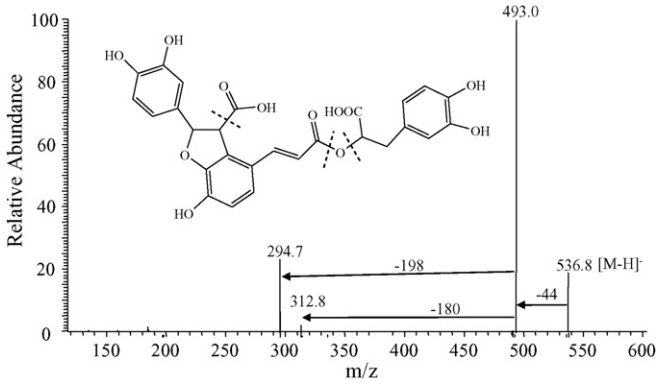



Fig. 7. Proposed structure and tandem mass spectra of Degradeate **8**.

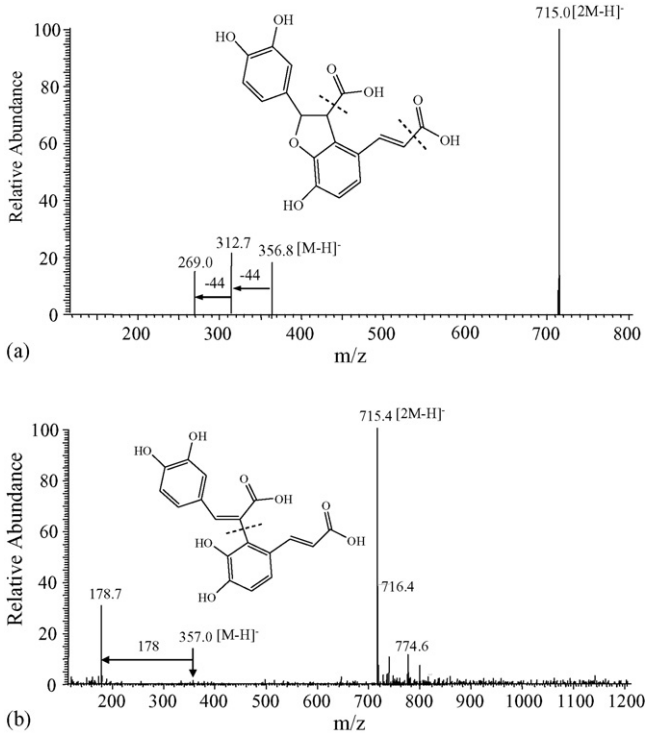


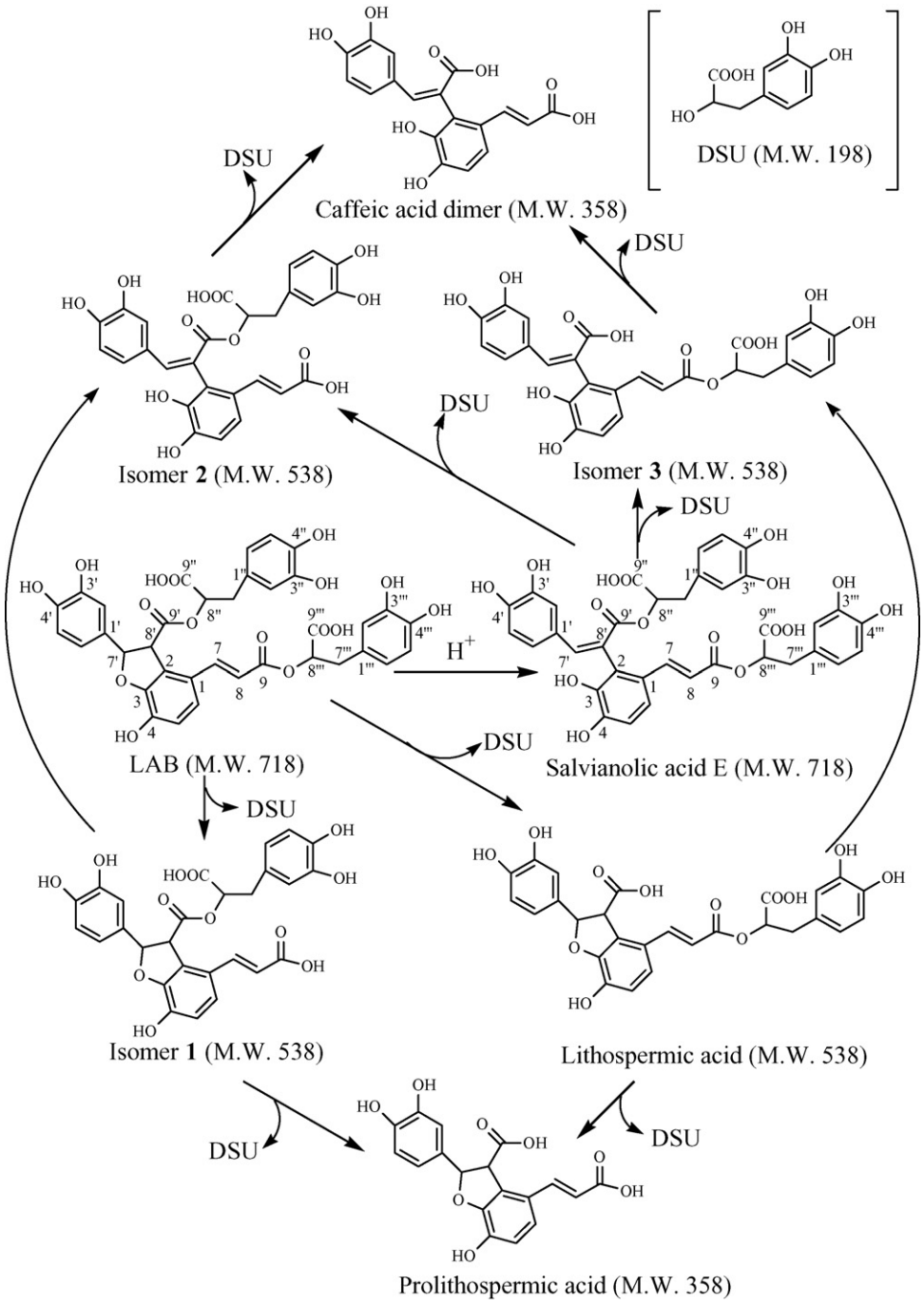

Fig. 8. Proposed structures and tandem mass spectra of Degradates **3** (a) and **4** (b).

The rate constants were estimated by the non-linear least squares regression by fitting Eq. (3) to the experimental rate constants at various pH: $k_{H1} = 0.36\,M^{-1}\,h^{-1}$, $k_{H2} = 41.49\,M^{-1}\,h^{-1}$ $k_{H3} = 4.45 \times 10^3\,M^{-1}\,h^{-1}$, $k_{H_2O} = 0.35\,h^{-1}$, $K_{OH} = 1.13 \times 10^5\,M^{-1}\,h^{-1}$, $K_{a1} = 7.30 \times 10^{-4}$ ($pK_{a1} = 3.14$) and $K_{a2} = 3.00 \times 10^{-5}$ ($pK_{a2} = 4.52$). The calculated values are in good fit with the experimental data

Table 2
Activation energies, the frequency factors and half-life for LAB in aqueous solution at pH 0.93 and 2.0

| pH | $E_a$[a] (kJ/mol) | ln $A$[a] | $R$[a] ($p < 0.06$) | $E_a$[b] (kJ/mol) | ln $A$[b] | 20 °C (half-life)[b] | $R$[b] ($p < 0.06$) |
|---|---|---|---|---|---|---|---|
| 0.93 | 55.99 | 15.88 | 0.99 | 80.39 | 24.35 | 162 | 0.99 |
| 2.0 | 43.75 | 11.32 | 0.99 | 72.14 | 21.23 | 124 | 0.99 |

[a] Data obtained on basis of the degradation rates for hydrolysis at temperatures of 70, 80 and 90 °C.
[b] Data obtained on basis of the degradation rates for hydrolysis at temperatures of 50, 60 and 70 °C.




Scheme 1. Proposed degradation pathway for LAB.

($r = 0.99$, $p < 0.05$), and this equation adequately describes the degradation kinetics in the pH range studied.

### 3.5. Influence of temperature

The rate constants at various temperatures in solution at pH 0.93 and 2.0 were shown in Table 1. The influence of temperature on reaction rate constant ($k_{obs}$) was given by Arrhenius equation as follows (7):

$$\ln k_{obs} = \ln A - \frac{E_a}{RT} \quad (7)$$

where $A$ is the frequency factor, $E_a$ the energy of activation, $R$ the universal gas constant, and $T$ is the absolute temperature. The linear relationship between $\ln k_{obs}$ and $1/T$ was investigated. The curve may be divided into two lines at 70 °C. This implied that the activation energy and frequency factor are dependent on the temperature. Table 2 compares the activation energies and frequency factors obtained at temperatures from 70 to 90 °C and from 50 to 70 °C, respectively. It shows that activation energies are higher at low temperatures than those at high temperatures. This might be dependent on the complex structure of LAB. If the mechanism is identical at below 70 °C, the theoretical half-life

of LAB at room temperature would be predicted according to the activation energy and frequency factor obtained. It is interesting that the extrapolated half-life value of 124 days at 20 °C is in agreement with an experimental value of 131 days obtained from sample at room temperature (approximately 20 °C) and pH 2.0. The short half-life suggests that LAB is not suitable for storage in aqueous solution for a long time.

### 3.6. Detection of degradation products

A tentative assignment of the degradation products was made on basis of quasi-molecular ion $[M-H]^-$ and fragment ions obtained from LC–MS experiments. It was supported by the above kinetic knowledge of ester group degradation processes and by comparison of their MS spectra with those reported in the literatures [14,15].

Degradates **1** and **2** were identified as DSU and protocatechuic aldehyde according to their MS with $[M-H]^-$ ions at *m/z* 197 and 137, respectively. In addition, they have been further determined by comparing the retention times with those of standard compounds.

Compared to LAB, Degradate **9** is non-polar as it elutes after the parent compound (Fig. 2). Further examination of the MS spectra associated with Degradate **9** shows $[M-H]^-$ ion at 717, which suggested that it is the isomer of LAB. Its characteristic fragments are detected at *m/z* 519, originating from the loss of DSU (198u), and at *m/z* 493 resulting from the concurrent losses of DSU (198u) and $CO_2$ (44u). Fragmentation patterns of Degradate **9** are slightly different from the parent compound LAB, which may be due to differences of chemical environment around benzofuran moiety. It was assigned as salvionolic acid E, which was formed via ring opening of benzofuran of LAB under mild acidic condition. An additional evidence for the structure assignment of Degradate **9** was obtained by usually detecting salvianolic acid E in decoction [16]. The tandem mass spectrum and the structure of Degradate **9** are shown in Fig. 6.

Degradates **5**,**6**,**7** and **8** are four major degradation products over a range of pH values (0.35–7.05). The same $[M-H]^-$ at *m/z* 537 suggested that they were isomers (M.W. 538) associated with cleavage of one of the two ester bonds from LAB or salvianolic acid E. Degradate **8** (Fig. 7) first lost 44u ($CO_2$) from the $[M-H]^-$ ion to form the abundant $[M-H-CO_2]^-$ ion at *m/z* 493, that then lost 180u (caffeic acid) or 198u (DSU) to yield *m/z* 313 and 295, respectively. The facile loss of one molecule of $CO_2$ from Degradate **8** in the MS/MS spectrum suggests that carboxyl substituented on the benzofuran ring. So it was assigned as lithospermic acid. No more MS information can identify the difference of other three products.

The MS of Degradates **3** and **4** show the same $[M-H]^-$ ions at 357, and $[2M-H]^-$ ions at 715. They are isomers (M.W. 358) associated with cleavage of two ester bonds from LAB or salvianolic acid E. The characteristic fragments of Degradate **3** (Fig. 8a) are detected at *m/z* 313 $[M-H-CO_2]^-$, and 269 $[M-H-2CO_2]^-$. Similarly as discussed previously for Degradate **8**, the facile loss of $CO_2$ from Degradate **3** in the MS/MS spectra suggests that Dgradate **3** has one carboxylic group substituented on the benzofuran ring, so it was assigned as prolithospermic acid. Degradate **4** (Fig. 8b) easily lost its caffeic acid moiety (178u) and formed stable fragment ion at *m/z* 179. It was speculated to be caffeic acid dimer, which is a compound commonly observed in the danshen decoction.

### 3.7. Proposed degradation mechanisms

After the identification of the various LAB degradation products, a tentative pathway for degradation of LAB in water at 90 °C is proposed (Scheme 1). LAB accepts a proton to form salvianolic acid E. They are hydrolyzed at the ester bond of C-9′ or C-9 to release DSU, lithospermic acid or its isomers labelled by 1, 2, and 3, respectively. Subsequently, isomer **1** and lithospermic acid can then undergo the ester hydrolysis to yield prolithospermic acid and DSUs. Alternatively, isomer **1** and lithospermic acid can also undergo the furan ring-opened to form isomers **2** and **3** of lithospermic acid in the mild acid solution, respectively. Isomers **2** and **3** are not stable and are hydrolyzed to release DSU and caffeic acid dimer.

Degradate **2** (protocatechuic aldehyde) could not be explained on basis of the above degradation route. It may be an oxidative product of DSU.

## 4. Conclusion

The hydrolysis of LAB obeyed a pseudo-first-order kinetics. It was observed that the degradation rate constants of LAB at 90 °C were highly dependent on pH. The optimal pH for stability was estimated to be pH 2. The catalytic effects of acetate and phosphate buffer on the rate constants were observed to increase linearly with the increase of buffer concentration. The hydrolysis rate constant is a function of temperature described as the Arrhenius equation. The activation energy at low temperature range was determined to be 80.39 and 72.14 KJ $mol^{-1}$ in aqueous solution at pH 0.93 and 2.0, respectively.

The parent compound LAB and its degradation products could be well separated and detected by the established HPLC. Nine degradation products were characterized by LC–MS and compared with the available standards. These degradation products are proposed to form through main pathway including the ring-open of benzofuran and the cleavage of the ester bonds in the parent compound LAB.

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