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Hydrolytic kinetics of lithospermic acid B extracted from roots of *Salvia miltiorrhiza*

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Abstract

The hydrolytic kinetics of lithospermic acid B (LAB) extracted from the roots of *Salvia miltiorrhiza* (Chinese herb: danshen) was investigated by using reversed-phase high-performance liquid chromatography (HPLC) with UV–vis detection. The influences of initial drug concentration, pH and temperature on hydrolysis of LAB were studied in aqueous solutions. The results showed that initial concentration of LAB has no effect on the degradation rate at pH 2.0. The hydrolysis followed pseudo-first-order kinetics at 90 °C. The log k_{obs}-pH profile indicated that the optimal stability range was at pH 2.0–5.0. The rate constant of overall hydrolysis as a function of temperature under the given conditions obeyed the Arrhenius equation. Analysis of the acid-induced degraded solution of LAB by liquid chromatography–mass spectrometry (LC–MS) revealed at least four degradation products $[M-H]^-$ ion at *m/z* 197, 137, 537 and 537, respectively. Three of these degradation products, i.e. danshensu (DSU), protocatechuic aldehyde (PRO), and lithospermic acid, were further identified by comparing the retention times with standard samples. According to the structure of LAB and its hydrolysis behavior in solution, the other product was proposed to be the isomer of lithospermic acid.

Keywords: Lithospermic acid B; Hydrolysis; Kinetics; LC/MS

1. Introduction

Danshen, a Chinese medicine, is the dried root and rhizome of *Salvia miltiorrhiza* Bunge [1]. Traditionally, danshen has been widely used for the treatment of coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrheal and neurasthetic insomnia [2,3]. The chemical constituents of *S. miltiorrhize* mainly include lipophilic diterpenoid quinines and water-soluble phenolic acids. Danshen is mainly used as a decoction in traditional Chinese medicinal prescription. Therefore the water-soluble phenolic acids should be responsible for the therapeutic effects of this medicinal plant. The pharmacological studies on phenolic acids of *S. miltiorrhiza* showed that polyphenolic acids such as salvianolic acid A and lithospermic acid B (LAB, also named salvianolic acid B) possessed more potent biological activities than single phenolic acids such as danshensu (DSU), caffeic acid and protocatechuic aldehyde (PRO) [4]. However, polyphenolic acids were not stable in aqueous solution, and the hydrolysis of polyphenolic acids resulted in the loss of the clinical efficacy of danshen products.

Among water-soluble components, LAB (see Fig. 1) is the most common in salvia species and the most abundant in decoction. Recent pharmacological studies indicated that LAB has strong antioxidant and free radical scavenging activity [5–8]. In addition, LAB has been indicated to protect against renal dysfunction, liver damage and lung fibrosis [9–11]. As a result, there is a great interest in LAB as a new drug in the pharmaceutical field. The pharmacokinetic and metabolic investigations for LAB in biological samples have been reported in the literature [12–14]. However, to our knowledge, no publication describing the kinetics and hydrolytic products of LAB in the aqueous solution was found.

Undoubtedly, the stability of polyphenolic acids in aqueous solution is very important for extraction from *S. miltiorrhiza*, drug formulation, storage, and analysis. The present study will

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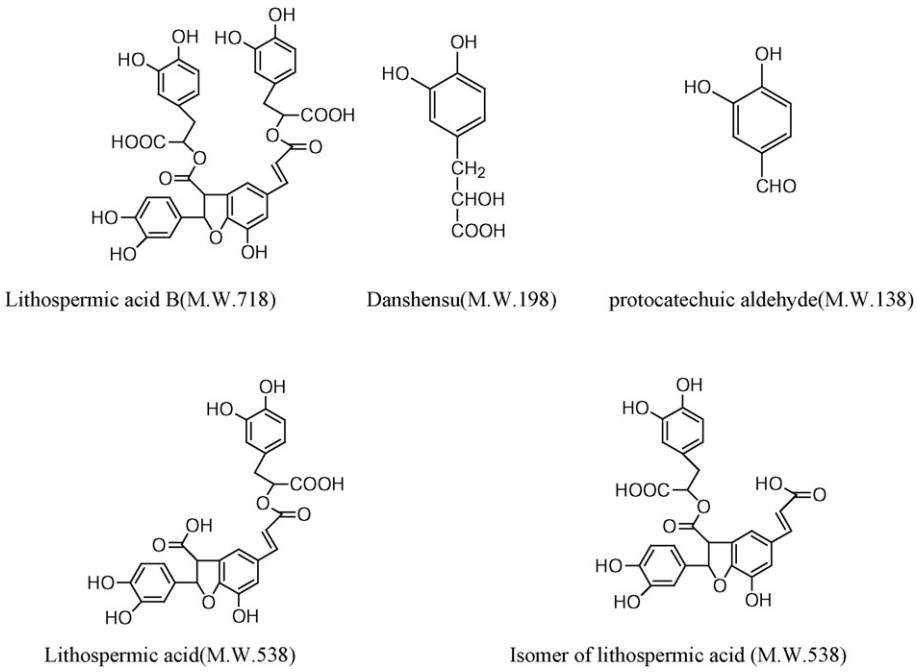

Fig. 1. Carbon skeletons of LAB and its degradation products.

investigate the hydrolytic kinetics of LAB and hydrolytic conditions, e.g. solvent, pH, temperature, initial concentration.

2. Materials and methods

2.1. Reagents and drugs

LAB, DSU and PRO were purchased from the National Institute for the Control of Biological and Pharmaceutical Drugs (Beijing, PR China), the purity of these compounds was above 98.5%. Acetonitrile (HPLC grade) was obtained from Honeywell international Inc. (USA), and methanol (HPLC grade) from TEDIA Company Inc. (USA). All the other chemicals, including buffer components, were analytical-reagent grade.

2.2. Instruments and conditions

HPLC-UV analysis was carried out on Agilent 1100 Series with G1314A UV–vis detector using a 5 μm Kromasil RP-18 (250 mm × 4.6 mm). The column was maintained at 30 °C. Detection wavelength was 288 nm. The flow rate was 1.00 ml/min. A linear gradient elution of A (acetonitrile:methanol = 3:2, v/v) and B (1% v/v aqueous acetic acid) were used starting with 20% A and 80% B to reach 30% A and 70% B at 68 min. Then the system was recovered by decreasing mobile phase B to 20% at 70 min, and the column was equilibrated with 20% A and 80% B for 8 min.

MS experiments were performed on a triple-quadrupole mass spectrometer (TSQ, Finnigan MAT, San Jose, USA) equipped with an APCI interface. Auxiliary gas at 20 arbitrary units, and sheath gas at 40 psi. Initially, the mass spectrometer was programmed to perform full scans between 120 and 1200 *m/z* in order to observe molecular ion signals as well as fragments or adducts in negative ion mode. Spray voltage was 4.5 kV, capillary temperature was 325 °C.

2.3. Validation procedures

The stock solution (600 μg/ml) was prepared by dissolving an appropriate amount of solid substance of LAB in methanol. The calibration curve was made using six standard solutions of different concentrations (30, 60, 100, 200, 300, 600 μg/ml). The standard solutions were prepared by diluting an appropriate volume of stock solution with methanol. Each solution was analyzed in twice. The peak area values were plotted against the corresponding analyte concentrations to obtain the linear calibration. Six injections of one concentration of LAB (300 μg/ml) were analyzed on the same day. The value of relative standard deviation of this assay was calculated to determine precision. Accuracy was evaluated as a percentage of recovery obtained from analysis of samples spiked with known amount of LAB (25, 65, 150 μg/ml).

2.4. Sample preparation

The root powder of *S. miltiorrhiza* Bunge (300 g) was immersed in 3000 ml of double distilled water for 5 h at room temperature. The mixture was then adjusted to pH 2.5 with HCl, and extracted at 90 °C for 40 min. The aqueous extract was purified using a DM301 macroporous resin. Each column was eluted with copious amounts of distilled water to remove the polysaccharide and protein, and was eluted with ethanol of 20, 50% to yield solution contained different compounds. The fraction of 20% aqueous of ethanol corresponding to salvianolic acid B was collected and evaporated under reduced pressure at 60–70 °C.

The residue was freeze-dried and the sample containing 70% LAB was obtained.

2.5. Hydrolytic experiments

LAB sample was dissolved in water to prepare a stock solution with a concentration of 5 mg/ml. The final concentration for all stability samples was 0.2 mg/ml, which was obtained by mixing a 4:96 ratio of stock solution with the appropriate experimental solution. Stability samples were sealed in screw-topped test tubes and then laid in a thermostat bath at 90 °C (stable to ±0.5 °C). Samples periodically withdrawn during a kinetic run were rapidly cooled in ice to quench the reaction, or diluted 1:2 with 0.2 M acetate at pH 2–3 when it was necessary. Solutions were stored in an ice bath for analysis within 12 h. A 20 μl of sample was injected into a reversed-phase HPLC column for analysis.

In the hydrolytic experiments, the influences of pH, temperature, buffer concentration and initial drug concentration on hydrolysis of LAB were investigated. The influence of pH on hydrolysis was determined at 90 °C in hydrochloric acid (pH 0.51–1.27) and phosphate buffer (pH 2.0–7.05). The ionic strength was adjusted to 0.5 using sodium chloride. All the pH measurements were performed on a pH meter (Sartorius, Germany) equipped with a combination electrode, which was calibrated with primary buffer solution of pH 4.01, 6.86 and 9.18. The effect of temperature on hydrolysis was investigated by using phosphate buffer solution at pH values of 2.0, 3.0 and 4.0, and the reaction rate constants were determined at 70, 80 and 90 °C, respectively. The hydrolysis rate constants from phosphate buffer solutions with three different buffer concentrations at 90 °C were analyzed by one-way ANOVA ($\alpha = 0.05$) to test whether the variation appeared in the hydrolysis rate constants was attributed from the concentration of buffer. The effect of initial drug concentration on hydrolysis was performed by using phosphate buffer (pH 2.0) at a drug concentration from 0.2 to 4.56 mg/ml at 90 °C.

3. Results and discussion

3.1. Method validation

The linearity of this method was proved using linear correlation of the peak area values and appropriate concentration of LAB in range of 30–600 μg/ml. The correlation coefficient of this dependence was calculated to be 0.999. The precision of method was determined to be 2.21. The results of method accuracy are presented in Table 1.

Table 1
Accuracy of this method

Concentration added (μg/ml)	Concentration calculated ± S.D.; R.S.D.%	Recovery (%)
251	248.05 ± 2.72; 1.10	98.825
115	116.43 ± 1.47; 1.27	101.24
45	44.89 ± 0.82; 1.84	99.762

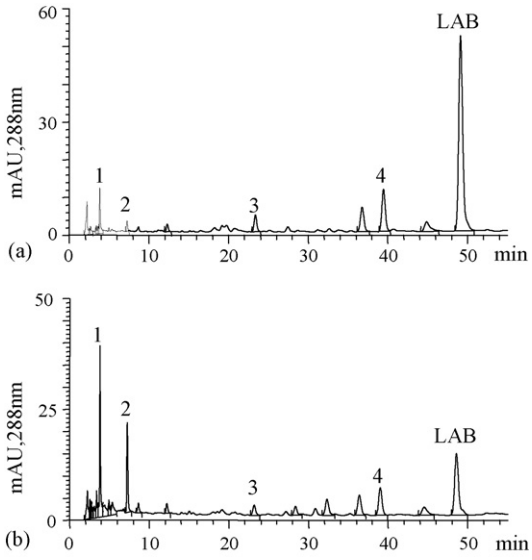

Fig. 2. HPLC chromatograms of danshen extract (a) and hydrolysate in solution at pH 5.99 for 7 h (b). The peaks from 1 to 4 represent DSU, PRO, isomer of lithospermic acid and lithospermic acid, respectively.

3.2. Identification of degradation products

Typical HPLC chromatograms of LAB and its hydrolytic products under optimal chromatographic conditions were shown in Fig. 2. Comparing chromatogram Fig. 2a with Fig. 2b, it was clearly observed that the mainly peaks 1 and 2 increased and peaks 3 and 4 and LAB decreased during the hydrolytic time-course at 90 °C. Based on the studies of heating solution of pure LAB, the compounds corresponding peaks 1–4 were determined as the main degradation products.

Samples were analyzed using LC–MS in a negative-ion mode over mass range from *m/z* 120 to 1200. Peak 1 was identified as DSU according to its MS with a quasi-molecular ion peak at *m/z* 197 $[M-H]^-$, and *m/z* 395 $[2M-H]^-$. Peak 2 was identified as PRO on the basis of its MS containing a quasi-molecular ion peak at *m/z* 138 $[M-H]^-$, and *m/z* 275 $[2M-H]^-$. In addition, compounds 1 and 2 have been further determined by comparison of retention times with those of reference compounds. Peaks 3 and 4 are two major products in all pH ranges, the same $[M-H]^-$ at *m/z* 537 and *m/z* 1075 $[2M-H]^-$ suggested that they were isomers. Peak 4 was confirmed as lithospermic acid by comparison of retention time with authentic compound. According to peak 3 behavior in the degradation process and Ref. [15], it was ascribed to isomer of lithospermic acid.

This hydrolytic process could be observed more clearly in the time-course for the change of objective components as shown in Fig. 3. It can be seen that DSU and PRO increased as LAB degraded, lithospermic acid and isomer of lithospermic acid increased firstly and then decreased. From the structure of LAB, the formation of lithospermic acid and its isomer are two prominent intermediates, because both are attributed to one of the two esters hydrolysis reaction of parent compound LAB. They can then subsequently undergo the ester hydrolysis to yield DSU.

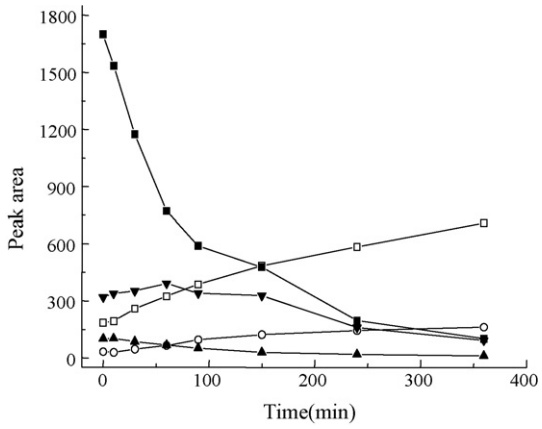

Fig. 3. Evolution of HPLC peak areas of LAB and degradation products as a function of hydrolysis time at pH 7.05 and 90 °C. (■) LAB; (□) DSU; (◯) PRO; (▼) lithospermic acid; (▲) isomer of lithospermic acid.

3.3. Determination of first-order rate constants

For a given starting concentration at constant temperature and pH, the hydrolysis of LAB followed a pseudo-first-order reaction kinetics because a linear relationship between natural logarithmic remaining percent of LAB and hydrolytic time existed as shown in Fig. 4. The observed first-order rate constant, k_{obs}, was determined from linear plots based on the following equation:

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

where t is time; $[C_0]$ and $[C_t]$ are the initial and time-dependent concentration of LAB, respectively. The apparent rate constants were obtained by linear regression ($r > 0.99$) for data in the range from $[C_0]$ to 0.25 $[C_0]$. Each study was comprised of eight or more assays spaced to provide changes of ~0.1 $[C_0]$ per samples.

The influence of initial concentration of LAB on the hydrolysis rate is shown in Fig. 5. It indicates that initial concentration of LAB has no effect on the degradation rate at pH 2.0 when the initial concentration of LAB is less than 5 mg/ml.

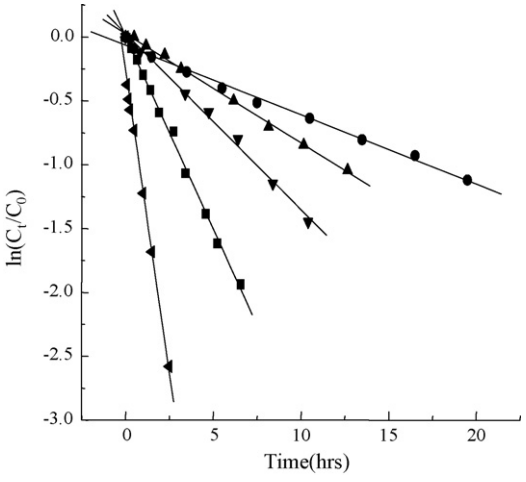

Fig. 4. Pseudo-first-order plots for hydrolysis of LAB at 90 °C in buffer solutions at pH (■) pH 0.51; (●) pH 2.0; (▲) pH 3.0; (▼) pH 4.87; (◄) pH 7.05.

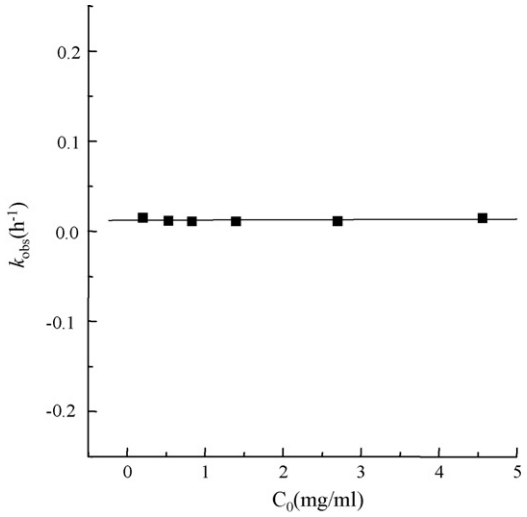

Fig. 5. Hydrolysis rate of LAB at different initial concentration in phosphate buffer at pH 2.0 and 90 °C.

3.4. Influence of pH on hydrolysis

The buffer concentrations and the corresponding hydrolysis rate constants in two pH values are shown in Table 2. The results showed that the significant effect of phosphate buffer concentration on the hydrolysis rate of LAB was observed in aqueous solutions at pH 2.0 and 3.0 ($P < 0.05$).

The pH-rate profile of LAB was obtained by plotting natural logarithm of k_{obs} versus pH values of solutions at 90 °C and a constant ionic strength of 0.5. As shown in Fig. 6, the plots contained three parts: proton- (pH < 2.0), hydroxyl- (pH > 6.0), and mixture-catalyzed hydrolysis (2.0 < pH < 6.0). It is easy to find the optimal pH for LAB stability, i.e. pH 2.0. However, danshen materials have traditionally been extracted at pH 5–7. As a result, a large amount of LAB is believed to be lost in the heating process, because the hydrolytic degradation of LAB was obviously enhanced in this pH range. Therefore, extraction of danshen should be performed at pH 2.0 to get a higher yield of LAB.

3.5. Influence of temperature on hydrolysis

The rate constants at various temperatures in phosphate buffer solution at pH 2.0, 3.0 and 4.0 are shown in Table 3. The influence of temperature on reaction rate constant was given by Arrhenius equation (2):

$$\ln k_{obs} = \ln A - \frac{E_a}{RT} \quad (2)$$

Table 2
Effects of concentration and pH of buffer on hydrolytic rate constants for LAB

pH	k_{obs} (h^{-1})[a]		
	0.05 M	0.10 M	0.20 M
2.0	0.0376 ± 0.0027	0.0394 ± 0.0068	0.0488 ± 0.0058
3.0	0.0877 ± 0.0053	0.1089 ± 0.0103	0.1228 ± 0.0111

[a] Results are presented as means ± S.D. for quadruplicate analyses.

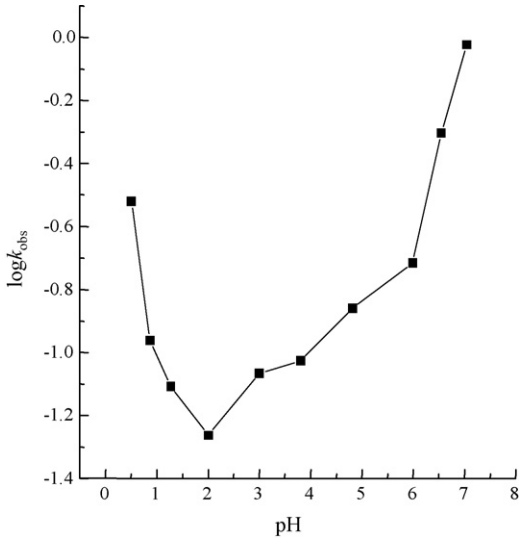

Fig. 6. Effect of pH on hydrolytic rate constant log k_{obs} of LAB at 90 °C and a constant ionic strength of 0.5.

Table 3
Rate constants from the reaction in 0.05 M phosphate at various temperatures (I = 0.5)

pH	k_{obs} (70 °C) (h^{-1})	r^a	k_{obs} (80 °C) (h^{-1})	r^a	k_{obs} (90 °C) (h^{-1})	r^a
2.0	0.0168	0.9896	0.0252	0.9885	0.0376	0.9909
3.0	0.0396	0.9962	0.0687	0.9984	0.0858	0.9988
4.0	0.0582	0.9987	0.1158	0.9984	0.1488	0.9974

[a] Correlation coefficient.

Table 4
Activation energies, frequency factors, k_{obs} (25 °C) and half-lives for LAB hydrolysis at various pH values

pH	E_a (kJ mol^{-1})	ln A	k (25 °C) (h^{-1})	Half-life (days)
2.0	41.72	10.54	1.84×10^{-3}	15.67
3.0	40.20	10.91	4.94×10^{-3}	5.85
4.0	48.83	14.33	4.66×10^{-3}	6.20

in which A represents frequency factor while E_a stands for activation energy. R is ideal gas constant (8.314 J mol^{-1} K^{-1}) and T is temperature (K). The data obtained were fitted with Eq. (2) and the results were shown in Table 4. These activation energies are in agreement with those reported [16,17] for different drugs containing ester groups in their structures, which suggested that hydrolysis of LAB is relevant to ester bond. If the hydrolysis mechanism in the interested temperature range was identical, the activation energy and the frequency factor could be further applied to predict the theoretical half-life of LAB. The theoretical rate constants at 25 °C were also shown in Table 4. These data demonstrate a very short shelf life for LAB when stored above pH 3.0 in aqueous solution. The formulation of aqueous injections should be avoided under normal circumstances. It is possible that similar degradation process may be occurred when moist granulation procedures are employed in the preparation of solid dosage forms.

4. Conclusion

The stability of LAB was investigated using a stability-indicating HPLC procedure. The method permits detection and quantification of LAB in the presence of its degradation products. The kinetic studies indicated that hydrolysis rate constants are highly dependent on pH value, and the maximal stability of LAB was observed at pH 2.0. The influence of phosphate buffer concentration on hydrolysis at pH 2.0, 3.0 was studied, and the significant effects were observed. Arrhenius equation for hydrolysis rate constant as a function of temperature was obtained, and the half-life at 25 °C was predicted to be 15.67 days in a solution at pH 2.0. Initial concentration of LAB has no effect on degradation rate at pH 2.0. Based on the results obtained in present work, it is possible to decide the optimal operating conditions of temperature and pH, under which storage and extraction process for LAB should be performed.

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