Structure elucidation of degradation products of *Z*-ligustilide by UPLC-QTOF-MS and NMR spectroscopy

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Abstract: *Z*-Ligustilide, a major phthalide isolated from a widely used traditional Chinese medicine *Ligusticum chuanxiong*, possesses various pharmacological activities including neuroprotective, anti-inflammatory, anti-proliferative and vasorelaxing effects. However, it is unstable and inclined to degrade in natural conditions, which limits its study and application greatly. In this study, degradation behavior of *Z*-ligustilide and its degradation products stored at room temperature under direct sunlight were investigated and structure elucidated by HPLC-UV, UPLC-QTOF-MS and NMR. *Z*-ligustilide degradation and total five degradation products were generated and detected. Two degradation products were unequivocally identified as senkyunolide I and senkyunolide H by comparison with reference compounds. Another two degradation products were further isolated by semi-preparative HPLC and structure elucidated as (*E*)-6, 7-trans-dihydroxyligustilide and (*Z*)-6, 7-epoxyligustilide by ^{1}H and ^{13}C NMR, respectively. The degradation pathways of *Z*-ligustilide were finally proposed. Oxidation, hydrolysis and isomerization are the major degradation reactions.

Key words: *Z*-ligustilide; degradation product; UPLC-QTOF-MS

CLC number: R917 **Document code**: A **Article ID**: 0513-4870 (2013) 06-0911-06

Z-藁本内酯降解产物的 UPCL-QTOF-MS 和 NMR 结构鉴定

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摘要: *Z*-藁本内酯是中药川芎中主要的苯酞类化合物, 具有神经保护、抗炎、抗增值及扩张血管等多种药理作用。但 *Z*-藁本内酯在自然条件不稳定且易于降解, 限制其研究和应用。本文采用 HPLC-UV、UPLC-QTOF-MS 和 NMR 对 *Z*-藁本内酯室温自然光照下的降解行为及降解产物进行考察和鉴定, *Z*-藁本内酯完全降解后生成 5 个降解产物, 2 个降解产物通过与对照品对比确定为洋川芎内酯 I 和洋川芎内酯 H, 2 个降解产物采用半制备 HPLC 分离后进行 ^{1}H 和 ^{13}C NMR 解析, 确定其结构为 (*E*)-6, 7-反式-双羟基藁本内酯和 (*Z*)-6, 7-环氧藁本内酯。最后推测了 *Z*-藁本内酯的降解途径, 氧化、水解和异构化是主要的降解反应。

关键词: *Z*-藁本内酯; 降解产物; UPLC-QTOF-MS

Rhizoma Chuanxiong, a famous traditional Chinese medicine, is used to treat cardiovascular diseases for centuries[1]. As most abundant phthalide in this herb, *Z*-ligustilide displays a wide range of pharmacological activities such as neuroprotective, anti-inflammatory, anti-proliferative and vasorelaxing activities[2−5]. It is considered as a major bioactive compound relevant to the therapeutic effects of Chuanxiong and has attracted great interest in recent years.

Received 2012-12-18; Accepted 2013-03-29.
Project supported by the National Science Foundation of China (No. 30973873); Key National Science and Technology Specific Projects (No. 2009ZX09502-023).
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However, with α, β-unstatured lactone in its structure, *Z*-ligustilide is a volatile and unstable compound. It can degrade into other phthalides through oxidation, isomerization or dimerization, *etc*, which limits its study and application greatly[6]. In order to explore better storage conditions and control its quality, affected factors including temperature, light, pH, co-solvents, antioxidants and methods of stabilization have been investigated and probed extensively[7−9]. Nevertheless, these studies can not resolve the unstable problem absolutely. At present, the degradation behavior of *Z*-ligustilide and its degradation products are scarcely studied and still unclear. Although 8 degradation products of *Z*-ligustilide were analyzed by GC-MS[10], these degradation products were only deduced by MS data and disagreed with another report, which indicated senkyunolide I, senkyunolide H and 4 dimmers were generated under different conditions[11]. Therefore, it is necessary to characterize the degradation products of *Z*-ligustilide and propose the clear degradation pathways, which will benefit for finding new bioactive compounds and further clarify the effective substance of *Z*-ligustilide and Chuanxiong.

In this study, degradation behavior of *Z*-ligustilide was investigated by HPLC-UV and five degradation products were tentatively identified by UPLC-QTOF-MS. Except for senkyunolide I and senkyunolide H, the other two degradation products were further isolated by semi-preparative HPLC and characterized by ^{1}H and ^{13}C NMR. Finally the degradation pathways of *Z*-ligustilide were proposed.

Materials and methods

Chemical and reagents HPLC-grade acetonitrile was purchased from Fisher Chemicals (Fisher Chemicals, USA). HPLC-grade methanol and formic acid were obtained from Yuwang (Yuwang, China). Water was purified with a Milli-Q system (Millipore, Bedford, USA). *Z*-ligustilide, senkyunolide I and senkyunolide H were isolated from *Ligusticum chuanxiong* and their structures were confirmed by comparing UV, MS and MS/MS data with literature. Their purities were determined to be above 95% by HPLC analysis. Other chemical reagents were all analytical-grade.

HPLC-UV analysis A Waters Alliance 2690 chromatographic system (Waters Corp, Milford, USA) was equipped with auto sampler, vacuum degasser and diode-array detector. Chromatographic separation was carried out on an Inertsil ODS-3 column (250 mm × 4.6 mm, 5 μm) with a flow rate of 1.0 mL·min^{-1}. The mobile phase consisted of acetonitrile (A) and water (B). A gradient program was adopted as follows: 5% − 10% A from 0 − 5 min, 10% − 70% A from 5 − 45 min, 70% − 100% A from 45 − 60 min. UV spectra were recorded from 210 nm to 400 nm and the detection wavelength was set at 280 nm.

UPLC-QTOF-MS analysis UPLC separation was carried out on a Zorbax Eclipse Plus C18 column (150 mm × 3.0 mm, 1.8 μm) at 40 ℃ using Agilent 1290 system (Agilent Technologies). The mobile phase consisted of acetonitrile (A) and 0.5% aqueous formic acid (B). Initial gradient conditions were 5% A with a linear rise to 100% A in 12 minutes with the flow rate of 0.6 mL·min^{-1}. Mass spectrometry was performed on Agilent 6520 QTOF-MS (Agilent Technologies) equipped with an ESI interface. The mass conditions were set as follows: drying gas, 8 L·min^{-1}; gas temperature, 320 ℃; nebulizer, 45 psi; capillary voltage, 4 kV; fragmentor, 120 V. It was programmed to perform full scan analysis over mass range of *m/z* 100 − 1 000 in positive ion mode. All the operation, acquisition and analysis of data were controlled by Agilent MassHunter Workstation software.

Semi-preparative HPLC Isolation of degradation products was carried out on a Waters Delta 4000 semi-preparative HPLC system (Waters Corp, Milford, USA). The samples were purified on C18 Nove-Pak and Chromatorex C18 column (250 mm × 20 mm, 10 μm). Methanol and water were used as mobile phase. The flow rate was 15 mL·min^{-1} and the detection wavelength was set at 280 nm.

NMR The ^{1}H NMR and ^{13}C NMR spectral data were generated using Varian NMR 500 MHz instrument. All sample solutions were prepared in deuterated chloroform. TMS was used as an internal standard.

Preparation of degraded samples 26 mg of *Z*-ligustilide (purity > 95%) was stored in room temperature under direct sunlight. After 60 and 120 days, the sample was resolved in methanol and injected to HPLC-UV for monitoring the degradation behavior.

Isolation of degradation products by semi-preparative HPLC 4.76 g of *Z*-ligustilide (purity > 85%) was stored at room temperature under direct sunlight for 120 days. It was firstly purified on C18 Nove-Pak column using methanol-water (70∶30) as mobile phase. Two fractions were obtained: fraction 1 (containing DP-1, 2 and 3) and fraction 2 (containing DP-4 and DP-5). It was discovered that DP-2 can

transform into DP-1 in natural conditions. Thus, fraction 1 was further stored at room temperature under direct sunlight for 4 months for the purpose of obtaining enough mount of DP-1. DP-1, DP-2 and DP-3 were finally purified on Chromatorex C18 column using methanol-water (50 ∶ 50) as mobile phase. Their purities were determined to above 98% by HPLC analysis. Fraction 2 was also purified on Chromatorex C18 column using methanol-water (50 ∶ 50) as mobile phase. DP-5 was obtained and its purity was determined to be above 98% by HPLC method. It is pity that due to its low content DP-4 was not obtained.

Results and discussion

1 Degradation behavior of *Z*-ligustilide by HPLC-UV analysis

The chromatograms of *Z*-ligustilide stored at room temperature under direct sunlight for 60 and 120 days are shown in Figure 1. After 60 days, *Z*-ligustilide (t_R: 44.03 min) degraded totally and five new degradation products named DP-1, 2, 3, 4, 5 were generated. Their peak area percentages are also listed in Figure 1. It is clear that DP-2 and DP-5 are the major degradation products. Compared with 60 days, after 120 days the peak area percentages of DP-2 and DP-5 decreased, while the peak area percentages of DP-1 and DP-4 increased, suggesting the possible transformation from DP-2 to DP-1, DP-5 to DP-4, respectively. In addition, DP-1 and DP-4 have the same maximum wavelength at 278 nm in UV spectra, suggesting DP-1 is structurally related to DP-4. Similarly, there is also relation between the structures of DP-2, DP-3 and DP-5 due to their same maximum wavelength of 276 nm.

2 Identification of the degradation products by UPLC-QTOF-MS

The exact molecular ion mass and fragment ion mass obtained by UPLC-QTOF-MS were used for identifying the degradation products. The UPLC-QTOF-MS data for Z-ligustilide and its degradation products are shown in Table 1.

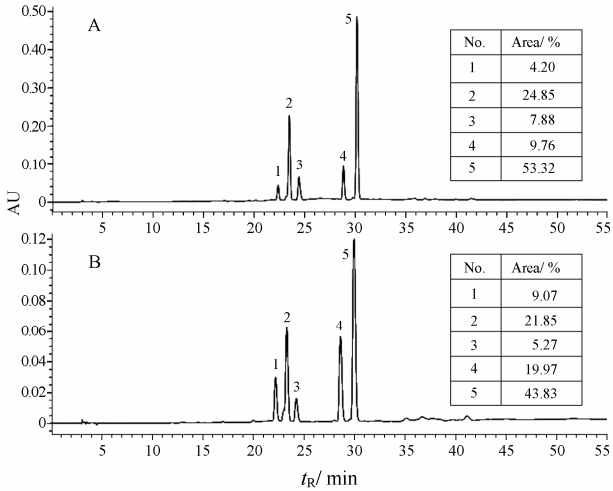

Figure 1 HPLC chromatograms at 280 nm of *Z*-ligustilide stored at room temperature under direct sunlight for 60 (A) and 120 days (B)

Z-ligustilide showed intense $[M+H]^+$ ion at *m*/*z* 191.107 7, indicating the accurate molecular weight of 190.099 4 and calculated formula of $C_{12}H_{14}O_2$. With the collision energy of 20 eV, major fragment ions at *m*/*z* 173.094 1 $[M+H-H_2O]^+$, 145.100 0 $[M+H-H_2O-CO]^+$, 117.070 3 $[M+H-H_2O-CO-C_2H_4]^+$, 105.069 6 $[M+H-H_2O-CO-C_3H_4]^+$ were generated, indicating *Z*-ligustilide inclined to losses of H_2O, CO and several alkyl groups. This fragmentation pattern will benefit for identifying the degradation products.

DP-5 shows the exact $[M+H]^+$ ion at *m*/*z* 207.101 8 and solvent adduct $[M+H+CH_3OH]^+$ ion at *m*/*z* 239.128 4, indicating calculated formula of $C_{12}H_{14}O_3$ and accurate molecular weight of 206.094 3, 16 Da higher than *Z*-ligustilide, suggesting the oxidation of *Z*-ligustilide. By losses of H_2O, H_2O+CO and H_2O+CO+C_2H_4, the product ions at *m*/*z* 189.090 6, 161.095 9 and 133.065 0 were generated. Since this fragmentation pattern was similar to that of ligustilide, it was induced that the degradation reaction didn't occur on easy cleavable group in mass spectrometer. There are two double bonds in the structure of *Z*-ligustilide. Double bond at C6−C7 position is relative active group and can

Table 1 UPLC-QTOF-MS data for Z-ligustilide and its degradation products

Compound	t_R/min	MS	MS/MS	Elemental composition	Identification
DP-1	5.075	225.111 7, 207.101 6	207.100 5, 189.090 9, 161.096 1, 133.064 5, 119.085 8, 105.070 5	$C_{12}H_{16}O_4$	(*E*)-6, 7-dihydroxyligustilide
DP-2	5.285	225.101 5, 207.101 6	207.101 5, 189.091 2, 161.096 7, 133.064 4, 119.085 8, 105.070 4	$C_{12}H_{16}O_4$	Senkyunolide I
DP-3	5.483	225.112 1, 207.101 6	207.100 9, 189.092 0, 161.096 0, 133.102 6, 119.086 2	$C_{12}H_{16}O_4$	Senkyunolide H
DP-4	6.422	207.102 2, 239.128 7	189.092 4, 161.097 0, 133.065 7, 119.086 8, 105.071 3	$C_{12}H_{14}O_3$	(*E*)-6, 7-epoxyligustilide
DP-5	6.694	207.101 8, 239.128 4	189.090 6, 161.095 9, 133.065 0, 119.085 6, 105.069 6	$C_{12}H_{14}O_3$	(*Z*)-6, 7-epoxyligustilide
Z-ligustilide	9.384	191.107 7	191.104 9, 173.094 1, 145.100 0, 128.061 1, 117.070 3, 105.069 6	$C_{12}H_{14}O_2$	*Z*-ligustilide

be oxidized easily. Thus, DP-5 with the exact mass of 206.094 3 is deduced as epoxyligustilide at C6−C7 position[12]. DP-4 was considered as the isomer of DP-5 due to their similar MS and MS/MS data.

DP-1, 2 and 3 exhibited weak $[M+H]^+$ ion at *m/z* 225.112 1 and intense $[M+H-H_2O]^+$ ion at *m/z* 207.101 6. Their molecular weights are 224.104 9, 18 Da higher than epoxyligustilide (DP-4 and DP-5) and 34 Da higher than *Z*-ligustilide, indicating one H_2O molecule and two hydroxyl groups, respectively. Therefore, DP-1, 2 and 3 may be generated by hydrolysis of DP-4 and DP-5. Except for the first loss of H_2O, the other fragment ions, such as 189, 161, 133, 119 and 105, were in accord with those of epoxyligustilide, suggesting the similar fragmentation patterns. Thus, they are assigned as the isomers of dihydroxylated ligustilide and possibly generated by hydrolysis of epoxyligustilide. Furthermore, DP-2 and DP-3 were unequivocally identified as senkyunolide I and senkyunolide H by comparison with reference compounds and Chuanxiong [13].

3 Structure elucidations of degradation products by NMR

^1H and ^{13}C NMR data of DP-1, DP-2, DP-5 and *Z*-ligustilide are shown in Table 2. By comparing DP-5 and *Z*-ligustilide, it is clear that there is no distinct chemical shift except for C6 and C7, suggesting the oxidation occurred at the C6−C7 position, not at C3−C10 position. The chemical shifts of C-6 and C-7 have an intense upfield shift from 129.9 to 73.9 ppm, 116.8 to 68 ppm, respectively. There is also upfield shift of 2.1 ppm at H-6 and H-7. The NMR data of DP-5 is also coherent to the reported data[14]. Thus, it was confirmed as (*Z*)-6, 7-epoxyligustilide.

DP-1, DP-2 and DP-3 are the isomers and the latter two were identified as senkyunolide I and senkyunolide H. Since senkyunolide H is the isomer of senkyunolide I at the C6-C7 position, DP-1, which can also be generated from senkyunolide I[15], might be the isomer of senkyunolide I at the C3-C10 position. The chemical shifts of DP-1 and DP-2 are similar except for a little difference at H-4 and H-10. It was reported that if the double bond at C10 position is *E*-form, the chemical shift of H-10 is about 5.80 ppm[14]. Thus, DP-1 was characterized as (*E*)-6, 7-trans-dihydroxyligustilide. Furthermore, DP-4 has the similar UV features with DP-1 and is the isomer of DP-5. Thus, DP-4 was deduced as (*E*)-6, 7-epoxyligustilide.

4 Degradation pathways of *Z*-ligustilide

From the above results, *Z*-ligustilide is likely to degrade into epoxyligustilide through oxidation, then transform into dihydroxyligustilide by further hydrolysis when stored at room temperature with direct sunlight. Among them, senkyunolide I (DP-2) and (*Z*)-6, 7-epoxyligustilide (DP-5) are the major degradation products, which could be further transformed into (*E*)-6, 7-trans-dihydroxyligustilide (DP-1) and (*E*)-6, 7-epoxyligustilide (DP-4) through isomerization, respectively. The proposed degradation pathways of *Z*-ligustilide are shown in Figure 2. Oxidation, hydrolysis and isomerization are the major degradation reactions. It suggests that *Z*-ligustilide should be kept in dry condition without oxygen and sunlight.

In addition, It has been reported that senkyunolide I and (*Z*)-6, 7-epoxyligustilide are also the *in vivo* metabolites of *Z*-ligustilide[16, 17]. In other words, they are not only the main degradation products, but also the

Table 2 ^1H and ^{13}C NMR data for DP- 1, 2, 5 and *Z*-ligustilide (*J* in Hz)

Position	DP-1		DP-2		DP-5		*Z*-ligustilide	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	−	168.6	−	169.1	−	169.4	−	167.5
3	−	150.8	−	152.8	−	154.0	−	147.0
4	2.67−2.82 (2H, m)	22.9	2.45−2.59 (2H, m)	19.1	2.53 (2H, m)	17.4	2.60 (2H, t, *J* = 5.0)	18.3
5	1.87−1.94 (1H, m)	27.2	1.85−1.93 (1H, m)	26.6	1.94−2.04 (2H, m)	24.5	2.48 (2H, m)	22.2
	2.12−2.17 (1H, m)		2.08−2.10 (1H, m)					
6	3.93 (1H, m)	71.4	3.98 (1H, br s)	71.7	3.97 (1H, m)	73.9	6.01 (1H, dt, *J* = 9.5, 4.0)	129.9
7	4.49 (1H, br d, *J* = 6.1)	68.5	4.48 (1H, br d)	67.8	4.16 (1H, d, *J* = 2.5)	68.0	6.27 (1H, dt, *J* = 9.5, 2.0)	116.8
8	−	128.3	−	125.9	−	124.4	−	123.7
9	−	147.6	−	148.0	−	148.6	−	148.4
10	5.80 (1H, t, *J* = 8.6)	118.3	5.25 (1H, t, *J* = 7.9)	114.3	5.27 (1H, t, *J* = 8.0)	113.5	5.25 (1H, t, *J* = 8.0)	112.9
11	2.31 (2H, q, *J* = 7.7)	28.0	2.35 (2H, q, *J* = 7.5)	28.1	2.36 (2H, q, *J* = 7.5)	28.1	2.38 (2H, q, *J* = 7.5)	28.0
12	1.53 (2H, sext, *J* = 7.3)	23.0	1.49 (2H, sext, *J* = 7.4)	19.1	1.49 (2H, sext, *J* = 7.4)	22.3	1.51 (2H, s, *J* = 7.5)	22.2
13	0.97 (3H, t, *J* = 7.4)	13.7	0.95 (3H, t, *J* = 7.4)	13.8	0.95 (3H, t, *J* = 7.4)	−	0.96 (3H, t, *J* = 7.5)	13.6

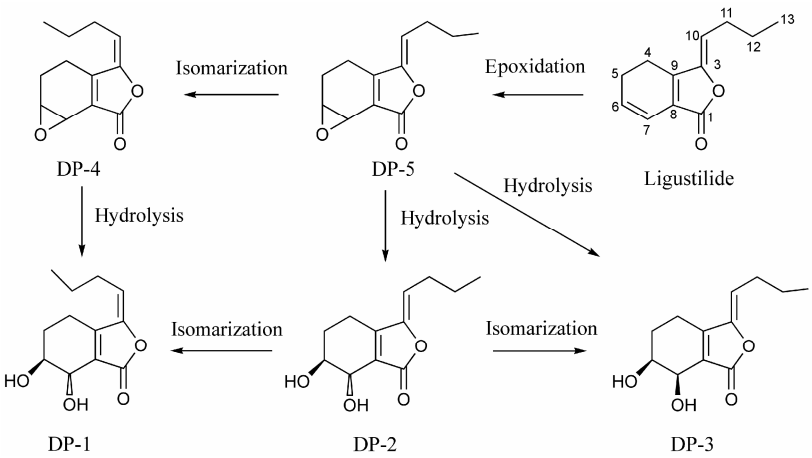

Figure 2 The proposed degradation pathways of *Z*-ligustilide

metabolites of *Z*-ligustilide, indicating they may be the effective substance of *Z*-ligustilide. Therefore, it is necessary to study and compare their pharmacological activities.

Senkyunolide I is also one of major bioactive phthalides of Chuanxiong. More and more pharmacological effects have been reported recently[18−20]. (*Z*)-6, 7-epoxyligustilide was only isolated from Danggui, another traditional Chinese medicine. There is no study about its pharmacological activities except for this patent, which indicated (*Z*)-6, 7-epoxyligustilide can enhance the sensitivity of drug-resistance tumor cells against chemotherapy and decrease the drug resistance of tumor cells[21]. It was reported that *Z*-ligustilide had inhibitory effect on rat vascular smooth muscle cells[22, 23]. Our laboratory indicated that the inhibitory effect of (*Z*)-6, 7-epoxyligustilide on HUVSMCs proliferation was higher than that of *Z*-ligustilide and senkyunolide I, indicating it is a promising bioactive compound. The other pharmacological effects are underway. In addition, (*Z*)-6, 7-epoxyligustilide is more stable than *Z*-ligustilide. Its purity scarcely decreased and was determined to be above 95% by HPLC analysis when stored at 4 ℃ or −80 ℃ for two years. The values of molecular weight, log *P*, hydrogen-bond donor and hydrogen-bond acceptor are 206.26, 2.09, 0 and 3, respectively. It is in accord with Rule of 5, suggesting it can be absorbed and distributed easily in the body.

Conclusion

The degradation behavior of *Z*-ligustilide stored at room temperature with direct sunlight for 60 and 120 days was monitored by HPLC-UV. Total five degradation products were detected, identified and characterized by UPLC-QTOF-MS and NMR. Finally the degradation pathways of *Z*-ligustilide were proposed. Oxidation, hydrolysis and isomerization are the major degradation reactions, suggesting *Z*-ligustilide should be kept in dry condition without oxygen and sunlight.

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